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The Occurrence and Development of Amylase Enzymes in Incubated, De-embryonated Maize Kernels[1]

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ABSTRACT

The development of amylase activity in extracts from de-embryonated and GA_3-treated de-embryonated maize kernels (*Zea mays* L.) was determined during a 10-day incubation period. The increase in activity was compared with activity extracted from endosperms dissected from germinating whole kernels. Chromatographic analysis of reaction products as well as physicochemical characterization demonstrated that the activities from GA_3-treated and nontreated tissue were comparable and that part of the activity was attributable to α-amylase.

Concomitant with the increase in activity was the appearance of a number of starch-degrading bands, as evidenced by polyacrylamide gel electrophoresis. Actinomycin-D (20 μg/ml) and cycloheximide (5 μg/ml), when present in the incubation medium at early periods of incubation, were capable of inhibiting the development of amylase activity and of preventing the appearance of the starch-degrading bands.

The results indicate that the development of α-amylase activity in de-embryonated maize kernels is independent of an embryo or an exogenous source of gibberellic acid and suggest that this process involved protein synthesis.

Amylolytic activity, readily extracted from the endosperms of incubating maize kernels, has been shown to consist of both α- and β-amylases (3, 6, 9). Bernstein (2) suggested that α- and β-amylases had separate origins in the maize kernel. It was proposed that α-amylase is inherited as an embryo characteristic, whereas the inheritance of β-amylase is a maternal characteristic and is formed *in situ* in the endosperm. In the mature grain, however, it has generally been concluded that β-amylase, which originates in the endosperm, is the only amylase present (6).

It has been reported that the development of amylolytic activity accompanying germination of cereal grains is in part attributable to production of α-amylase, which is dependent on some embryonic tissue (embryonic axis or scutellum or both) (6). Ingle and Hageman (12) concluded that GA_3 is essential for the complete hydrolysis of starch in excised endosperms of corn. This information, along with the results of extensive studies with barley (13, 18, 23), has led to the suggestion that GA_3 is the embryonic factor responsible for the development of α-amylase in germinating cereal grains. This general concept has been expanded to show that GA_3 is capable of inducing *de novo* synthesis of α-amylase and protease in isolated aleurone layers (8, 14, 24). These enzymes are then secreted into the starchy endosperm where α-amylase degrades the reserve starch and where protease is believed to liberate inactive β-amylase. There have been reports, however, of differences in the requirement for and source of GA_3 for the induction of amylase in certain cereals (4, 17).

In this report, we describe the development of amylase activity in maize kernels and examine the extent to which the embryo and GA_3 are involved. We also report the effect of RNA and protein synthesis inhibitors on development of amylase activity.

MATERIALS AND METHODS

Plant Material. Seed of Seneca Chief sweet corn (*Zea mays* L.) was purchased from the Joseph Harris Seed Company (Rochester, New York). The mature, dry kernels, as obtained commercially, were physically de-embryonated using a file and a scalpel.

Tissue Incubation. The kernels, either whole or de-embryonated, were surface sterilized for 15 min in a 1% (w/v) aqueous hypochlorite solution. After rinsing thoroughly with 10 changes of sterile, deionized H_2O, the tissue was placed aseptically in 9-cm Petri plates with 8 ml of an aqueous incubation medium containing 40 μg of Penicillin G and 2.4 μg of Streptomycin sulfate. The tissue was incubated at 30 C in the dark.

Enzyme Extraction. Enzyme extracts were prepared by homogenizing the sample and any remaining incubation medium with 2 ml of 0.05 M tris-HCl buffer, pH 7.6, for 2 min in a "Virtis 45" homogenizer. The homogenate was centrifuged for 10 min at 20,000*g* and the supernatant fraction was collected. The pellet was resuspended in an additional 4 ml of tris buffer and centrifuged as before. The combined supernatants were decanted through three layers of cheesecloth and adjusted to a volume of 22 ml.

