HORMONAL REGULATION OF PROTEIN SYNTHESIS IN BARLEY ALEURONE LAYERS

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This is to certify that the

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Major professor

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ABSTRACT

HORMONAL REGULATION OF PROTEIN SYNTHESIS
IN BARLEY ALEURONE LAYERS

By

Warren H. Evins

Exogenous addition of gibberellic acid (GA) to barley aleurone cells induces synthesis of α-amylase and other proteins following an 8 to 10 hour lag period. During the lag period endoplasmic reticulum (ER) synthesis and polyribosome formation occur, starting at 2 to 4 hours after hormone treatment. Indirect evidence suggests that the GA-induced protein synthesis occurs on ER membrane-bound polysomes and that the polysomes isolated are membrane-bound.

Polysome formation reaches a maximal level at 12 to 15 hours after hormone addition. A linear increase in the proportion of ribosomes present as polysomes is seen, reaching a maximum of 76% polysomes at 10 to 11 hours. Total polysomes increase over 2.5 fold, while the percent polysomes, total ribosomes, number of active ribosomes, and rate of protein synthesis double. Although the number of active ribosomes (ribosomes capable of synthesizing

nascent polypeptides measured by the formation of acid insoluble ^{3}H-peptidyl puromycin) doubles, the proportion of the total ribosomes that are active is not affected by the hormone. The rate of protein synthesis *in vivo* was measured with ^{14}C-amino acids.

The high tryptophan/tyrosine ratios of the bulk of the GA-induced proteins was used as a chemical tag to identify the polysomes isolated as the polysomes responsible for the synthesis of the induced enzymes. Polysomes isolated from hormone-treated cells and nascent polypeptides released by puromycin from these polysomes have higher tryptophan/tyrosine ratios than polysomes and nascent peptides isolated from control tissues.

Various treatments which inhibit synthesis of the hormone-induced enzymes inhibit polysome formation. The plant hormone abscisic acid (ABA) prevents GA-induced polysome formation. The removal of GA by washing or the mid-course addition of ABA (2.5×10^{-7} M) cause a 10 to 15% decrease in the percent polysomes within 2 hours. When ABA is added at the start of the incubation period with 1 μM GA, a 10% decrease in the percent polysomes and no polysome formation occur. Anaerobiosis and actinomycin D added at the start of the incubation period inhibit both α-amylase synthesis and polysome formation. Fluoro-uracil inhibits both GA responses to a lesser extent.

GA increases ER synthesis 4 to 8 fold, as measured by determining the amount of ^{14}C-choline incorporated into a lipid extractable, acid insoluble, semi-purified ER fraction.

6-Methylpurine does not inhibit the recovery of α-amylase synthesis when GA is added back to aleurone layers following GA removal. However, the application of 6-methylpurine to aleurone layers 6 hours prior to the readdition of GA (following GA removal) completely inhibits any recovery of α-amylase synthesis. The inhibition is reversed if 6-methylpurine is removed when GA is added back.

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LIST OF ABBREVIATIONS

The standard abbreviations used in "Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry" of the IUPAC-IUB Combined Commission on Biochemical Nomenclature, published in (1966) Biochemistry 5: 2485 are used in this thesis. Some of the more frequently used abbreviations are as follows:

ABA	abscisic acid
Act D	actinomycin D
ATP	adenosine triphosphate
c	curie
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane or (dichlorodiphenyltrichloroethane)
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
ER	endoplasmic reticulum
FU-5	fluoro-uracil
g	gravity
GA, -GA, + GA	Gibberellic acid, without GA, with GA
GB	grinding buffer
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid
lys	lysine
PEP	phospho-enol-pyruvate
POPOP	1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
RB	ribosomal buffer
RNA, m, r, t	ribonucleic acid, messenger, ribosomal, transfer
RNAase	ribonuclease
S	Svedberg unit (sedimentation coefficient)
TCA	trichloroacetic acid
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet

I

THE INDUCTION OF POLYSOME FORMATION AND OF TRYPTOPHAN-RICH PROTEINS BY GIBBERELLIC ACID

Introduction

The barley aleurone is a highly specialized tissue of non-dividing cells of a single type in which the production of certain enzymes is dependent on the plant hormone gibberellic acid (GA) (Varner *et al.*, 1965; Crispeels and Varner, 1967a) and with the exception of abscisic acid (ABA) not influenced by other plant hormones (J. E. Varner, unpublished observations; Cleland and McCombs, 1965). The aleurone tissue produces and releases enzymes that degrade the starchy food reserves of the endosperm to supply the embryo with energy and metabolites necessary for the development of the young seedling. These enzyme activities appear or increase in response to GA, and include: α- and β-amylases, catalase, β-hydroxymethyl cellulase, endo-β-glucanase, endopentosanase, peptidases, protease, RNAase, transaminase, etc. (Brian, 1966; Chrispeels and Varner, 1967a; Paleg, 1960; Yomo 1960a,b,c,d). The increases in α-amylase and

protease have been shown to be due to de novo synthesis. They occur, however, only after an 8 to 10 hour lag period following GA addition (Chrispeels and Varner, 1967a; Filner and Varner, 1967; Jacobsen and Varner, 1967).

Two possible control points for GA action are transcription and translation. The evidence that transcription of new RNAs is required for the hormone response is inconclusive. Washing out GA arrests α-amylase synthesis (Chrispeels and Varner, 1967b) so that GA does not act only as a trigger, possibly indicating a requirement for continuous RNA synthesis. Inhibition of α-amylase production by 6-methylpurine, an inhibitor of RNA synthesis (Chrispeels and Varner, 1967b) may be due to an effect on the level or action of some nucleotide cofactor. Act D inhibits enzyme synthesis when it is added during the lag period, although addition after the lag period inhibits secretion (Chrispeels and Varner, 1967b). However, Act D has effects other than the inhibition of RNA synthesis, such as the inhibition of phospholipid synthesis (Pastan and Friedman, 1968).

Two observations suggest that GA might act during translation. First, the rate of α-amylase synthesis depends on the GA concentration in the range of 10^{-7} to 10^{-11} M. Another lag period occurs before the rate of α-amylase synthesis increases following an increase in GA concentration (Chrispeels and Varner, 1967a,b).

Second, although GA induces enzyme synthesis, it was reported that there is no increase in the rate of total protein synthesis at 12 hours following hormone treatment (Varner et al., 1965).

These results suggested further investigation of the possible involvement of GA in one of the steps of translation. One approach is to find out what is happening during the 8 to 10 hour lag period before α-amylase production starts. Perhaps some fundamental biochemical parameter responds to GA earlier than α-amylase synthesis.

In addition, it now appears that the previous measurements of protein synthesis were low because of isotope dilution and proteolysis. Two ways to check the increase in protein synthesis not dependent on isotope dilution are to measure the polysomal levels after various treatments and to determine the number of active ribosomes.

I now report that polysome formation starts 3 to 4 hours after hormone treatment. The rate of protein synthesis (^{14}C-amino acid incorporation) doubles toward the end of the lag period and the number of ribosomes increases. The hormone has no effect on the proportion of ribosomes active in protein synthesis. The polysomes are probably bound to the endoplasmic reticulum. The trp-tyr ratio of nascent peptides [α-amylase and other GA-induced proteins are trp-rich (Fischer and Stein, 1960; J. E. Varner, unpublished observations)] was used as a

characteristic identification tag to show that the polysomes were responsible for synthesis of the GA-induced proteins.

Methods

Incubation

Barley (Hordeum vulgare var. Himalaya, supplied by Drs. R. A. Nilan, B. V. Conger, and the Agronomy Club at Washington State University, Pullman) half-seeds were prepared by making 2 transverse incisions, one incision removing the embryo and the other removing the distal tip. The half-seeds were sterilized in about 0.5 ml/half-seed of a sodium hypochlorite solution (reagent grade, diluted 1:5, 4-6+ % NaOCl) in groups of 70-120 half-seeds. After 25 minutes, the solution was stirred, and rinsed 6 times with copious amounts of sterile distilled H_2O. The half-seeds were preincubated 3 days on moist sterile sand in Petri dishes wrapped in aluminum foil. Two sterile stainless steel spatulas, one with flexible rounded ends, and the other with a rigid square blade, were used to dissect the aleurone layers from the starchy endosperm. All operations were performed in a sterile hood equipped with a UV light.

Between 30 and 40 aleurone layers were shaken on a Dubnoff metabolic shaker with 5 ml of incubation medium containing 1 m$\underline{M}$ Na acetate, pH 4.8, 20 m$\underline{M}$ $CaCl_2$, and

where required, 1 $\mu\underline{M}$ K^+ gibberellic acid (GA_3), in a 50 ml flask (approximately 80 to 100 oscillations/minute, 25°). At the end of the incubation period, the layers were rinsed 6 times with copious volumes of sterile H_2O and blotted on sterile paper towels. All further operations were carried out in the cold room. Care was taken not to warm the homogenate with the hands. All pipets and glassware were handled with plastic gloves and not touched where they would be in contact with the layers or cell fractions, in order to prevent contamination with ribonoclease which might cause degradation of polysomes.

Polyribosome Isolation

The procedures of Wettstein, Staehelin, and Noll (1963, 1964) as modified using ideas of Leaver and Key (1967) were used for polysome isolation. A prechilled (-20°) porcelain mortar was half-filled with liquid N_2 and the layers were added. After the liquid N_2 evaporated, the layers were powdered by rapid grinding. The layers were transferred to a chilled homogenizer in an ice bucket (Duall tissue grinder, size E, Kontes Glass Co., Vineland, N.J., empty capacity without pestle approximately 125 ml), which had previously been hand ground with medium, fine, then very fine abrasive (Zip grinding compound, Zip Abrasive Co., Cleveland). As polysome distributions differed with the amount of grinding used to prepare the homogenizer, the same homogenizer was used for all samples.

Four ml of "GB" [grinding buffer, containing: 450 m$\underline{M}$ sucrose, 100 m$\underline{M}$ HEPES, pH 7.55, with 50 m$\underline{M}$ K^+, 2 m$\underline{M}$ Mg acetate, 7 m$\underline{M}$ 2-mercaptoethanol (0.5 µl/ml total volume)] was then added to the homogenizer. The powder was allowed to thaw for 5 minutes and then ground with 2 to 3 strokes and 3 to 5 quarter turns/stroke to the same "feel" and visible consistency. The homogenate was decanted into a cold centrifuge tube. The homogenizer and pestle were rinsed twice with 3 ml of GB.

The homogenate was centrifuged at 0° in the Sorvall SS-34 rotor (2 k$\underline{g}$ for 10 minutes). The supernatant was decanted and spun at 10 k$\underline{g}$ for 15 minutes (Method 2). (Earlier studies used centrifugal spins of 5 and then 27 k$\underline{g}$ (Method 1), but greater polysome recovery and a greater detergent effect are seen with the slower centrifugations.) The supernatant was centrifuged 2 hours through a discontinuous sucrose gradient in the Beckman 65 rotor at full speed. The discontinuous gradient was composed of a bottom layer of 3.5 ml of 1.6 $\underline{M}$ sucrose buffer [RNAase-free sucrose, Mann Research Laboratories, New York, containing: 50 m$\underline{M}$ HEPES, pH 7.55, with 25 m$\underline{M}$ K^+, 2 m$\underline{M}$ Mg acetate, and 7 m$\underline{M}$ 2-mercaptoethanol (0.5 µl/ml total volume)], a middle layer of 3.0 ml of 0.6 $\underline{M}$ sucrose buffer, and the top layer being the 10 k$\underline{g}$ supernatant in 0.45 $\underline{M}$ sucrose. The pellet produced will be referred to as the polysomal pellet.

The polysomal pellet was resuspended in 0.3 ml "RB" (ribosomal buffer, same as above sucrose buffers, but without any sucrose) with a ground glass pestle (Pyrex no. 7725, supplied by Sargent-Welch Scientific Supply Co., Chicago).

