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Influence of Cotyledons upon α-Amylase Activity in Pea Embryonic Axes

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ABSTRACT

α-Amylase activity remained relatively low in the axes of intact etiolated pea seedlings; the activity was predominantly confined to the epicotyl. Starch accumulated slightly. When the cotyledons were removed and the axes cultured on medium containing no carbon source, the starch reserve in the axes disappeared within a few days. This was accompanied by a 10- to 15-fold increase in α-amylase activity, in the absence of additional epicotyl growth. The phenonemon was observed for axes throughout early growth, although the relative accumulation of α-amylase activity in cultured axes was less for older seedlings. This change was attributed to a reduced response by nongrowing tissues. There was no corresponding change in β-amylase activity. These observations, described for several varieties of peas, demonstrate the control of cotyledons upon the utilization of stored reserves within the axis, with α-amylase as a key enzyme.

Seeds are ideal systems for studying the interaction of one part of an organism with another. The regulation of carbohydrate mobilization can be monitored to investigate specific aspects of this interaction. In the more thoroughly studied grains, it is generally believed that the hydrolytic enzymes in the endosperm are synthesized in response to gibberellins produced by the embryo (4, 33, 36). Thus, we have an instance of a positive control mechanism whereby the presence of the embryo promotes the mobilization of reserve materials in the storage organ.

The situation is less clear for dicot seeds (28) where the major storage organs are the cotyledons. Although α-amylase activity was low in cotyledons of ungerminated pea, it increased 5- to 10-fold during the 2nd or 3rd week of seedling growth (5, 16, 26, 27, 29, 32, 37). The increased activity was associated with a rapid loss of starch in peas (1, 16, 26) and lentils (30). When cotyledons were cultivated in the absence of the embryonic axis the amounts of amylase activity were often lower than for cotyledons from intact seedlings (17, 27, 32) although the difference was often small or nonexistent (2, 37). Although gibberellins tended to have no influence upon amylase activity when applied to intact peas (27 32), there was a slight effect when dwarf varieties were tested (13), suggesting that the gibberellin content in the cotyledons of tall varieties was not limiting. (This appeared to be the situation for corn also [15].) Furthermore, light-inhibited α-amylase activity in bean cotyledons was reversed by exogenous gibberellic acid (31). Cytokinins have also been reported to be effective in replacing the embryonic axis (14, 17, 31) or when applied to intact seeds (26). Most of the above investigations, along with the studies on other cotyledons (9, 21, 22), are at least consistent with the concept that the embryo axis exerts a positive control over the cotyledons.

Since smaller amounts of storage materials are also present in the embryonic axis (11), I hypothesized that the storage organ exerts a negative control over these reserves; in other words, the presence of the storage organ would suppress the utilization of reserves in the axis. Although the absence of the cotyledons has multiple effects upon the pea embryonic axis, few papers have examined the biochemical responses related to utilization of stored materials. Amylase activities were higher in axes of intact dwarf pea seedlings treated with gibberellic acid (19), and were reported to increase in excised bean hypocotyls incubated with kinetin (8). Neither of the above papers can be used to assess the existence of a negative control from the cotyledons.

In this paper excised pea embryonic axes were cultured on medium containing no carbon source. The increased α-amylase activity, occurring in the absence of epicotyl elongation, is consistent with the hypothesis that the cotyledons control the utilization of starch reserves within the axis and that α-amylase is a key enzyme in this control system.

MATERIALS AND METHODS

Plant Materials. Dry pea seeds (*Pisum sativum*, obtained from Atlee Burpee Co.; var. Early Alaska unless noted otherwise) were rinsed in 70% ethanol, surface-sterilized for 15 min in 20% (v/v) commercial bleach, rinsed repeatedly, and imbibed for 16 to 20 h at 20 C in sterile distilled H_2O. Ungerminated seeds (time zero) were at the end of imbibition period. Embryonic axes were removed aseptically and cultured in plastic Petri dishes containing Raghavan and Torrey's (23) basal medium minus the carbon source. Intact seeds were germinated aseptically on the same medium in larger glass containers. Both intact seedling and cultured axes were grown in the dark at 20 C.

Amylase Assays. α-Amylase activity was estimated by the β-limit dextrin method as described previously (11). β-Amylase was estimated by the increase in reducing materials (3) in the presence of 10 mM EDTA using hydrolyzed starch (Connaught) as the substrate to extend the linear part of the curve. Since this EDTA concentration eliminated all activity as measured by the β-limit dextrin method, the difference between the rates of reducing material formation with and without the chelator was used as an independent method of measuring α-amylase activity.