Enzyme Assay. Total amylase activity was routinely assayed by the method of Bernfeld (1). The complete reaction mixture contained 3.9 ml of 0.05 M acetate buffer, pH 5, 1 ml of a 2% Merck soluble starch solution, and 0.1 ml of enzyme. (The volumes of buffer and enzyme were adjusted according to the activity of the sample so as to maintain a constant volume of 5 ml.) After 5 and 10 min of incubation at 30 C, 1-ml aliquots were withdrawn from the reaction mixture and added to 3 ml of 3,5-dinitrosalicylic acid reagent. Reducing power was determined spectrophotometrically at 575 nm according to Sumner and Somers (20). Standard curves using maltose were prepared and enzyme activity was expressed as mg of maltose released per unit time.

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Protein Determination. The protein content of the extracts was determined using the Folin-Ciocalteu reagent according to the method of Lowry *et al.* (16) with BSA as a standard.

Reaction Products. Amylase reaction products were determined using a partially purified enzyme preparation. The crude enzyme extract was made 66% (v/v) with respect to acetone. After thorough mixing, the aqueous acetone solution was stored at −10 C for 20 min and then centrifuged at 35,000*g* for 15 min. The supernatant fraction was discarded, and the pellet was resuspended in 1 to 2 ml of 0.05 M tris-HCl buffer, pH 7.6, and served as the source of enzyme in the standard reaction mixture. Aliquots were withdrawn at intervals of increasing reaction time and boiled for 5 min to inactivate the enzyme. They were then cooled, stoppered, and stored frozen (−10 C) until analyzed. One hundred microliters were spotted on strips of Whatman No. 1 chromatography paper. The chromatograms were developed with H_2O-95% ethyl alcohol-nitromethane (21:44:35) for 14 hr by descending flow. The reaction products were visualized according to the method of Robyt and French (19) by dipping the papers first in a solution containing 0.5 ml of saturated $AgNO_3$ in 100 ml of acetone. After drying, the papers were dipped in a second solution containing 0.1 ml of 10 N NaOH in 100 ml of 95% ethyl alcohol.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was employed to study the complement of starch-degrading enzymes present in the enzyme extracts. A modified procedure of Davis (5) was used in which 20 ml of an 8% gel solution were prepared to include 2.5 ml of 4% iodometric, soluble starch. Gels were run vertically at 3 mamp/tube at 4 to 6 C for approximately 1 hr or until the bromophenol blue tracking dye had migrated to the anodic end of the tube. The gels were extruded from the tubes, incubated for 40 min in a 4% iodometric soluble starch solution at room temperature, and stained with iodine (12 g KI and 1.2 g I/l). Areas of amylase activity appeared as clear zones against a dark blue background.

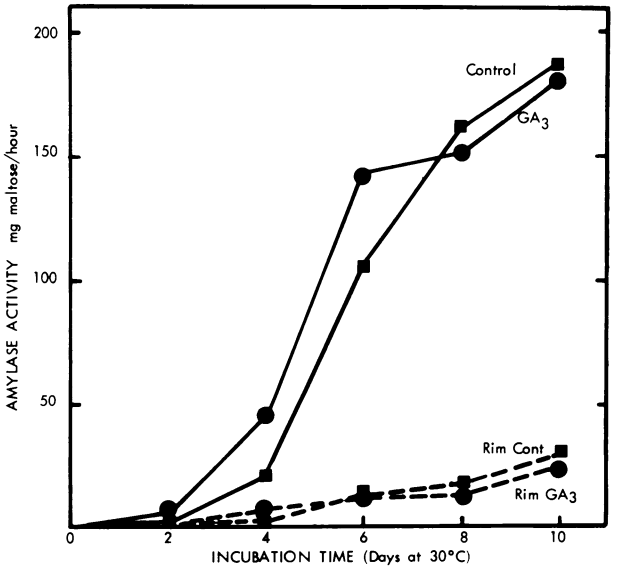

FIG. 1. Effect of GA_3 on the time course development of amylase activity in incubated, de-embryonated maize kernels. De-embryonated kernels were incubated with 1 μM GA_3 or as controls at 30 C. At the respective time periods, crude enzyme extracts were prepared and amylase activity was determined. Aliquots of residual incubation medium (Rim) from both treatments were also assayed for amylase activity.

