ABSTRACT

ON THE MECHANISM OF HORMONE CONTROLLED ENZYME FORMATION IN BARLEY ALEURONE LAYERS

By

Tuan-hua David Ho

Ribonucleic acid containing polyadenylic acid (poly A-RNA) is present in barley aleurone layers. The poly A-RNA is polydisperse in size and the 3'-OH end of the molecule is occupied by the poly A segment which is about 80 to 200 nucleotides long. The poly A-RNA becomes labeled with radioactive precursors of RNA during the incubation of isolated aleurone layers with or without gibberellic acid (GA_3). However, the rate of synthesis of poly A-RNA is enhanced by GA_3. This enhancement begins within 3-4 hours after addition of the hormone and reaches a maxium, which is about 50-60% over the control, 10-12 hours after the addition of the hormone.

Cordycepin, 3'-deoxyadenosine, inhibits synthesis of total RNA as well as poly A-RNA in barley aleurone layers. However, cordycepin inhibits the hormone-controlled formation of α-amylase only if it is added 12 hours or less after GA_3. The rapid accumulation of

α-amylase after 12 hours of GA_3 is due to the de novo synthesis of the enzyme molecule, i.e. accumulation of an α-amylase precursor does not precede the appearance of α-amylase activity, as examined by ^{13}C-amino acid density labeling experiment. Cordycepin has no effect on the turnover of α-amylase. Therefore, it is suggested that α-amylase is translated from stable mRNA which is synthesized during the first 12 hours of GA_3 treatment and the control mechanism of α-amylase synthesis 12 hours after the addition of GA_3 appears to be strictly post-transcriptional.

The accumulation of α-amylase activity after 12 hours of GA_3 treatment can be effectively decreased by abscisic acid (ABA). However, the accumulation of α-amylase activity is sustained or quickly restored when cordycepin is added simultaneously or soem time after ABA, indicating that the response of aleurone layers to ABA depends on the continuous synthesis of a short-lived RNA. Analysis of the newly synthesized proteins by gel electrophoresis with sodium dodecylsulfate showed that the synthesis of α-amylase is decreased in the presence of ABA while the synthesis of most of te other proteins remains unchanged. From the rate of resumption of α-amylase production in the presence of cordycepin and ABA, it appears that ABA does not have a measurable effect on the stability of α-amylase mRNA.

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PART I

ON THE MECHANISM OF HORMONE CONTROLLED ENZYME FORMATION IN BARLEY ALEURONE LAYERS

INTRODUCTION

Mobilization of endosperm reserves during the germination of cereal grains supplies nutrients for the growth of the embryo. This is accomplished by several hydrolases, including α-amylase (E.C.3.2.1.1.) and protease (E.C.3.4), which are synthesized in the aleurone tissue that surrounds the endosperm. Aleurone tissue of barley consists of three layers of homogeneous non-dividing triploid cells. These cells respond to gibberellic acid (GA_3), which is formed in the embryo during the early stage of seed germination, by a series of morphological and biochemical changes (66,68). The most prominent among these changes is the increase in α-amylase and protease activities after an eight and ten hour lag period (10,29). The GA_3 enhanced activities of α-amylase, protease, and ribonuclease (E.C.3.1.4) have been found to be due to the de novo synthesis of the enzyme proteins (3,17,29), and most of the α-amylase and protease are secreted into endosperm after their synthesis (68). Besides the synthesis and secretion of α-amylase, protease,a and ribonuclease, GA_3 also enhanced the secretion and to a less extent the synthesis of α-1,3-glucanase (E.C.3.2.1.6) (34) and the release of acid

phosphatase (E.C.3.1.3.2) from the cell wall (2).

α-Amylase becomes the most predominent protein (40%) synthesized in barley aleurone layer after several hours of GA_3 treatment (66). Therefore, it is usually used as a marker in studies of the mechanism of the hormone controlled formation of enzyme in this system. Inhibitors of RNA synthesis, such as actinomycin D (10,21), 6-methylpurine (10), or cordycepin (3'-deoxyadenosine) (this study) can block the GA_3 enhanced α-amylase synthesis. Incorporation into salt-soluble RNA is enhanced by GA_3 (8). Zwar and Jacobsen (69) demonstrated that the incorporation of radioactive nucleotides into polydisperse RNA was increased in the presence of GA_3, and it was reported recently that GA_3 enhanced the synthesis of rapidly labeled RNA species in barley aleurone layer (62). These observations tend to support a previous suggestion that the synthesis of α-amylase may depend on the GA_3 mediated synthesis of their mRNA (10).

During the lag period before the production of hydrolases, there is an extensive proliferation of cellular membranes, princepally endoplasmic reticulum (ER) (32,33), and the formation of membrane bound polyribosomes (13). Concurrrently the incorporation of choline and of ^{32}P into membrane phospholipids is also enhanced by GA_3 starting at about 4 hours after the addition of GA_3 (38). Furthermore, two enzymes, phosphorylcholine cytidyl transferase (E.C.2. 7.7.15) and phosphorylcholine glyceride transferase

(E.C.2.7.8.2), which are involved in the synthesis of lecithin (phosphatidylcholine), are activated within minutes of GA_3 addition (4,31). All these events seem to indicate that membrane proliferation is a major GA_3 effect before the rapid increase of α-amylase activity. α-Amylase is a secretory protein and there is ample evidence for the participation of rough ER in the synthesis and exportation of secreted proteins in eucaryotic cells (53, 60). Thus, a possible post-transcriptional model to account for the GA_3 enhanced synthesis of α-amylase has been proposed by Johnson and Kende (31). They reasoned that α-amylase specific mRNA be present and turning-over in the aleurone cell before GA_3 treatment and the synthesis of α-amylase may soly depend on the availability of proper membrane for the attachment of polysome that carry the α-amylase specific mRNA (31).

It is now well established that mRNA (with the exception of histone mRNA) in eucaryotic cells contains a convalently linked polyadenylic acid (poly A) segment (5,22). The occurrence of poly A-RNA in higher plants has been reported in mung bean (24), rice (41), corn (61), barley (25,30), Vicia faba (56), soybean (57), and pea (67). Since poly A can be hybridized with immobilized poly U or oligo dT, mRNA togeher with a certain fractions of heterogeneous nuclear RNA can be easily isolated from the other RNA species. There is recent evidence

which indicates that up to 40% of the mRNA in certain mammalian cells does not contain a poly A segment (45). However, it has been reported that in higher plant essentially all the translatable messages are poly A containing RNA species (67).

In order to elucidate the mechanism of hormonal control of enzyme formation in the barley aleurone cell, that up to 40% of the mRNA in certain mammalian cells does not contain a poly A segment, a detailed study of the relationship between the metabolism of RNA, especially that of mRNA, and the formation of α-amylase becomes necessary. The approaches used in this study are :

1) to determine the effect of hormones on the synthesis of poly A-RNA.
2) to check the stability of α-amylase mRNA by monitoring the synthesis of α-amylase in the presence of specific transcription inhibitors.

MATERIALS AND METHODS

Sources of Seed and Chemicals

Barley seeds (Hordeum vulgare L.cv. Himalaya, 1969 crop) were supplies by Department of Agronomy, Washington State University, Pullman, Wash. in 1972 and stored in the cold room since then. GA_3, ABA (mixture of equal amounts of cis-trans and trans-trans isomers, all concentrations mentioned in this study refer to that of cis-trans isomer only), cordycepin, and azocasein were obtained from Sigma Chemical Co., St. Louis, Mo. Diethyl pyrocarbonate was obtained from Calbiochem., La Jolla, Calif. Potato starch for the α-amylase assay was obtained from Nutritional Biochemical Co., Cleveland, Ohio. ^{13}C-Labeled amino acids mixture (hydrolyzate of alal proteins) was obtained from Merck Inc. Radioactive labeled compounds were obtained from New England Nuclear, Boston, Mass., Amersham/Searle Co., Arlington Heights, Ill. NCS tissue solubilizer was obtained from Amersham/Searle Co. and a mixture of 9 parts of full strength of solubilizer nd one part of water was used. Poly A and poly U were supplied by Sigma Chem. Co. and Milts Laboratory. Oligo dT cellulose (T-2 and T-3) was obtained from Collaborative Research, Waltham, Mass. All the other reagents used in

this study reagent grade.

Preparation of Aleurone Layers

The method described by Chrispeels and Varner (10) was followed. The embryos of dry barley seeds were mechanically separated from the endosperm by cutting with a dissecting knife. The embryo-less half of the seeds (half seeds) were surface sterilized by sodium hypochlorite (5 fold dilution of commercial bleach) for 20 min. After a thorough rinse with sterilized deionized water, the half seeds were imbibed on a sand plate moistened with sterilized water in a gless petri dish. The aleurone layer can be readily peeled from the endosperm with two spatulas after three days of water imbibition at room temperature. Ten,twenty, or fifty aleurone layers were put in a 25 ml, 50 ml, or 125 ml foil capped sterilized Erlenmyer flask containing 2 ml, 4 ml, or c0 ml of 2 mM acetate buffer, pH 5.0, respectively. The concentration of GA_3 in this study was 1 uM, and one drop of chloramphenicol (500 ug/ml) was usually added to every 2 ml of buffer in order to prevent bacterial contamination. All solutions used were sterilized either by autoclaving or by filtration through a Millipore filter (VC,pore size: 0.13 u). The flasks containing aleurone layers were shaken in a reciprocal metabolic shaker (120 oscillations/min) at 25 ^{o}C.

Extraction and Assays of Enzymes

In all the experiments dealing with α-amylase and protease assays, duplicate 10 layer samples were used. After incubation, the medium was decanted and the layers were rinsed with 3 ml deionized water. The medium and rinse solution were combined (5 ml total). The layers were homogenized with 5 ml water in a mortar and pestle. Because α-amylase is fairly stable, the enzyme preparation can be stored at 2-5 °C, but not frozen, for several hours without significant loss of activity. On the other hand, protease is very unstable and it was assayed immediately after extraction. The α-amylase assay was a modification of that of Varner and Mense (65). The starch solution was prepared by boiling 150 mg of potato starch in 100 ml of 50 mM KH_2PO_4, pH 4.2; 10 mM $CaCl_2$ for 1 min. After centrifugation at 12,000g for 15 min, the upper two thirds of the supernatant was used. The iodine solution was prepared by adding 0.3 ml iodine stock solution (600 mg and 6 g KI/loo ml water) to 100 ml 0.01 N HCl. The assay mixture contains 5 to 100 ul of enzyme, 0.5 ml starch solution and 1.0 ml deionized water. After incubation at room temperature (22 °C) from 1 to 5 min the reaction was stopped by adding 1.0 ml iodine solution and the absorbance at 620 nm was measured in a Coleman Junior II A spectrophotometer. The starch solution was diluted with water in order to have an A_{620} close to 1.0 after reaction with iodine.

The amount of enzyme and the length of incubation can be varied in order to give an $A_{620\ nm}$ between 0.55 to 0.75. The unit of α-amylase was defined as a change of one absorbance unit per minute. Alpha-amylase activity is proportional to enzyme concentration under these assay conditions (Figure 1). Protease was assayed in 20 mM sodium acetate, pH 5.0; lo mM β-mercaptoethanol. The mixture was incubated at 30 °C for one hour and the reaction was stopped by adding 0.5 ml 50% trichloroacetic acid (TCA). After being cooled in an ice bucket for 10 min, the mixture was centrifuged at 12,000g for lo min. The supernatant was decanted and absorbance at 330 nm was measured. The enzyme unit for protease was defined as one tenth of an absorbance unit per hour. The protease assay also is proportional to enzyme concentration under the assay conditions used (Figure 2).

Total RNA Extraction

The aleurone layers were ground with 100 mM Tris-HCl buffer pH 7.6 containing 1% SDS and 0.1% diethyl pyrocarbonate (1 ml buffer per 10 layers) in a prechilled mortar. An equal volume of phenol (redistilled and saturated with deionized water); chloroform (1;1) mixture was added to the homogenate ad the whole mixture was stirred vigorously at cold for at least 10 min. After centrifugation at 12,000g for 10 min, the aqueous phase was

Figure 1.--Validation of α-Amylase Assay.

The concentration of α-Amylase in the medium of a $+GA_3$ sample was assigned as "100". Serial dilutions were then made and the α-amylase activity in each diluted sample was assayed.

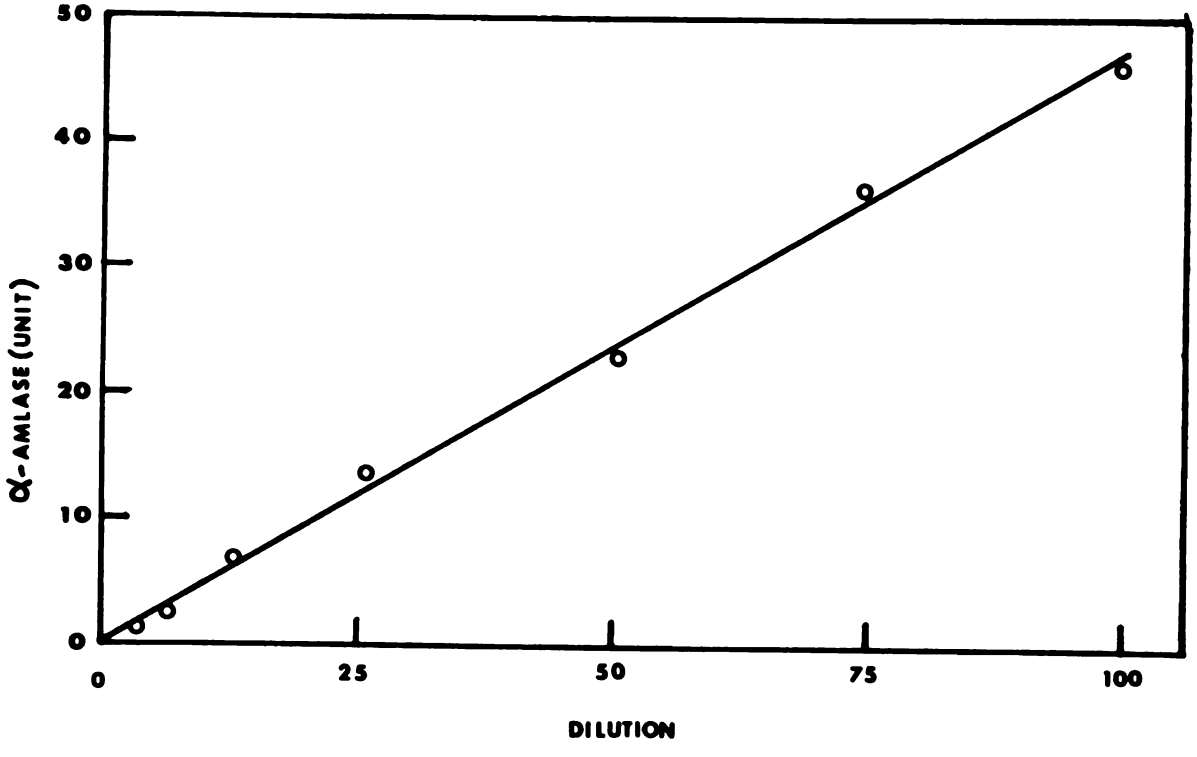
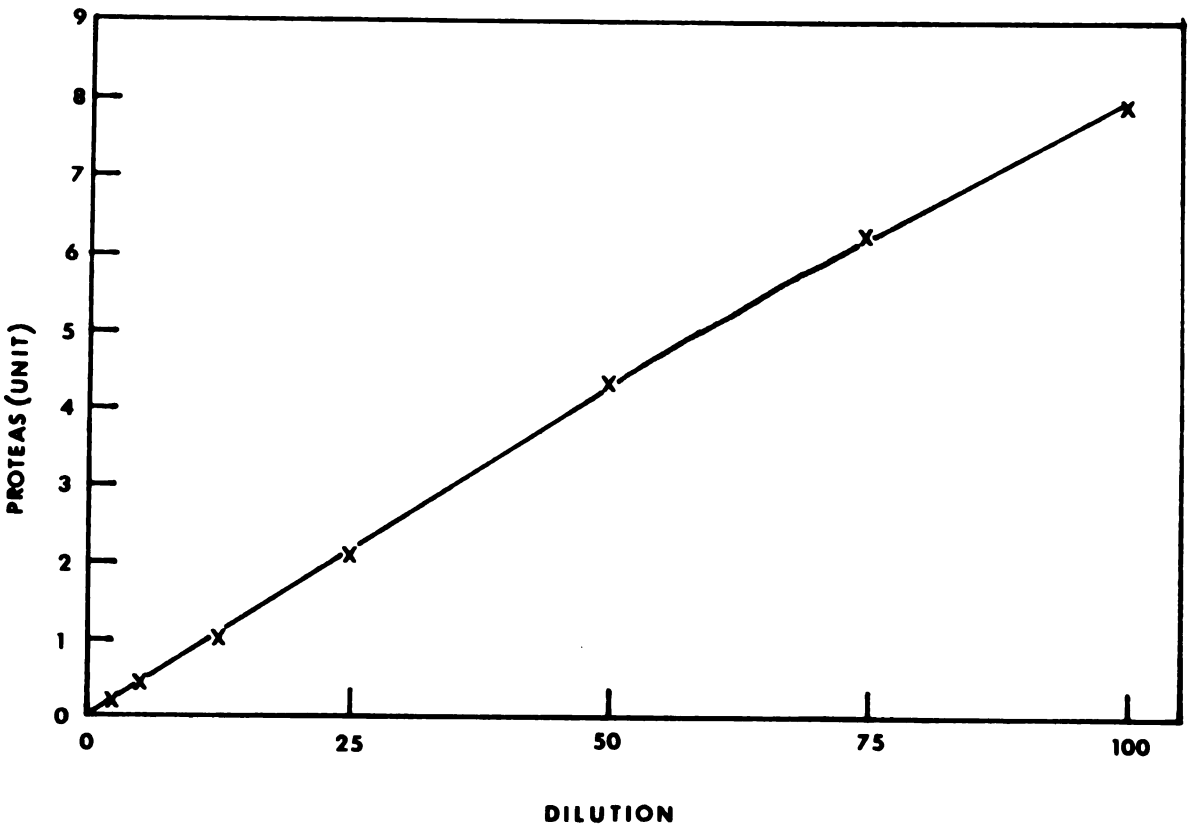

50
40
30
20
10
0
α-AMLASE (UNIT)
0
25
50
75
100
DILUTION

Figure 2.--Validation of Protease Assay.

The concentration of protease in the medium of a $+GA_3$ sample was assigned as "100". Serial dilutions were made and then the protease activity in each diluted sample was assayed.


PROTEAS (UNIT)
0
1
2
3
4
5
6
7
8
9
0
25
50
75
100
DILUTION

decanted and the non-aqueous phase was rextracted by stirring with 100 mM Tris-HCl, pH 9.0 (0.8 ml per 10 layers), and the two phases were again separated by centrifugation. All aqueous phases were combined and further extracted with phenol:chloroform mixture. Total nucleic acid was precipitated by adding one tenth volume of 1 M NaCl and 2.5 volumes of absolute ethanol and storing overnight at -20 °C. The precipitated nucleic acid was collected by centrifugation (12,000g for 30 min) and then dissolved in either deionized water or buffer as indicated in some experiments. RNA was further pufified by DNase digestion. However, barley aleurone cells were found to incorporate little radioactivity from RNA precursors into DNA. Therefore, the DNase digestion step was disocontinued.

Isolation of Poly-A RNA

Oligo dT celluloase column (1 x 5 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 7.6, containing 0.5 M KCl (binding buffer). RNA samples were dissolved in binding buffer and applied to the column. After thorough washing with binding buffer and the same concentration of Tris buffer containing 0.1 M KCl, the bound RNA was then eluted with buffer alone. Preparation of the fiberglass filter with immobilized poly-U and the filtration procedure were modified from that of Sheldon et al (59). A 0.15 ml volume of poly-U solution (1 mg/ml

10 mM Tris-HCl, pH 7.5) was added to a fiberglass filter (Whatman GF/C, 2.4 cm diameter) supported on a plastic grid. The filter was then dried at 37 °C and irradiated for 3 min on each side at a distance of 20 cm from a 30 Watt Sylvania germicidal lamp. The filter can be stored in the cold for a month. Just before use each filter was rinsed with 50 ml of deionized water to remove unbound poly-U. The filter was then equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.12 M NaCl. RNA samples were dissolved in the same buffer and filtration was done under unit gravity. The filter was washed ith 20 ml buffer followed by 20 ml 10% TCA, and 10 ml ethanol (95%). After drying, the radioactivity on the filter was measured.

Isolation of Poly-A Segment

Bound RNA eluted from the oligo dT cellulose column was made to 10 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM $MgCl_2$. After digestion with pancreatic ribonuclease A (2 ug/ml) and T_1 RNase (10 units/ml) for 30 min, diethylpyocarbonate or SDS was added to inactivate the enzymes. After phenol:chloroform extraction, the RNase resistant fragment was precipitated by ethanol.

Polyacrylamide Gel Electropnoresis for Poly-A

The method described by Laemmli (40) was followed. Ten cm sample gels (10%) with 0.1% SDS were used. One

hundred ul of RNA solution was applied on each gel. The electrophoresis was run under constant power supply (0.5 Watt/tube) until the tracking dye reached the bottom of the gel. The gel was sliced into 1 mm pieces and each piece was digested with 0.5 ml NCS solubilizer at 50 °C for 2 hours before radiioactivity was measured.