Homogenates were diluted so that the slope could be computed from four to five A readings on the straight line portion of the curve; all points represent the average of five determinations on individual axes or sections. Semilog plots were used to show relative changes. Samples anticipated to have high activities were routinely assayed at two dilutions to verify the proportionality between dilution and rate. Activities are expressed per axis or per section.

Starch. Starch was extracted according to the method of Shannon (25) using dimethylsulfoxide to enhance sedimentation of cell debris and protein. The starch concentration was measured in properly diluted supernatants by the anthrone reagent (20). The starch standard was treated the same as the homogenates.

RESULTS

Ungerminated Axes. Axes of intact seeds started to grow as soon as they were sown (Fig. 1A). The starch content showed a slight but reproducible reduction during early growth, then accumulated slowly (Fig. 1B). As demonstrated previously (11), the α-amylase activity was constant for several days, then increased as the epicotyl elongated.

In contrast, when the cotyledons were removed and the axes cultured on medium containing no carbon source, the fresh weight increased for approximately 3 to 5 days and then remained constant at a total of about three times the initial size (Fig. 1A). The starch content continued to decline until it was below the sensitivity of the method by around 7 days (Fig. 1B). The α-amylase activity increased until 7 days when the activity was over an order of magnitude higher than in the initial ungerminated axis (Fig. 1C). For comparison by 7 days the cultured axis in Figure 3 was only 1 cm long while the axis from the intact seedling was more than 20 cm long.

The identification of this activity as α-amylase was confirmed by several methods used previously for axes from intact seeds: change in absorption spectra for the iodine-starch complex, increase in reducing materials relative to the decrease in starch, and complete inhibition of β-limit dextrin activity with 10 mM EDTA. Furthermore, when homogenates were subjected to electrophoresis in Noble agar at pH 7, the increased activity was observed to migrate to the same position as the α-amylase from germinated cotyledons (method of Shain and Mayer [24]).

Several experiments were designed to investigate the kinetics of accumulation of the α-amylase activity in cultured axes. The increase in activity had a temperature-dependent lag phase (Fig. 2). In extended experiments the activity reached a peak at approximately 7 days in culture (Fig. 3), corresponding to the time when the starch in the axes was completely depleted (Fig. 1B). There was subsequently a gradual loss of activity (Fig. 3).

The changes in activity varied somewhat from experiment to experiment. In five different experiments the maximum relative accumulation after 5 to 7 days in culture had a range of 15.6 to 23.2; the $\bar{X} \pm$ SD was 18.8 ± 3.7.

Several varieties of pea were chosen for their different seed characteristics. All excised embryonic axes had limited growth when cultured on medium containing no carbon source, and all had comparable increases in α-amylase activity (Table I). The variation among different varieties was within that observed for different experiments using Alaska variety.

One possibility would be that all enzymes in the axis responded to the excision and culture. However, this was not true, as indicated by the lack of response of β-amylase (Fig. 4). The α-amylase activity observed in this experiment, as assayed by the increase in reducing materials (total activity minus that resistant to EDTA), showed both the same general kinetics and the same relative increase as observed using the β-limit dextrin method. These results were confirmed by electrophoresis: the band for β-amylase remained constant while that for α-amylase increased.

The slight growth of cultured axes was due primarily to extension of the radicle. When the axes were subdivided into radicle, hypocotyl, and epicotyl and assayed independently, it became evident that although the activity doubled in the root (as had the fresh weight), the order of magnitude changes in activity were in both the epicotyl and hypocotyl.

Germinated Seedlings. The above experiments demonstrate the influence of the cotyledons upon α-amylase activity in the axis of ungerminated seeds. Is this mechanism maintained during seedling development? For comparison, seedlings were grown for 2 or 4 days before the cotyledons were removed. The relative growth rates were comparable for all cultured axes (Fig. 5A). Inasmuch as the α-amylase activity in axes from intact seedlings is associated