Polysome Sedimentation

The resuspended polysomal pellets were layered on 0.3-1.0 M isokinetic sucrose gradients as described by Noll (1967). Samples were centrifuged at 0° in Beckman SW65 or SW56 rotors at full speed. The 4.8 ml (with sample added) gradients were centrifuged for 33 minutes in the SW65 rotor, while 40 minutes were required to centrifuge 3.8 ml gradients spun in the SW56 rotor.

After centrifugation the bottom of the polyallomer tube was punctured by means of a puncturing device (J&I Technical Specialties, 1535 Cornelia Ave., Waukegan, Illinois 60085). The gradient was pumped through a Helma straight-through 4 mm flow cell in a Gilford spectrophotometer as described by Noll (1969). Polysome profiles were obtained by recording the absorbancy of material in the gradient at 260 nm on the Gilford-Honeywell recorder. (The scan speed was 1 in/min and the pump flow rate was 0.8 ml/min.)

Polysome Quantitation

The areas of the polysome and monosome regions of the absorbancy scans were determined with a Keuffel

and Esser planimeter graduated in area units of 0.1 square cm. The areas were measured 3 times and averaged. In most samples, 40 aleurone layers were used and the absorbancy scans were recorded at 0.750 A_{260} units as full scale absorbancy. However, the number of aleurone layers and the full scale absorbancy used for recording the various scans are not the same in all experiments. Therefore, all areas are expressed as the number of area units (0.1 cm^2) per 100 aleurone layers, recorded when using 1.0 absorbancy units as the full scale chart deflection (approximately 0.0025 A_{260} units/area unit).

The polysomal distribution or the "percent polysomes" was calculated by dividing the area of the polysome (P) region of the sucrose gradient scans by the sum of the areas of the polysomal and monosomal (M) regions (% P/P+M).

RNA Determination

RNA was determined by the spectrophotometric method of Warburg and Christian (1942) in the Cary 15 double-beam spectrophotometer using an aliquot of the resuspended polysomal pellets. The results are expressed as µg RNA per 100 aleurone layers.

Amino Acid Incorporation

Incorporation of ^{14}C-amino acid was performed with 10 aleurone layers and 0.5 µc of a mixture of 15 ^{14}C-uniformly labeled amino acids (reconstituted algal protein

hydrolysate, Volk Radiochemical Corp. or International Chemical and Nuclear Corp., Irvine, Cal.). Carrier-free amino acid was added to the aleurone layers at the start of the incubation period. The aleurone layers were ground and the homogenate of the aleurone layers and the medium were prepared as described by Chrispeels and Varner (1967a). In one experiment, an ethanol extract was prepared by washing the pellets 3 times with 1 ml of 70% ethanol and combining the washings. Carrier bovine serum albumen (crystalline, A grade, Calbiochem, Los Angeles, Cal.) was added to each fraction (25 µg). The homogenate, medium, and extract were brought to "10%" TCA by the addition of 50% (w:v) TCA and allowed to sit overnight in the cold room. The precipitate was collected on Millipore filters (0.45 µ, 25 mm diameter, Millipore Filter Corp., Bedford, Mass.), washed with at least 30 ml of 5% TCA containing carrier amino acids (neutralized casein acid hydrolysate, Calbiochem), dried for several hours at 70°, and counted with 10 ml of scintillation fluid "A" (4g PPO + 100 mg POPOP per 1 of toluene) in a Beckman scintillation counter.

In one experiment, amino acid incorporation was determined in the presence of carrier amino acids. Tryptophan, phenylalanine, and cysteine were added to a mixture of the 17 other protein amino acids to make a freshly prepared solution. Each amino acid (A grade, Calbiochem) was present at a concentration of 5×10^{-4} M.

Trp/Tyr Ratio Determination

One μc of L-^{14}C-tyr (uniform label, New England Nuclear Corp., Boston, sp. act. 362 mc/mmole) and 5 μc of L-^{3}H-trp (general label, New England Nuclear Corp., sp. act. 5.4 c/mmole, supplied in 50% ethanol solution) were added to the aleurone layers for 2 hours, beginning 8 hours after the start of incubation. In some experiments, the labels were present for all 10 hours. Cell fractions were prepared as described above (see amino acid incorporation).

In another series of experiments, a 16 minute pulse of labeled trp and tyr was given to the aleurone layers so that the aleurone layers were incubated a total of 10 hours. ^{3}H-trp and ^{14}C-tyr were used to determine the trp/tyr ratio. The reverse experiment was also performed using L-trp-3-^{14}C (side chain label, New England Nuclear Corp., sp. act. 22.8 mc/mmole) and L-tyr-3,5-^{3}H (New England Nuclear Corp., sp. act. 16.5 c/mmole, supplied in sterile 50% ethanol solution). The pulse was given in order to label nascent peptides that are attached to the polysomes. At 9 hours 45 minutes after the start of incubation, the aleurone layers were transferred to a clean, sterile 25 ml erlenmeyer flask after rinsing 3 times with sterile H_2O and blotting on sterile paper towels. One hundred μl of a mixture containing the carrier-free trp and tyr labels and incubation medium ±GA was spread evenly over the 40 aleurone layers. All

transfer operations were performed in the sterile transfer hood. The flask was replaced on the Dubnoff metabolic shaker at 25°. It was removed from the shaker after the labeling period, rinsed 6 times with sterile H_2O, and the layers were blotted. The polysomal pellet was prepared as described.

The polysomal pellet was resuspended in 0.2 ml of RB and 0.15 µl of a 10^{-2} M stock solution of puromycin (nutritional Biochemical Corp., Cleveland) was added (7.5×10^{-4} M). The suspension was allowed to react with the puromycin for 30 minutes at 0° in an ice bucket as described by Redman and Sabatini (1966) and Redman (1967).

The suspension was layered onto a discontinuous sucrose buffer gradient and spun in the Beckman SW65 or SW56 rotors at full speed for 2 hours. The discontinuous gradient consisted of a bottom layer of 1.6 M sucrose buffer, a middle layer of 0.3 M sucrose buffer, and the sample in buffer alone. The middle layer was 2.0 ml in the SW56 rotor tube, and 2.5 ml in the SW65 rotor tube; the total volumes were 3.8 and 4.8 ml, respectively.

After centrifugation, the top 2 layers were removed with a disposable Pasteur pipet, the bottom layer was decanted, and the pellet was taken up in 5% TCA. The top and bottom supernatant fractions were counted with 18 ml of Bray's scintillation fluid (Bray, 1960) in the Beckman liquid scintillation counter. The pellet was

collected on a Millipore filter, washed with 25 ml of 5% TCA, dried for several hours at 70°, and counted with 10 ml of scintillation fluid "A".

In some experiments, 5×10^{-4} M L-trp and 5×10^{-4} M L-tyr were added to the first discontinuous sucrose ultracentrifugal gradients before preparation to the polysomal pellet. These carrier amino acids were added in order to remove some of the background counts present in the supernatant fractions.

Polysomal RNA Preparation

The polysomal pellet was resuspended with a ground glass homogenizer in 0.5 volumes of 0.1 M EDTA · Na_2 and 0.5 volumes of RB + 2-mercaptoethanol (total volume 0.3 ml) and was incubated for 30 minutes with EDTA at 0°. No further release of polysomal RNA occurs after this time.

The suspension was layered onto continuous 15-30% isokinetic 10 mM EDTA · Na_2-sucrose buffer gradients and spun in the SW65 or SW56 rotors of the Beckman ultracentrifuge at full speed. A spin of 3.75 hours was used to separate the heavy and light ribosomal subunits from 5 S and tRNA. In order to separate the mRNA region, the heavy ribosomal subunit was pelleted during 9-13 hour runs. The sucrose gradients were prepared by the Noll method using 15% and 37% sucrose buffer-EDTA solutions.

The tube was punctured as described and scanned in the Gilford spectrophotometer. Fractions were collected with an LKB fraction collector or by hand. Twenty-five µg of carrier DNA was added to each fraction, followed by 50% (w:v) TCA to a final concentration of 10%. The sample was allowed to sit in the cold room overnight. The precipitate was collected on Millipore filters washed with at least 30 ml of 5% TCA with 0.1 M carrier phosphate, dried at 70°, and counted in the Beckman scintillation counter.

Formation of ^{3}H-peptidyl Puromycin

The first step in the assay of the number of active ribosomes is the reaction between ^{3}H-puromycin and the polysome-bound nascent peptidyl tRNA. The methods of Wool and Kurihara (1967) were used. Ribosomes isolated from aleurone cells were incubated at 37° in a reaction mixture (0.500 ml total volume) that contained: 37.7 µM HEPES, pH 7.55, with 18.8 µM K^{+}, 50 µM Mg acetate, 7.3 mM 2-mercaptoethanol (0.5 µl/ml total volume), 5 mM ATP, 50 µM GTP, 1 mM PEP, and 10 µg pyruvate kinase. In most experiments 5 µc of puromycin (puromycin methoxy-^{3}H (N)-dihydrochloride, specific activity 1.11 c/mmole) was used. The reaction was terminated by the addition of 5 ml of 10% TCA, filtered on a Millipore filter, and washed with 50 ml of 5% TCA containing 5×10^{-5} M carrier puromycin · HCl. The filter was dried at 70°, and counted with 10 ml

of scintillation fluid in the Beckman scintillation counter. Most of the control experiments performed by Wool and Kurihara with rat muscle ribosomes were repeated with aleurone layer ribosomes.

The amount of RNA present in each of the resuspended polysomal pellets assayed for active ribosomes was measured spectrophotometrically by A_{260}/A_{280} ratios as previously described. This allowed the determination of the specific activity of active ribosomes or the cpm ^{3}H-peptidyl puromycin formed per µg RNA.

The following parameters were determined or calculated: (a) total number of ribosomes, (b) total molecules of peptidyl puromycin formed, and (c) the ratio (b)/(a) to obtain the molecules ^{3}H-peptidyl puromycin/ribosome from which the number or percent active ribosomes is easily obtained.

Results

Protein Synthesis

The incorporation of a carrier-free mixture of ^{14}C-amino acids was measured in order to estimate the rate of protein synthesis (Table 1). At all times, there is more labeled protein in the homogenates of the control samples; whereas, the hormone treated samples have more labeled protein in the medium. After 8 hours of

TABLE 1

The Effect of GA on ^{14}C-Amino Acid Incorporation by Barley Aleurone Layers*

Time of Incubation	Treatment	cpm (x 10^{-3}) ^{14}C-Amino Acid Incorporated Into TCA Precipitable Material			
		Total	Medium	Homogenate	Extract
8 hours	+GA	261.1	194.8	66.3	. .
	-GA	129.3	17.3	112.0	. .
16 hours	+GA	141.6	72.2	69.5	. .
	-GA	157.7	51.8	105.8	. .
24 hours	+GA	132.0	91.4	40.6	. .
	-GA	198.4	46.2	152.2	. .
8 hours, with carrier amino acids	+GA	168.7	13.0	91.1	64.6
	-GA	140.2	11.8	70.2	58.1

*Duplicate samples of 10 barley aleurone layers each were incubated at 25° without carrier amino acids and 0.5 µc carrier-free reconstituted algal protein hydrolysate ^{14}C-amino acid mixture (15 amino acids), or with the addition of a mixture containing 5 x 10^{-4} M of each of 20 amino acids and 2 µc ^{14}C-amino acid mixture in 1 mM Na acetate, pH 4.8 buffer, 20 mM $CaCl_2$, and 10^{-6} M GA_3 if +GA, total volume 2 ml.

incubation 2.0 times more label is incorporated into TCA-precipitable material in the GA-treated tissue. At 16 hours, there are more total cpm of TCA-precipitable ^{14}C-amino acid mixture incorporated into the control tissue because of increasing protein turnover and amino acid pool sites in the hormone-treated samples. The total cpm incorporated continues to increase at 24 hours in the absence of the hormone, but decreases further with GA.