Sucrose Density Gradient Centrifugation for RNA

A linear density gradient of 15% to 30% sucrose dissolved in 10 mM Tris-HCl, pH 7.6, 1% SDS, 5 mM EDTA was prepared in a nitrocellulose centrifuge tube. The RNA sample was dissolved in the same buffer without sucrose, heated at 65 °C for 10 min, and cooled rapidly in an ice bucket in order to dissociate the aggregated RNA molecules. One to two hundred ul of the solution was applied on the top of the gradient. Centrifugation was carried out at 25 °C in a Beckman L-4 ultracentrifuge equipped with SW-50 L rotor at 47,500 rpm for 7 hours. After centrifugation the tube was punctured and 5 drop fractions were collected. The RNA in each fraction was precipitated by TCA and collected by filtration through nitocellulose filters. After washing with 20 ml 10% TCA the filters were dried and subjected to scintillation counting.

Base Composition Analysis of RNA

The RNA sample labeled with ^{32}P was hydrolyzed with 0.3 N KOH at 37 °C for 18 to 20 hours. After neutralization with perchloric acid the hydrolysate was subjected to paper electrophoresis as modified from that of Sebring and Salzman (58). RNA hydrolysate was applied on a 7.5 x 16 in electrophoresis paper and electrophoresis was carried out in pyridine-acetate buffer, pH 3.5, containing 10 mM EDTA at 500 V for about 51/2 hours immersed in CCl_4 as coolant. After electrophoresis, the pyridine was evaporated and the paper was dried by autoclaving for 3 min. The ultraviolet light-absorbing region on the paper was cut off and radioactivity was determined by scintillation counting. Alternatively the radioactivity on the paper could be scanned directly by a Packard strip scanner.

Chemical Labeling of the 3'-OH End of the RNA Molecule

The method derived by Randerath et al (51) was followed. To a solution of about 50 ug of RNA in 100 ul of water was added 20 ul of an aqueous solution containing 2 nmoles of $NaIO_4$. The oxidation was allowed to proceed for 2 hours in the dark at room temperature. Then 5 ul of 0.1 N NaB^3H_4 (50 uCi) in 0.1 N KOH was added and the reaction mixture was kept in the dark at room temperature for 2 hours. A drop of 1 M acetic acid was added at the end of 2 hours to convert excess NaB^3H_4 into boric acid

and tritium gas. The last step was done in a well ventilated fume hood. The labeled RNA was collected by ethanol precipitation.

Double Labeling of RNA for Checking the Effect of GA_3 on Poly-A RNA Synthesis

Labeling was carried out by addition of 50 uCi of ^{3}H-adenoisne to a sample (20 layers) containing GA_3 and a sample without GA_3, and 2 uCi of ^{14}C-adenosine to two other samples without GA_3 at specific times. After 2 hours of further incubation, ^{3}H-labeled layers were mixed with ^{14}C-labeled layers (i.e., ^{3}H-labeled containing GA_3 with ^{14}C-labeled sample without GA_3; ^{3}H-labeled sample without GA_3 with ^{14}C-labeled sample without GA_3; see Figure 3) and rinsed extensively with ice cold carrier adenosine solution (10 mMO). RNA was then extracted as described previously.

Induction of Nitrate Reductase and Its Assay

Nitrate reductase was induced by incubating aleurone layers with 2 mM sodium acetate buffer pH 5.0, containing 10 mM KNO_3. The intact tissue assay of nitrate reductase developed by Ferrari and Varner (16) was used. Ten induced aleurone layers were rinsed twicd with 4 ml of 50 mM KNO_3, then placed in a 25 ml Erlenmeyer flask with 2 ml of 0.1 M potassium phosphate buffer, pH 7.5, 20 mM KNO_3, and 5% ethanol. The flask was deaerated by bubbling

Figure 3.--Diagram Showing the Double Labeling Procedure Used to Check the effect of GA_3 on Poly A-RNA Synthesis.

2 Hours

+/- + GA_3 Sample — 50 uCi ^{3}H-Adenosine + 2 uCi ^{14}C-Adenosine → Mix → → Poly A-RNA → $^{3}H/^{14}C$

+/- Control Sample — 50 uCi ^{3}H-Adenosine + 2 uCi ^{14}C-Adenosine → Mix → → Poly A-RNA → $^{3}H/^{14}C$

$$GA_3 \text{ Enhancement (\% over Control)} = \frac{^{3}H/^{14}C \text{ ratio of +/- sample}}{^{3}H/^{14}C \text{ ratio of -/- sample}} - 100$$

N_2 gas for 1.5 min and then stoppered tightly. After being shaken at 30 ^{o}C in darkness for 30 min, 0.5 ml of the medium was taken out and mixed with 0.3 ml each of 1% sulfanlamide in 3 N HCl and 0.02% N-1-naphtylethylene-diamine dihydrochloride. After standing in darkness for 20 min the absorbance at 540 nm was measured.

Density Labeling of Proteins with ^{13}C-Amino Acids

Twenty aleurone layers were incubated in 2 mM sodium acetate buffer pH 5.0, 10 mM $CaCl_2$, 1 uM GA_3, 5 mM $KBrO_3$ containing either casein hydrolyzate (C^{12}-amino acids) or ^{13}C-labeled hydrolyzate of algal proteins. The concentration of both hydrolyzates was 10 mg/ml, supplemented with 0.5 mg tryptophan (C^{12}) per ml. The incubaton of enzyme were carried out as described previously. The enzyme preparation was mixed with a ^{14}C-protein marker and saturated CsCl solution to make a density of 1.3 g/ml. After being centrifuged in a Beckman L-4 centrifuge equipped with either Ti 50 or SW 50 L rotor at 40,000 rpm for 65 hours, the centrifuge tube was punctured and 4 drop (when SW 50 L rotor was used) or 12 drop (when Ti 50 rotor was used) fractions were collected. Refractive index of every eighth fraction was measured with a Bausch and Lomb Abbe-32 refractometer to monitor the density gradient. α-Amylase activity and radioactivity in the fractions were then measured to determine the distribution

of α-amylase and newly synthesized proteins in the gradient.

Liquid Scintillation Counting

Toluene based scintillation fluid was generally used (18.34 g PPO + 0.34 g POPOP/gal. of toluene). For aqueous samples, either Tritosol as described by Fricke (18) (3 g PPO/257 ml Triton X-100, 37 ml ethyl glycol, 106 ml ethanol, and 600 ml xylene) or the combination of NCS solubilizer and toluene scintillation fluid was used. For the latter case, 1 ml of NCS solubilizer was mixed with less than 100 ul of aqueous sample and incubated at 50 ^{o}C for 2 hours before 10 ml of toluene scintillation fluid was added to the sample. A Packard Tri-Carb Model 3385 liquid scintillation counter was used in this study. The counting efficiencies are shown in TABLE 1.

TABLE 1.--Effect of Scintillation Counting

Sample	^{3}H	^{14}C	$^{3}H(^{14}C)$	$^{14}C(^{3}H)$	^{14}C Spillover in ^{3}H channel
NCS Solubilizer and "Toluene only"	49	94	41	58	16
Tritosol	29	88	28	26	69
Nitrocellulose filter and "Toluene only"	20	82	19	23	91
Fiber glass filter and "Toluene only"	30	88	25	53	17

Note: # Numbers indicate the percentages of the ^{14}C counts in the preset $^{3}H(^{14}C)$ channel to that in the $^{14}C(^{3}H)$ channel.

"Toluene only" is the toluene only scintillation fluid described in the "Materials and Methods".

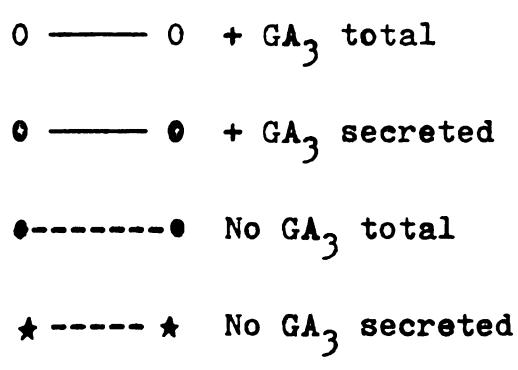
Millipore HAWP nitrocellulose filter and Millpore AP 2002500 fiberglass filter were used.

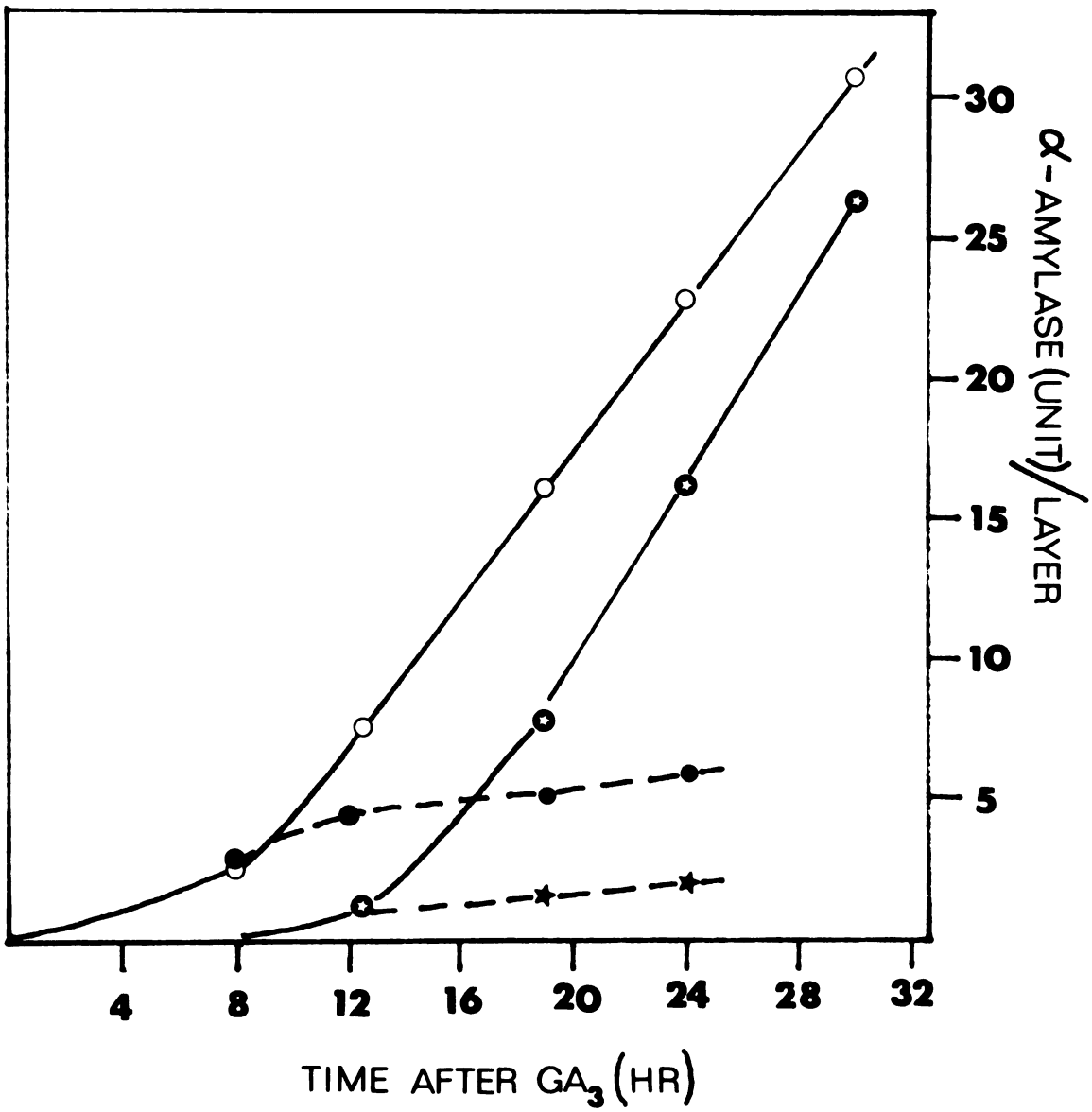
RESULTS

Time Course of the GA_3 Enhanced α-Amylase Production

The GA_3 enhanced α-amylase production has a lag period of about 8 to 10 hours (Figure 4). From 12 to 36 hours after the addition of GA_3 there is a linear accumulation of α-amylase activity indicating a constant rate of net α-amylase formation (Figure 4). After 36 hours of GA_3 treatment the production of α-amylase gradually alows down perhaps due to the senescence of the tissue. Most of the α-amylase (over 80%) is secreted into the bathing medium and this process starts about the same time as rapid accumulation of α-amylase (about 12 hr after GA_3 addition). On the other hand, most of the enzyme in the control tissue (- GA_3) is retained. The GA_3 enhancement of α-amylase production as measured after 24 hr of hormone treatment is 3 to 10 fold if sodium acetate buffer is used. However, the enhancement is greatly improved if sodium succinate buffer is used (see Part II). Usually there is some α-amylase (about 2 units per aleurone layer) present in the freshly peeled aleurone layer, this may represent enzyme already present in the dry seed or that formed during the 3 day imbibition period. The

Figure 4.--Time Course of the GA_3 Enhanced α-Amylase Formation in Barley Aleurone Layers.




30
25
20
15
10
5
α-AMYLASE (UNIT)/LAYER
4
8
12
16
20
24
28
32
TIME AFTER GA3 (HR)

activity of the α-amylase present at zero time was substracted from the data presented.

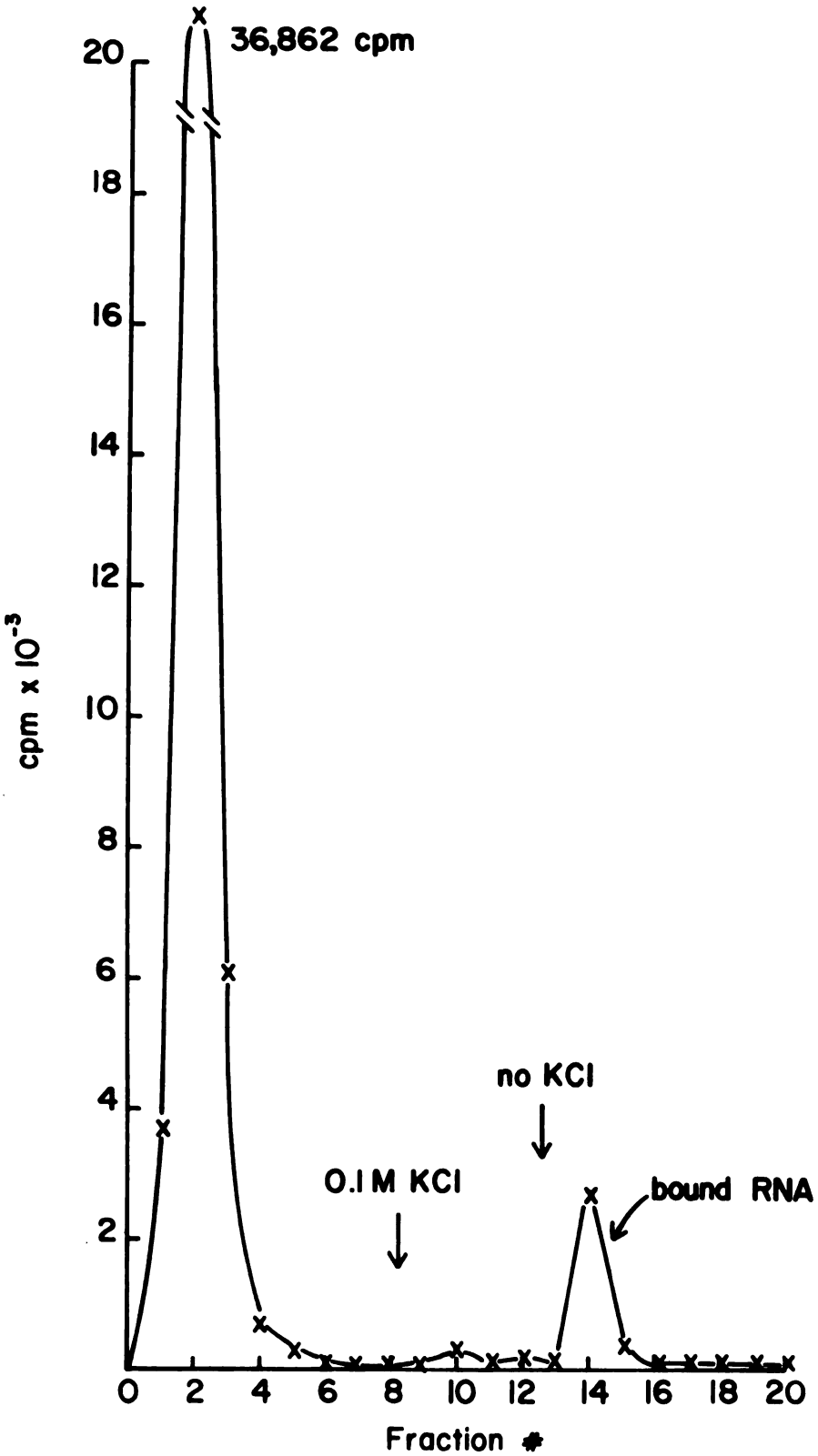
Occurence and Characterization of Poly A-RNA

It has been reported that poly A-RNA can bind to nitrocellulose or plain cellulose, or be hybridized with immobilized oligo dT or poly U, the following methods have been tried in this study and all of them successfully bind a certain fractions of total RNA extracted from barley aleurone layers: oligo dT cellulose column chromatography, poly U cellulose column chromatography, cellulose (plain) column chromatography, poly U filtration, nitrocellulose filtration. However, only oligo dT cellulose column chromatography (for preparative purpose) and poly U fltration (for analytical purpose) were used in later studies.

From an oligo dT cellulloce column (Figure 5) two major RNA peaks are eluted, one by high salt buffer (binding buffer), the other by Tris buffer without salt. A third peak eluted by buffer containing 0.1 M KCl was occasionally found may be nonsepcifically bound RNA. The recovery of oligo dT cellulose column chromatography varies in different batches of oligo dT cellulose. However, the specific batch used most often in this study had a recovery greater than 98%. The bound RNA is about 5 to 8% of the total RNA. The eluted peak by high salt

Figure 5.--Chromatography of total Barley RNA on Oligo (dT)-Cellulose.

Fractions of 4.5 ml were collected.


36,862 cpm
20
18
16
14
12
10
8
6
4
2
cpm x 10^{-3}
no KCl
0.1 M KCl
bound RNA
0
2
4
6
8
10
12
14
16
18
20
Fraction #

contains rRNAs and tRNA (Figure 6A) as checked by sedimentation analysis with a sucrose density gradient. The bound RNA is heterogeneous with an average size of 14 to 17 S (Figure 6B). Since no detectable rRNA and tRNA were observed, this fraction presumably represents mRNA and probably some heterogeneous nuclear RNA. The estimation of RNA size distribution probably was not hampered by the aggregation of RNA, because the 65 ^{o}C heat treatment of RNA sample prior to sedimentation analysis described under "Material and Methods" has been used successfully to dis aggregate ovalbumin mRNA without reducing its ability to direct ovalbumin synthesis in a cell free system (23). The AMP content of bound RNA is higher than that of total RNA (TABLE 2). After RNase digestion with bound RNA, a RNase resistant segment was recovered which contain more than 90% AMP (TABLE 2 and Figure 7). Analyzing the size of this RNase resistant segment by polyacrylamide gel electrophoresis, the size of this segment was found to be heterogeneous, ranging from 80 to 200 nucleotides (Figure 8). However, based on the AMP to adenosine ratio the poly A segment was estimated to have an average size of 156 nucleotides (TABLE 3). Using the AMP content of poly A-RNA and the poly A segment as well as the size of poly A segment, one can calculate the average chain length of poly A-RNA as shown in the following diagram:

Figure 6.--Size Distribution of Barley Aleurone RNA in a Sucrose Density Gradient.