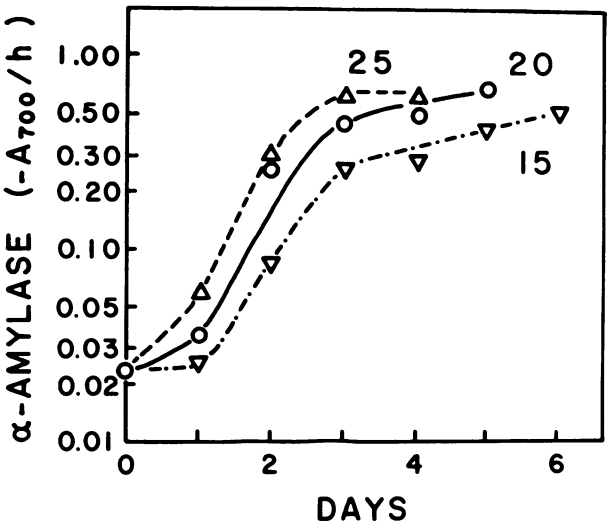

FIG. 2. Effect of temperature upon accumulation of α-amylase activity in cultured embryonic axes.

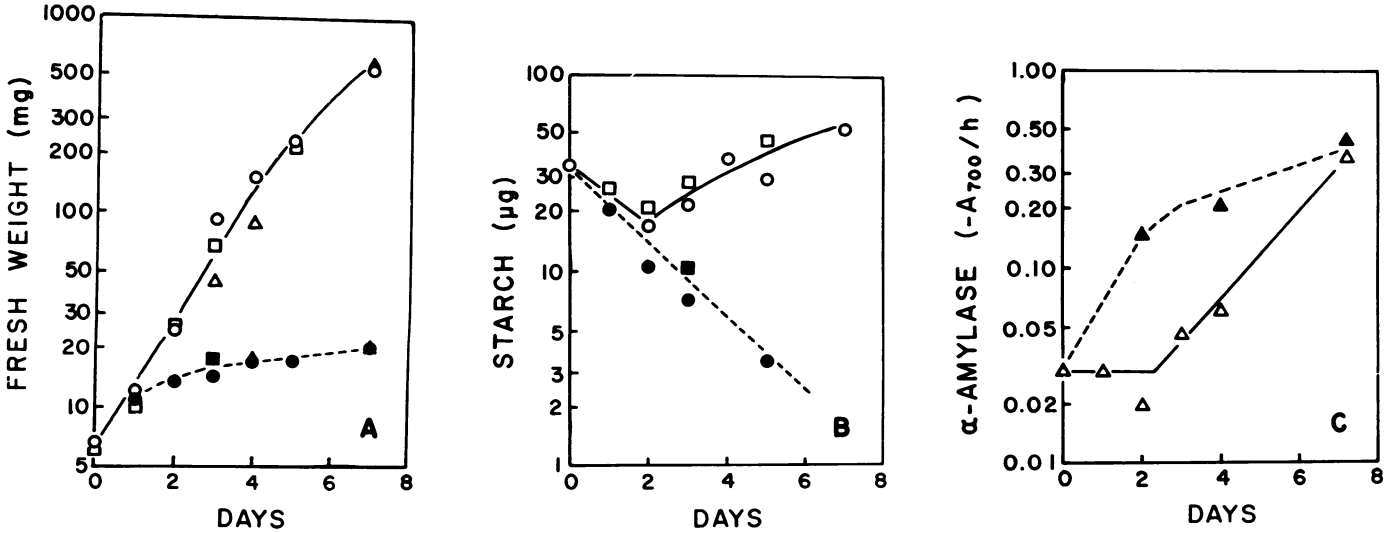

FIG. 1. Comparison of changes in embryonic axes of intact seedlings (○, △, □) with those of excised axes cultured on medium containing no carbon source (●, ▲, ■); different symbols represent different experiments. A: fresh weight; B: starch; C: α-amylase activity.