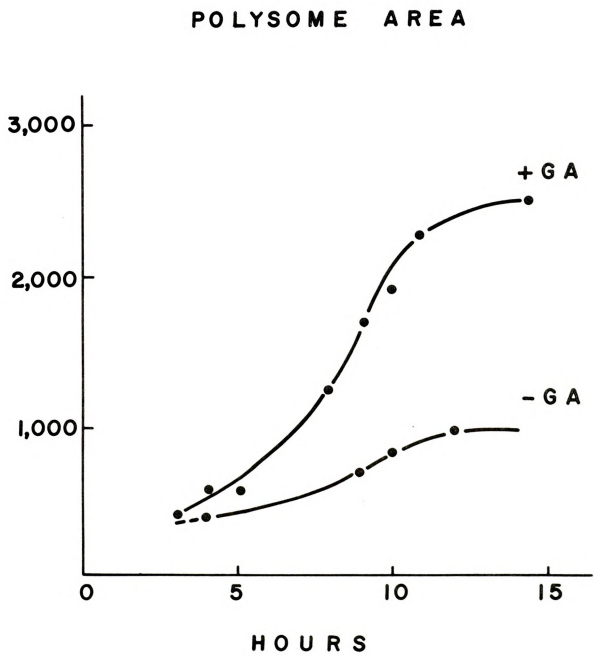
It seems that the increase in protein synthesis due to GA can be seen only at early times.

Other experiments were performed with carrier amino acids present. Each of the 20 common amino acids was present at a concentration of 5×10^{-4} M. Four times as much label was added as in the previous experiments. In addition, the 70% ethanol extract was isolated and counted. After 8 hours of incubation, there were more cpm of TCA-precipitable ^{14}C-amino acids incorporated in the presence of GA.

Polysome Formation

More polysomes can be isolated from GA-treated aleurone layers than from the control layers (Figure 1). Polysome formation starts at 3-4 hours of incubation and continues until 12-15 hours both in the presence and absence of GA. The increase in control tissues may be

Figure 1.--The effect of GA on polysome formation. [The absolute amount of polysomes/100 barley aleurone layers was determined by centrifuging ribosomes in 0.3 to 1.0 M isokinetic sucrose gradients. Forty aleurone layers were incubated at 25° for various times in 1 mM acetate buffer pH 4.8 with 20 mM $CaCl_2$ and either +GA = 1 μM gibberellic acid (GA_3) or −GA = without GA. The polysome area was measured with a planimeter. Area measurements are in relative units. Each point represents the average of duplicate samples.]


POLYSOME AREA
3,000
2,000
1,000
+ GA
- GA
0
5
10
15
HOURS

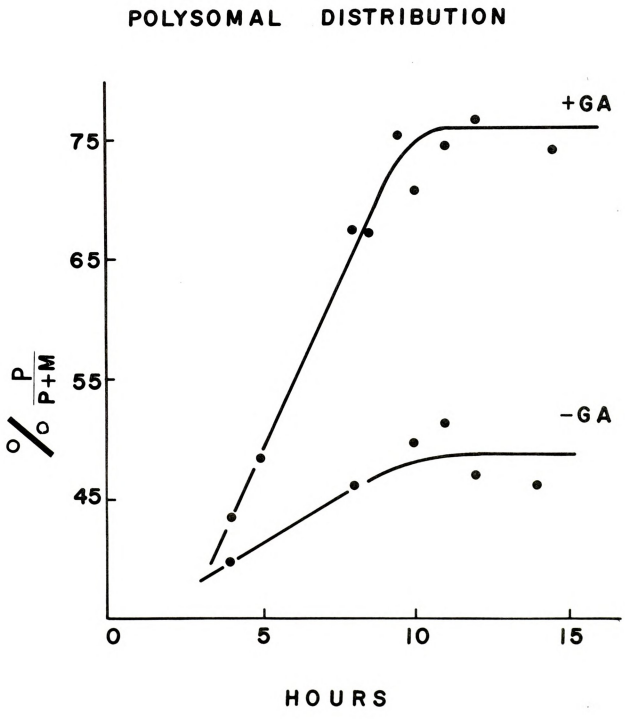
due to endogenous gibberellins. At longer times of incubation, the amount of polysomes found decreases from the maximal levels seen at 12-15 hours in both the control and hormone-treated tissues. Over 2.5 times more polysomes were found after 12 hours of hormone treatment. Polysome formation appears to follow a sigmoid curve.

Polysomal Distribution

Not only does GA increase polysome formation, but it also causes an increase in the distribution of ribosomes in polysomes or the percent polysomes (Figure 2). A 10% increase in the proportion of ribosomes in polysomes occurs in the control tissue. However, the proportion of ribosomes in polysomes in the hormone-treated tissue more than doubles by 10 hours after the start of incubation. The percent polysomes increases linearly starting at 3-4 hours, i.e., at the same time that polysome formation starts (Figure 1). A maximum level of about 76% polysomes is reached at approximately 10 hours after the start of incubation. At longer times of incubation, the percent polysomes decreases as does the amount of polysomes found.

The percent polysomes in a preparation also depends on the clearance between the homogenizer and pestle used for homogenization of the powdered aleurone layer. The homogenizer was hand ground to an empirically determined clearance and then tested experimentally. One homogenizer

Figure 2.--The distribution of ribosomes in polyribosomes. [The distribution of ribosomes in polyribosomes was determined in the presence and absence of 1 μM GA_3. The polysomal distribution % (P/P+M) was calculated by determining the area of the polyribosome (P) and monosome (M) regions of the A_{260} profile with a planimeter. Each point represents the average of duplicate samples.]


POLYSOMAL DISTRIBUTION
+GA
-GA
75
65
55
45
% P/P+M
0
5
10
15
HOURS

yielded a maximum of 86% polysomes. The samples plotted in Figures 1 and 2 were prepared with the same homogenizer.

Total Ribosomes

When examined in the electron microscope the polysomal pellet is relatively clean and contains ribosomes and membranes (examination performed with the help of Dr. Gordon Spink, Mrs. Rhoda Papaioannou, and Dr. William V. Dashek). The ratio of A_{260}/A_{280} absorbancy was 1.7-1.9. A typical ribosomal absorbancy profile is usually seen when the resuspended polysomal pellet is scanned in the Cary spectrophotometer.

A number of short term labeling experiments were performed with carrier-free ^{32}P-ortho-phosphoric acid in order to measure the rate of ribosome synthesis. ^{32}P was present during the last 30 to 60 minutes of incubation. ^{32}P (1.5-2.5 mc) in very small volumes of incubation medium (±GA) was evenly distributed over the aleurone layers. Polysomal RNA was prepared as described. Incorporation of ^{32}P occurs into RNAase digestible RNA throughout the period of polysome formation, both in the control and GA-treated tissue. Most of the ^{32}P counts incorporated appear in isolated rRNA. The amount of ^{32}P incorporated into isolated rRNA was greater in GA-treated rather than control tissue. Therefore, in the presence of the hormone, more ribosome synthesis occurs.

Spectrophotometric measurements of the amount of RNA present in the polysomal pellet show an increase, both in the control and hormone-treated tissues as shown in Figure 3. This increase represents primarily the synthesis of new ribosomes. The control tissue shows an increase of about 25%. At longer times of incubation, there are fewer ribosomes present in the control tissue than before incubation. In the presence of the hormone, the amount of ribosomes almost doubles. The maximum number of ribosomes is present about 12 hours. The effect of ribosome turnover increases after 12 hours as a decrease in the amount of ribosomes is seen in the presence of the hormone. The +GA curve is sigmoidal.

Effects of RNAase

Typical polysome profiles are obtained after 8 hours of incubation with 10^{-6} M GA (Figure 4a) or in the absence of GA (Figure 4b) using Method 1 (see Methods). The polysome profiles obtained after 8 hours of incubation with 10^{-6} M hormone (Figure 5a) or in the absence of the hormone (Figure 5b) improve with Method 2 (see Methods). The latter isolation procedure increases the percent of the absorbing material recovered in the polysomal region and reduces the ratio of maximal monosome/polysome peak heights.

Figure 3.--The effect of GA on the total number of ribosomes present in the polysomal pellet. [The μg RNA/100 aleurone layers was determined spectrophotometrically using the method of Warburg and Christian (1942) by measuring the A_{260}/A_{280} ratios of ribonuclease sensitive material in the polysomal pellet.]

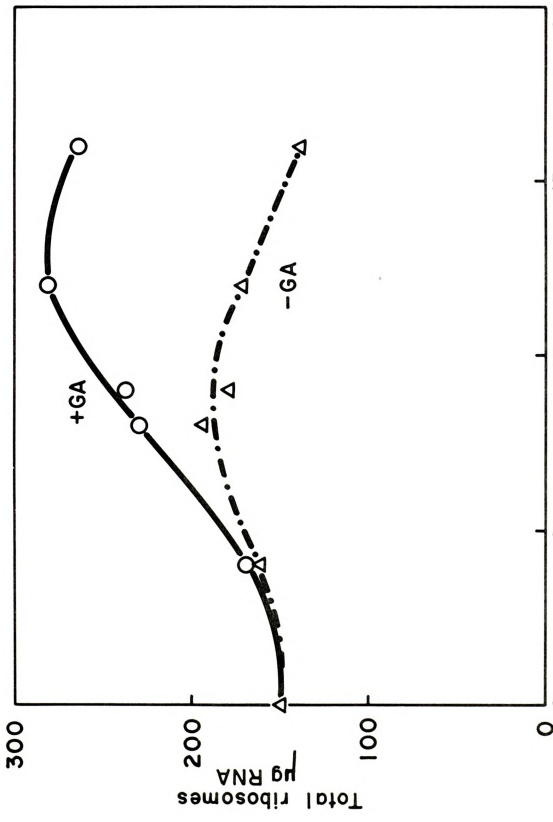
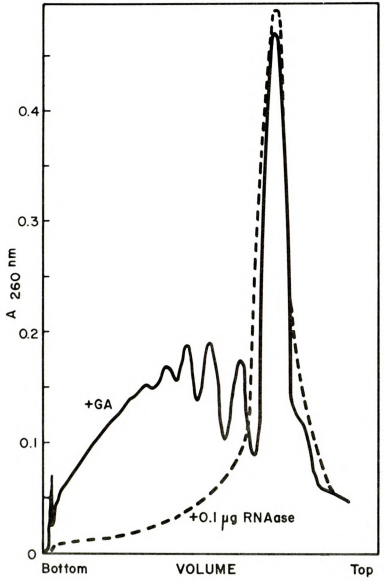

Total ribosomes
µg RNA
300
200
100
0
0
5
10
15
20
Time (hrs)
+GA
-GA

Figure 4.--Typical polysome profiles (older isolation method) and the effect of RNAase. [Polysomes were isolated from 40 barley aleurone layers incubated for 8 hours at 25° using the older isolation procedure with a 24 to 27 kg second centrifugal spin. The polysome profiles were determined by pelleting the polysomes through 0.3 to 1.0 M isokinetic sucrose gradients: (a) in the presence of 1 μM GA alone = +GA, and following 5 minutes incubation with 0.1 μg RNAase at 37° = +RNAase, or (b) without GA = -GA.