A. RNA species not retained by oligo (dT)-cellulose

B. RNA species retained by oligo (dT)-cellulose

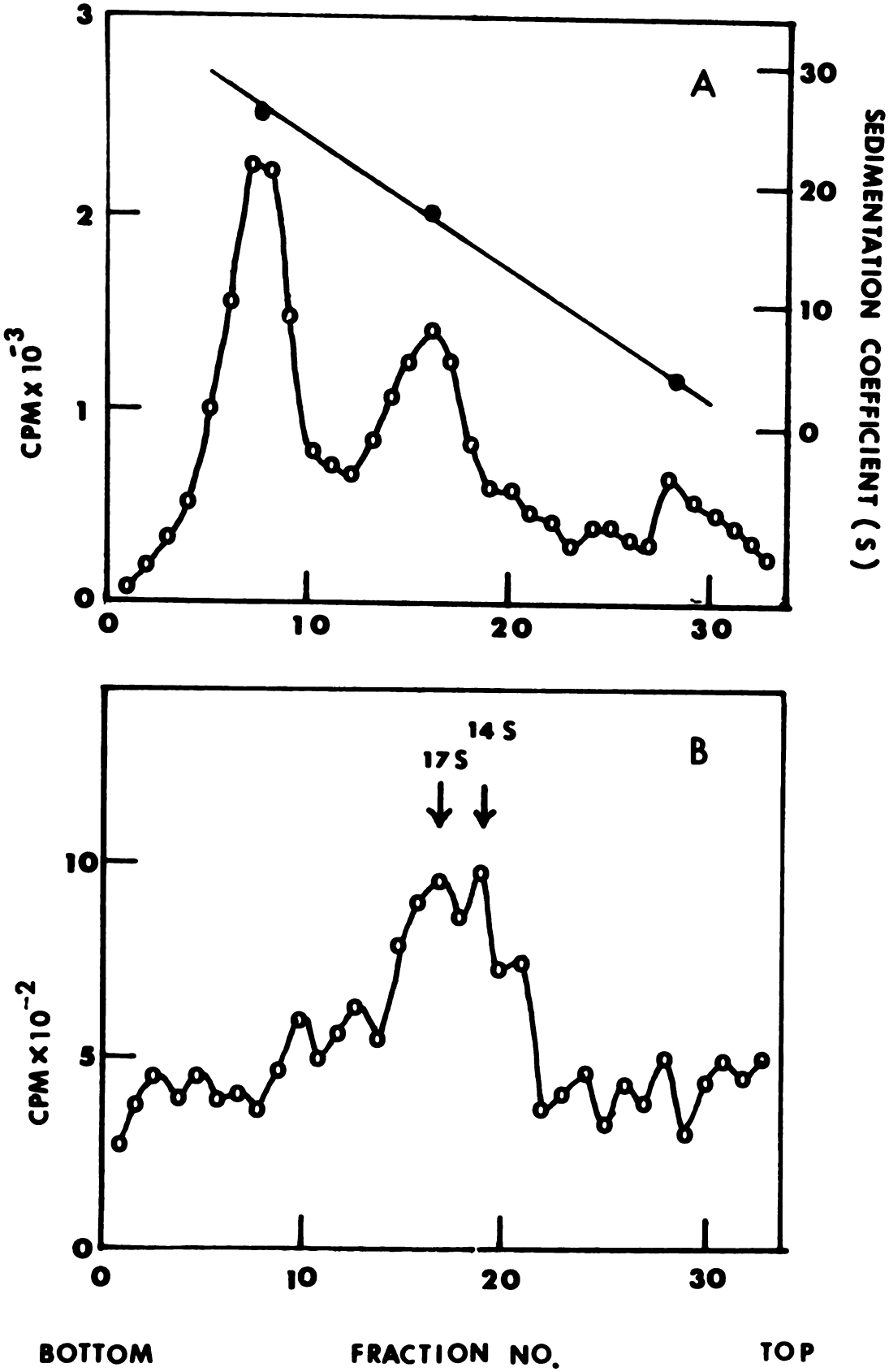

A
B
CPM x 10^-3
CPM x 10^-2
SEDIMENTATION COEFFICIENT (S)
17S
14S
BOTTOM
FRACTION NO.
TOP

TABLE 2.--Base Composition of RNA Species Isolated from Barley Aleurone Layers

Sample	CMP	AMP	GMP	UMP
Total RNA	25.1	22.9	33.5	18.5
Bound RNA (Poly(A)-RNA)	23.7	30.2	25.1	21.0
RNase resistant (Poly(A) segment)	0.8	94.1	3.6	1.5

Figure 7.--Radioelectrophoretogram of the Hydrolyzate of the RNase Resistant RNA Segment Isolated from Barley Aleurone Layers.

RNase resistant RNA segment isolated from ^{32}P RNA was described under "Materials and Methods". A 2 mm slit and linear scale were used during the scanning.

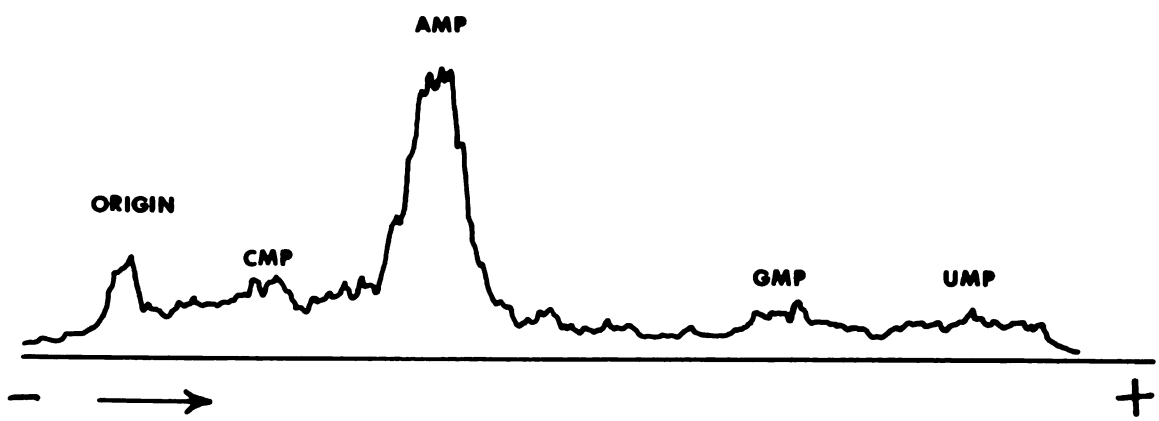

AMP
ORIGIN
CMP
GMP
UMP
−
+

Figure 8.--Polyacrylamide Gel Electrophoresis of Poly (A) from Barley Aleurone Layers.

Poly (A) segment labeled with ^{3}H-adenosine was isolated as described in " Materials and Methods" and then analyzed on 10% polyacrylamide gel. Tritiated tRNA, analyzed on a separate gel, was used as a marker.

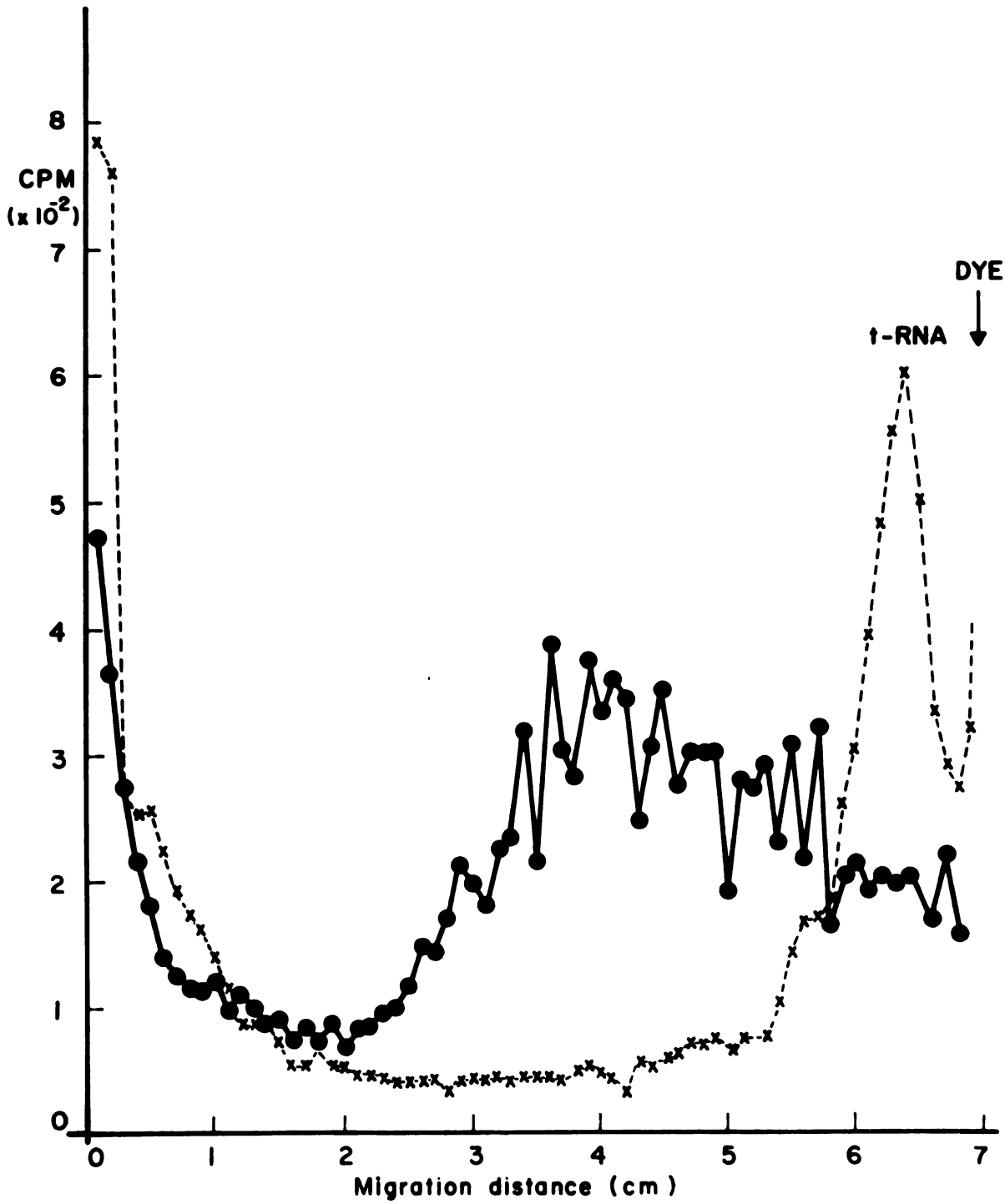
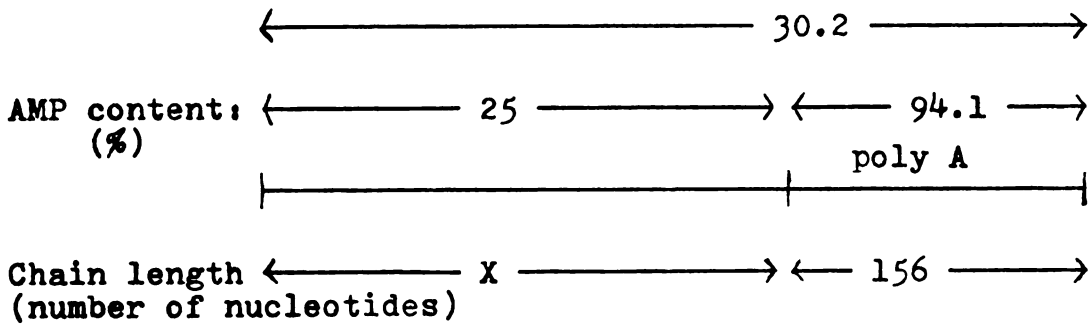

8
CPM
(x 10⁻²)
7
6
5
4
3
2
1
0
DYE
t-RNA
0
1
2
3
4
5
6
7
Migration distance (cm)

TABLE 3.--Estimation of the Size of Poly (A) Segment by the Ratio of AMP to Adenosine

	AMP	Adenosine	Average Size of Poly A Segment (AMP/Adenosine)
CPM	37,440	239	156

Note: Poly (A) segment isolated from aleurone layers incubated with ^{3}H-adenosine was used.



$$(X + 156) \times 30.2 = 25X + 156 \times 94.1$$

$$X = 1920$$

Therefore:

Chain length of poly A-RNA: 1920 + 156 = 2076

M.W. of poly A-RNA: 5.6×10^5

Predicted S value of poly A-RNA: about 15 S

The calculated size of poly A-RNA is in agreement with that obtained by direct sedimentation analysis (Figure 6).

In order to study the localization of poly A segment on the RNA molecule, the 3'-OH end of RNA was selectively labeled by $NaIO_4$ oxidation followed by NaB^3H_4 reduction as described under "Materials and Methods". Barley aleurone poly A-RNA as well as the chemically synthesized poly A from Sigma Chemical Co. retained their label after RNase digestion (TABLE 4) indicating that the 3'-OH end of all the RNA is occupied by a poly A segment. However, the results of this experiment do not show that all poly A segment are located adjacent to the 3'-OH

TABLE 4.--Localization of Poly(A) Segment on RNA Molecule.

Sample	Counts per minute		% Remained
	Before RNase treatment	Agter RNase treatment	
Poly (A) from Sigma	4996.5	4493.5	89.9
E. Coli t-RNA	65025.0	4911.5	7.6
Barley poly (A)-RNA	73656.0	70340.5	95.5

Note: Barley samples were chemically labeled at the 3'-OH end as described in "Materials and Methods" before ribonuclease treatment.

end- the possible occurence of internal poly A-segments is not ruled out.

Effect of Hormones on Poly A-RNA Synthesis

Fiberglass filters with immobilized poly U were used to analyze a sample with little RNA because the filtration takes only about ten minutes and several filtrations can be handled simutaneously. As shown in TABLE 5, the poly U filter retains labeled poly A, but not poly U, and it has a maximal capacity to retain about 8 ug of poly A which is more than sufficient to analyze the small quanity of RNA used in the later experiments. The poly U filter retains a certain fraction of the short-termed labeled barley aleurone RNA and the binding can be competed out by cold poly A but not tRNA (TABLE 5). The double labeling technique described in "Materials and Methods" maximizes the sensitivity of the experiment; this design also eliminates possible artifacts generated by differential degradation during the isolation of poly A-RNA. The latter consideration is important because GA_3 is known to enhance ribonuclease activity in barley aleurone layers (11). Although a combination of several potent protein denaturants were used during RNA isolation, i.e. diethy pyrocarbonate, SDS, and phenol-chloroform, a trace of RNase activity could be still detected in the RNA preparations. From the $^{3}H/\ ^{14}C$ ratio of RNA retained

TABLE 5.--Retention of RNA by Poly U Filtration

RNA Sample	Input cpm	Retained cpm	%
1 ug poly A(^{3}H)	3859.1	3859.1	99.8
2.5 ug poly A(^{3}H)	9647.8	9678.8	100.3
20 ug poly A(^{3}H)	77182.7	33130.5	42.9
100 ug poly U(^{3}H)	184766.1	278.6	0.1
0.5 ml barley RNA	10099.7	1305.5	12.9
1 ml barley RNA	20199.3	2667.7	13.2
1 ml barley RNA + 200 ug cold poly A	20199.3	330.2	1.6
1 ml barley RNA + 50 ug cold poly A	20199.3	452.4	2.2
1 ml barley RNA + 200 ug tRNA (E.coli)	20199.3	2636.6	13.1

Note: Barley RNA was extracted from 100 aleurone layers labeled with ^{3}H-uridine for 1 hr in the absence of GA_3. The extracted RNA was dissolved in 10 ml filtration buffer (0,12 M NaCl, 10 mM Tris-HCl, pH 7.5).

on poly U filter, one can calculate the hormonal effect as the percent enhancement of poly A-RNA synthesis over the control. For example, at 12 hr of GA_3, the $^3H/^{14}C$ ratio of the mixture of the sample containing GA_3 and the sample without GA_3 is 38.05 and that of the mixture of samples both without GA_3 is 24.22; therefore, the GA_3 enhancement is 7%. It is observed that GA_3 enhances the synthesis of poly A-RNA with a lag period 3 to 4 hours. This enhancement reaches a maximum, which is about 50 to 60% over control, at 10 to 12 hours, then decreases afterwards (Figure 9). This GA_3 effect can still be observed if uridine is used as the labeled precursor, indicating that the synthesis of the portion of the poly A-RNA molecules not containing poly A is also enhanced by GA_3. Abscisic acid, a naturally occurring plant hormone that antagonizes GA_3 mediated hydrolase synthesis in barley aleurone layers (68), prevents the GA_3 effect on poly A-RNA synthesis (TABLE 6).

The aleurone cell has a thick and rigid cell wall, and the wall is foftened in the presence of GA_3 probably due to the hormone enhanced pentosanase activity (6). This fact leads to possible argument that the bserved enhancement of poly A-RNA synthesis is an artifact resulting from the preferential extraction of RNA from hormone treated tissue. However, because drastic grinding in a porcelain mortar with pestle and sharp sand was used in

Figure 9.--Effect of GA_3 on poly (A)-RNA Synthesis in Barley Aleurone Layers.

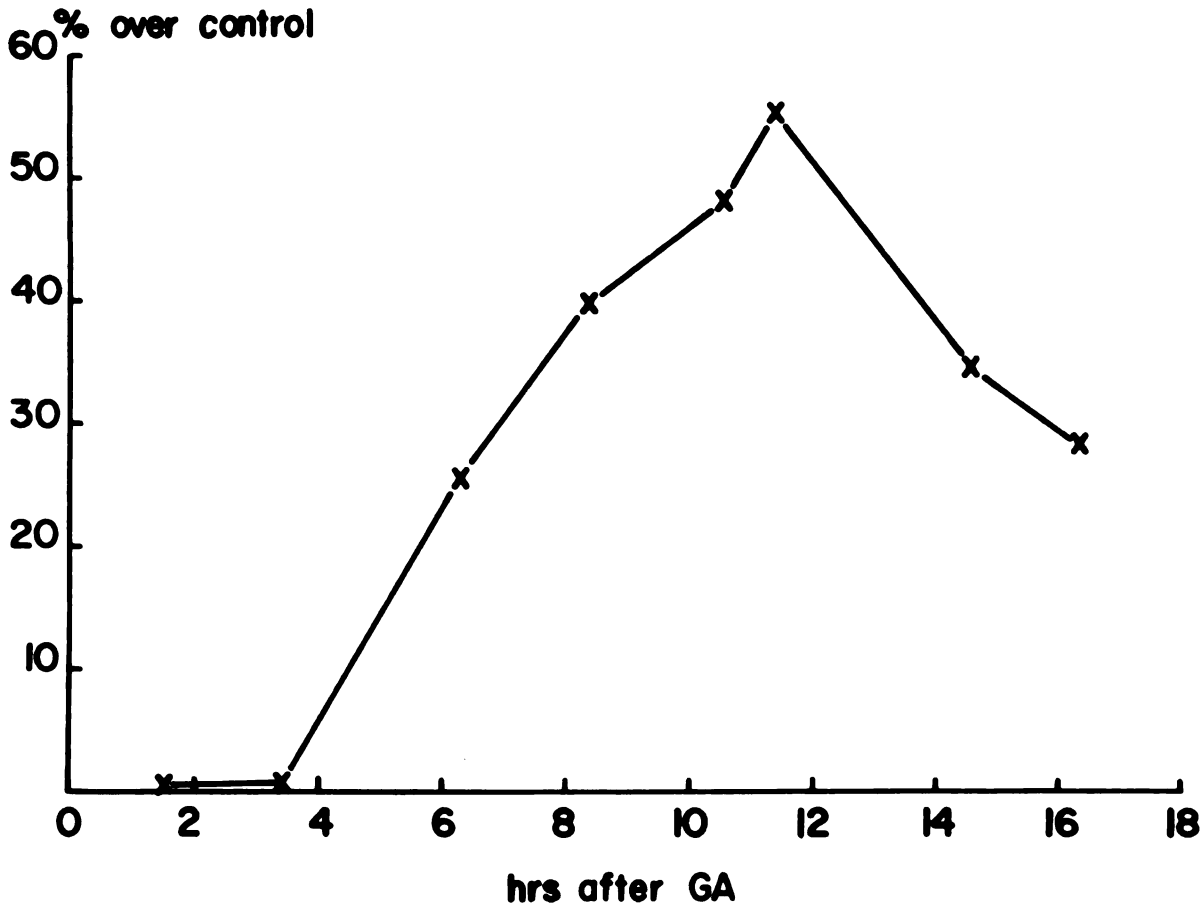

% over control
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
hrs after GA

TABLE 6.--Effect of ABA on the GA_3 Enhanced Poly (A)-RNA Synthesis

Treatment	#^3H/ ^{14}C	% over Control
Control (no GA_3)	25.70	0
+ GA_3	38.89	51
+ GA_3 + ABA (5 uM)	28.72	12

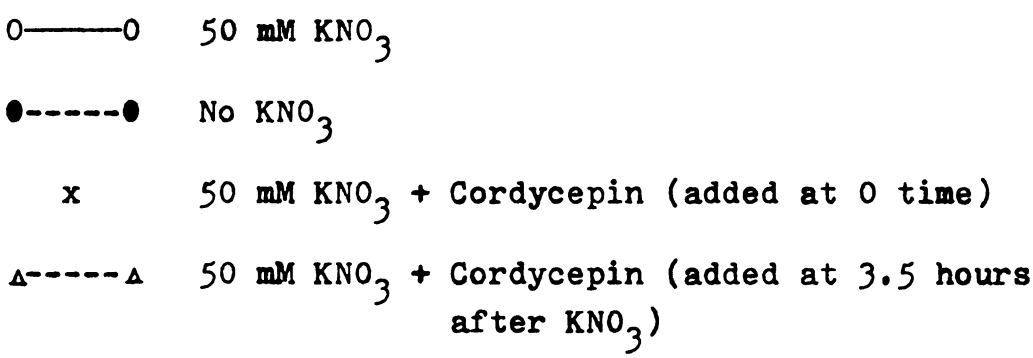
Note: #The experimental design was the same as described under "Materials and Methods". ABA was added at the same time as GA_3.

this study, the possibility of preferential extraction is unlikely.