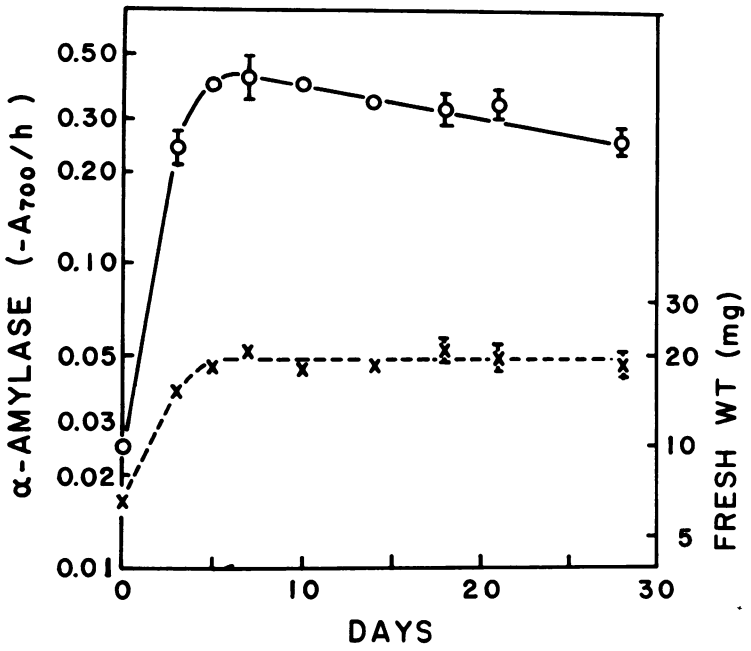

FIG. 3. Changes in fresh weight (×) and α-amylase activity (○) in long term cultures of excised axes; bar represents SE.

Table I. Changes in cultured axes from different varieties. Imbibed, ungerminated axes excised and cultured 3 days on medium containing no carbon source.

Variety	Initial	Cultured		Initial	Cultured	
	mg fresh weight			α-amylase activity		
Alaska	7.0 ± 0.2[1]	17.5 ± 0.7	(2.5)[2]	0.025 ± 0.001	0.398 ± 0.018	(15.6)[2]
Burpeeana	7.0 ± 0.4	15.5 ± 0.9	(2.6)	0.031 ± 0.003	0.321 ± 0.023	(10.4)
Fordhook Wonder	7.6 ± 0.3	20.1 ± 1.4	(2.6)	0.031 ± 0.002	0.499 ± 0.089	(16.1)
Little Marvel	7.8 ± 0.3	15.1 ± 1.4	(1.9)	0.038 ± 0.003	0.274 ± 0.062	(7.2)
Sweetpod	6.7 ± 0.4	17.5 ± 2.0	(2.7)	0.026 ± 0.003	0.382 ± 0.012	(14.7)
Dwarf Gray Sugar	5.6 ± 0.3	15.4 ± 2.7	(2.7)	0.025 ± 0.002	0.279 ± 0.077	(11.2)

[1] mean ± standard error

[2] increase relative to initial amount

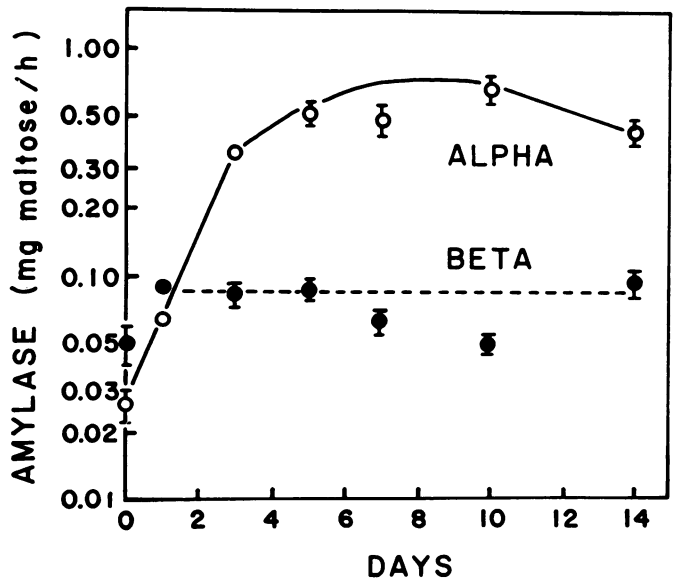

FIG. 4. Changes in α- (○) and β-amylase (●) activities in cultured axes; bars represent SE.

with epicotyl development (11), it is important to note that there was little additional epicotyl growth once the cotyledons had been removed (Fig. 5B). Any change in enzyme activity would suggest a control mechanism rather than a secondary response to a developmental change (as distinguished by Ching [7]). Indeed, the α-amylase activity did increase in each instance (Fig. 5C), although the relative increase dropped from 22 to 16 to 6 when the cotyledons were removed after 0, 2, or 4 days, respectively.