0.4
0.3
A 260 nm
0.2
0.1
0
+GA
+0.1 µg RNAase
Bottom
VOLUME
Top

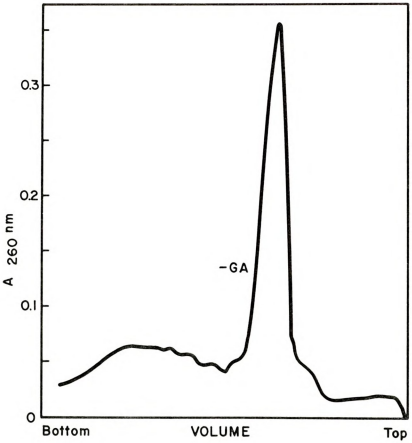
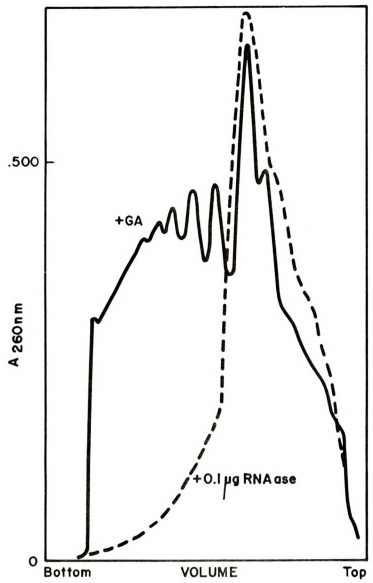
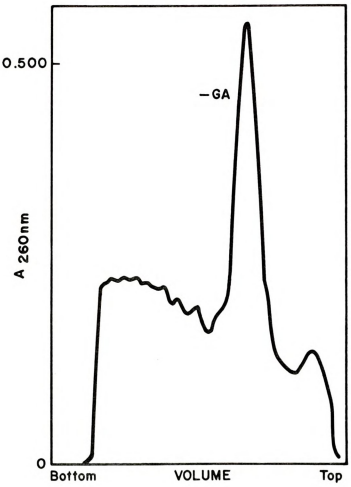

0.3
A 260 nm
0.2
0.1
0
-GA
Bottom
VOLUME
Top

Figure 5.--Typical polysome profiles (newer isolation method) and the effect of RNAase. [Polysomes were isolated from 40 barley aleurone layers incubated for 8 hours at 25° using the newer isolation procedure with centrifugal spins (see Methods) of 4 kg and 10 kg. The polysomal profiles were determined by pelleting the polysomes through 0.3 to 1.0 M isokinetic sucrose gradients: (a) in the presence of 1 μM GA alone = +GA, and following 5 minutes incubation with 0.1 μg RNAase at 37° = +RNAase, or (b) without GA = -GA.


.500
0
A 260nm
+GA
+0.1 µg RNAase
Bottom
VOLUME
Top


0.500
0
A 260nm
-GA
Bottom
VOLUME
Top

After 5 minutes of incubation with 0.1 μg of pancreatic RNAase at 37°, the polysomes are entirely degraded (Figures 4a and 5a). Similar degradation occurs in control samples.

Is the lack of polysomes in the absence of GA due to a greater degree of polysome degradation when the hormone is not present? In order to answer this question, mixing experiments were performed using polysomes isolated from rat livers, and polysomes isolated from aleurone layers incubated for 8 hours in the presence or absence of 10^{-6} M GA. Rat liver polysomes were isolated following the methods of Wettstein, Staehelin, and Noll (1963, 1964), concentrated, and stored in small aliquots at -70°.

A substantial amount of degradation occurs when polysomes isolated from aleurone layers and rat livers are mixed and allowed to sit for short times at 0° (Table 2). It is reasonable to suspect that there is more RNAase present in polysome preparations from aleurone cells treated with the hormone as RNAase is a GA-induced enzyme (Chrispeels and Varner, 1967b). Significantly fewer polysomes are recovered when +GA polysomes are mixed with rat liver polysomes than when -GA polysomes are mixed with rat liver polysomes.

The polysomal distribution seen after mixing polysomes isolated from rat livers and non-treated aleurone

TABLE 2

Recovery of Polysomes from Mixtures of Rat Liver and Aleurone Layer Ribosomes*

	Polysome Area*	% $\frac{\text{Polysomes}}{\text{Polysomes \& Monosomes}}$	Polysome Distribution Due to Rat Liver Polysomes	Area of Rat Liver Polysome	% of Rat Liver Polysomes Recovered
Rat Liver Polysomes	1244	84.5	. .	1244	100.0
8h + GA + RLP	986	59.0	-4.0	89	7.2
8h - GA + RLP	880	61.0	+6.8	298	24.0
8h + GA	897	63.0	. .		
8h - GA	582	54.2	. .		

*Duplicate samples of 40 barley aleurone layers (±GA), of 20 barley aleurone layers + rat liver polysomes (+ GA + RLP), or of 2X rat liver polysomes (RLP) were incubated at 25° in the presence and absence of 1 μM GA_3. The polysome and monosome areas were determined by planimetry.

cells shows an increase compared to the distribution in the control aleurone tissue. However, when rat liver polysomes are mixed with hormone-treated aleurone layer polysomes, the resulting polysomal distribution is lower than that in the unmixed aleurone tissue. This reduction is due to the high percentage breakdown of polysomes and the consequent increase in monosomes. It is clear that there is greater polysome breakdown in polysomes from the hormone-treated tissue. Therefore, the lack of polysomes in the control samples is not due to greater polysome degradation.

Sedimentation Coefficients

In order to further characterize the polysomes and determine the size and number of ribosomes present in the polysomal peaks the sedimentation coefficients of the barley polysomes, monosomes, and large ribosomal subunit (Table 3) were determined by comparison with the known sedimentation values of the rat liver polysomes, monosome, and large ribosomal subunit (Noll, 1967) by the method of Stutz and Rawson (1968). Calibration of the isokinetic gradients used with rat liver polysomes (Figure 6) and mixing experiments were performed.

Active Ribosome Determination

Barley aleurone ribosomes incubated with ^{3}H-puromycin formed radioactive ^{3}H-peptidyl puromycin rapidly (Figure 7). The formation of ^{3}H-peptidyl

TABLE 3

Sedimentation Coefficient of Polysomes*

n-mer	Rat Liver	Barley Aleurone
Large ribosomal subunit	60	60
1	80	80.5
2	119	117
3	152	150
4	180	179
5	207	207
6	230	233

*The sedimentation coefficients of barley aleurone polysomes were determined by comparison with the known sedimentation values of rat liver polysomes using isokinetic gradient centrifugation and mixing experiments. Similar results were obtained in 3 experiments.

Figure 6.--Calibration of isokinetic gradients with rat liver polysomes. [Rat liver polysomes of known sedimentation coefficients were centrifuged through 0.3 to 1.0 M sucrose gradients in the Beckman SW56 rotor. The position of each peak was plotted vs. its sedimentation coefficient. A straight line was obtained when gradients in the SW65 rotor were used. Each point represents the average of 6 replicate samples. Similar curves were obtained in 5 experiments (although some curves showed less deviation from linearity near the top of the gradient).]

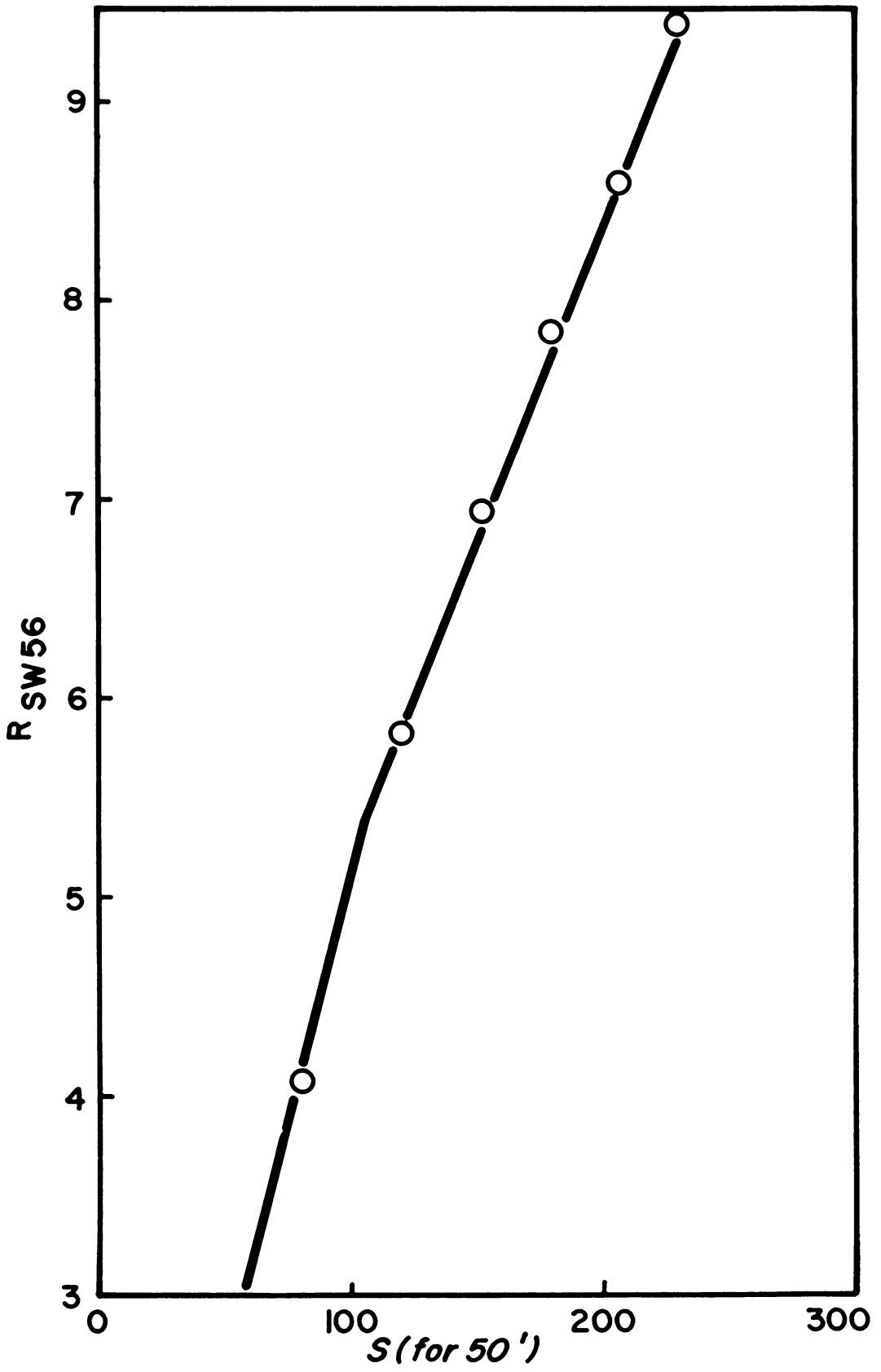
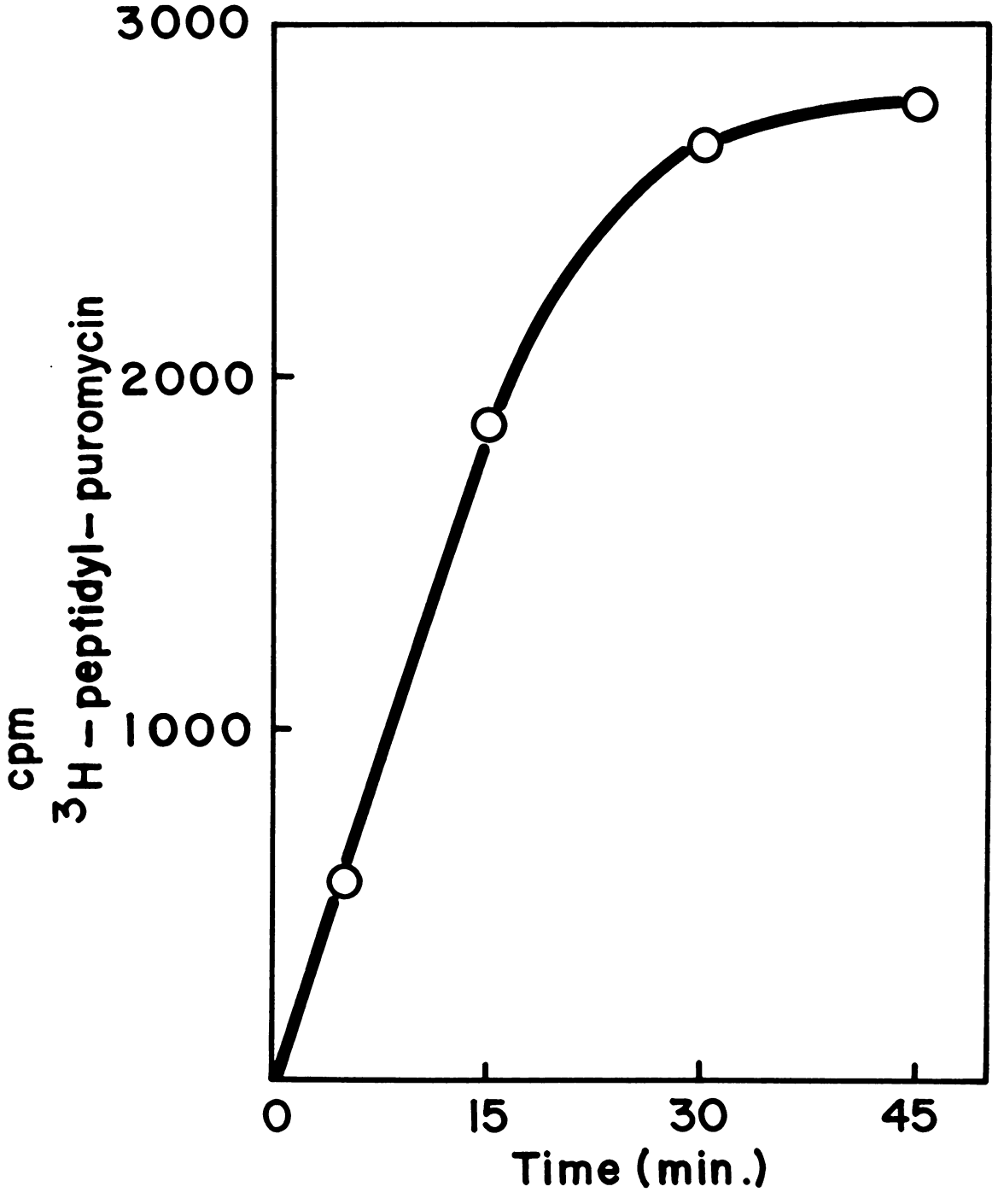