Effect of Cordycepin on the Production of α-Amylase

Cordycepin, 3'-deoxyadenosine, is believed to work as a chain terminator during RNA synthesis. In animal tissue, cordycepin preferentially inhibits poly A synthesis during short-termed incubation (12). However, it also inhibits the formation of major RNA species if the incubation is longer than a few minutes. In order to check the effectiveness of the cordycepin effect on enzyme induction in plant tissue, I tested the effect of cordycepin on the induction of nitrate reductase in barley aleurone layers, because it is known that nitrate reductase induction depends on the continuous synthesis of RNA (15). As shown in Figure 10, cordycepin, no matter whether added at the same time or 3.5 hours after KNO_3 quickly abolishes the induction. Therefore, together with the data of cordycepin effect on RNA synthesis (to be presented in the next section), this result indicates that cordycepin is quickly taken up by the tissue and is quickly effective. As for the GA_3 enhanced α-amylase formation, cordycepin inhibits most of the hormone increased α-amylase activity if it is added at the same time as the hormone. On the other hand, 2'-deoxyadenosine, even at much higher concentration, is not inhibitory (TABLE 7).

Figure 10.--Effect of Cordycepin on the Substrate Induction of Nitrate Reductase.



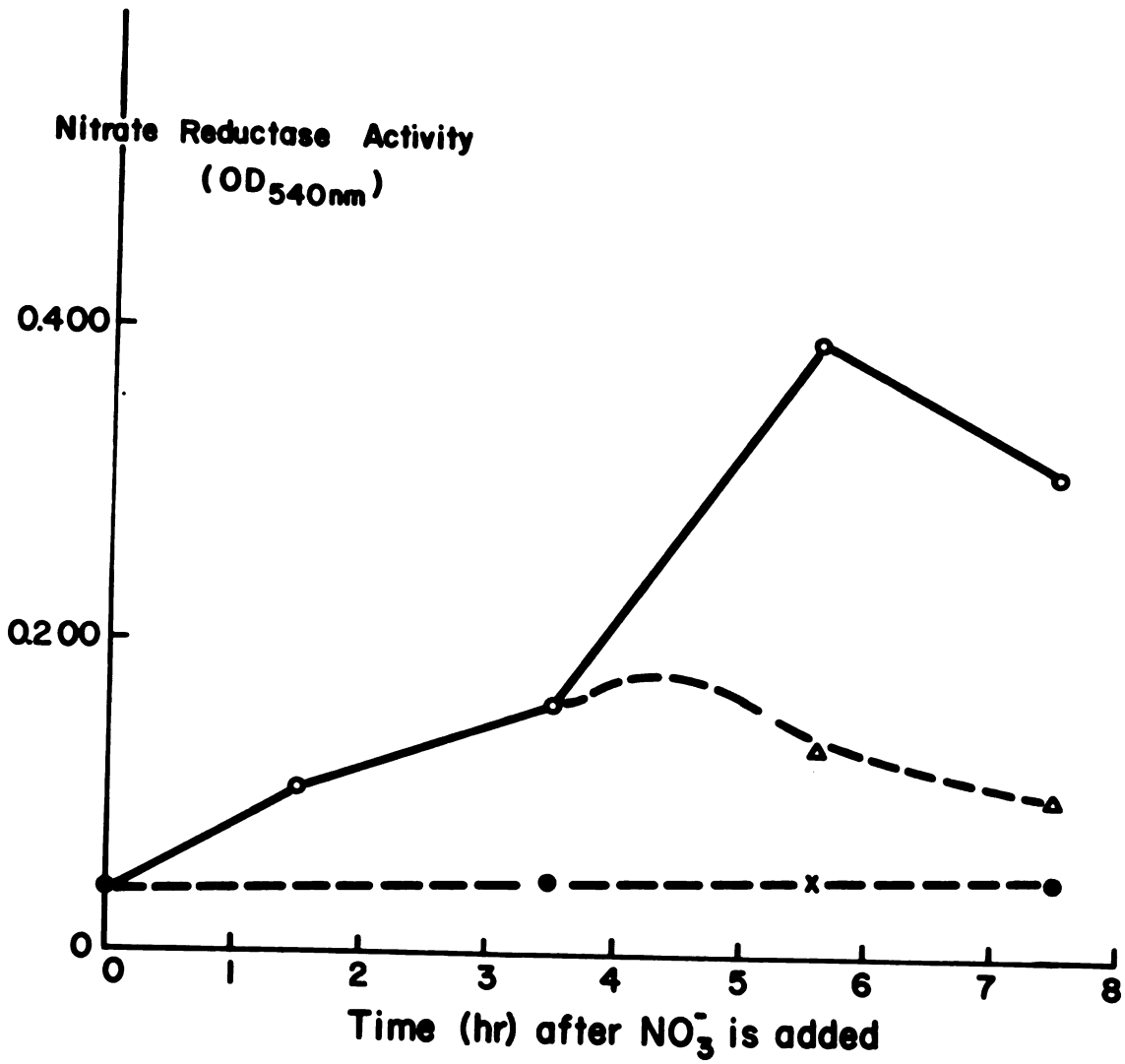

Nitrate Reductase Activity
(OD540nm)
0.400
0.200
0
0
1
2
3
4
5
6
7
8
Time (hr) after NO3- is added

TABLE 7.--Effect of Cordycepin and 2'-Deoxyadenosine on the GA_3 Enhanced Formation of α-Amylase in Barley Aleurone Layers

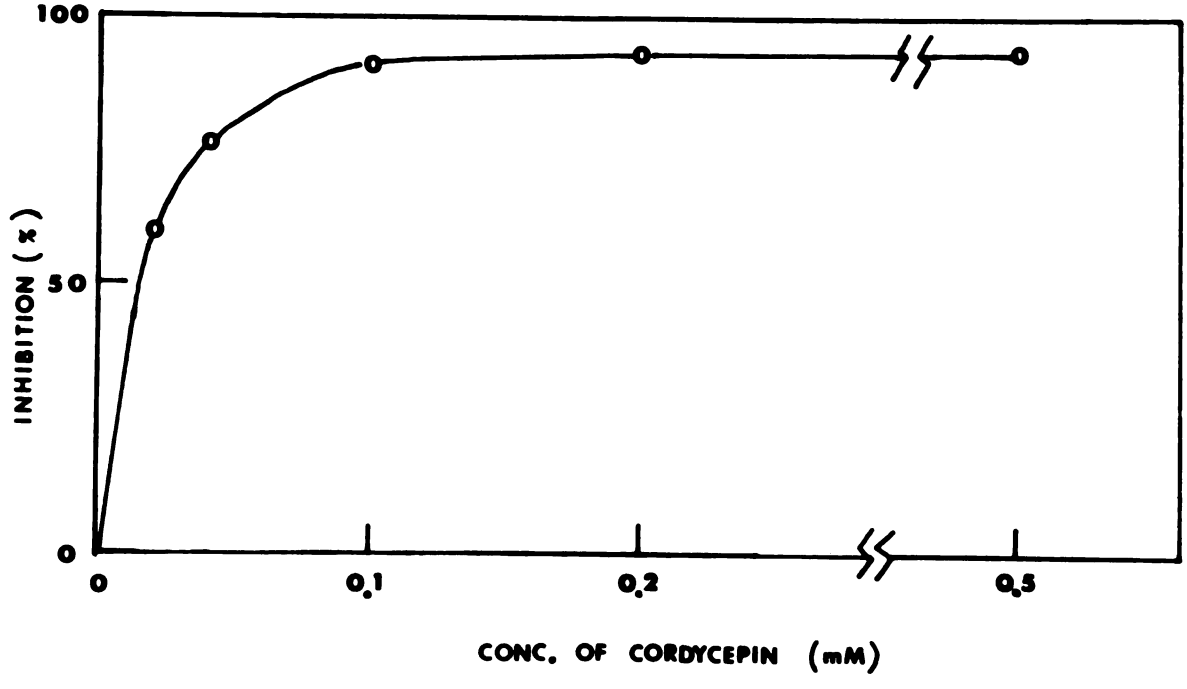
Treatment	α-Amylase Activity (units/layer)	%
+ GA_3 only	39.6	100
0.1 mM Cordycepin	6.3	15.9
0.1 mM 2'-Deoxyadenosine	36.1	91.1
01.0 mM 2'-Deoxyadenosine	33.0	83.3

Note: Cordycepin or 2'-deoxyadenosine was added at the same time as GA_3 and enzyme was assayed 24 hours after addition of the hormone.

Cordycepin at a concentration of 0.1 mM (about 25 ug/ml) can give maximal effect (Figure 11), therefore, 0.1 mM was used in most of the experiments. Cordycepin treated tissue usually retains more of the α-amylase in the layer (as much as 50%). The inhibitory effect of cordycepin on α-amylase production is not fully reversible. However, results to be shown in Part II of this thesis strongly suggest that the inhibitory effect of cordycepin is not via general toxicity to the cell. If cordycepin is added after GA_3, the inhibitory effect becomes progressively less and less as the time of addition is delayed (Figure 12). There is seentially no inhibitory effect on α-amylase production if the cordycepin is added 12 hours or

Figure 11.--Inhibition of α-Amylase Formation by Various Concentrations of Cordycepin.

Cordycepin was added at the same time as GA_3 and α-amylase was assayed at 24 hours after the addition of GA_3.


100
50
0
INHIBITION (%)
0
0.1
0.2
0.5
CONC. OF CORDYCEPIN (mM)

more after GA_3 (Figure 12). Similar inhibitory effects of cordycepin were observed on the GA_3-enhanced protease production (TABLE 8). Since the length of incubation with cordycepin becomes shorter and shorter when the inhibitor is added later and later after GA_3 addition, one might argue that the decreasing inhibitory effect results from the shortening incubation time with the inhibitor. However, this has been shown not the case, because no inhibiroey effect was observed if cordycepin was present 12 to 30 hours after GA_3 (18 hr incubation) while cordycepin added at 6 hr after GA_3 (6 to 24 hr; also 18 hr of incubation) inhibited α-amylase production by 40% (TABLE 9). Increasing the concentration of cordycepin up to 0.7 mM (about 175 ug/ml) caused no inhibition when the cordycepin was added 12 hours after the addition of GA_3 (TABLE 10). Occasionally α-amylase production is even higher when cordycepin is added after 12 hours of GA_3 treatment (TABLE 10). However, the mechanism of this "superinduction" is not known.

All the data presented in this section suggest that the α-amylase after 12 hours of GA_3 treatment is translated from a stable mRNA. In the next three sections evidence will be presented to answer the following three questions which are necessary to prove the above suggstion :

1). Does cordycepin effectively inhibit the synthesis of RNA, especially mRNA (poly A-RNA)

Figure 12.--Effect of Cordycepin on α-Amylase Activity.

Cordycepin was added at different times after GA_3 as indicated, and aleurone layers were further incubated until 24 hours after the addition of GA_3, when the activity of α-amylase was assayed.

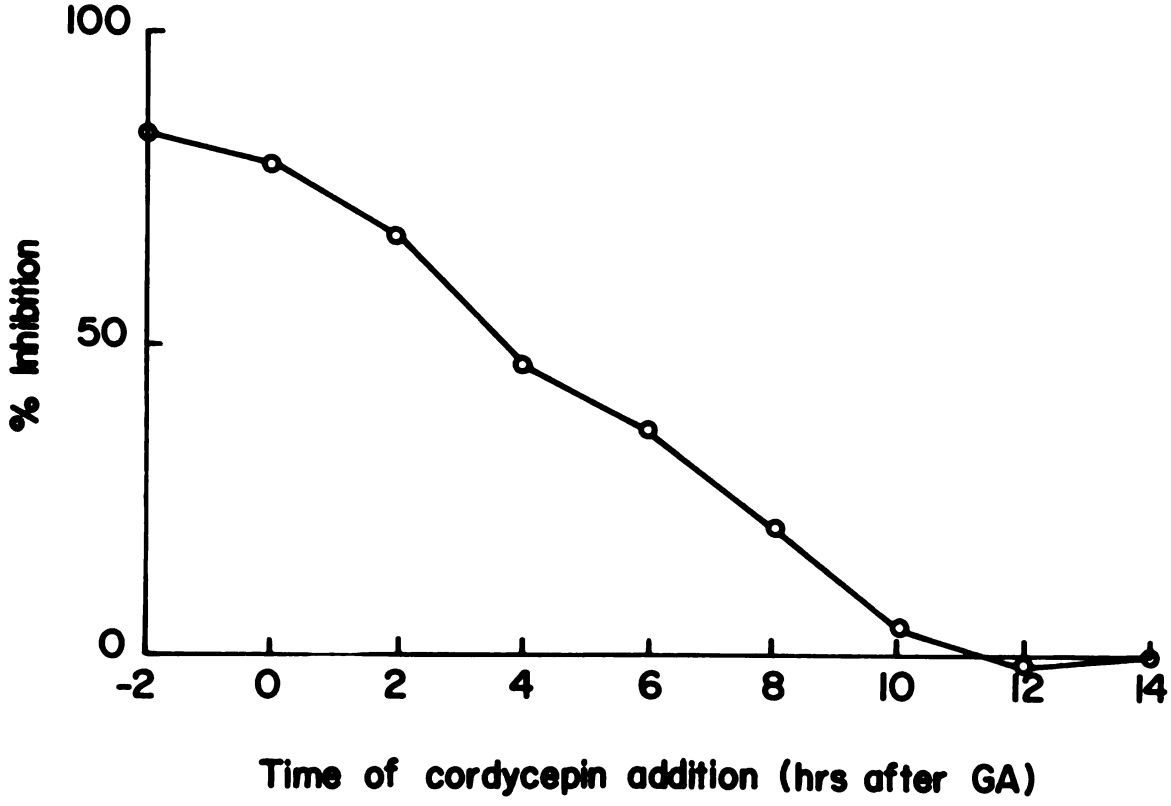

100
50
0
% Inhibition
-2
0
2
4
6
8
10
12
14
Time of cordycepin addition (hrs after GA)

TABLE 8.--Effect of Cordycepin on the GA_3-Enhanced Protease Formation

Time of Cordycepin Addition (hours after GA_3)	Protease Activity (units/10 layers)	% Inhibition
+ GA_3 only	7.47	0
0 time	2.66	64.4
2	2.54	66.0
4	2.97	60.0
6	4.31	42.3
8	4.43	40.7
10	4.88	34.7
12	6.52	12.7
14	6.90	7.6

Note: Cordycepin was added at different times after GA_3 as indicated, and aleurone layers were further incubated until 24 hours after the addition of GA_3, when the activity of protease was assayed.

TABLE 9.--Comparison of the Effect of Cordycepin Added at Different Times after GA_3 on the Production of α-Amylase

Treatment	α-Amylase Activity (units/layer)	%
+ GA_3 only (24 hr)	24.9	100
+ GA_3 + Cordycepin (6 to 24 hr)	15.1	60
+ GA_3 only (30 Hr)	34.2	100
+ GA_3 + Cordycepin (12 to 30 hr)	34.8	102

TABLE 10.--Effect of Various Concentrations of Cordycepin on the Production of α-Amylase

Concentration of Cordycepin (mM)	α-Amylase Activity (units/layer)	%
0	16.1	100
0.1	22.0	136
0.2	19.4	120
0.4	20.8	129
0.7	16.8	104
1.0	12.7	79

Note: Cordycepin was added 12 hours after GA_3 addition, and the aleurone layers were further incubated until 24 hours after GA_3, when the enzyme was assayed. α-Amylase produced before 12 hours of GA_3 was subtracted.

when added 12 hours after GA_3 addition ?

2). Is the rapid increase of α-amylase activity after 12 hours of GA_3 treatment due to the de novo synthesis of the enzyme molecule ?

3). Does cordycepin inhibit both the synthesis and degradation of α-amylase which tend to compensate with each other ?

Effect of Cordycepin on RNA Synthesis

Cordycepin, at a concentration of 0.1 mM, has a profound effect on the synthesis of total RNA in barley aleurone layers as measured by uridine incorporation (Figure 13). Cordycepin has no significant effect on the uptake of the radioactive presursor. When it is added at the same time as GA_3, the inhibitory effect of cordycepin on RNA synthesis is about 60%. However, the inhibitor causes nearly complete inhibition of RNA synthesis in one hour if it is added 12 hours after GA_3 (Figure 13). The more effective inhibition of cordycepin after 12 hours of GA_3 may be due to the faster uptake of the inhibitor. Poly A-RNA and on-poly A-RNA are inhibited by cordycepin to 84% and 93% respectively when the inhibitor is added 12 hours after GA_3 (Figure 14). Thus, unless the analog has some unexpected selectivity one must conclude that the formation of all the RNAs is inhibited and that at 12 hours α-amylase message is present in non-limiting amounts and is stable for an additional 18 hours.

Figure 13.--Effect of Cordycepin on the Incorporation of Uridine into RNA.

The amount of ^{3}H-uridine used was 2.5 uCi/ml. RNA was extracted at specific times as described under "Materials and Methods".

A. No Preincubation with GA_3 -- Cordycepin was added at the same time as GA_3.

B. Preincubated with GA_3 for 12 hours -- Cordycepin was added 12 hours after GA_3.

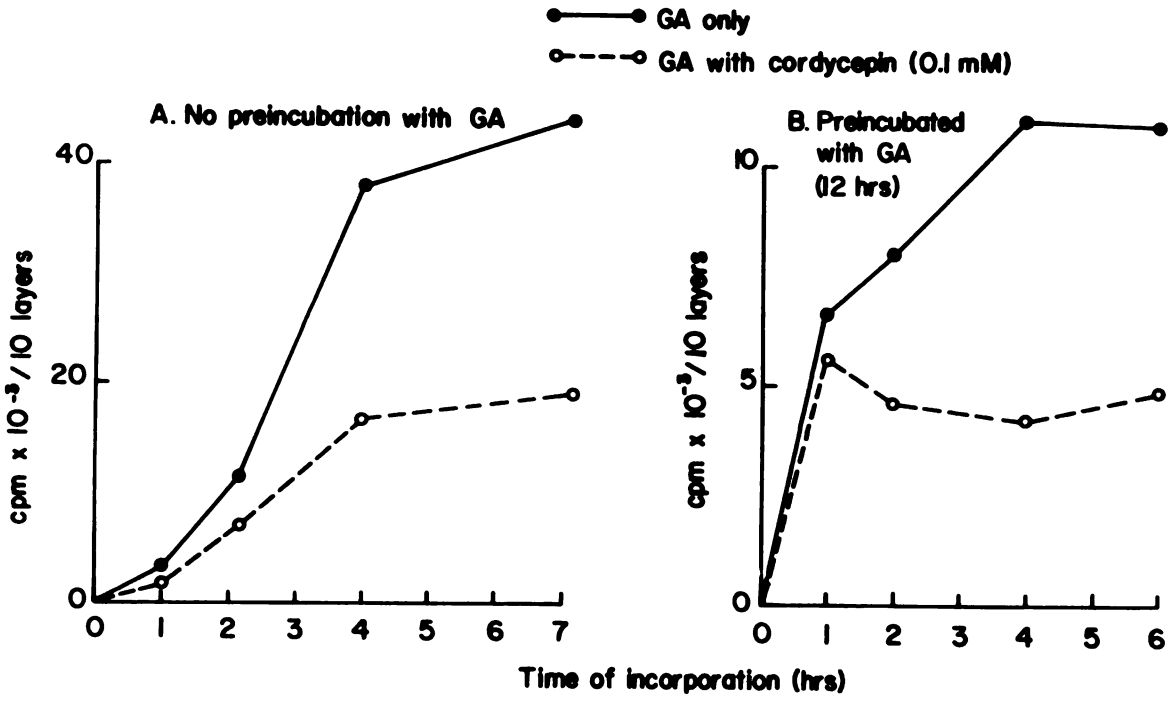
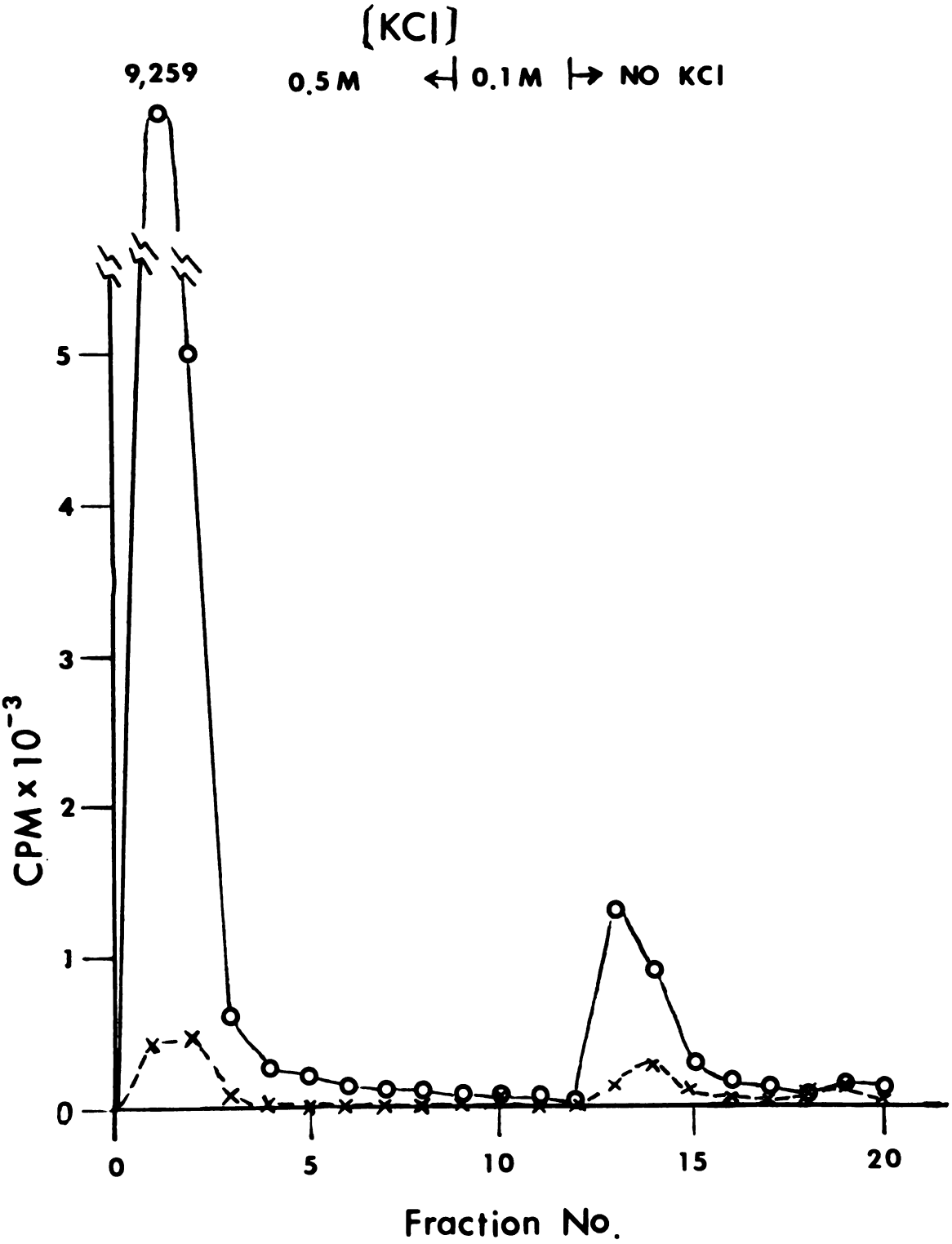

GA only
GA with cordycepin (0.1 mM)
A. No preincubation with GA
B. Preincubated with GA (12 hrs)
cpm x 10⁻³/10 layers
Time of incorporation (hrs)

Figure 14.--Effect of Cordycepin on RNA Synthesis.