RESULTS

Incubation Studies. The time course development of amylase activity in de-embryonated maize kernels is shown in Figure 1. After a slow initial rise, the level of activity dramatically increased between three and five days after initiation of incubation and continued to increase for at least 10 days of incubation. Inasmuch as all the embryo had presumably been removed before incubation, this increase in activity could not have been stimulated by some embryonic factor during incubation. During physical removal of the embryo from the kernel a noticeable difference in texture between the sott embryo and the hard endosperm starch surrounding the embryo was observed and used as the criterion that no residual embryonic tissue remained. To insure that the time course development of amylase activity was independent of the presence of an embryo, kernels were obtained from field-grown material 60 days after pollination. At this time, the kernels were sufficiently soft to allow the removal of the whole, intact embryos with the aid of a scalpel. These de-embryonated kernels were incubated and the development in activity was monitored. The initial rate was somewhat slower than that for the tissue prepared from dry kernels but a sharp increase in activity occurred after 4 to 6 days and the absolute level of activity attained in both tissues was comparable.

Ingle and Hageman (12) demonstrated in some cultivars of maize that the presence of GA_3 is necessary for the initiation of sugar production. It was of interest to determine the effects of GA_3 on our de-embryonated tissue system. Figure 1 shows that after 4 days of incubation, the presence of 1 μm GA_3 in the incubation medium resulted in some 2-fold greater activity compared to the nontreated tissue. This difference, however, diminished with increased time of incubation, and after a total of 8 days, the level of activity in both tissues was comparable.

GA_3 has also been reported to cause the release of amylase activity from the tissue into the incubation medium (23). To test this effect, aliquots from the incubation medium for both treated and nontreated tissue were sampled during the course of incubation. No difference in the appearance of the amylase activity in the residual incubation medium between the GA_3-treated and nontreated tissue (Fig. 1) was observed.

To determine the type of amylolytic activity present (α or β), the products of the reaction catalyzed by the partially purified, extracted amylases from the control and GA_3-treated tissue were compared. The action patterns revealed by chromatographic analysis (Fig. 2) illustrate the marked similarity between the treated and nontreated tissues. The control tissue, like the GA_3-treated tissue, produced maltose as well as a number of different-sized glucose oligomers.

Amylase activity was also determined in endosperms and embryos separated from intact, germinating kernels during incubation (Fig. 3). After 6 days of incubation, the level of activity in dissected endosperms was similar to that of the filed endosperms (Table I). The reaction product patterns produced by the activity isolated from endosperms that were dissected from germinating kernels were analogous to the patterns for both the GA_3-treated and nontreated filed endosperms. This consistency of similar products for the respective time course incubation periods suggests that similar amylase activities are involved.

Polyacrylamide Gel Electrophoresis Analysis. Concomitant with the time course increase in activity was the appearance of a number of starch-degrading bands as shown by polyacrylamide gel electrophoresis using an activity stain (Fig. 4). Between 3 and 4 days of incubation, several starch-degrading bands became evident, and after 6 days, the full complement of