9
8
7
6
5
4
3
R_{SW56}
0
100
200
300
S (for 50')

Figure 7.--The time course of ^{3}H-peptidyl puromycin formation by barley aleurone layer ribosomes. [The ribosomes (from 10 layers) were incubated with 5 μc of ^{3}H-puromycin (see Methods) and the ^{3}H-puromycin peptides were precipitated and washed with TCA and collected on Millipore filters. Each point represents the average of 4 replicate samples. Similar results were obtained in 2 experiments.]


3000
2000
1000
cpm
^{3}H-peptidyl-puromycin
0
15
30
45
Time (min.)

puromycin is essentially completed after a reaction period of 30 minutes, which was used as the standard 37° incubation time for all of the assays.

Does GA cause an activation of the ribosomes? An active ribosome is a ribosome that is synthesizing nascent protein and consequently has nascent peptidyl tRNA as a structural part of the ribosome. Puromycin added in vitro inhibits protein synthesis, but allows an active ribosome to make 1 peptide bond releasing the nascent puromycin peptide. However, a few active ribosomes could react with puromycin again if reinitiation occurs. Previous results indicated that in vitro amino acid incorporation was dependent on the addition of a pH 5.0 enzyme fraction to isolated microsomes. Puromycin inhibited 80% of the cell-free amino acid incorporation (Mrs. Rhoda Papaioannou and Evins, unpublished observations). It is likely, therefore, that only one peptide bond is formed per active ribosome.

Ribosomes were isolated from aleurone cells that were incubated for various times in the presence or absence of GA. The amount of RNA present in duplicate aliquots was determined. The sample was then divided into 4 equal parts: 2 assays and 2 blanks. Two duplicates were assayed for the amount of ^{3}H-peptidyl puromycin formed during a 30 minute incubation period. The peptidyl puromycin formation reaction in the other 2 duplicates

was immediately terminated with the addition of 5 ml of 10% TCA as soon as the ^{3}H-puromycin was added (zero time blank). Control samples with ^{3}H-puromycin and assay medium were incubated without ribosomes. The no ribosome and zero time blank incorporations were subtracted from the counts incorporated during the 30 minute incubation period.

The effect of GA on the change in the number of active ribosomes in the barley aleurone cells with time was measured. The specific activity of TCA precipitable ^{3}H-peptidyl puromycin presented in Figure 8 is a relative measure of the number of active ribosomes. A peak of ribosome activity occurs around 4 hours after the start of incubation. This increase in ribosome activity is, however, not due to hormone action as both the control and GA-treated tissues show the same time course of ribosome activation; it may be a hydration or injury effect. Subsequent to 4 hours, the ribosome activity decreases in both the control and hormone-treated aleurone cells. The results expressed in Figure 8 are on a per ribosome basis. There is more total ribosome activity in the hormone-treated tissues. There are also more ribosomes present, but the activity of each ribosome does not depend on the presence of GA.

Table 4 shows the effect of GA and time of incubation on the percent of active ribosomes. Although there

Figure 8.--Time course of ribosome activity. [The specific activity of ^{3}H-peptidyl puromycin (cpm/µg rRNA), a measure of the number of active ribosomes present, was determined (see text and Methods). Duplicate samples were assayed at 37° following 0 minute (blank) or 30 minute incubations with 5 µc of ^{3}H-puromycin. TCA insoluble ^{3}H-peptidyl puromycin was collected on Millipore filters and washed with 5% TCA containing carrier puromycin. The amount of RNA in an aliquot of each sample was measured spectrophotometrically. Each point represents the average of 4 replicate samples. Similar results were obtained in 2 experiments.]

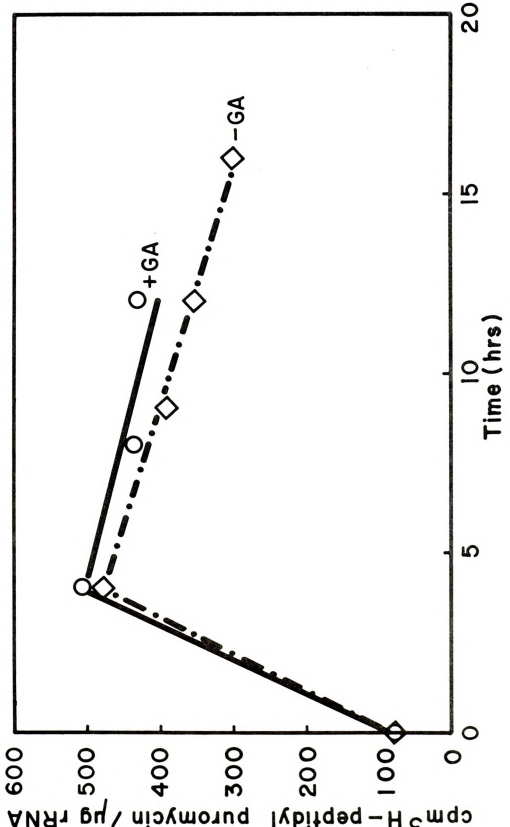

Active ribosomes
cpm ^{3}H – peptidyl puromycin / µg rRNA
600
500
400
300
200
100
0
0
5
10
15
20
Time (hrs)
+GA
-GA

TABLE 4

Formation of Peptidyl-Puromycin by Ribosomes from Barley Aleurone: Effect of GA and Time of Incubation*

Treatment	Incubation Time (hrs)	Molecules ^{3}H-Peptidyl Puromycin Formed	µg RNA	% Active Ribosomes
-GA	0	$.40 \times 10^{13}$	150.0	9.7
	4	2.52	162.2	56.8
	8	2.91	193.6	54.9
	12	1.95	170.9	41.7
	16	1.35	138.4	35.6
+GA	0	$.40 \times 10^{13}$	150.0	9.7
	4	2.74	169.4	59.1
	8	3.25	230.3	57.5
	12	3.90	280.6	50.7
	16	1.66	264.2	22.9

*The number of molecules of ^{3}H-peptidyl puromycin formed and the percent active ribosomes were calculated from previous data (see text and Methods). All results are the average of two duplicates. Similar results were obtained in 2 experiments.

is slightly more ribosomal activity present in the GA-treated cells early in the incubation period, the activity per ribosome decreases faster in the hormone-treated cells. At 16 hours the control tissue has 55% more activity per ribosome than the hormone-treated tissue.

It is clear that the hormone causes an increase in total ribosomes and also in total polysomes. The expected hormone-mediated increase in the number of active ribosomes is seen in the above experiments. However, these results indicate that the activity of each ribosome is not affected by the hormone; the hormone does not cause ribosome activation.

Identification of Some of the GA-Induced Enzymes by Use of the Trp/Tyr Ratio

Fischer and Stein (1960) noted that although α-amylases display no striking similarities in their rather average amino acid composition, they are rich in trp and tyr which accounts for their high extinction coefficients at 280 nm. The tyr-to-trp ratios of α-amylases vary markedly, however, providing them with distinctive absorption spectra. Many hydrolytic enzymes are rich in trp (Table 5). Barley α-amylase has a high trp/tyr ratio (J. E. Varner, personal communication).

The distribution between medium and homogenate of ^{3}H-trp incorporated into TCA-precipitable protein of