The two samples, 80 aleurone layers each, were pretreated with GA_3 for 12 hours and cordycepin was then added to one of the samples. Label (^3H-uridine, 5 uCi /ml) was introduced from 13.5 to 18 hours after GA_3. RNA was extracted and fractionated by oligo dT cellulose chromatography as described under "Materials and Methods". The first peak (fractions 1 to 4) eluted by 0.5 M KCl consists of RNA species not containing poly A segment. The peak (fractions 13 to 15) eluted in the absence of KCl contains poly A-RNA.

O———O Control (+ GA_3 only)

x-----x Cordycepin traeted


[KCl]
9,259
0.5 M
0.1 M
NO KCl
CPM x 10⁻³
0
1
2
3
4
5
0
5
10
15
20
Fraction No.

De Novo Synthesis of α-Amylase

The GA_3 enhanced α-amylase activity has been shown to be due to the de novo synthesis of the enzyme molecule after addition of hormone (17,63,64). However, since α-amylase activity does not increase until 8 to 10 hours after GA_3, one can raise the possibility that an inactive precursor of α-amylase is formed in the lag period and is converted into active form when the rapid increase of α-amylase is observed. In order to check this possibility, a density labeling experiment was performed in which ^{13}C-amino acids were introduced to the aleurone layers either before or during the rapid increase of α-amylase activity, and the buoyant density of α-amylase molecules was then measured by isopycnic centrifugation in a CsCl density gradient. The amino acids necessary to support protein synthesis in barley aleurone layers are derived from the degradation of reserve proteins in this tissue (17). Therefore, the degradation of reserve proteins had to be slowed down in order to have an effecient use of the exogenously supplied ^{13}C-amino acids. This is accomplished by including 5 mM $KBrO_3$ in the incubation medium, because $KBrO_3$ inhibits barley aleurone protease effectively (42). The production of α-amylase (TABLE 11) and the inhibitory effect of cordycepin (TABLE 12) were unchanged in the presence of 5 mM $KBrO_3$ if exogenous amino acids were supplied. As shown in Figures 15 to 19 and summarized

TABLE 11.--Effect of Bromate and Amino Acids on the Production of α-Amylase in Barley Aleurone Layers

Treatment	α-Amylase (units/layer)			
	Medium	Layer	Total	%
GA_3 only	32.1	4.7	36.8	100.0
GA_3 + Bromate	7.0	2.2	9.2	25.0
GA_3 + Bromate + Amino Acids	29.4	10.3	39.7	108.0

Note: The concentration of bromate was 5 mM and the concentration of amino acids was 10 mg casein hydrolyzate plus 0.5 mg tryptophan per ml.

TABLE 12.--Effect of Cordycepin on α-Amylase Production in the Presence of Bromate and Amino Acids

Treatment	α-Amylase (units/layer)	%
GA_3 + Amino Acids	35.4	100
GA_3 + Amino Acids + Cordycepin	43.0	121
GA_3 + Amino Acids + Bromate + Cordycepin	44.9	127

Note: The concentration of bromate and amino acids are the same as described in TABLE 11.

in TABLE 13, because density of α-amylase molecule was much higher if ^{13}C-amino acids were present after 12

Figure 15.--Density Labeling of Barley Aleurone Proteins with ^{13}C-Amino Acids (I).

Control: Aleurone layers were incubated with ^{12}C-amino acids for 24 hours. Fifty uCi of ^{3}H-leucine were added after 12 hours of GA_3.

. . . . . ^{3}H- Radioactivity

0———0 α-Amylase activity peak

x———x Density gradient

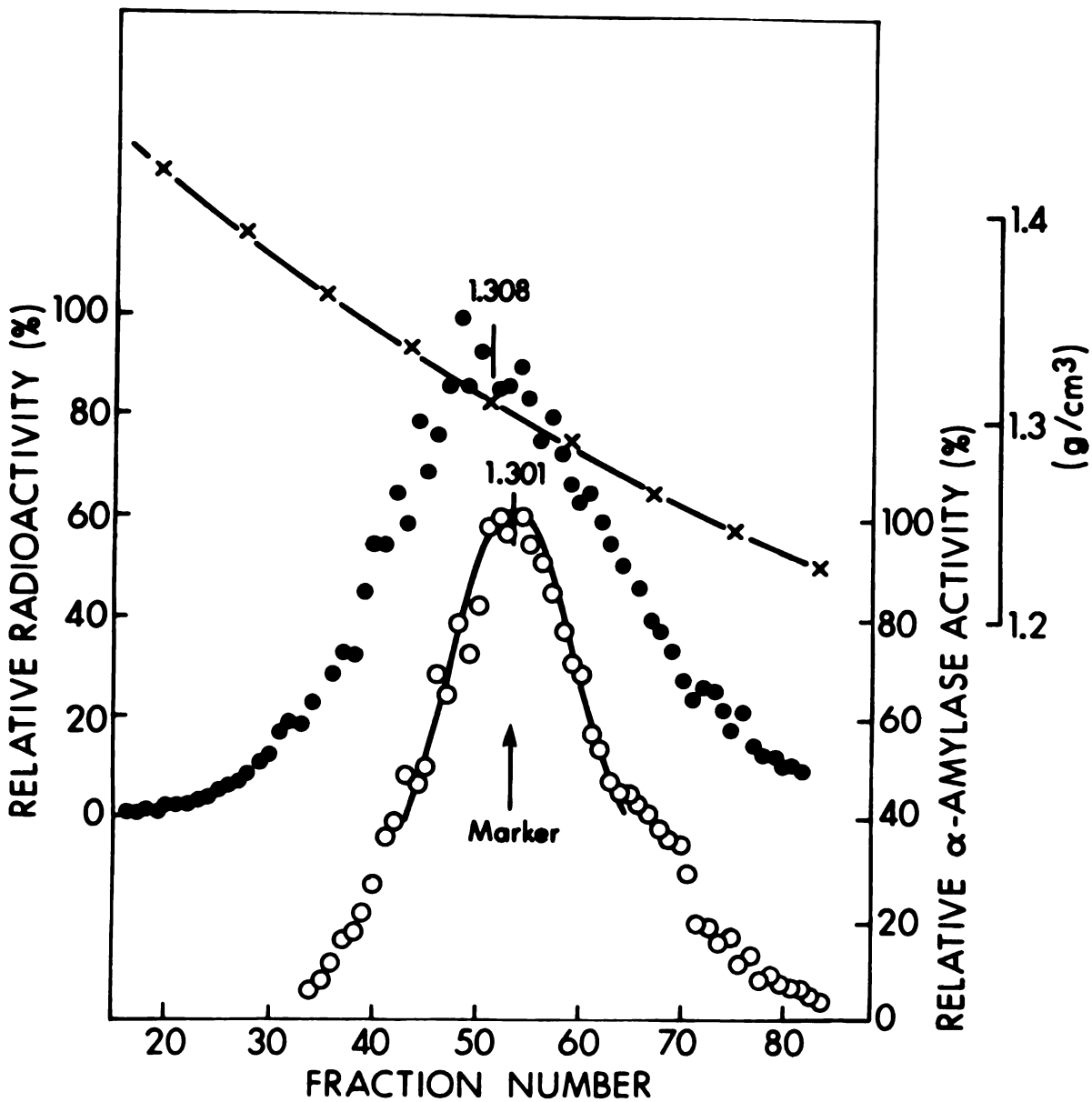

RELATIVE RADIOACTIVITY (%)
100
80
60
40
20
0
1.308
1.301
Marker
20
30
40
50
60
70
80
FRACTION NUMBER
RELATIVE α-AMYLASE ACTIVITY (%)
100
80
60
40
20
0
1.4
1.3
1.2
(g/cm3)

Figure 16.--Density Labeling of Barley Aleurone Proteins with ^{13}C-Amino Acids (II).

Aleurone layers were incubated with ^{13}C-amino acids for 24 hours. Fifty uCi of ^{3}H-leucine were added to the incubation medium at 12 hours after the addition of GA_3.

• • • • • ^{3}H-Radioactivity

0———0 α-Amylase activity peak

x———x Density gradient

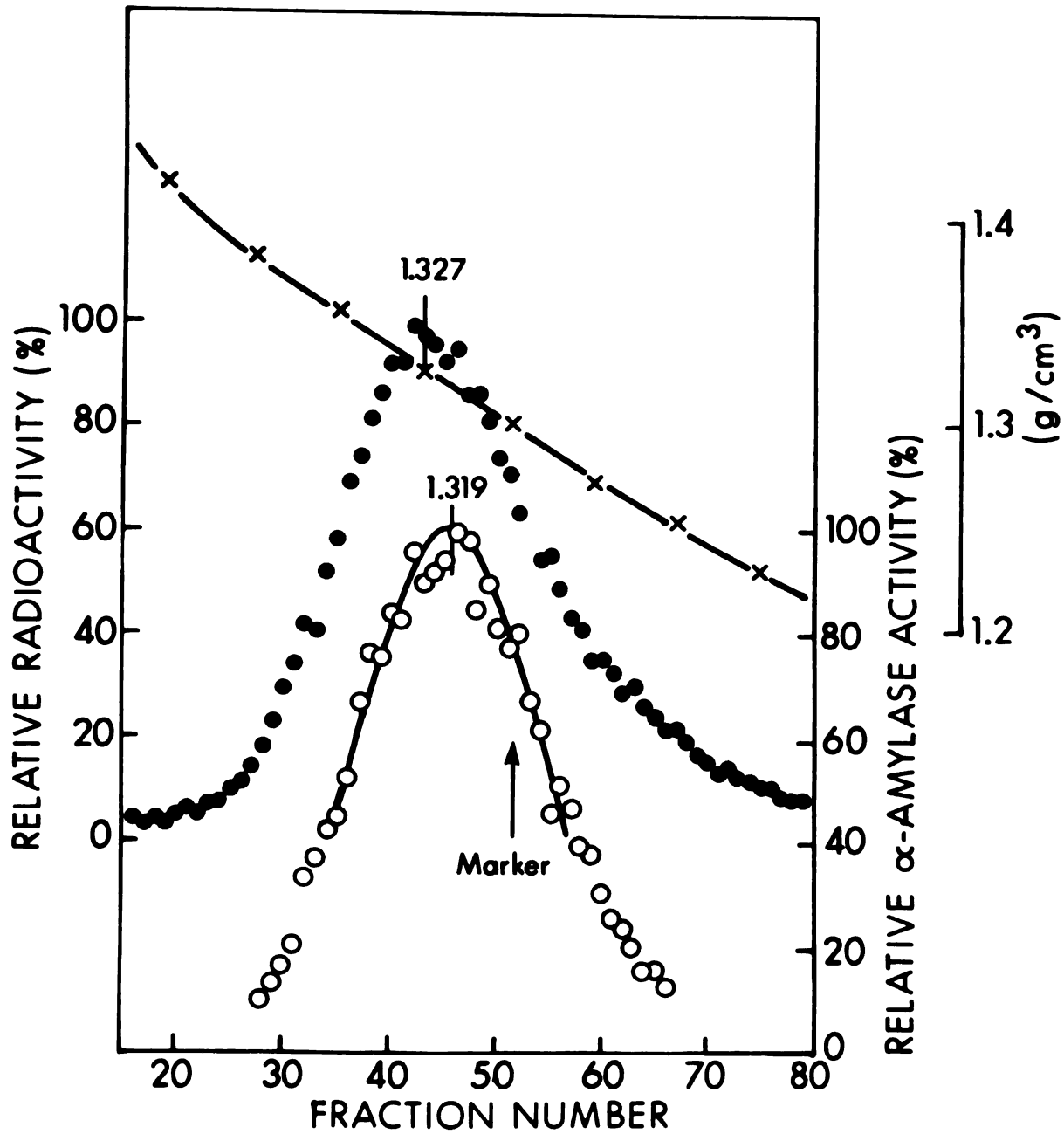

1.327
1.319
Marker
RELATIVE RADIOACTIVITY (%)
RELATIVE α-AMYLASE ACTIVITY (%)
(g/cm³)
FRACTION NUMBER
0
20
40
60
80
100
1.2
1.3
1.4
20
30
40
50
60
70
80

Figure 17.--Density Labeling of Barley Aleurone Proteins with ^{13}C-Amino Acids (III).

Aleurone layers were incubated with ^{12}C-amino acids for 12 hours and then further incubated with ^{13}C-amino acids for another 12 hours. Fifty uCi of ^{3}H-leucine was added to the incubation medium at 12 hours after the addition of GA_3.

• • • • • ^{3}H-Radioactivity

0————0 α-Amylase activity peak

x————x Density gradient

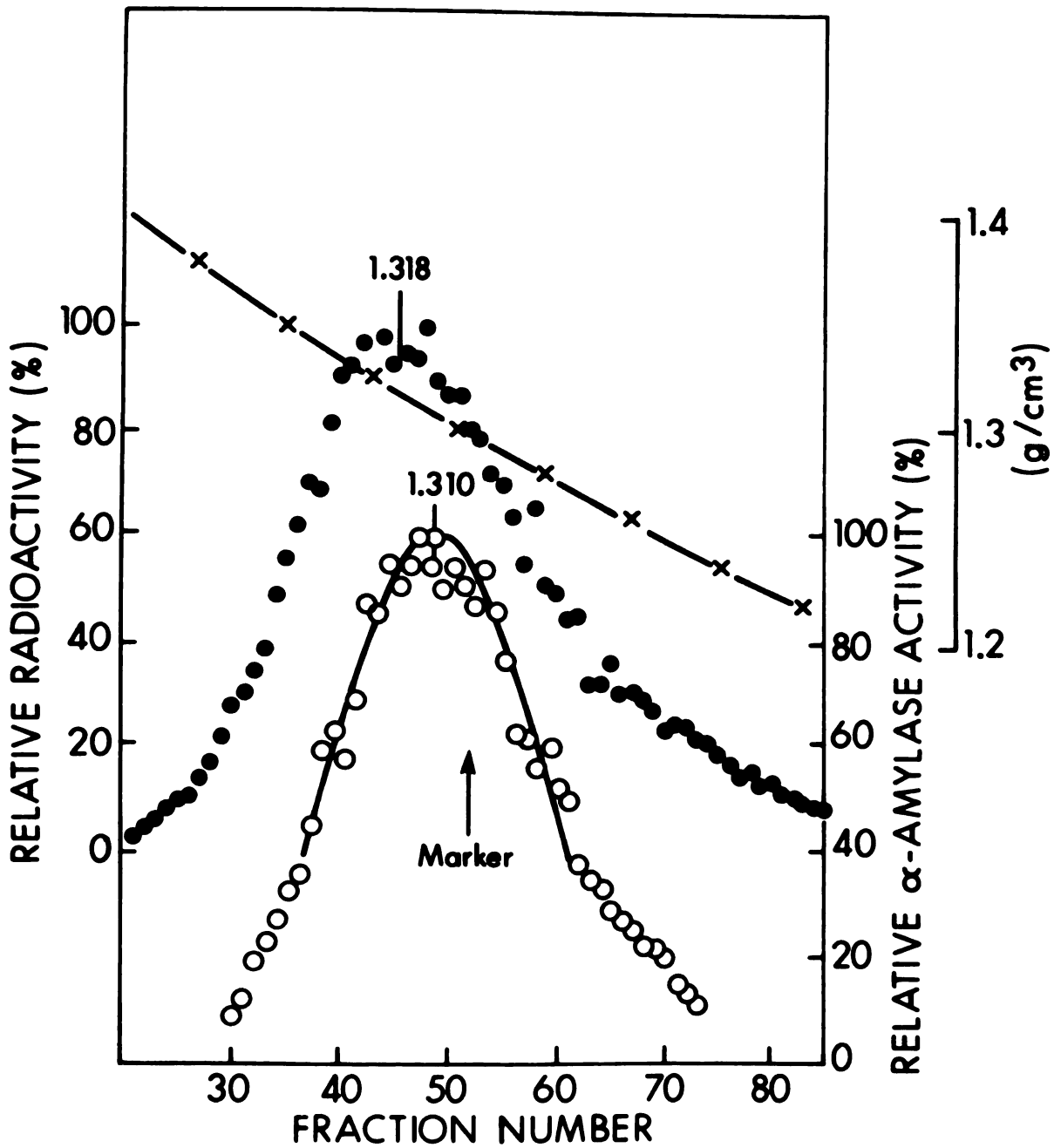
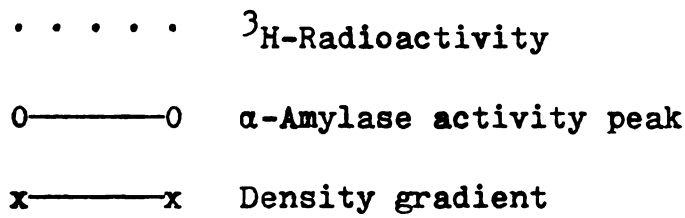

1.318
1.310
Marker
RELATIVE RADIOACTIVITY (%)
RELATIVE α-AMYLASE ACTIVITY (%)
(g/cm³)
FRACTION NUMBER
0
20
40
60
80
100
30
40
50
60
70
80
1.2
1.3
1.4

Figure 18.--Density Labeling of Barley Aleurone Proteins with ^{13}C-Amino Acids (IV).

Aleurone layers were incubated with ^{13}C-amino acids for 12 hours and then further incubated with ^{12}C-amino acids for another 12 hours. Fifty uCi of ^{3}H-leucine was added to the incubation medium at 12 hours after the addition of GA_3.