The reduction in relative accumulation of α-amylase activity was investigated by culturing 1-cm sections of stem removed from just below the epicotyl hook. When the stem sections were removed from epicotyls after 4, 6, and 8 days germination, the length in each case increased slightly during the first 2 days only (mean values from 27 mg fresh weight at time of excision to 35–38 mg after 2 days). In contrast, the α-amylase activities in each case increased approximately an order of magnitude. It appears that the ability to respond did not change in tissues of comparable physiological age.

In contrast, when 1-cm sections were removed from the apical (just below the epicotyl hook) and the basal (just above the hypocotyl) portions of the stem, the responses were somewhat different. First, although the basal sections were slightly thicker (about 50 mg fresh weight), they did not elongate in culture. Second, the sections contained reduced amounts of α-amylase, as

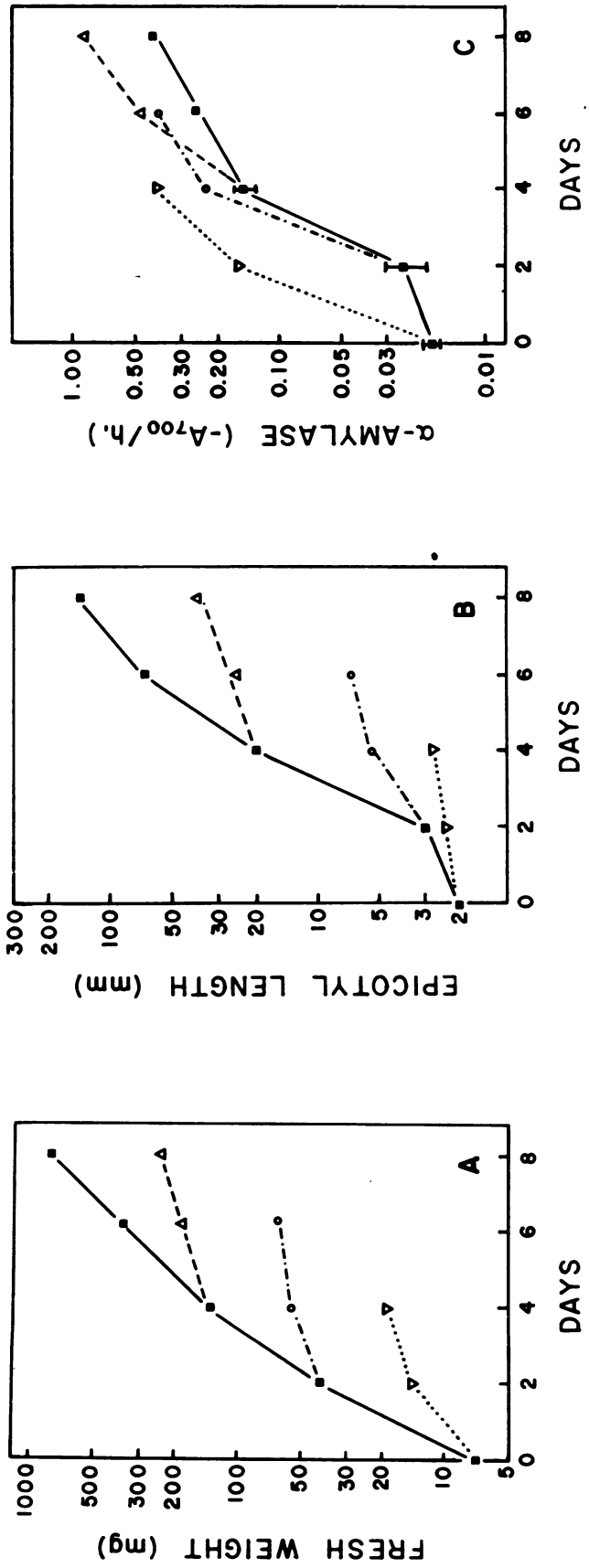

FIG. 5. Effect of removing cotyledons after zero (▽), 2 (○), and 4 (△) days of germination, compared to intact seedlings (■). A: fresh weight; B: epicotyl length; C: α-amylase activity. SE shown where it exceeded size of symbol.

noted previously (11). Most important, the relative accumulation of α-amylase activity in the basal sections had a distinct lag phase, a slower rate of accumulation, and a lower final amount than observed for apical sections (Fig. 6). I concluded that the ability to respond changes as the tissue "matures."