TABLE 5

Tryptophan and Tyrosine Composition of Some Proteins

Protein	Reference	Source	MW ($\times 10^3$)	Total N	Moles of Amino Acid in 100,000 g of Protein		Trp/Tyr
					Trp	Tyr	
Catalase	1	Equine liver	250	nd	nd	34.4	. .
Trp Synthetase	1	Escherichia coli	29.5	17.5	0	23.9	0
Carbonic Anhydrase-I	1	Bovine erythrocytes	31	14.9	nd	21.0	. .
Carbonic Anhydrase-II	1	Bovine erythrocytes	31	16.1	nd	19.4	. .
RNAase	1	Bovine pancreas	12.7	17.8	nd	42.3	. .
RNAase	1	Bovine pancreas	12.7	nd	nd	40.7	. .
Enolase	1	Yeast	67.2	17.3	7.8	22.4	.35
Enolase	1	Yeast	67.2	nd	10.7	21.6	.50
α-Amylase	8	Bacillus subtilis	48.7	16.23	30.5	50.0	.61
Papain	1	Papaya	20.34	16.10	22.9	81.3	.28
Papain	1	Papaya	20.34	16.10	20.6	66.7	.31
Chymotrypsinogen A	1	Bovine	25.1	16.5	27.9	16.3	1.7
Chymotrypsinogen B	1	Bovine	24	16.2	25.7	12.6	2.0
Carboxypeptidase A	1	Bovine	34.3	15.4	17.7	57.2	.31
Carboxypeptidase A	1	Bovine	34.3	nd	16.9	51.0	.33
Carboxypeptidase B	1	Bovine	34	nd	29.1	64.0	.45
Carboxypeptidase B	1	Porcine	34.3	15.5	25.9	57.6	.45
Pepsin	1	Bovine	35	14.9	17.2	51.9	.33
Insulin	1	Bovine	5.7	nd	0	66.0	0
Insulin A-component	1	Bovine	. .	15.88	0	69.5	0
Insulin B-component	1	Bovine	. .	15.63	0	67.4	0
Glucagon	1	Bovine	3.6	17.45	28.0	58.6	.48
Hemoglobin α-Chain	1	Human	15.1	nd	5.9	18.7	.32
Hemoglobin β-Chain	1	Human	15.9	nd	11.1	17.8	.62
Apoferritin	1	Horse spleen	480	16.3	4.4	27.8	.16
Cytochrome C	1	Horse heart	12.5	16.8	7.5	27.2	.28
Cytochrome C	1	Horse heart	12.1	15.98	8.3	29.7	.28
Cytochrome C	1	Horse heart	12.1	15.91	8.3	31.4	.26
Serum albumin	1	Human	68	nd	0.9	25.7	.04
β-Lactoglobulin AB	1	Bovine	37.7	nd	12.8	21.5	.60
β-Lactoglobulin A	1	Bovine	37.7	nd	12.8	21.4	.60
β-Lactoglobulin B	1	Bovine	37.7	nd	12.7	20.9	.61
Lactalbumin-α	1	Bovine	15.5	15.86	34.3	29.7	1.2
α-Casein	1	Bovine	. .	15.1	9.8	40.3	.24
Histone	1	Calf Thymus	15.5	17.4	0	20.4	0
Histone A	1	Calf Thymus	15.5	15.4	0	4.1	0
Histone B	1	Calf Thymus	15.5	16.9	0	24.1	0
Nucleohistone	1	Calf Thymus	10.0	16.9	nd	20.5	. .
SilkFibroin	1	Bombyx mori	. .	18.3	2.0	66.6	.03
Tropomyosin	1	Finna	50	18.1	0	0	0
Collagen	1	Python skin	. .	16.19	0	1.7	0
Collagen-Elastoidin	1	Shark fin	. .	18.2	0	9.5	0
α-Amylase	2	Bacillus mascerans	140	. .	22*	56*	.39
α-Amylase	3	Aspergillus niger acid stable	58	. .	11*	31*	.35
α-Amylase	3	Aspergillus niger acid unstable	61	. .	12*	34*	.35
α-Amylase	4	Aspergillus oryzae	51.8	. .	10*	31*	.32
α-Amylase	5	Barley aleurone	45	. .	10*	10*	1.0
Cytochrome C	6	Various	12.1	. .	1-2*	4-5*	.20-.50
Ferredoxin	6	Clostridium pasterianum	6	. .	0*	1*	0
Ferredoxin	6	C. butyricum	6	. .	0*	1*	0
Glucose Oxidase	7	Aspergillus niger	150	. .	22*	5*	4.4
Hemoglobin α	6	Various	15.1	. .	1*	3*	.33
Hemoglobin β	6	Various	15.9	. .	2*	2-4*	1.0-0.5
Hemoglobin γ	6	Human		. .	3*	2*	1.5
Hemoglobin δ	6	Human		. .	2*	3*	.66
Lysozyme	6	Chicken	14.3	. .	6*	3*	2.0
Myoglobine	6	Sperm whale	17.8	. .	2*	3*	.66
RNAase	6	Bovine	12.7	. .	0*	6*	0
RNAase T1	6	Aspergillus oryzae	11.1	. .	1*	9*	.11
Trypsinogen	6	Bovine	24	. .	4*	10*	.40

*Residues of amino acid in the total protein.

References: (1) Tristam and Smith (1963); (2) Pinto and Campbell (1968); (3) Minoda et al. (1969); (4) Stein et al. (1960); (5) J. E. Varner; (6) Dayhoff and Eck (1968); (7) Pazur et al. (1965); (8) Fischer and Stein (1960).

barley aleurone cells is shown in Table 6. Proteins formed in the presence of labeled trp and tyr have 36% higher trp/tyr ratios in the homogenate, 3.5 times higher trp/tyr ratios in the medium, and more trp incorporated in hormone-treated aleurone layers. These results suggest that the bulk of the GA-induced proteins are trp-rich and have trp-tyr ratios. The results demonstrate that the trp/tyr ratio can be used to identify at least some of the GA-induced enzymes.

Double label experiments were performed to show that the polysomes isolated from GA-treated cells were the polysomes responsible for synthesis of the GA-induced proteins (Figure 9). Growing polypeptide chains of the GA-induced hydrolases should have a higher trp/tyr ratio than nascent peptides synthesized in the absence of the hormone. Puromycin release and recovery of the GA-induced peptides would demonstrate that they have higher trp/tyr ratios than nascent peptides of the control polysomes.

Table 7 shows that ribosomes from GA-treated cells have significantly higher trp/tyr ratios than ribosomes from control cells. Puromycin release of the nascent peptides causes a large decrease in the trp/tyr ratio of the ribosomes isolated from GA-treated cells. The released puromycin peptides are recovered in the supernatant and have a higher trp/tyr ratio. Contaminating labeled amino acids are also found in the supernatant. Release of

TABLE 6

Location of Acid Precipitable ^{3}H-Tryptophan in Barley Aleurone*

Treatment	Homogenate		Medium	
	cmp	Trp/Tyr Ratio	cmp	Trp/Tyr Ratio
+GA	48,000	.87	9600	4.78
-GA	32,400	.64	5200	1.35

*Triplicate samples of 10 aleurone layers were incubated at 25° in the presence and absence of 1 μM GA. Between 8-10 hours 5 μc of ^{3}H-tryptophan (trp) and 1 μc of ^{14}C-tyrosine (tyr) were added. Cell fractions were prepared (see Methods), and the TCA precipitates were collected on Millipore filters and washed with 5% TCA containing carrier amino acids. The samples were counted on a Beckman 3 channel liquid scintillation counter and the trp/tyr ratios were calculated using specially prepared ^{3}H and ^{14}C standards.

Figure 9.--Diagram of expected trp/tyr ratio results following hormone treatment. [Aleurone layers were incubated during the last 16 minutes of a 10 hour incubation period with ^{3}H-tryptophan (trp) and ^{14}C-tyrosine (tyr) or ^{14}C-trp and ^{3}H-tyr (reverse experiment) in the presence or absence of GA. The polyribosomal pellet was isolated. The ribosomes should have a greater trp/tyr ratio in the presence of the hormone (+GA > -GA) if polysomes synthesizing GA-induced enzymes were isolated. Puromycin was used to release nascent peptides. The polysomes were collected again by centrifugation through a discontinuous sucrose gradient (P). Nascent peptidyl-puromycin remained in the supernatant (S). The expected results are shown. The actual results agree with the predictions.]

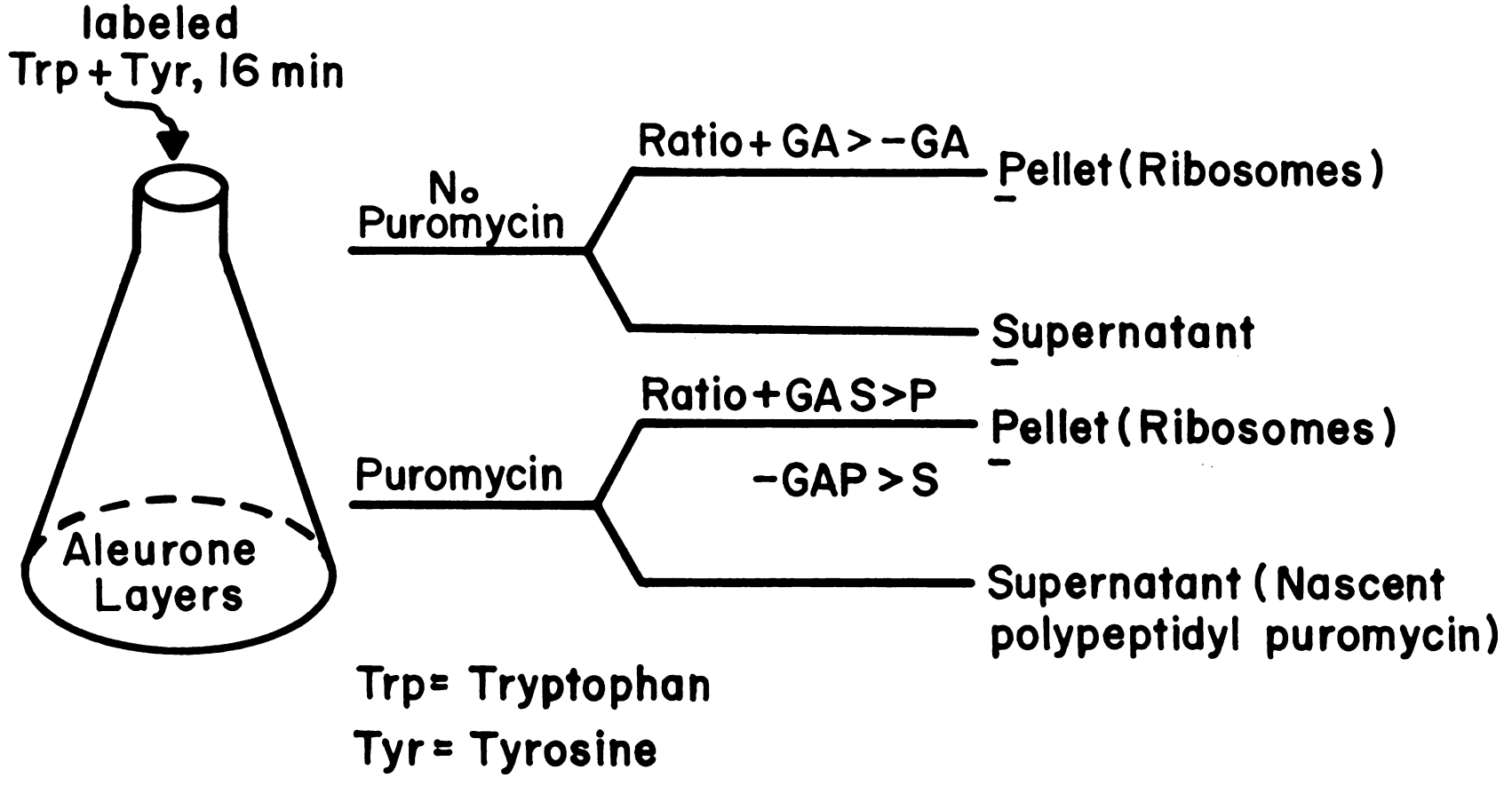

labeled
Trp + Tyr, 16 min
Aleurone Layers
No Puromycin
Ratio + GA > − GA
Pellet (Ribosomes)
Supernatant
Puromycin
Ratio + GA S > P
− GAP > S
Pellet (Ribosomes)
Supernatant (Nascent polypeptidyl puromycin)
Trp = Tryptophan
Tyr = Tyrosine

TABLE 7

Location and Puromycin Release of Tryptophan-rich Nascent Polypeptides*

Treatment	Label Ratio	Trp/Tyr Ratio	
		Pellet	Supernatant
+GA	$^{3}H/^{14}C$	1.35 ± 0.34	1.55 ± 0.58
+GA + Puromycin		0.47 ± 0.24	2.19 ± 0.59
-GA	$^{3}H/^{14}C$	0.317 ± 0.315	2.34 ± 0.72
-GA + Puromycin		0.17 ± 0.16	1.53 ± 0.20
+GA	$^{14}C/^{3}H$	1.59 ± 0.24	0.44 ± 0.02
+GA + Puromycin		1.02 ± 0.12	0.70 ± 0.08
-GA	$^{14}C/^{3}H$	0.91 ± 0.07	0.56 ± 0.18
-GA + Puromycin		0.96 ± 0.08	0.34 ± 0.09

$P < 0.1$ for significant differences.

*Forty aleurone layers were incubated during the last 16 minutes of a 10 hour incubation period with 25 µc of ^{3}H-tryptophan (trp) and 5 µc of ^{14}C-tyrosine (tyr) or in the reverse experiment with 4 µc of ^{14}C-trp and 20 µc of ^{3}H-tyr at 25° in the presence and absence of 1 µM GA. The polysomal pellets were treated with 7.5×10^{-4} M puromycin and the released nascent peptidyl puromycin was separated from the ribosomes by centrifugation through a discontinuous sucrose gradient. In the reverse experiment, 5×10^{-4} M L-trp and 5×10^{-4} M L-tyr were added to the first discontinuous gradients to reduce background counts. The samples were counted on a Beckman 3 channel liquid scintillation counter (the pellet was precipitated with 10% TCA, collected on a Millipore filter, and washed with 5% TCA containing carrier amino acids--see Methods, and the supernatant was counted directly with Bray's scintillation fluid) and the trp/tyr ratios were calculated using specially prepared ^{3}H and ^{14}C standards.

nascent peptides from non-treated cells reduces the trp/tyr ratio in the supernatant.