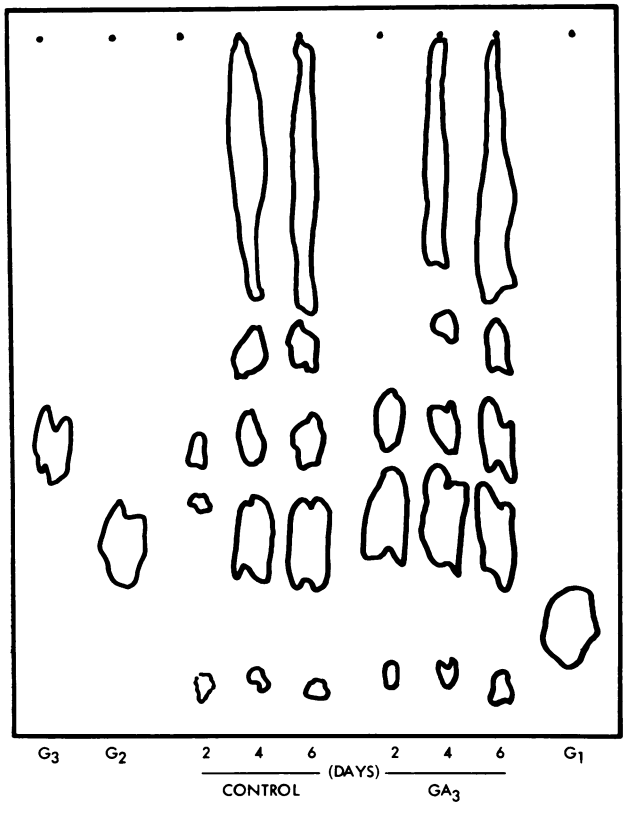

FIG. 2. Paper chromatograms of reaction product patterns of amylase activity from nontreated and GA_3-treated, incubated, de-embryonated maize kernels. Acetone-purified enzyme extracts were prepared from 2-, 4-, and 6-day incubated control and GA_3-treated de-embryonated maize kernels. Aliquots of enzyme were added to complete reaction mixtures which were equilibrated at 30 C. After 60 min, 1-ml aliquots were withdrawn, boiled for 5 min to inactivate enzyme activity, and then subjected to analysis by paper chromatography.

starch-degrading bands was observed. Since longer incubation did not result in an increase in the number of bands although it did produce further increases in activity, it seems reasonable that increases in the amount(s) of one or more of the enzyme bands were responsible for the observed increase in activity.

Physicochemical Characterization of the Amylase Activity. A number of physicochemical criteria have been used to differentiate between α- and β-amylases (3, 6, 13, 19, 21, 23). A comparative analysis using some of these criteria was used to characterize the activities of de-embryonated kernels that were incubated for 3 and 6 days with or without GA_3 (Table II). $CaCl_2$ added to a final concentration of 1 mM had little or no effect on the *in vitro* activity. This result plus the fact that the amylase activity was almost completely stable against heat treatment with or without added Ca suggests that the endogenous Ca^{2+} level present in the crude extract was sufficient for the full expression and protection of that amylase activity normally requiring Ca^{2+}. The loss of some activity (9 to 33%) by heat treatment is attributed to the heat-sensitive β-amylase activity that is not afforded protection by Ca^{2+}.

Sulfhydryl groups are reportedly essential for the expression of β-amylase activity (22). Thus the use of heavy metal ions as well as thiol reagents may be used to determine the involvement of free SH groups, and therefore the contribution of β-amylase to the total activity. The 3-day control activity was completely inhibited by Hg^{2+}. The thiol reagent, 1 mM dithiothreitol, was able to restore all of the Hg^{2+}-inactivated activity, thus supporting the idea that free sulfhydryl groups capable of fully reversible oxidation and reduction were involved.

The comparative data for the GA_3-treated and nontreated tissue (Table II) support the idea that GA_3 induces an earlier onset of development of amylase activity. The physiocochemical characteristics obtained for the 3-day GA_3-treated tissue appear to correspond closely to those for the 6-day control. Thus the addition of GA_3 to the incubation medium may allow for earlier development of amylase activity.