Axes from 6-day-old seedlings were subdivided into hook, stem, hypocotyl, and root and each cultured independently for 4 days. Although only the root grew, the α-amylase activity increased significantly in each case. Although this experiment was not very quantitative (inadequate number of points for kinetic interpretation, lack of assessment for mature tissues, etc.), it does demonstrate that all portions of the axis have the ability to respond to cotyledon excision. Because there was no change in the α-amylase activity in the basal stem sections when the upper portion of the epicotyl (but not the cotyledons or root) had been removed, I concluded that the response was to the cotyledon and not simply to the surgical procedure.

The response of the apical stem sections was comparable to that of the ungerminated axes in two other ways. Several varieties of peas were chosen for their different growth habits. In all cases there was a substantial increase in α-amylase activity in cultured sections (Table II). Although there was much more β-amylase activity in the stem sections, this activity did not change in culture. The change in α-amylase activity was more difficult to assess in the later experiment because of the greater variability of the tissue from experiment to experiment, the higher background of β-amylase activity, and the reduced reliability of the procedure itself. However, the change observed by this method was comparable to that observed in other experiments using the β-limit dextrin method.

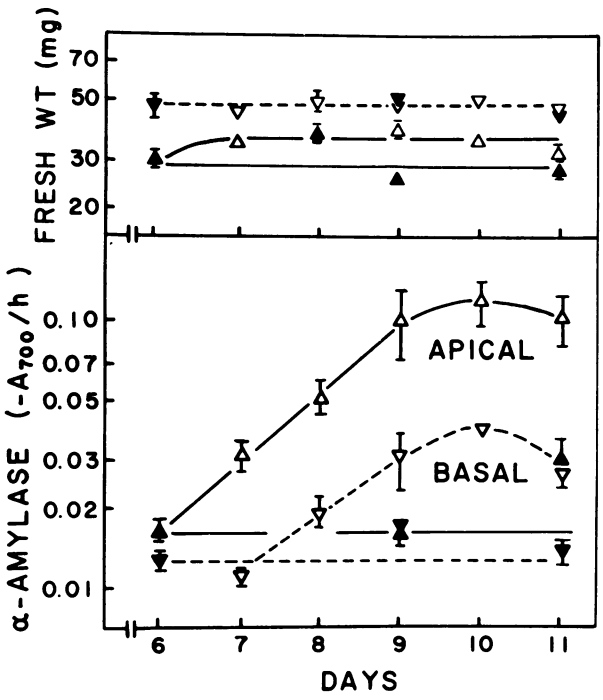

FIG. 6. α-Amylase activity for stem sections removed from apical (△) and basal (▽) sections of the stem; (△, ▽): cultured sections; (▲, ▼): sections from intact seedlings.

Nature of Increased Activity. Although changes in enzyme activity may reflect a change in amount of enzyme, several other mechanisms could result in these observations. The following possibilities were tested using homogenates from ungerminated and cultured axes.

Enzymes associated with starch metabolism are known to be adsorbed onto starch granules (*e.g.*, refs. 12 and 35). Therefore, the difference could be due simply to the presence of starch granules in ungerminated but not in cultured axes, the enzyme being in the pellet in the first instance but not in the second. (The homogenates were cleared by centrifugation before assayed.) Ungerminated axes were homogenized and a sample removed before the rest was centrifuged. The activities for the centrifuged and uncentrifuged samples ($\bar{X}$ ± SE) were 0.019 ± 0.001 and 0.023 ± 0.001; the *t* test for paired observations was 0.29 (N= 5). Comparable results were obtained in several experiments. When homogenates of ungerminated and cultured axes were mixed before and after centrifugation, the rates were identical (0.122 ± 0.006 and 0.120 ± 0.006, respectively). No additional activity was observed when ungerminated axes were homogenized in 10 mM NaCl or in 0.4 M sucrose (the latter removed phosphorylase from starch granules; 14). No activity was lost when homogenates of cultured axes were incubated with granules of soluble starch. Therefore, the increased activity in cultured axes cannot be due to the adhesion of α-amylase to starch granules.

A second possibility could be that the α-amylase was trapped within the amyloplast membrane that was ruptured after excision. Homogenates of ungerminated axes were subjected to up to three freeze-thaw cycles with no change in enzyme activity. Sonic oscillation was also without effect. Both methods are known to disrupt membranes, as is the osmotic shock of homogenizing the samples in distilled H_2O. (These experiments are important in that they demonstrate the stability of the enzyme.)