The reverse experiments shown in Table 7 give the same results. The ratios in the supernatant are lower primarily because of the reduction of contaminating label by the addition of carrier trp and tyr. All important differences are significant at $P < 0.1$.

Discussion

An increase in polysomes is one of the earliest events to be seen after the application of GA and is presumably responsible for the hormone induction of specific enzyme synthesis. A silent period of 3 to 4 hours precedes polysome formation during which the synthesis of the ER to which the polysomes are attached, occurs (section 3). The actual synthesis of α-amylase occurs at 8 to 10 hours, although under certain conditions α-amylase production can be seen at 6 hours (P. C. Lin, personal communication). This short lag period between polysome formation and the appearance of measurable α-amylase activity might be related to the secretion processes.

GA causes a 2.5 fold increase in polysome formation within 12 to 15 hours after hormone addition. The large amount of polysome formation induced by GA is due to the fact that the aleurone system consists of a

single cell type and that every cell most probably responds to the added hormone.

Polysome formation has been demonstrated in a number of growing systems, after fertilization (Humphreys, 1969; Monroy, 1970), during germination (Marcus et al., 1966), and during H_2O imbibition (Marcus and Feeley, 1965; Barker and Rieber, 1967; Sturami et al., 1968; Mascarenhas and Bell, 1969). But hormone-induced polysome formation generally occurs at long times after addition of the hormone, during which time growth and development of the cells continue. Many hormones induce the synthesis of specific enzymes. The more direct effects of the hormone on polysome formation are minimal, in that the polysomes responsible for the synthesis of the hormone-induced enzymes represent only a small percent of the total polysomes. For example, lactogenic hormone causes a doubling of polysomes over a 96 hour period (Gaye and Denamur, 1969).

The increase in the number of ribosomes isolated as polysomes is due both to ribosome synthesis and aggregation (increase in the percent polysomes). It is necessary to be reasonably certain that the increase is not due to changes in recovery, in spite of the fact that there is more RNAase in the hormone-treated cells. No increase in recovery was seen with different isolation methods, including the use of various RNAase inhibitors,

detergents, and different methods of isolation, centrifugation, and homogenization (unpublished results).

Polysome recovery increases in GA-treated cells following isolation in 0.5% Na deoxycholate when gentle homogenization is used. Not all cells are broken and the total polysome recovery is less than that following normal homogenization. The relationship between the amount and distribution of polysomes isolated from GA-treated and control tissue does not change.

The polysomes responsible for the synthesis of GA-induced enzymes are probably ER-bound. Electron micrographs show a considerable number of bound ribosomes (van der Eb and Nieuwdorp, 1967; Jones, 1969a,b; Vigil and Ruddat, in press). Secretory proteins are thought to be synthesized on membrane-bound polysomes (Palade and Porter, 1966; Siekevitz and Palade, 1966; Redman, 1968; Priestley et al., 1969; Takagi et al., 1969; Andrews and Tata, 1969; Ganoza and Williams, 1969). The increased recovery of polysomes following membrane solubilization by detergents can be used to estimate the amount of ER-bound ribosomes. However, the cell walls of aleurone cells are very thick, up to 1/2 of the cell by volume. Fairly vigorous homogenization is necessary to break the cells. This homogenization probably breaks membranes and releases membrane-bound polysomes.

The rate of protein synthesis doubles following hormone treatment. This increase was measured by the addition of a mixture of 15 amino acids. Varner et al. (1965) did not see any increase; however, they measured the incorporation of only one amino acid into half-seeds (endosperms) at later times.

The number of active ribosomes also doubles. This determination of the rate of protein synthesis is unaffected by the problems of isotope dilution affecting the above measurements.

Many different types of experiments have established that the polysomes isolated were those responsible for GA-induced enzyme synthesis. The high trp/tyr ratio of the nascent peptides released from polysomes isolated from hormone-treated cells indicates that nascent GA-induced proteins are bound to these polysomes. Polysomes isolated from hormone-treated cells have substantially more α-amylase activity associated with them than the control polysomes (Evins, unpublished observations). In addition, anaerobic conditions inhibiting α-amylase synthesis inhibit polysome formation, washing out GA reduces the number of ribosomes in polysomes, ABA, a plant hormone that prevents α-amylase synthesis but not RNA or protein synthesis, prevents polysome formation, and inhibitors of α-amylase synthesis inhibit polysome formation (section 2).

The events occurring during the lag period before the appearance of hormone-induced enzyme activity are now better understood. However, further investigation is needed before the primary site and mechanism of gibberellin action in the aleurone system is completely understood.

Summary

Gibberellic acid (GA) causes the formation of polysomes and an increase in the proportion of ribosomes present as polysomes during the 8 to 10 hour lag period of α-amylase induction. Polysome formation starts at 3 to 4 hours and reaches a maximal level at 12 to 15 hours after hormone addition. A linear increase in the percent polysomes is seen between 3 to 4 hours and 10 to 11 hours, reaching a maximum of 76% polysomes. The percent polysomes more than doubles following GA treatment, while polysome formation increases over 2.5 fold. An increase in total ribosomes of almost twofold occurs.

The recovery of polysomes from mixtures of rat liver and aleurone layer ribosomes was much less using polysomes isolated from hormone-treated aleurone cells indicating that the stimulation of polysome formation occurs despite an increase in the amount of ribonuclease present in GA-treated cells. Indirect evidence suggests that the GA-induced protein synthesis occurs on endoplasmic reticular membrane-bound polysomes and that the polysomes isolated are membrane-bound.

Although the number of active ribosomes (ribosomes capable of synthesizing nascent polypeptides, measured by the formation of acid insoluble ^{3}H-peptidyl puromycin) doubles at 12 hours following hormone treatment, the proportion of the total ribosomes that are active is not affected. The incorporation of ^{14}C-amino acids into acid insoluble material was used to show a doubling in the rate of protein synthesis within 8 hours of hormone treatment.

The bulk of the GA-induced proteins are tryptophan-rich and have high tryptophan/tyrosine (trp/tyr) ratios. Polysomes isolated from hormone-treated cells and nascent polypeptides released by puromycin from these polysomes have higher trp/tyr ratios than polysomes and nascent peptides isolated from control tissues.

II

GIBBERELLIC ACID CONTROLLED POLYSOME FORMATION: PREVENTION BY ABSCISIC ACID AND ANTI-METABOLITES AND FUNCTIONAL STUDIES

Introduction

The response of barley aleurone layers to exogenous gibberellin has been described in recent articles (Chrispeels and Varner, 1967a,b; Filner et al., 1969; Varner and Johri, 1968). A dramatic increase in the de novo synthesis of α-amylase and protease follows the addition of the hormone after an 8 to 10 hour lag period (Filner and Varner, 1967; Jacobsen and Varner, 1967). Recently, it has been demonstrated that GA-treatment also increases the formation of polysomes, the percentage of ribosomes present in polysomes, the rate of protein synthesis, and the rate of synthesis of the endoplasmic reticulum (ER). Most of these effects occur within 2 to 4 hours after hormone application, that is preceding the induction of hydrolytic enzymes (sections 1 and 3). Using the distinctive trp/tyr ratio of α-amylase and the high trp content of some of the other GA-induced proteins as a

chemical identification tag, it was moreover shown that the polysomes produced in the presence of GA were, in fact, the polysomes responsible for the synthesis of the GA-induced proteins. Several lines of evidence have suggested that the polysomes are membrane-bound.

ABA (recent review, Millborrow, 1969), a plant hormone recently characterized and synthesized in 1965 (Cornforth et al., 1965; Ohkuma et al., 1965), acts antagonistically to GA (Thomas, 1965; Aspinall et al., 1967). In many systems ABA may inhibit specific RNA synthesis (Khan and Anojulu, 1970; Khan and Heit, 1969; Ihle and Dure, 1970). Inhibition of polysome formation in Fraxinus excelsior by the inhibition of RNA synthesis was indicated by electron microscopical and autoradiographic studies (Villiers, 1968).

One method to realize the primary goals of the study of hormone action, the determination of both the mechanism and the location of the primary site of action, is to investigate early effects of hormone administration. The early release of soluble carbohydrate and several phosphatases has been noted (Pollard and Singh, 1968). I have tried to trace the cause of an early fundamental biochemical change, the de novo synthesis of α-amylase. The present results show that the formation of polysomes and the control of ER synthesis, which occur near the beginning of the lag period, are required steps necessary for the synthesis of α-amylase in response to GA. I now

report that inhibitors of α-amylase synthesis, such as anaerobiosis, specific metabolic inhibitors, and removal of GA inhibit or decrease polysome formation. The addition of ABA to hormone-treated aleurone layers prevents polysome formation.

Methods

Aleurone layers were prepared as described in Section 1 and incubated for various times with 1 m$\underline{M}$ Na acetate pH 4.8, 20 m$\underline{M}$ $CaCl_2$, and where required 1 μ$\underline{M}$ K^+ GA_3, in a 50 ml flask on a Dubnoff metabolic shaker at 25°. The homogenate and medium fractions and α-amylase assays were performed as described by Chrispeels and Varner (1967a). Polysomes were prepared as described previously (Section 1). Wash out experiments were performed by removing the flask containing the aleurone layers from the shaker, rinsing the aleurone layers 6 times with sterile distilled H_2O under a UV sterilized hood, blotting on sterile paper towels, rinsing and shaking 10 times with sterile distilled H_2O, and then rapidly shaking the layers for 30 minutes in buffer without GA. The wash out and transfer steps were repeated 5 times.

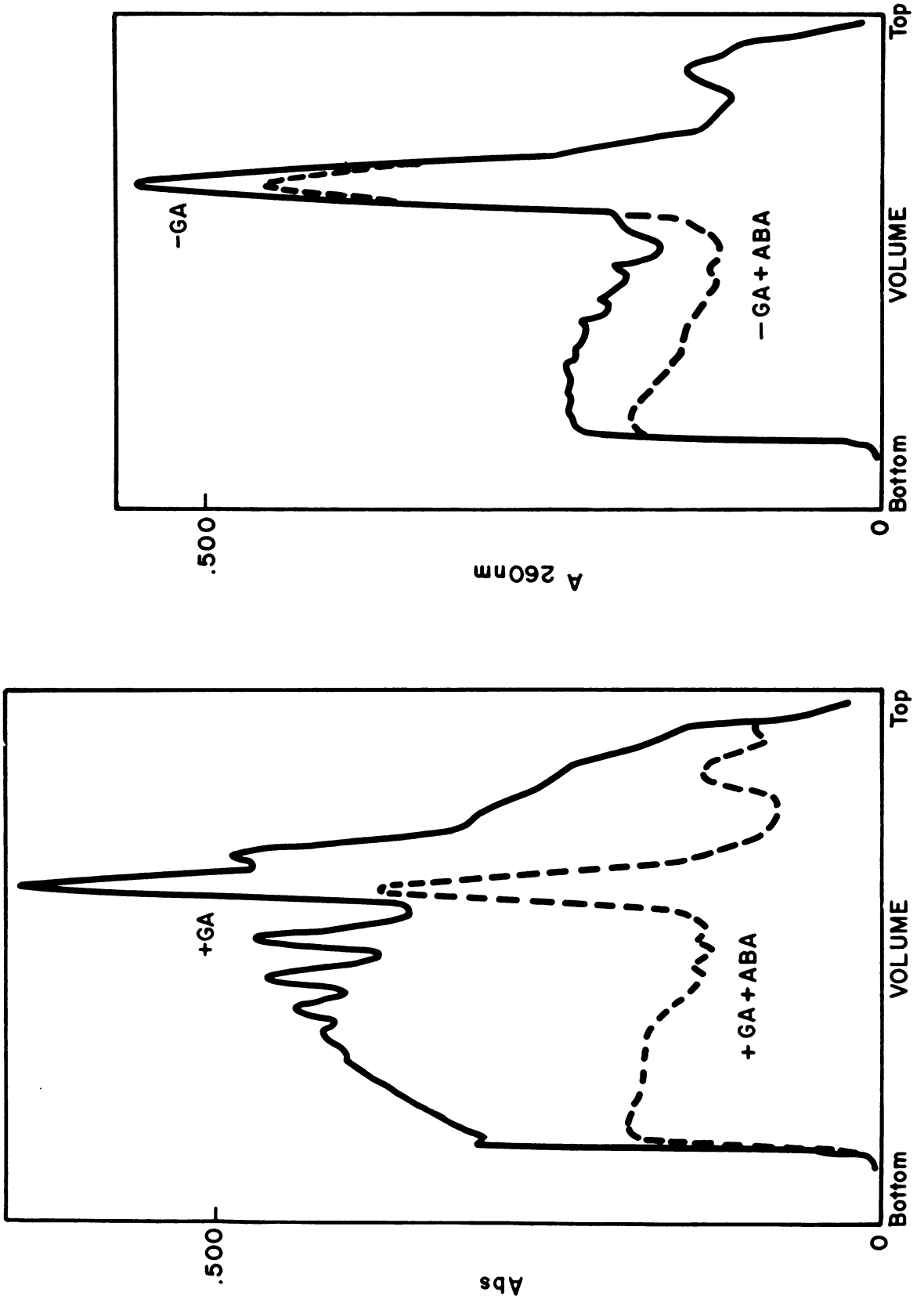
Incorporation experiments were performed with carrier-free ^{14}C-amino acid mixture (a mixture of 15 amino acids from an algal protein hydrolysate, International Chemical and Nuclear Corp.) and carrier-free