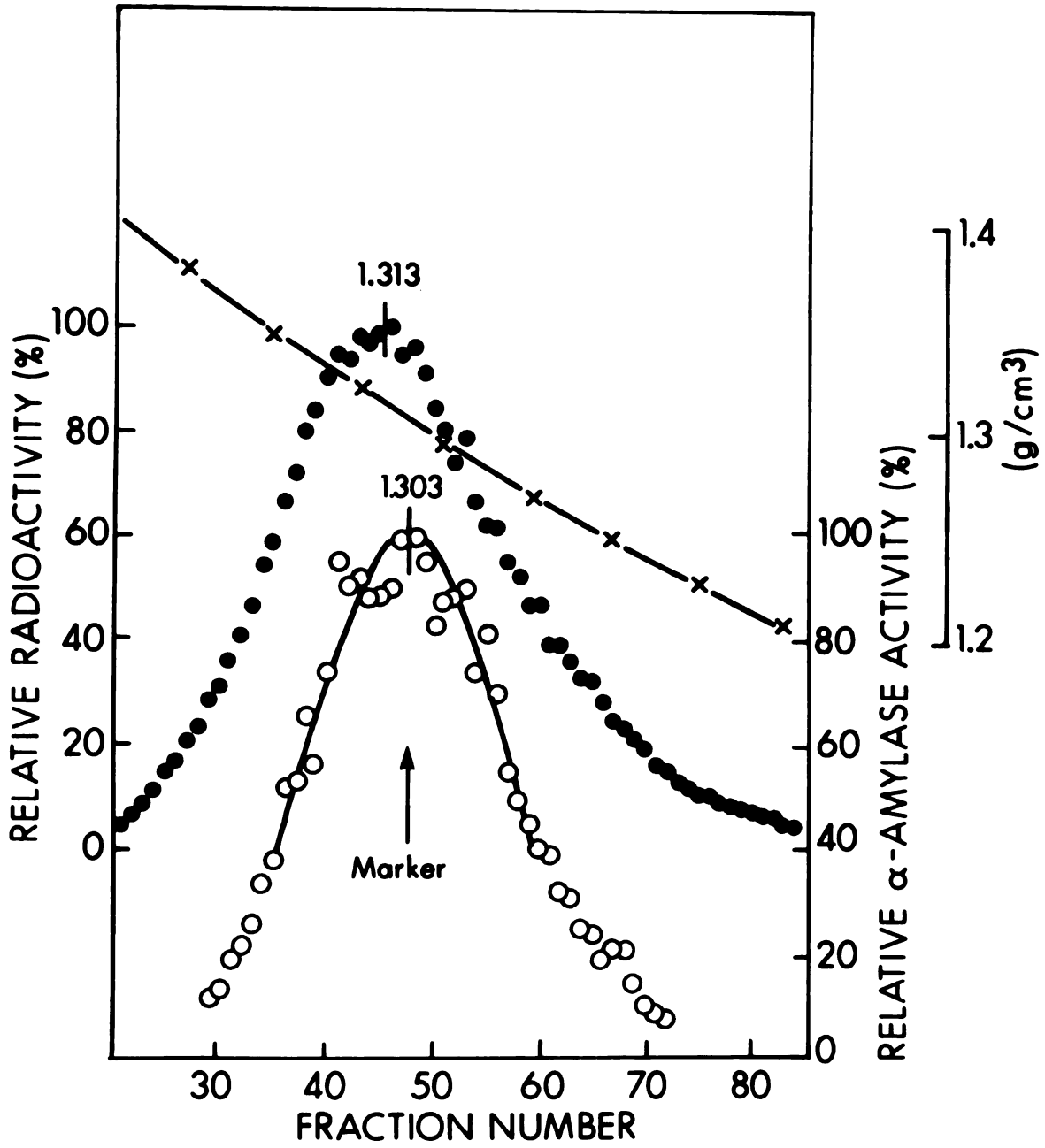
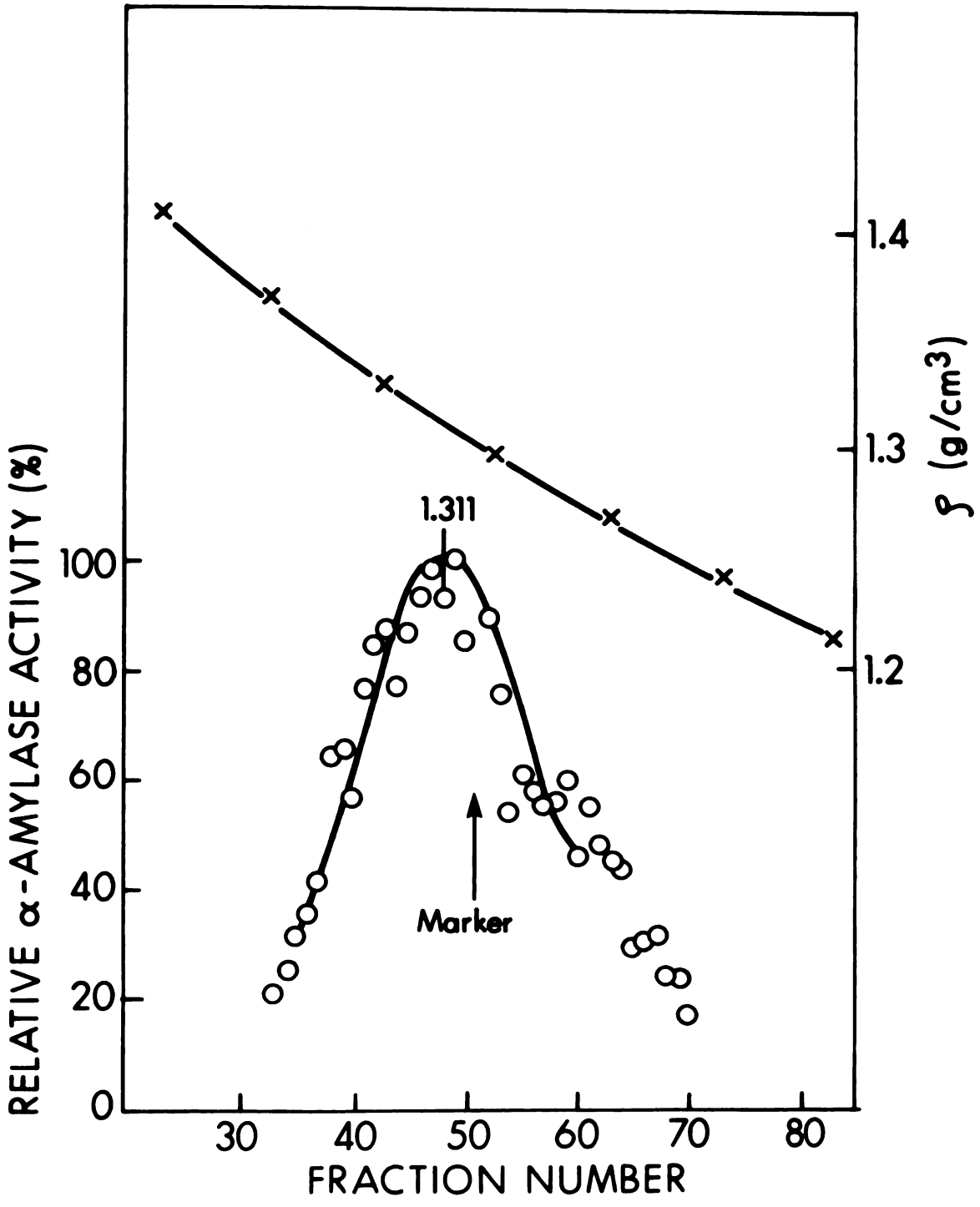

1.313
1.303
Marker
RELATIVE RADIOACTIVITY (%)
RELATIVE α-AMYLASE ACTIVITY (%)
(g/cm³)
FRACTION NUMBER
0
20
40
60
80
100
30
40
50
60
70
80
1.2
1.3
1.4

Figure 19.--Density Labeling of Barley Aleurone Proteins with ^{13}C-Amino Acids (V).

The experimental procedures were the same as described in Figure 18, except the aleurone layers were incubated with ^{12}C-amino acids for 12 hours and then further incubated with ^{13}C-amino acids and cordycepin for another 12 hours.

O———O α-Amylase activity peak

x———x Density gradient


RELATIVE α-AMYLASE ACTIVITY (%)
100
80
60
40
20
0
30
40
50
60
70
80
FRACTION NUMBER
1.311
Marker
1.4
1.3
1.2
ρ (g/cm³)

hours of GA_3 treatment, the rapid increase of α-amylase activity after 12 hours of GA_3 is due to the de novo synthesis of the enzyme molecule. Furthermore, by also adding a radioactive amino acid during the experiment but after 12 hours of GA_3 treatment, it was possible to measure the density shift of the newly synthesized proteins which would represent the maximal density shift (100% de novo synthesis) for that period. By comparing the density shift of α-amylase and that of the total newly synthesized proteins (radioactive peak), one should be able to estimate the extent of de novo synthesis of α-amylase molecule. As shown in TABLE 13, essentially all α-amylase activity that appears after 12 hours is due to the de novo synthesis.

Lack of Cordycepin Effect on the Degradation of α-Amylase

The degradation of α-amylase, measured at the time when its synthesis was shut down by cycloheximide is moderate with a half life about 13 hours (Figure 20). The dissappearance of α-amylase in the medium is much slower than that of α-amylase in the layer; however, disappearance from the layer might result from the continuous secretion of α-amylase even though protein synthesis was inhibited (65). Cordycepin does not slow down the degradation of α-amylase. Therefore, the insensitiveness of α-amylase production to cordycepin after 12 hours of GA_3

TABLE 13.--Summary of the ^{13}C-Amino Acids Density Labeling Experiments

Treatment	α-Amylase Peak (g/cm^3)	α-Amylase Peak Half-height width x 10	Radioactivity peak (ρ)	% of α-Amylase synthesized
^{12}C (o to 24 hr)	1.300	0.603	1.308	
^{13}C (o to 24 hr)	1.317	0.640	1.326	95%
^{12}C (0 to 12 hr) then ^{13}C (12 to 24 hr)	1.310	0.652	1.318	100%
^{13}C (0 to 12 hr) then ^{12}C (12 to 24 hr)	1.302	0.624	1.311	
^{12}C (0 to 12 hr) then ^{13}C (12 to 24 hr) with cordycepin	1.311	0.706		

Figure 20.--Effect of Cordycepin on the Degradation of α-Amylase.

Cordycepin was added at 12 hours after GA_3 and cycloheximide (10 ug/ml) was added at 15 hours after GA_3. α-Amylase activity was assayed at specific times after the addition of cycloheximide and the activity of α-amylase at 15 hours after GA_3 was assigned as 100%.

A. O——O + GA_3 only (α-amylase in the medium)

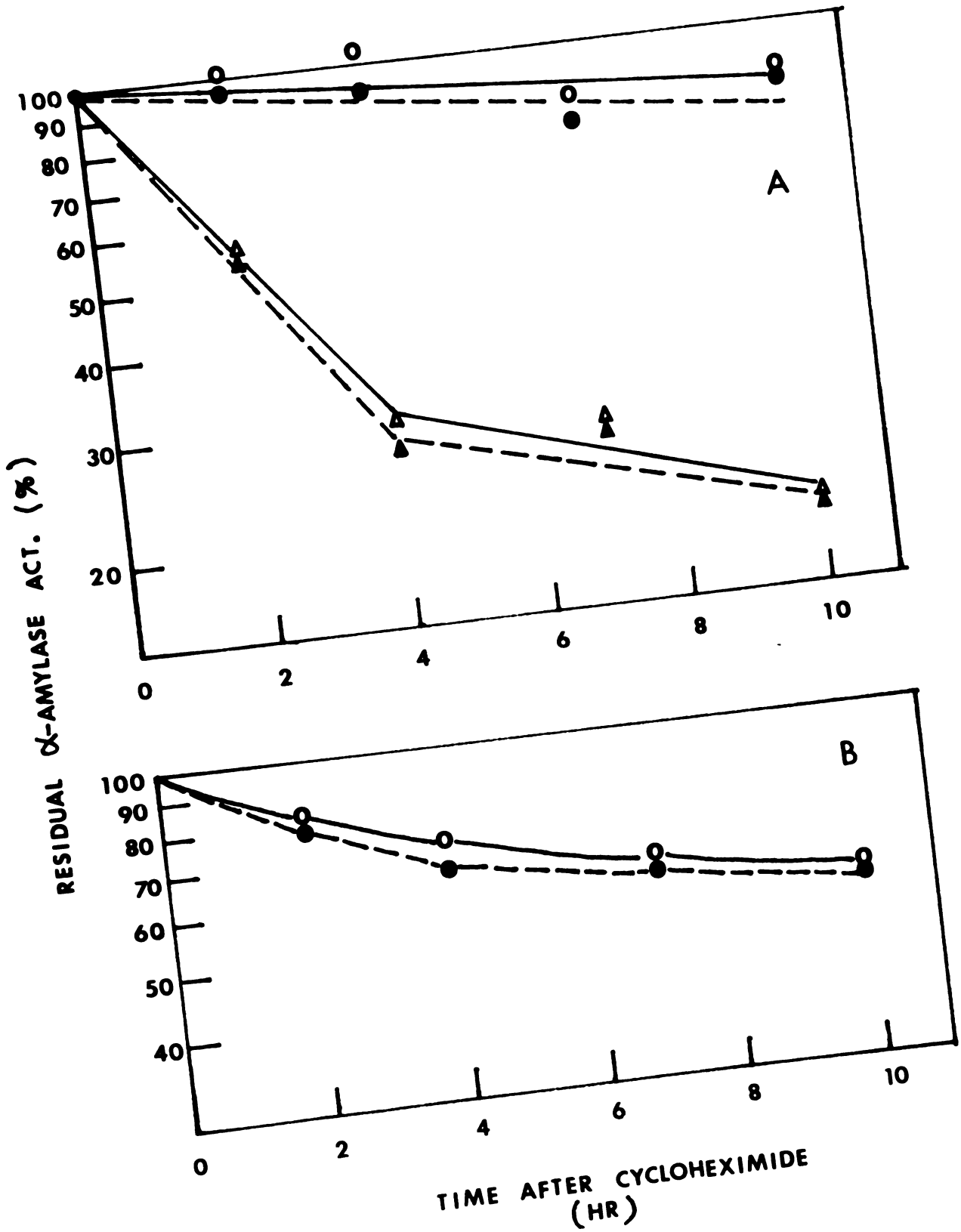
Δ——Δ + GA_3 only (α-amylase in the layer)

●-----● + GA_3 and cordycepin (α-amylase in the medium)

▲-----▲ + GA_3 and cordycepin (α-amylase in the layer)

B. O——O + GA_3 only (total α-amylase)

●-----● + GA_3 and cordycepin (total α-amylase)


A
B
100
90
80
70
60
50
40
30
20
0
2
4
6
8
10
RESIDUAL α-AMYLASE ACT. (%)
TIME AFTER CYCLOHEXIMIDE (HR)

cannot be explained by a slowing of α-amylase degradation by this inhibitor, which in turn would compensate for a slower rate of synthesis.

Lack of Effect of Osmoticum on the Stability of α-Amylase mRNA

It has been shown in mammalian tissue that the average half life of mRNA associated with membrane bound polysomes appears to be relatively short compared to mRNA associated with free polysomes (47). Since α-amylase is a secrdtory protein which is synthesized by membrane bound polysome, it would be interesting to know whether stripping the polysomes from the membrane has any effect on the stability of α-amylase mRNA as measured by the sensitivity of α-amylase synthesis to an RNA synthesis inhibitor such as cordycepin. It has been known that high concentrations of osmotica, such as mannitol or polyethylene glycol, can prevent protein synthesis in barley aleurone layers (35) and other plant tissues (26) by exerting water stress on the cells. Electonmicroscopic and biochemical evidences indicate that water stress induced by these osmotica is to reduce the binding of ribosomes to the endoplasmic reticulum and this reduction in membrane bound polysome formation does not result from reduced ribosome activity as measured by peptidylpuromycin formation (1). When aleurone layers were treated with 0.8 M mannitol together with cordycepin between 12 to 24 hours after GA_3 addition

and mannitol was removed afterwards, the production of α-amylase between 24 to 36 hours had the same rate as that of layers treated with 0.8 M mannitol but without cordycepin (TABLE 14). Since the mannitol effect on the binding of ribosomes to endoplasmic reticulum is reversible and the presence of cordycepin does not influence the resumed α-amylase production after mannitol is removed it appears that stripping ribosomes from ER by osmotic stress does not affect the stability of α-amylase mRNA.

It was recently reported that mRNA of membrane-bound polysomes in mammalian cells remain associated with the membrane even after ribosomes are removed (39) and the poly A segment appears to be attached to the membrane directly (39,44). If a similar situation occurs in plant cells, then one would expect that stripping off membrane bound ribosomes would not influence the association of α-amylase mRNA with microsome membrane.

TABLE 14.--Effect of Water Stress on the Sensitivity of α-Amylase Synthesis to Cordycepin Treatment

Treatment	α-Amylase (units/layer)	%
+ GA_3 only (26 hr)	44.4	100
- GA_3 (26 hr)	11.4	26
+ GA_3 and Cordycepin (0 to 26 hr)	12.6	28
+ GA_3 and Cordycepin (12 to 26 hr)	45.4	102
+ GA_3 ⟶ + GA_3 and Mannitol (12 to 26 hr) ⟶ + GA_3 only (26 to 36 hr)	16.0	100
+ GA_3 ⟶ + GA_3 and Mannitol and Cordycepin (12 to 26 hr) ⟶ + GA_3 and Cordycepin (26 to 36 hr)	17.0	106

Note: The concentration of mannitol was 0.8 M. For the mannitol treated layers only the activity of α-amylase produced between 26 to 36 hr after GA_3 is shown.

DISCUSSION

This study shows that poly A-RNA is present in barley aleurone layers. The poly A-RNA is polydisperse in size and its synthesis is enhanced in the presence of GA_3. This enhancement begins after a lag period of 3-4 hours and reaches a maximum at 10 to 12 hours after the addition of GA_3. ABA can prevent the GA_3 enhancement on poly A-RNA synthesis. The barley aleurone layer is a non-dividing tissue, and neither its respiration nor its energy charge is changed significantly by the application of hormone (9). Therefore, the GA_3 enhanced poly A-RNA synthesis can not be a nonspecific re sult of hormone-enhanced growth or hormone-enhanced energy metabolism.

In barley aleurone layers which have been treated with GA_3, about 40% of the newly synthesized protein is estimated to be α-amylase (66). The percentage of the sum of all kinds of hydrolases in terms of total protein is even higher. Thus, if GA_3 causes the synthesis of mRNAs for hydrolases, there should be a detectable enhancement of the synthesis of total mRNA. Gibberelic acid also causes an increase in the activity of two enzymes, phosphorylcholine cytidyl transferase and phosphorylcholine-glyceride transferase, which are required for the

biosynthesis of lecithin, a major component of membrane phospholipids (38). However, GA_3 appears to enhance the activity of these two enzymes by some activation process not involving protein synthesis or RNA synthesis (4). Therefore, it may be that enhanced synthesis of poly A-RNA is not necessary for the observed membrane proliferation.

In principle, the control of hormone-enhanced specific proteins could be either on the transcriptional or the post-transcriptional level. It has been shown that hormone-controlled synthesis of egg white proteins in chick oviduct is due to the accumulation of sepecific mRNAs (7,54). Palmiter (48) has found that ovalbumin has a fairly stable mRNA whose synthesis is enhanced by estrogen. It was recently concluded that withdrawal of estrogen will decrease the half life of ovalbumin mRNA, indicating that this mRNA is somehow stabilized in the hormone-treated tissue (50).

In barley aleurone layers the GA_3 enhanced synthesis of α-amylase is no longer sensitive to a transcriptional inhibitor such as cordycepin after 12 hours of exposure to the hormone. Therefore, a post-transcriptional control is proposed because the synthesis of α-amylase specific mRNA cannot be rate-limiting at this stage. This is not analogous to the situation in terminally differentiated cells such as eryhrocyte or galea cells in silk moth, in which the transcriptional control mechanism

ceases to exist when the cells engage in the rapid and massive synthesis of cell specific proteins. However, transcriptional control mechanisms can still exist in the barley aleurone after the first 12 hours of GA_3 addition. The evidence for this is that the synthesis of α-amylase in barley aleurone cells afters of GA_3 treatment can be prevented by another hormone, ABA, whose effect appears to be dependent on the continuous synthesis of a short-lived RNA (Part II). Thus, although α-amylase synthesis at this stage is under post-transcriptional control, yet the aleurone cells apparently maintain their potential for control the synthesis of specific RNAs.

Two concurrent events take place in barley aleurone cells during the first 12 hours of GA_3 treatment, namely the proliferation of membrane and the enhanced mRNA formation. The membrane proliferation, which is a prerequist for later secretory protein synthesis, is initiated by the hormone mediated activation of phosphorylcholine transferases. The hormone enhanced formation of mRNAs presumably those specific for α-amylase and other hydrolases, must reflect the existence of a transcriptional control mechanism at this stage.

Another fact to be considered in the study of the control mechanism in barley aleurone cells is that GA_3 does not increase the total protein synthesis in these cells. This is not necessary contradictory to the

discovery of hormone enhanced mRNA formation, because hydrolases have long lived mRNA. Therefore, a selective destruction of other enzymes' mRNAs would maintain the total mRNA population at a relatively constant level while the population of hydrolase specific mRNAs would be greatly increased. This is a situation analogous to the recent concept of Palmiter and Schimke (49) in which they suggest that more efficient translation of a long-lived mRNA would lead to an increased rate of production of its corresponding protein. This increase in efficiency would be due to more favorable competition for some rate limiting factor once the more labile meassages started to disappear. Since mRNA associated with membrane bound polysomes are more stable than those associated with free polysomes, a selective stabilization of hydrolase (secretory protein) specific mRNAs by their association with the ER which is abundant in the hormone treated tissue, could occur in barley aleurone cells leading to the enhancment of relative population of hydrolase mRNAs.

In mammalian hepatoma cell cultures, an RNA synthesis inhibitor, such as Actinomycin D, not only slows down the synthesis of tyrosine aminotransferase but also to a greater extent causes a decrease of the degradation of this enzyme (37). However, this does not occur in the cordycepin treated aleurone layer because cordycepin does not influence the degradation of α-amylase. Thus the

evidence presented in this study indicates that α-amylase and probably protease are translated from stable mRNAs. Since the synthesis of α-amylase is not influenced by the presence of cordycepin for at least 12 hours one would expect the half life of α-amylase mRNA exceeds 50 hours. This would not be an astonishing because the biosynthesis of cell specific proteins is usually associated with long lived messages. For example, the mRNA for cocoonase of silk moth has a half life of 100 hours (36), that for ovalbumin of chick oviduct is 18 hours (48) and reticulocytes can synthesize hemoglobin for at least 48 hours after extrusion of the nucleus (55).