Effects of Actinomycin-D and Cycloheximide on Amylase Development. As a preliminary means of determining the extent to which the development of amylase activity was dependent upon RNA and protein synthesis, the inhibitors actinomycin-D and cycloheximide were used. When added to the incubation medium at 4 days or earlier, they almost completely prevented the development of amylase activity (Fig. 5). However, the addition of either inhibitor after 5 days of in-

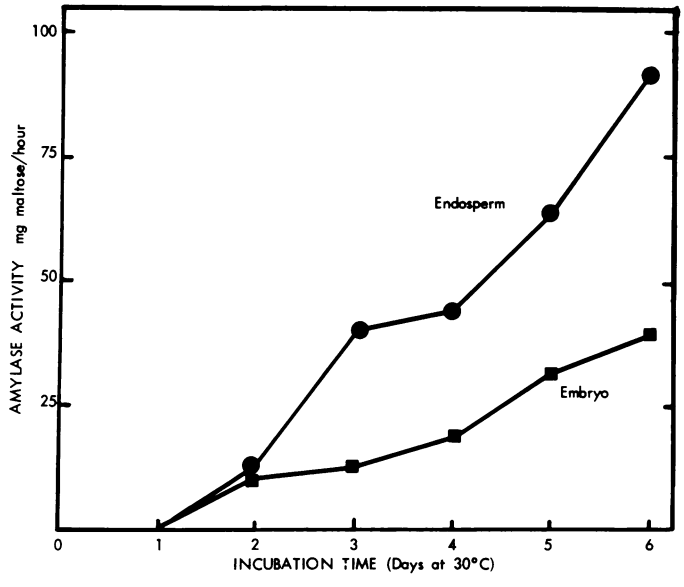

FIG. 3. Time course development of amylase activity in endosperms and embryos dissected from incubated, germinating, whole maize kernels. Whole, intact kernels were incubated at 30 C. At specified times embryos including developing roots and shoots were dissected from the germinating kernel. Crude enzyme extracts were then prepared from the embryo and endosperm tissues separately, and their respective amylase activities were determined.

Table I. *Amylase Activity of Incubated Zea mays Kernels*

Values were determined after a total of 6 days of incubation at 30 C.

Incubation Condition	Amylase Activity
	mg maltose released/ unit of tissue·hr
De-embryonated (filed) endosperms	105
Whole kernels separated after incubation into:	
Endosperm	92
Embryo	40