There are numerous examples of inhibitors of α-amylase, and changes in enzyme inhibitors may occur during germination. Alternately the increased activity could be due to the presence of a promoter or activator in the cultured axes. However, when homogenates of ungerminated and cultured axes were mixed in

Table II. Changes in excised stem sections from different varieties. Apical sections, 1 cm long, were removed from 6-day-old etiolated seedlings and cultured 4 days on medium containing no carbon source.

Variety	Initial	Cultured		Initial	Cultured	
	mg fresh weight			α-amylase activity		
Alaska	30.0 ± 1.6[1]	41.2 ± 1.2	(1.4)[2]	0.017 ± 0.004	0.081 ± 0.004	(4.8)[2]
Burpeeana	46.0 ± 2.7	59.4 ± 3.0	(1.3)	0.012 ± 0.001	0.128 ± 0.007	(10.7)
Little Marvel	38.2 ± 3.2	46.6 ± 3.7	(1.2)	0.021 ± 0.004	0.159 ± 0.016	(7.6)
Sweetpod	26.3 ± 1.8	29.9 ± 1.1	(1.1)	0.020 ± 0.002	0.083 ± 0.007	(4.2)
Dwarf Gray Sugar	29.3 ± 1.8	42.0 ± 1.6	(1.4)	0.015 ± 0.004	0.093 ± 0.012	(6.2)
Early Dwarf	40.6 ± 2.2	43.5 ± 2.5	(1.1)	0.024 ± 0.005	0.182 ± 0.026	(7.6)

[1] mean ± standard error

[2] increase relative to initial amount

different ratios, the observed rate for the mixture was the same as that predicted from the separate samples (t test for paired observations; N = 5). This was true for homogenates from axes cultured for 2 days (when the enzyme activity first increased) as well as for axes cultured for 7 days (when the peak enzyme activity was observed). These results are also inconsistent with the hypothesis that the increased activity was due to activation of a zymogen, as observed for β-amylase from grains (reviewed in ref. 10) and amylopectin-1,6-glucosidase from pea cotyledons (18, 24, 34).

DISCUSSION

Starch reserves of the embryonic axis accumulated in the intact pea seedling (Fig. 1B). In contrast, the starch disappeared upon removal of the cotyledons (Fig. 1B), demonstrating that the cotyledons have negative control over the utilization of this reserve. It appears that α-amylase is a key enzyme in this control system since the activity remained relatively low in axes of intact seedlings but increased more than an order of magnitude in cultured axes (Fig. 1C). (There was evidently enough enzyme in the ungerminated axis to account for the initial drop in starch in both intact and cultured axes.) This control was observed throughout the initial phases of germination (Fig. 5), although the ability to respond may be reduced in nongrowing relative to growing tissues (Fig. 6). The changes in activity were interpreted to be part of a control mechanism and not simply secondary effects related to developmental changes since the accumulation of α-amylase activity was observed in the nongrowing hypocotyl seedlings where the epicotyl growth was quite small after excision (Fig. 5), and in stem sections where the growth was small relative to change in enzyme activity (Fig. 6).

Several experiments are consistent with the hypothesis that the changes in α-amylase activity were the result of changes in enzyme synthesis. The increase was temperature-dependent (Fig. 2) and did not occur in submerged axes (unpublished observation), demonstrating the need for active metabolism. The changes in activity were not due to the production of a promoter, the loss of an inhibitor, or the release from amyloplasts or starch granules.

Having demonstrated that the cotyledons exert a negative influence upon the amount of α-amylase activity in the axis, one must now return to the original concept that the axis has a positive control over the enzyme activity in the cotyledons. Since multiple amylases have been demonstrated for pea cotyledons (16, 37) and stems (19), one might hypothesize that the amylase genes active in the cotyledons are controlled by different regulators than the amylase genes active in the axial tissues. A more basic problem involves the culturing of excised cotyledons under the same conditions as used for growing intact seedlings. When pea cotyledons were assayed for ribonuclease and acid phosphatase activities it was observed that although the activities for cotyledons cultured in Petri dishes were less than those for cotyledons from intact seedlings, the enzyme activities were actually highest for excised cotyledons cultured in large glass culture vessels (6). The results in these experiments were interpreted to demonstrate that the influence of the embryonic axes was a negative one. The resolution of this problem will depend upon the identification of the chemical nature of the signals exchanged between the cotyledons and the growing axis.

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