PART II

THE RESPONSE OF BARLEY ALEURONE LAYERS TO ABSCISIC ACID

INTRODUCTION

Abscisic acid is known to reverse or prevent many responses of barley aleurone layers to GA_3. These include the GA_3 enhanced syntheses of α-amylase (9,28) and protease (29), membrane bound polysome formation (14), incorporation of ^{32}P into membrane phospholipids (38), poly A-RNA synthesis (25), and the activation of phosphorylcholine transferases (4,31). However, abscisic acid has no effect on general cellular metabolism as measured by oxygen consumption (9).

Although ABA can prevent the response to GA_3, no direct effects of ABA in aleurone clells have been observed. However, the failure of the aleurone cells to respond to GA_3 in the presence of ABA does not result from simple competition between these two hormones because a high concentration of GA_3 cannot completely overcome the ABA effect (9,28).

Because most of the effects of GA_3 in aleurone cells depend on cellular metabolism, elgl transcription and translation, it has been difficult to study the mode of action of ABA because application of metabolic inhibitors would inhibit GA_3 effects directly. It has been demonstrated in the first part of this study that α-amylase is

translated from stable mRNA that is synthesized before the rapid increase of α-amylase activity. Because α-amylase synthesis after 12 hours of GA_3 treatment is no longer sensitive to transcription inhibitors such as cordycepin (3'-deoxyadenosine), while ABA at this stage still effectively inhibits α-amylase production, it provides us a good opportunity to study whether or not there is a requirement for RNA sythesis for the effect of ABA when it prevents the tissue's response to GA_3.

MATHERIALS AND METHODS

Source of Chemicals and Seed

Same as described in Part I.

Preparation and Treatment of Aleurone Layers

The metods described in Part I were modified. Embryo-less half-seeds are surface sterilized by sodium hypochlorite (5-fold dilution of commericial bleach) for 20 min and rinsed several times with sterile deionized water, and further stirred in 0.01 N HCl for 10 min to destroy any trace of sodium hypochlorite remaining on their surface. After thorough rinsing with water and with 20 mM sodium succinate buffer, pH 5.0, containing 10 mM $CaCl_2$, the half-seeds were imbibed on sterilized sand moistened with the same sodium succinate buffer. Aleurone layers were peeled from 3-day imbibed half-seeds and incubated with sodium succinate buffer, with different combinations of hormones and inhibitors, in a reciprocal metabolic shaker (120 oscillations/min) at 25 ^{0}C. The concentration of GA_3 used in this work was 2.5 uM. Using sodium succinate buffer slightly increases the production of α-amylase in the presence of GA_3. Further, it also decreases the base level of α-amylase production (in the absence of GA_3) by more than

50% (TABLE 15). Thus the GA_3 enhancement of α-amylase production in succinate buffer can reach about 25 to 40 fold intead of the 3 to 10 fold usually obtained in acetate buffer. Less α-amylase is released into the medium in the presence of succinate buffer (TABLE 15). The reason for these effects is unclear.

Extraction and Assays of Enzyme

α-Amylase was extracted and assayed as described in part I.

Sodium Dodecylsulfate (SDS) Gel Electrophoresis

The procedures were modified from those of Laemmli (40). Gels with 2.5 cm long stacking gel (4%) and 10.5 cm long separation gel (12%) were used. Samples for gel electrophoresis were prepared by grinding 30 to 40 aleurone layers with 0.5 ml 0.2 M NaCl containing 10 mM $KBrO_3$ with a mortar and pestle. The mortar was rinsed with another 1 ml of NaCl solution and the solutions were cmbined. After centrifuging for 30 min at 12,000g, the supernatant was decanted and 1.0 ml SDS reagent containing 0.125 M Tris-HCl, pH 6.8, 4% SDS and 10% β-mercaptoethanl was added to the supernatant. This supernatant is referred to as salt-soluble protein preparation. To the washed pellet 1.0 ml SDS reagent was added. After storage at room temperature for several days, the pellet with SDS reagent was centriguged. The resuting supernatant was diluted with an equal volume of distilled water and referred to as the salt

TABLE 15.--GA_3 Enhancement of the Production of α-Amylase in Different Buffers

Treatment		α-Amylase (units/layer)			GA_3 Enhancement (fold)
		Medium	Layer	Total	
2 mM Na Acetate	+GA_3	20.6	2.0	22.6	9.8
	No GA_3	1.6	0.7	2.3	
20 mM Na Succinate	+GA_3	17.2	8.6	25.8	25.8
	No GA_3	0.4	0.6	1.0	

Note: The pH of the buffers was 5.0 and α-amylase produced in the first 24 hours was assayed.

insoluble protein preparation. A 100 ul sample was applied to each gel and electrophoresis was carried out with constant voltage of 80 V and an initial current of 40 mA per 12 gels. The molecular weights of the radioactive bands in the gels were determined by comparing their mobility with those of marker protins of known molecular weight (Figure 21).

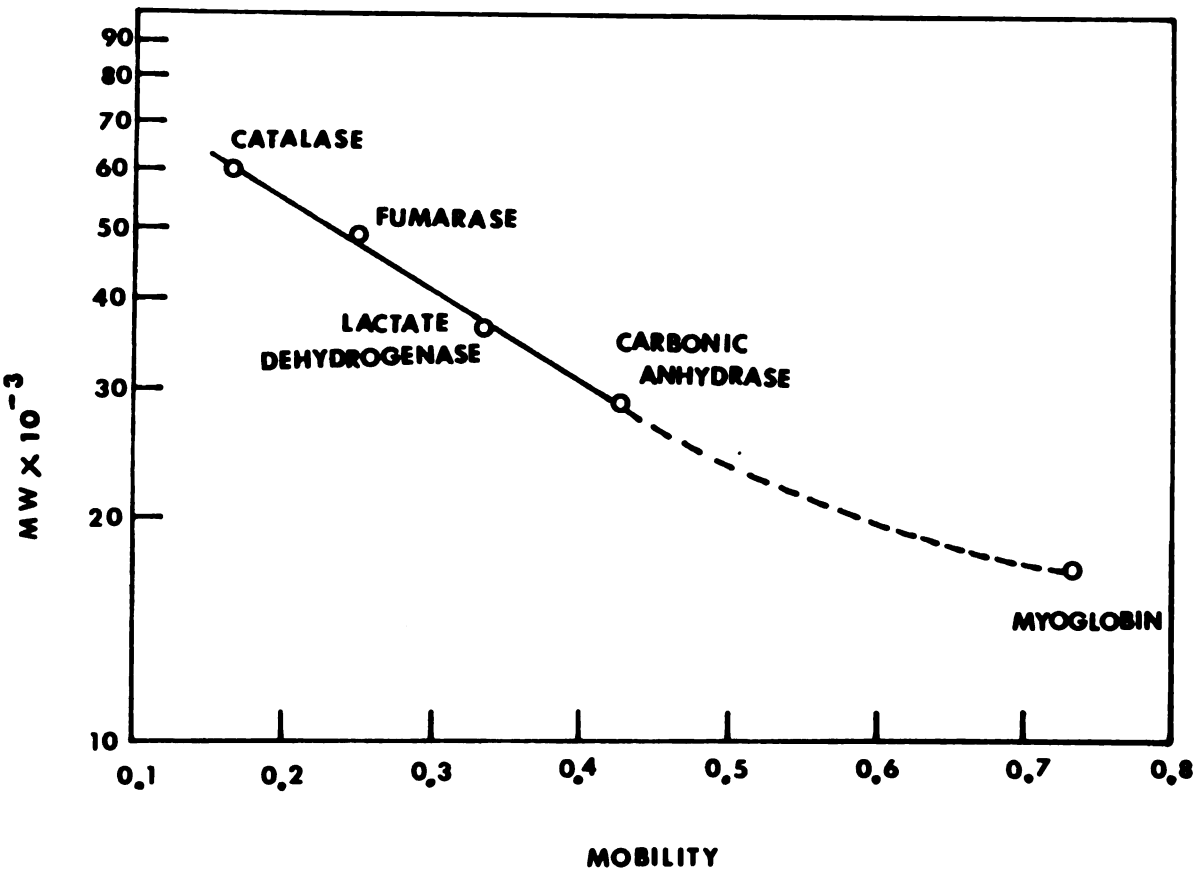
Figure 21.--Calibration Curve for Molecular Weight Determination on SDS Gel Electrophoresis.

Marker proteins with known molecular weight of their subunits were treated with SDS reagent and then subjected to electrophoresis as described under "Materials and Methods". The mobility is described as:

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

The swelling of gel during staining and destaining was insignificant, therefore, no correction was made.

The source and molecular weights of the marker proteins (subunit) are: Catalase (Beef liver, M.W. 60,000); Fumarase (Pig heart, M.W. 49,000); Lactate Dehydrogenase (Chicken heart, M.W. 36,000); Carbonic Anhydrase (Bovine erythrocyte, M.W. 29,000); Myoglobin (Equine skeletal muscle, M.W. 17,200).


90
80
70
60
50
40
30
20
10
MW X 10^-3
CATALASE
FUMARASE
LACTATE DEHYDROGENASE
CARBONIC ANHYDRASE
MYOGLOBIN
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
MOBILITY

RESULTS

Effect of Cordycepin on GA_3 Enhanced α-Amylase Formation

Cordycepin acts as a chain terminator during RNA synthesis and inhibits in barley aleurone layers the formation of both poly A-RNA and RNA species not containing the poly A segment (Part I). Cordycepin is equally effective as a transcription inhibitor whether it is added at the same time or 12 hours after GA_3 (Part I). As in acetate buffer, the inhibitory effect of cordycepin on GA_3 enhanced α-amylase synthesis in succinate buffer is less and less as cordycepin is added later and later after the hormone and no inhibitory effect is observed if cordycepin is added 12 hours or later after the addition of GA_3 (TABLE 16). In fact, an enhancement of α-amylase formation by cordycepin added 12 hours after GA_3 was occasionally oberved (TABLE 16). Because the α-amylase molecules are synthesized at the time that this increase in enzyme activity is observed (Part I), and cordycepin has no effect on the degradation of the enzyme, it is concluded that α-amylase is translated from stable mRNA.

TABLE 16.--Effect of Cordycepin on the GA_3 Enhanced Synthesis of α-Amylase

Treatment	α-Amylase Activity (units/layer)		%
	Total	Increase over Control	
GA_3 only	27.7	26.6	100
Control (No GA_3)	1.1		
GA_3 + Cordy. (added at 0 time)	6.0	4.9	18
GA_3 + Cordy. (added at 4 hours after GA_3)	10.9	9.8	37
GA_3 + Cordy. (added at 8 hours after GA_3)	25.0	23.9	85
GA_3 + Cordy. (added at 10 hours after GA_3)	27.6	26.5	100
GA_3 + Cordy. (added at 12 hours after GA_3)	31.2	30.1	113

Note: Cordycepin (Cordy., 0.1 mM) was added at different time as indicated and the aleurone layers were further incubated for a total of 24 hours of GA_3 treatment when α-amylase was extracted and assayed.

Effect of ABA on α-Amylase Formation

Abscisic acid, at a concentration of 5 uM, effectively inhibits α-amylase formation if it is added at the same time asGA_3. However, higher concentrations of ABA (10 to 25 UM) are needed to prevent α-amylase formation when ABA is added 12 hours after GA_3. It has been reported that ABA, at a concentration of 38 uM forms a complex with fungal α-amylase, resulting in inhibition of the enzyme activity (43,46). However, as shown in TABLE 17, ABA, at concentrations up to 50 uM, has no significant effect on barley aleurone α-amylase activity in a cell free enzyme preparation after a 23 hour incubation at 25 ^{o}C. Since the concentration of ABA we used for this study was 25 uM and the time span of the experiment was about 12 hours (from 12 hours to about 24 hours after GA_3), we conclude that the ABA effect on α-amylase production must be via a physiological process.

Effect of Cordycepin on the Action of ABA

Abscisic acid added 12 hours after GA_3 gradually inhibits further accumulation of α-amylase (Figure 22) (9). Because the accumulation of α-amylase in response to GA_3, both before and after 12 hours is due to <u>de novo</u> synthesis (Part I) and because α-amylase accumulation after 12 hours of GA_3 is not inhibited by cordycepin, it is clear that ABA inhibits translation of α-amylase and not transcription of α-amylase mRNA. However, when ABA is added 12 hours after

TABLE 17.--Lack of Direct Effect of Abscisic Acid on α-Amylase Activity

Incubation Time (at 25 °C)	No ABA		10 uM ABA		50 uM ABA	
	α-amylase	%	α-amylase	%	α-amylase	%
0 hr	41.0	100	41.0	100	41.0	100
3 hr	41.6	101	40.9	99.8	40.8	99.6
8 hr	40.2	98	39.5	96	37.2	91
23 hr	38.6	94	37.2	91	37.5	91

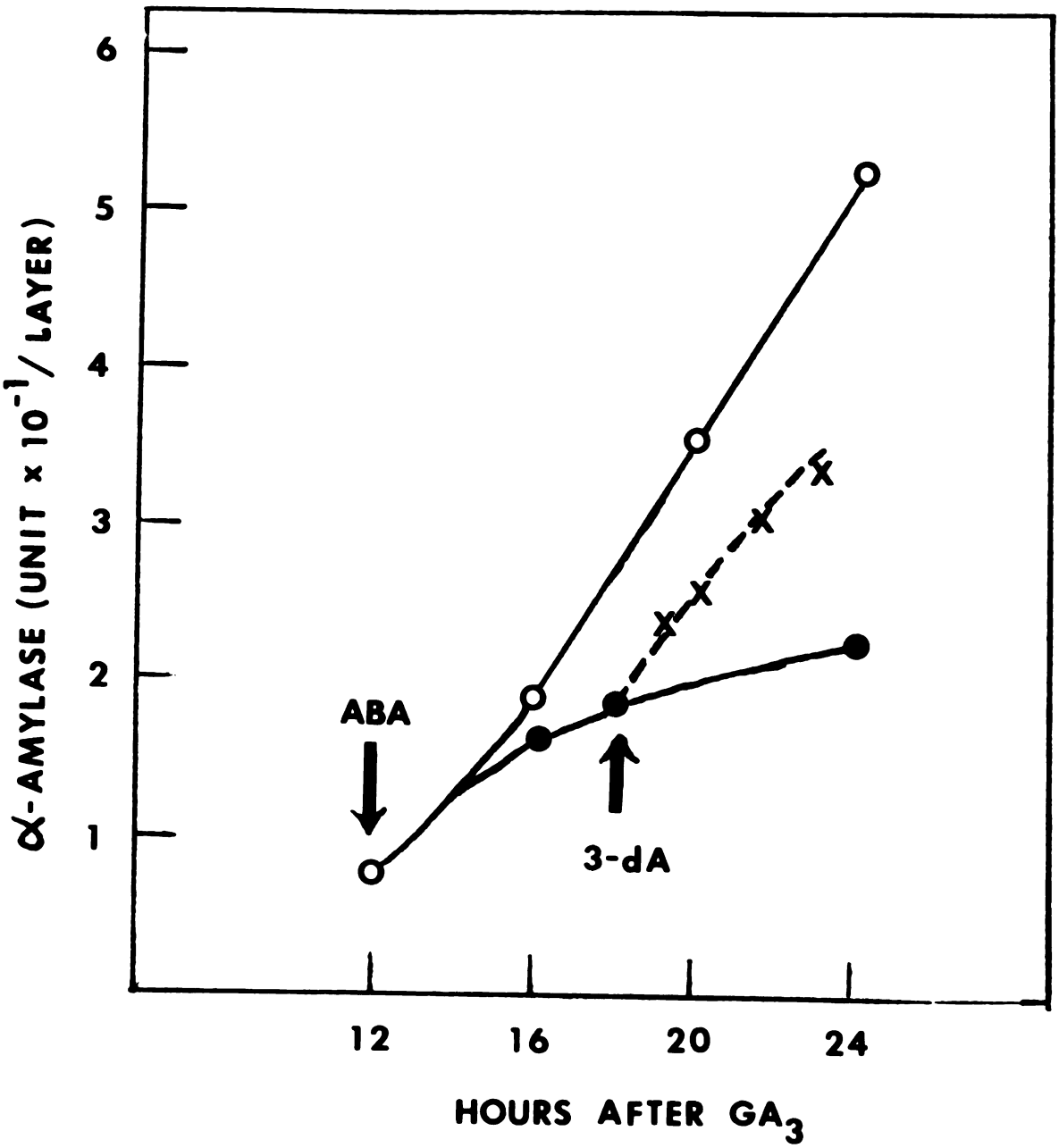
Note: α-Amylase extracted from 24-hour GA_3 treated aleurone layers was used. The unit of α-amylase is A_{620}/ min, ml.

Figure 22.--Effect of Midcourse Addition of ABA and Cordycepin on the Synthesis of α-Amylase.

o——o GA_3 only

●——● GA_3 and ABA

x——x GA_3, ABA, and Cordycepin (3-dA)


6
5
4
3
2
1
α-AMYLASE (UNIT x 10⁻¹/ LAYER)
ABA
3-dA
12
16
20
24
HOURS AFTER GA3

GA_3 and cordycepin is added at the same time or later, the accumulation of α-amylase either does not stop, or is quickly resumed (Figures 22 and 23). The effect of cordycepin cannot be due to a generally toxic effect because α-amylase production remains normal in the presence of cordycepin without ABA. Thus the effect of ABA depends on the continuous synthesis of a short-lived RNA which is inhibited by cordycepin. Because the rate of α-mylase accumulation after cordycepin addition is close to that of tissue treated with GA_3 alone (Figures 22 and 23), it appears that the amount of α-amylase mRNA is not limiting in the presence of ABA and cordycepin, i.e. ABA has no significant effect on the stability of α-amylase mRNA.

In order to determine whether ABA specifically prevents the synthesis of α-amylase (and perhaps other GA_3 enhanced hydrolases as well) or whether ABA slows down protein synthesis in general, the profile of newly synthesized proteins was chceked by SDS gel electrophoresis. α-Amylase is detected as the predominant radioactive band on SDS gel with a molecular weight of about 50,000 daltons. As shown in Figure 24 for salt-soluble proteins and Figure 25 for salt-insoluble proteins, the synthesis of α-amylase is substantially decreased in the presence of ABA, while the amount of radioactivity in most of the minor bands remains essentially the same as in control tissue ($+GA_3$-ABA).

Figure 23.--Effect of Cordycepin Added at Different Times on the Synthesis of α-Amylase in the Presence of both GA_3 and ABA.

ABA was added at 12 hours after GA_3. The arrows indicate the time of cordycepin addition.