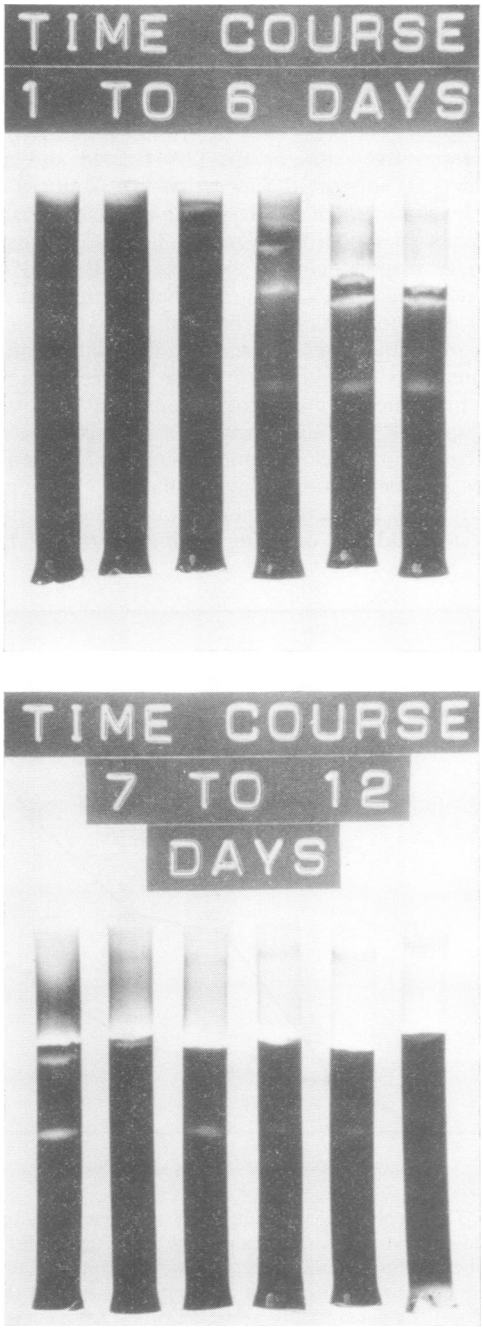

FIG. 4. Polyacrylamide gel electrophoresis zymograms of starch-degrading bands from the time course development of amylase activity from incubated, de-embryonated maize kernels. During the time course development of amylase activity, crude enzyme extracts prepared from incubated, de-embryonated maize kernels were applied to 8% gels and electrophoresis was performed.

cubation resulted in approximately 90% of the total activity being expressed.

The corresponding polyacrylamide gel electrophoresis patterns revealed that the inhibitors also prevented the appearance of the starch-degrading bands normally accompanying the increased activity (Fig. 6). It seems clear that the development of amylase activity in the incubated de-embryonated kernel is correlated with the appearance of a number of starch-degrading bands and this process is dependent upon both RNA and protein synthesis.

DISCUSSION

The data indicate that an increase in amylase activity in de-embryonated *Zea mays* kernels (var. Seneca Chief) requires incubation and that this development is independent of the presence of an embryo or an exogenous source of GA_3. In contrast to these results, Dure (6) and Ingle and Hageman (12) concluded that the hydrolysis of starch reserves in the endosperm of maize during incubation requires some embryo factor, either the embryonic axis (12) or scutellum (6). In both studies

Table II. *Physicochemical Analysis of the Amylase Activity Extracted from Nontreated and GA_3-treated De-embryonated Maize Kernels Incubated at 30 C*

Incubation Condition	Time	Amylase Activity: Treatment					
		Ca^{2+}[a]	Heat[b]	Ca^{2+} + Heat[c]	Hg^{2+}[d]	Dithiothreitol[e]	Hg^{2+} → dithiothreitol[f]
	Days	*% of control*					
Control	3	100	80	90	0	140	110
Control	6	115	72	83	42	100	100
GA_3	3	117	67	78	45	113	113
GA_3	6	100	86	85	45	92	100

[a] Aliquots from the respective enzyme extracts were incubated with 1 mM $CaCl_2$ for 1 hr at 0 to 4 C.

[b] Aliquots from the respective enzyme extracts were heated for 15 min at 70 C in a constant temperature water bath.

[c] Enzyme extracts were treated as in *a* followed by treatment as in *b*.

[d] Aliquots from the respective enzyme extracts were incubated with 1 mM $HgCl_2$ (final concentration) for 15 min at 0 to 4 C.

[e] Same procedure as in *d* but treated with 1 mM dithiothreitol.

[f] Treated as in *d* followed by treatment as in *e*.

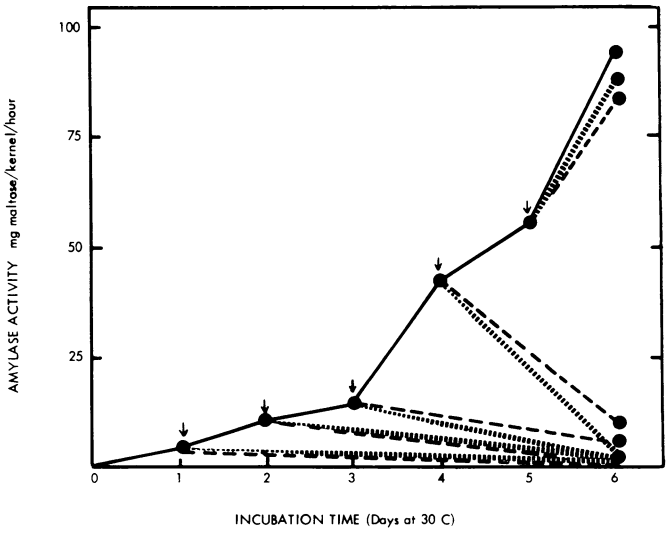

FIG. 5. Effects of actinomycin-D and cycloheximide on the development of amylase activity from incubated, de-embryonated maize kernels. De-embryonated kernels were incubated as controls (——) and either actinomycin-D (▮▮▮) (20 μg/ml) or cycloheximide (– – –) (5 μg/ml) was added at the times indicated (↓). After a total of 6 days of incubation, crude enzyme extracts were prepared and amylase activity was determined.

the intact kernals were first allowed to imbibe water for at least several hours before the dissection of the embryo (embryo axis plus scutellum). Khan *et al.* (15), using embryoless wheat grains, showed that some protein synthesis occurs in the embryoless grain during the early hours of imbibition. The biochemical events triggered by imbibition of water by the kernals before the removal of the embryo may affect the subsequent amylase development. Figure 3 shows that the amylase activity also increases in the embryos dissected from germinating kernals and may contribute to the activity expressed later on in the endosperms.

It seems that the effect of GA_3 on the development of amylolytic activity in our system is a quantitative one, and is seen only in an earlier, enhanced level of activity at the onset of incubation. Non-GA_3-treated tissue and the endosperms dissected from the germinating intact kernels (Figs. 1 and 3) show a similar pattern in amylase development which is lower than that of the GA_3-treated tissue (Fig. 1). This fact lends support to the idea that an embryonic factor is not critical to the development of activity, at least not during the earlier periods of incubation. The earlier appearance or the shorter time-lag in the development of amylase activity displayed by the GA_3-treated tissue suggests that the amount of GA_3 may be the critical factor. Thus the endogenous level of GA_3 present in the de-embryonated kernels used in this study may have been sufficient for the development of amylase activity. Support for this hypothesis is provided by the results in Figure 2 and Table II, which show the excellent similarity between the activity of GA_3-treated and nontreated tissue when reaction products and physicochemical characteristics are compared. The fact that the greater activity in the GA_3-treated tissue over the nontreated tissue is seen only early in the time course and diminishes with increased incubation suggests that the naturally occurring endogenous level of GA_3 is less than that required for the full development. The nontreated tissue may also possess the capacity to synthesize GA. This would increase the endogenous level of GA up to that critical level necessary for the full expression of activity.

An alternate explanation is that some endogenous inhibitor leaches out thus lowering the level of endogenous GA giving maximal amylase activity. This possibility has been briefly outlined by Harvey and Oaks (11). They suggest that addition of GA is required to overcome the inhibitory effects of ABA on α-amylase development. The results of the physicochemical data (Table 2) and the reaction product analysis (Fig. 3) indicate that a portion of the developing activity is attributable to α-amylase. Similar results have recently been reported by Harvey and Oaks (11), but as observed for other studies (6, 12), their tissue was prepared first by imbibing the intact kernel.

The development of amylase activity resulting during incubation is accompanied by the appearance of a number of starch-degrading bands. Such electrophoretic variants from maize endosperm tissue have been termed isoenzymes (3, 9). Although evidence may later justify the use of the term isoenzymes in our system, we feel that amino acid composition, sequencing, and genetic studies are first necessary. The prevention of both the development of activity and the appearance of the electrophoretic variants by actinomycin-D and cycloheximide suggest that the two are mutually related processes requiring protein synthesis. Actinomycin-D and cycloheximide, when added to the standard assay reaction mixture or incubated with a crude enzyme extract overnight at 4 C, had no inhibitory effect on the *in vitro* activity. This suggests that the inhibitors do not interact directly with the amylase enzymes but more likely act, respectively, at the levels of transcription and translation.