O———O GA_3 only

●———● GA_3 and ABA

x-----x GA_3, ABA, and cordycepin

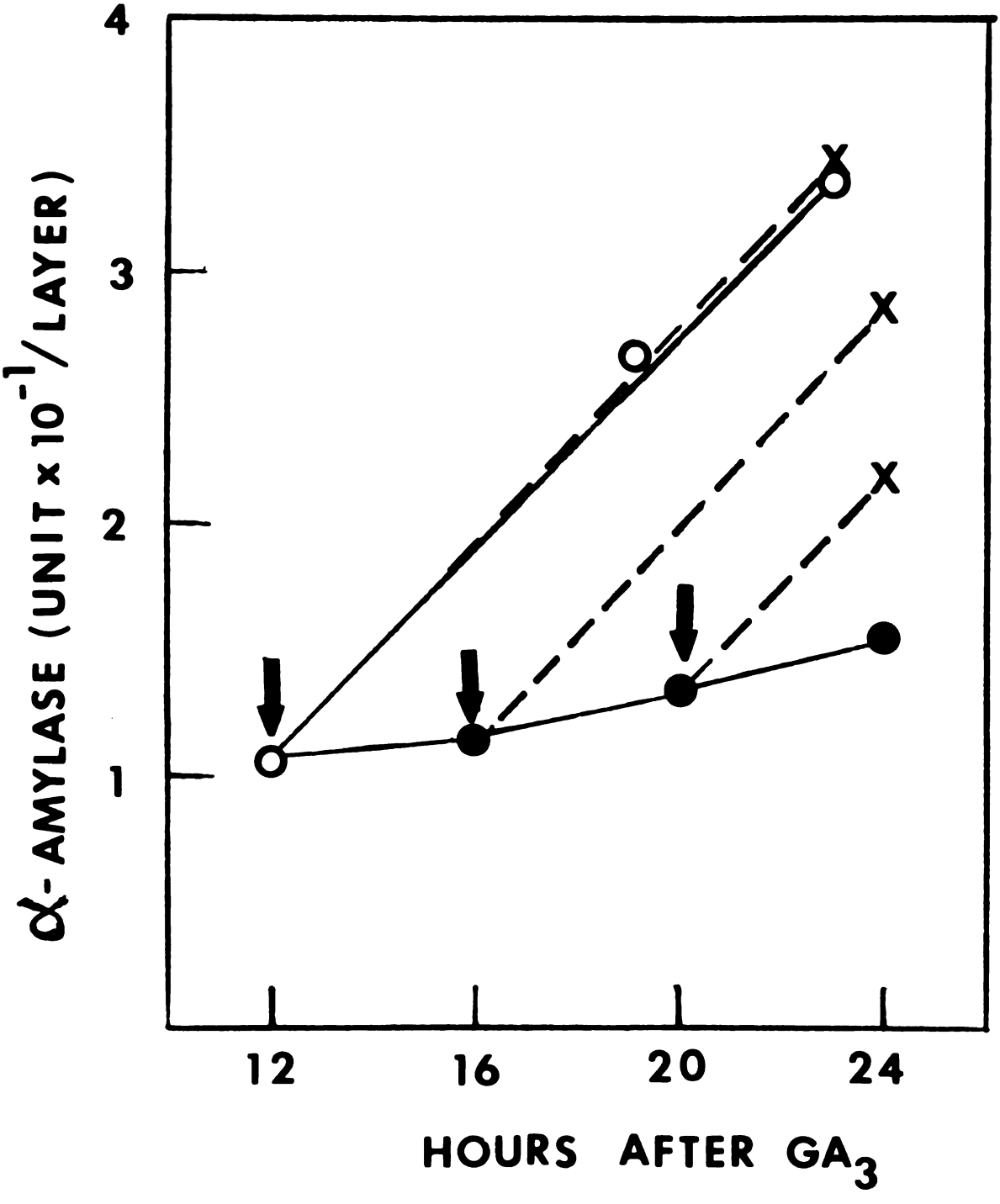
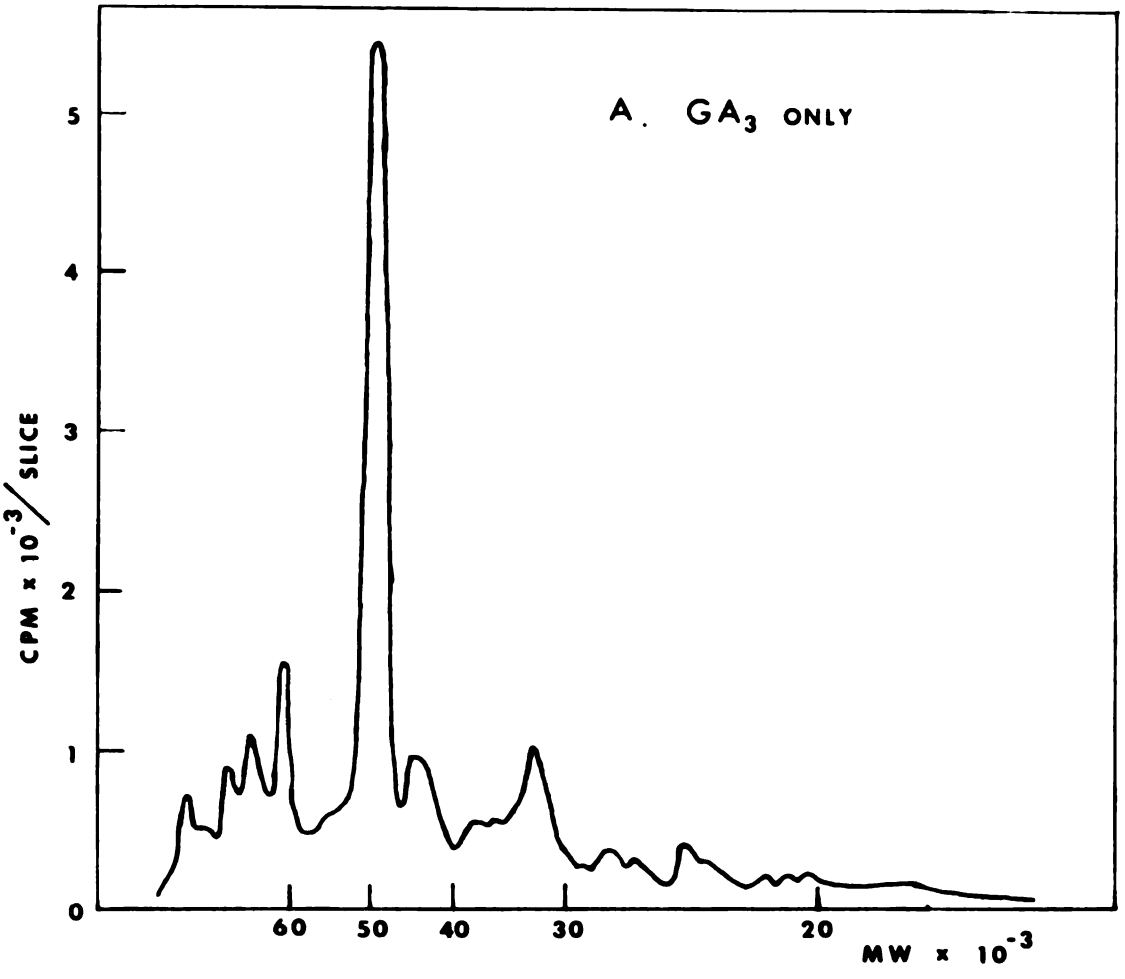

4
3
2
1
α- AMYLASE (UNIT x 10⁻¹/LAYER)
12
16
20
24
HOURS AFTER GA3

Figure 24.--Profile of Newly Synthesized Salt-Soluble Proteins on SDS Gel.

Aleurone layers (30 to 40) were labeled with ^{3}H-leucine (15 uCi/ml) for 2 hours (18 to 20 hours after GA_3). Salt-insoluble proteins were extracted as described under Materials and Methods. One mm thick gel pieces were sliced and digested in 0.5 ml NCS solubilizer (9 parts of full strength NCS solubilizer and 1 part of distilled water) at 50 ^{o}C for two hours. Ten milliliter of toluene based scintillation fluid (6 g PPO and 75 mg POPOP/liter)were used in each sample.


A. GA3 ONLY
CPM x 10-3/SLICE
0
1
2
3
4
5
60
50
40
30
20
MW x 10-3

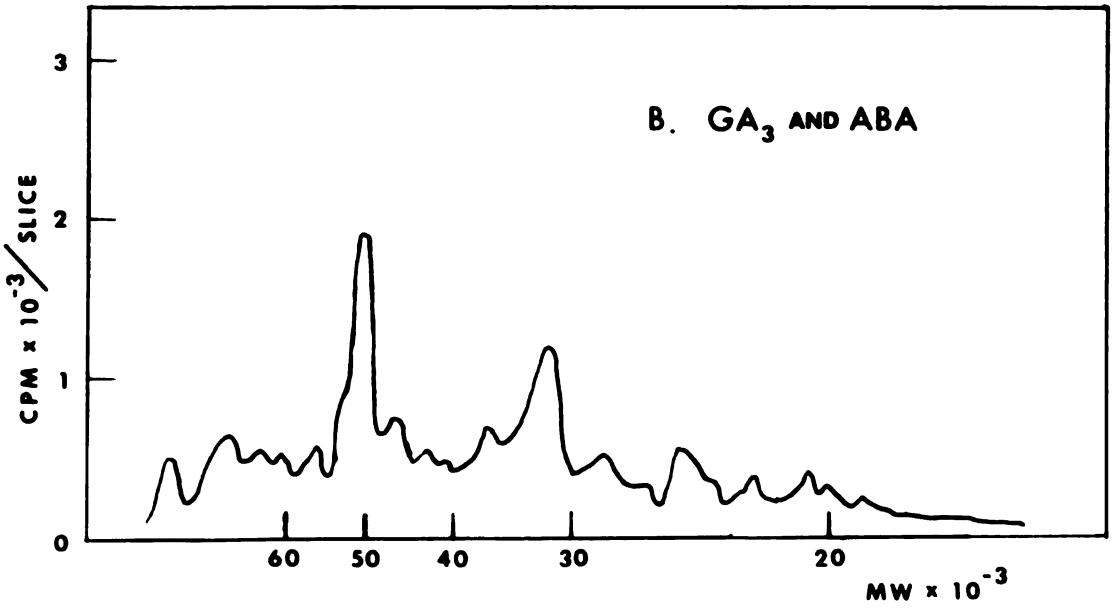
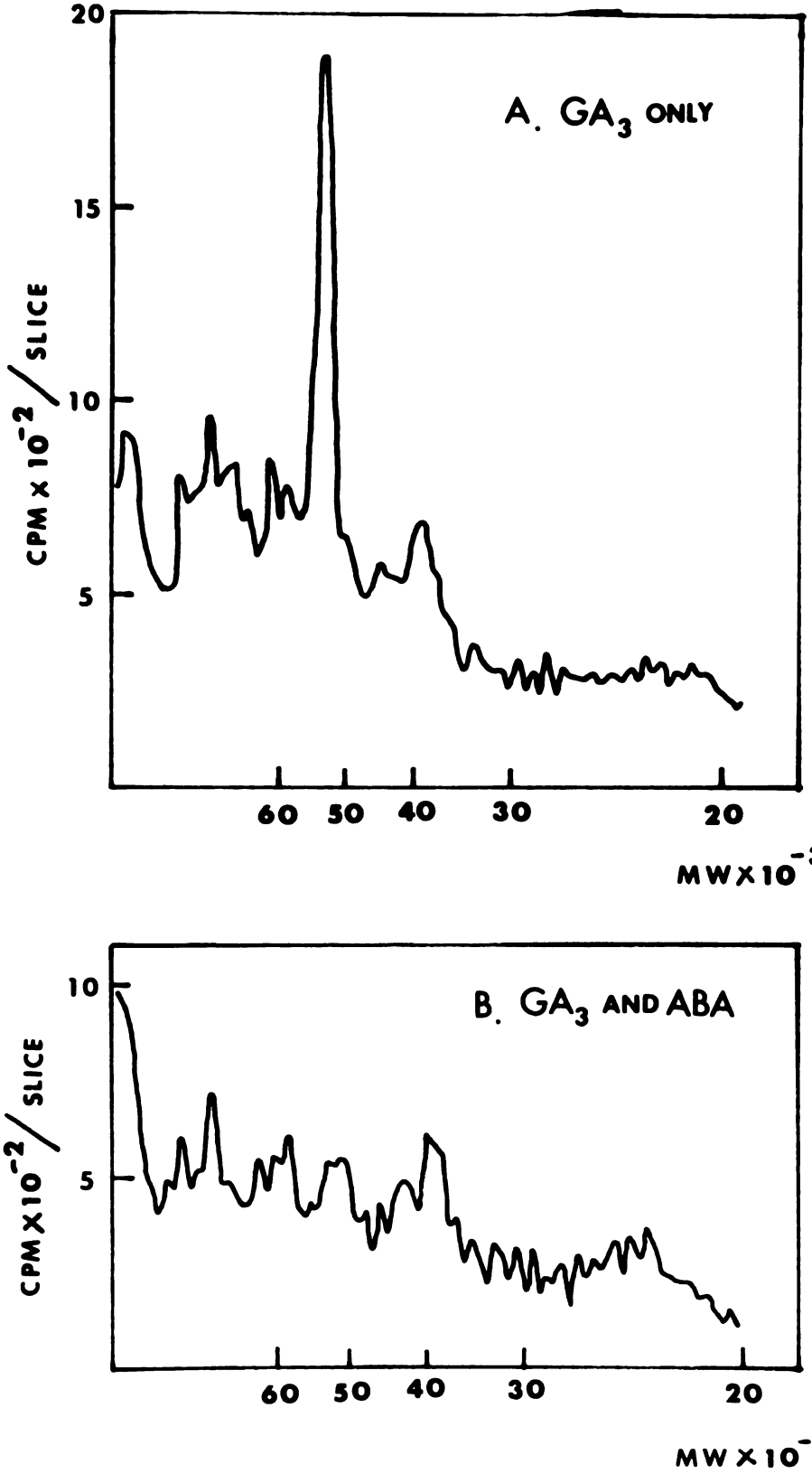

B. GA3 AND ABA
CPM x 10-3/SLICE
0
1
2
3
60
50
40
30
20
MW x 10-3

Figure 25.--Profile of Newly Synthesized Salt-Insoluble Proteins on SDS Gel.

Experimental methods were the same as described in Figure 24.


A. GA3 ONLY
CPM X 10-2 / SLICE
20
15
10
5
60
50
40
30
20
MW X 10-3
B. GA3 AND ABA
CPM X 10-2 / SLICE
10
5
60
50
40
30
20
MW X 10-3

The major predominant salt-insoluble protein (Figure 25) has a molecular weight of about 52,000 daltons, which is slightly larger than that of α-amylase. Whether this protein is a precursor of α-amylase or an α-amylase covalently linked to cell wall or certain membrane fractions is unknown.

DISCUSSION

Although the synthesis of α-amylase after 12 hours of GA_3 treatment is no longer subject to transcriptional control, the inhibitory effect of ABA on α-amylase production at this same sage apparently depends on the continuous synthesis of short-lived RNA (regulator-RNA in Figure 4). I propose that this regulator-RNA, or its translation product, can decrease the rate of translation of α-amylase mRNA without influencing protein synthesis in general. The mRNA of α-amylase is stable for at least 12 hours after exposure of the tissue to GA_3, and its stability is maintained in the presence of ABA.

Ihle and Dure (27), working with precociously germinating cotton embryos, obtained evidence that the translation of carboxypeptidase mRNA was inhibited by ABA. Because they found that actinomycin D prevented the ABA inhibition, they proposed that a suppressor molecule and had to be formed to bring about the ABA inhibition (27).

The action of ABA thus appears to be similar in the two systems, the precociously germinating cotton embryos where gibberellins probably have no regulatory role, and the mobilization of reserve nutrient in the germinating

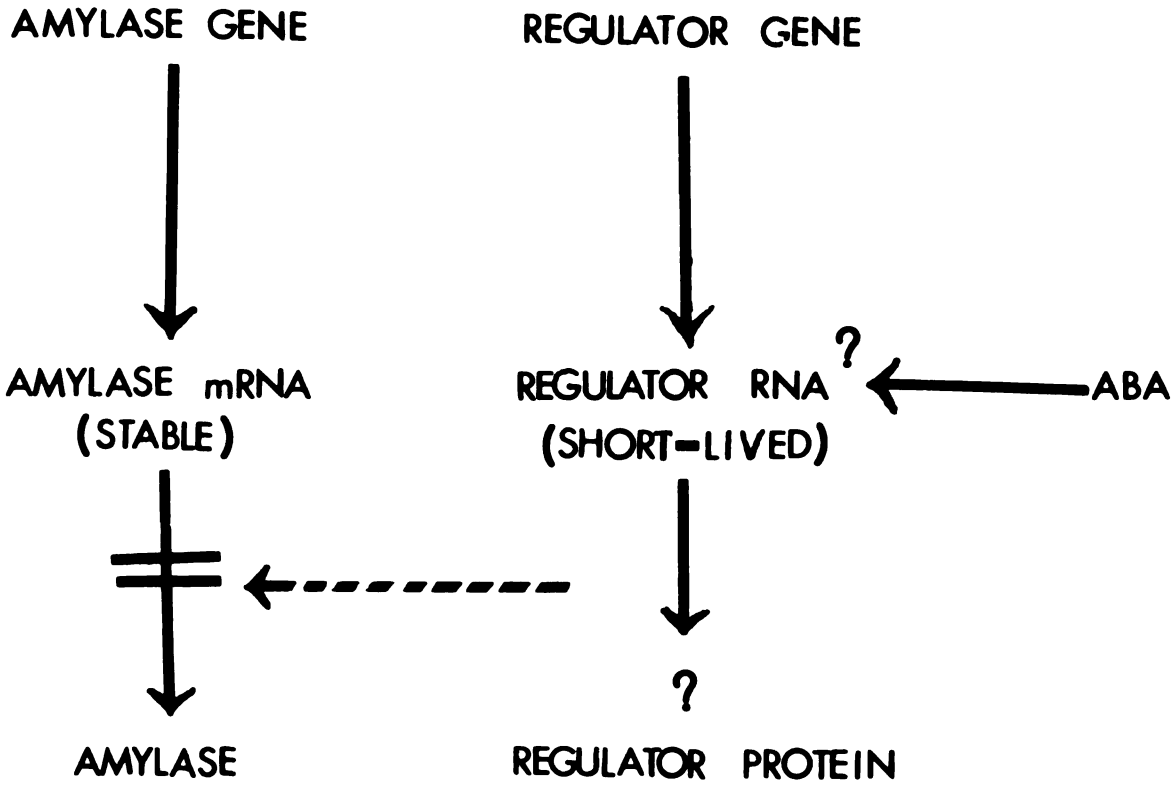
barley seed where ABA prevents the GA_3 enhanced α-amylase production in the aleurone cells. Therefore, it seems reasonable to suggest that some ABA effects depend on transcription although there is no evidence for a requirement of transcription in some fast effects of ABA such as that of preventing the activation of phosphorylcholine glyceride transferase (4) and that of stomatal closure (52).

There are two alternative sites of action for ABA (Figure26): 1) ABA might derepress the regulator-RNA and cause the synthesis of regulatory-RNA and/or regulator-protein, or 2) the regulator-RNA is under continuous turnover, ABA activates it or works with it to prevent the translation of α-amylase mRNA. In human reticulocytes a new species of low molecular weight RNA has been reported to be able to preferentially stimulate the synthesis of one of the globin chain (19,20).

The mechanism proposed here is by no means the only mode of action of ABA one might propose in barley aleurone cells. It has been shown that ABA prevents the GA_3 enhanced poly A-RNA synthesis (Part I) indicating besides the translational control ABA can also stress its effect on the transcriptional machinery before 12 hr of GA_3.

Because of the many convenient properties of barley aleurone tissue and because of the ability of ABA to modify the tissue's response to GA_3, as reported in this study, it is feelt that aleurone tissue is an important system

Figure 26.--Postulated Mechanism of ABA Action.


AMYLASE GENE
REGULATOR GENE
AMYLASE mRNA
(STABLE)
REGULATOR RNA
?
ABA
(SHORT-LIVED)
?
AMYLASE
REGULATOR PROTEIN

for the further study of how ABA modifies a tissue's response to GA_3.

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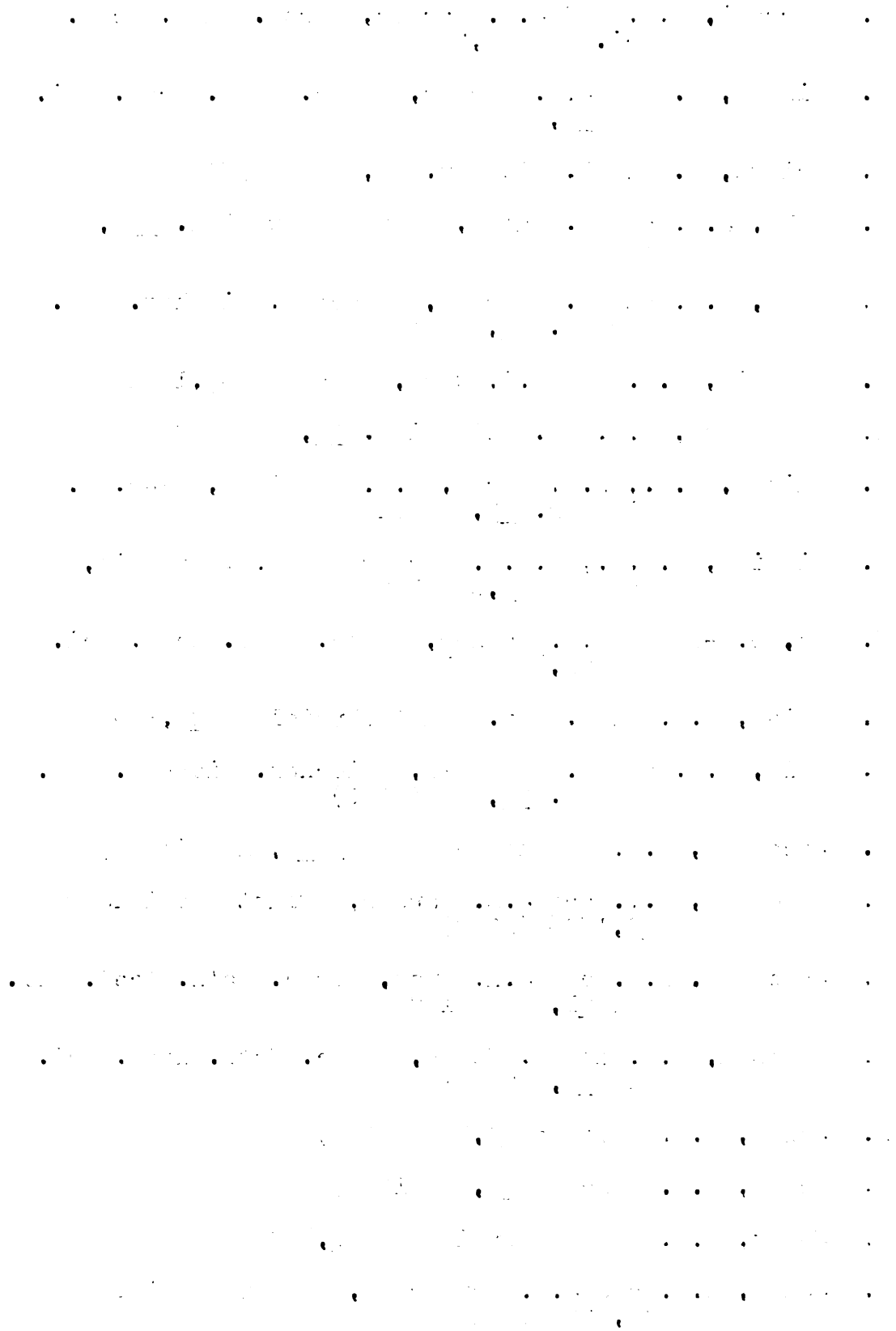

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