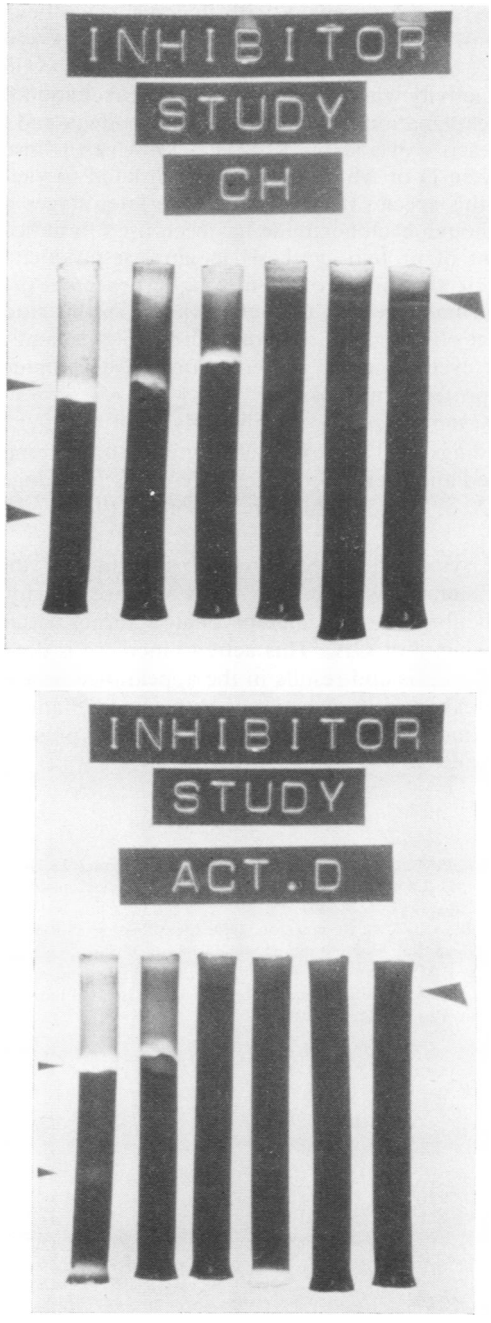

FIG. 6. Polyacrylamide gel electrophoresis zymograms of amylase activity from actinomycin-D-treated and cycloheximide-treated, incubated, de-embryonated maize kernels. Crude enzyme extracts prepared after 6 days of incubation from actinomycin-D-treated (20 μg/ml) and cycloheximide-treated (5 μg/ml), deembryonated maize kernels were applied to 8% gels and electrophoresis was performed. Inhibitors were added to the incubating kernels at the following times during incubation: from left, after 5, 4, 3, 2, 1, and 0 days.

The fact that additions of either inhibitor during the early stages of the time course (Fig. 5) prevent the development of activity indicates that some synthesis of RNA as well as protein is required for the observed development of amylase activity. With barley aleurone layers it was demonstrated (10) that actinomycin-D had a high degree of specificity for inhibit-

ing α-amylase development, with the conclusion being that actinomycin-D probably works by inhibiting RNA synthesis. In our study, both inhibitors failed to prevent the development of amylase activity when added at 5 days of incubation (Fig. 5). A possible explanation, also suggested by Goodwin and Carr (10), is the presence of a stable RNA species which is insensitive to actinomycin-D or which is rapidly translated to yield a stable protein; this species may appear at the later stages of incubation. Although cycloheximide has been used to indicate the involvement of protein synthesis in amylase-producing systems (11, 15), it may affect other metabolic processes (7). The response of maize kernels to cycloheximide in this study is similar to that observed for actinomycin-D (Fig. 5) and may indicate that cycloheximide prevents amylase development by inhibiting protein synthesis.

The reason the results of this study seem to differ from previous findings (6, 12) may be attributable to differences in cultivars used and therefore reflect differences in endogenous levels of GA_3 or certain inhibitors or both in the de-embryonated kernel.

In the system reported here, using kernels of the cultivar Seneca Chief, it has been shown that amylase activity increases during incubation of a de-embryonated kernel without an exogenous source of GA_3. This activity increase is dependent on protein synthesis and results in the appearance of a number of amylases exhibiting properties of both α- and β-amylase. A detailed characterization of this activity will be presented in another paper.

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