^{32}P-ortho-phosphoric acid (ICN). The layers were removed from the flask in the sterile hood, rinsed with sterile distilled H_2O, blotted on sterile paper towels, and transferred to a sterile 25 ml flask. ^{32}P-ortho-phosphoric acid or ^{14}C-amino acid mixture were mixed with medium with or without the hormone in a total volume of 100-150 µl/sample and spread evenly over the layers during the last 60 or 30 (^{32}P), or 16 or 7 (^{14}C) minutes of the incubation period. Fractions were collected dropwise by hand, 25 µg of carrier DNA or bovine serum albumen were added, 50% TCA (w:v) was added to a final concentration of 10%, and the samples were allowed to sit overnight in the cold room. The precipitates were collected on Millipore filters, washed with 30 ml of 5% TCA containing either 0.1 M phosphate or casein acid hydrolysate, dried at 70°, and counted on a Beckman scintillation counter in scintillation fluid "A" (4 g PPO + 100 mg POPOP per 1 of toluene).

Results

ABA added at the start of the incubation period prevents the GA-enhanced polysome formation and also causes a decrease in the amount of polysomes present in the control tissue (Figure 1). Not only is there a change in the amount of polysomes, but the percent of the ribosomes that can be isolated as polysomes decreases by 10% in the GA-treated samples, whereas no effect is seen in the control tissue. Similar results were seen when ABA is

Figure 1.--The effect of ABA on polyribosome formation. [Polysomes were isolated from 40 barley aleurone layers that were incubated for 10 hours: (a) in the presence of 1 μM GA (+GA) or GA + 2.5×10^{-7} M ABA (+GA+ABA), or (b) in the absence of GA (-GA) or with 2.5×10^{-7} M ABA (-GA+ABA) at 25° on a Dubnoff metabolic shaker. Polysome profiles were determined in 0.3 to 1.0 M isokinetic sucrose gradients. Similar results were obtained in 3 experiments.


A 260nm
.500
0
Bottom
VOLUME
Top
-GA
-GA + ABA
Abs
.500
0
Bottom
VOLUME
Top
+GA
+GA + ABA

added to aleurone layers incubated with GA during the mid-course of formation and α-amylase synthesis (Table 1). Following a 2 hour incubation with GA and ABA, there is a 16% decrease in the number of ribosomes found in polysomes.

Both 5-FU and Act D partially inhibit α-amylase synthesis and secretion (Table 2). Although some polysome formation occurs in the presence of GA and Act D or 5-FU, Act D prevents polysome formation to a greater extent than 5-FU.

Aleurone layers were incubated in flasks containing varying amounts of medium. Anaerobiosis is known to inhibit α-amylase synthesis (Varner, 1964). Decreased production of α-amylase is seen when larger volumes of medium are added to the flask. Polysome formation is also inhibited by larger volumes of medium.

Functional Studies on Polysomes

The polysomes isolated from control and hormone-treated tissues show incorporation of ^{32}P into TCA-precipitable, RNAase degradable material, and ^{14}C-amino acids into puromycin releasable nascent proteins (Figure 2). The specific activity (cpm/A_{260}) of the ^{32}P and ^{14}C-amino acids incorporated is higher in the polysomal region than in the monosomes in both control and hormone-treated tissue, although there is less difference in specific activities of the incorporation between polysomal and

TABLE 1

Effect of Mid-Course Addition of ABA
on GA-Induced Polysome Formation*

Treatment	Polysomal Distribution % $\frac{P}{P+M}$	% Change in $\frac{P}{P+M}$ from	
		-GA	+GA
12 hrs -GA	49.4	0	. .
11 hrs +GA	75.0	+51.8	. .
13 hrs +GA	75.7	+53.2	0
11 hrs +GA ⟶ 2 hrs +GA + ABA	63.8	+29.1	-15.8

*Forty barley aleurone layers were incubated at 25° with 20 mM $CaCl_2$, 1 mM Na Acetate pH 4.8, and, if present, 10^{-6} M GA_3 and 2.5×10^{-7} M ABA. All numbers are the average of duplicate samples. Similar results were obtained in 2 experiments.

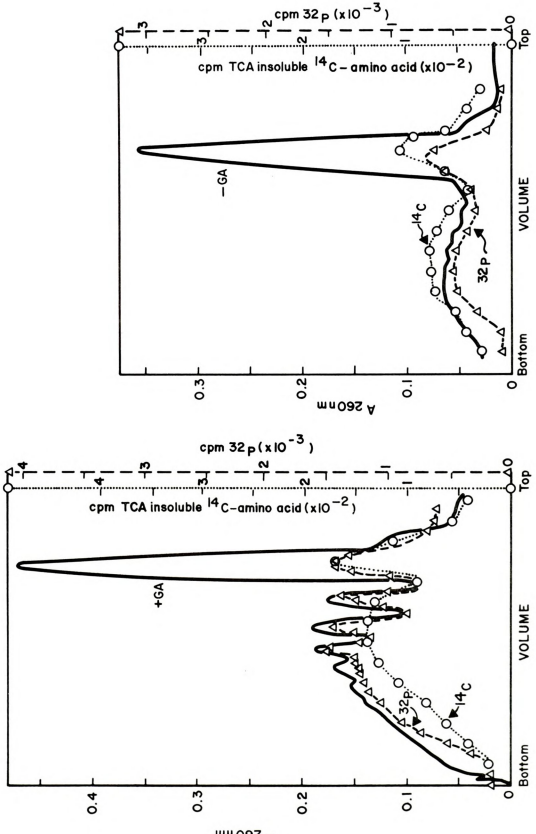
TABLE 2

Effect of Fluorouracil and Actinomycin D on α-Amylase Production*

Treatment	μg α-Amylase			% α-Amylase in Medium	% Inhibition
	Medium	Homogenate	Total		
+GA	78.4	7.5	85.9	91.3	0
+GA + 2.5 m$\underline{M}$ FU	29.3	48.1	77.4	37.9	9.9
+GA + 100 μg/ml Act D	19.1	26.1	45.2	42.2	47.4

*Ten aleurone layers were incubated 24 hours with GA (10^{-6} $\underline{M}$) and Fluorouracil (FU) or Actinomycin D (act D) or with GA alone. All numbers are the average of duplicate samples. Similar results were obtained in 2 experiments.

Figure 2.--Incorporation of ^{32}P-ortho-phosphoric acid and ^{14}C-amino acids into polysomes. [Polysomes were isolated from 40 barley aleurone layers and profiles were determined as described in Figure 1. Carrier-free radioactive ^{32}P-ortho-phosphoric acid or ^{14}C-amino acid mixture (a mixture of 15 amino acids from an algal reconstituted protein hydrolysate) with ±GA medium in 100 to 200 µl total volume was spread evenly over the layers in a 25 ml Erlenmeyer flask for 60 or 7 minutes, respectively. Fractions were collected on Millipore filters. Figure 2 shows the absorbancy profile, ^{14}C-amino acid counts incorporated into nascent polypeptides, and ^{32}P-ortho-phosphoric acid counts incorporated into RNA in polysomes prepared from layers incubated: (a) in the presence of the hormone (1 µM GA), or (b) in the absence of the hormone. Similar results were obtained in 3 experiments.]


cpm 32P (x10⁻³)
cpm TCA insoluble 14C-amino acid (x10⁻²)
+GA
32P
14C
VOLUME
Bottom
Top
A 260nm
cpm 32P (x10⁻³)
cpm TCA insoluble 14C-amino acid (x10⁻²)
-GA
14C
32P
VOLUME
Bottom
Top
A 260nm

monosomal regions in control tissues. The appearance of ^{14}C-amino acid counts in the monosome region and the region of smaller polysomes is probably due to degradation.

More α-amylase activity is found in the polysomal pellets isolated from GA-treated cells. However, it is difficult to show that this increase in activity is due to enzyme bound to the polysomes and not enzyme contamination from another fraction.

Removal of GA

GA was removed from the aleurone cells by numerous washings in an attempt to correlate the resulting decrease in the amount of α-amylase synthesized (see below) with an effect on polysomes.

Removal of GA by washing causes a reduction in the number of ribosomes isolated as polysomes (Table 4). Adding back GA causes the percent polysomes to return to the same level found in polysomes isolated from unwashed GA-treated tissue.

The amount of α-amylase synthesized decreases when GA is removed. The amount of α-amylase present in the medium (Figure 3a) parallels the total α-amylase production in all cases (not shown). When GA is added back to the layers following its removal, α-amylase synthesis starts without the original 8-10 hour lag period. 6-Methylpurine (1 mM) does not prevent the recovery of α-amylase synthesis upon readdition of the hormone.

TABLE 3

The Distribution of α-Amylase in the Medium and Homogenate of Barley Aleurone Layers*

Treatment	Time (hours)	μg α-Amylase Produced		
		Total	Medium	Homogenate
-GA	8	4.0	3.2	.8
	16	12.4	10.8	1.6
	24	17.5	15.2	2.3
+GA	8	7.4	5.8	1.6
	16	101.0	88.5	12.5
	24	133.2	125.6	7.6
+GA + 1 mM 6-Methylpurine	24	3.8	2.8	1.0

*Ten aleurone layers were incubated at 25° for the times specified in the medium described in Table 1 with 1 μM GA_3 and 1 mM 6-methylpurine, if present. All results are the average of duplicate samples. Similar results were obtained in 3 experiments.

TABLE 4

The Effect of Removal and Readdition of GA on the Distribution of Ribosomes in Polysomes*

Treatment	Total Time of Incubation (hours)	Time of Removal of GA (hour-hour)	Time of Readdition of GA (hour-hour)	Polyribosomal Distribution % $\frac{P}{P+M}$
+GA	10	. .	. .	75.0
	15	. .	. .	75.7
+GA ⟶ -GA	12	10-12	. .	68.6
	15	10-15	. .	66.8
+GA ⟶ -GA ⟶ +GA	15	10-12	12-15	75.8
-GA	10	. .	. .	45.3
	15	. .	. .	48.7

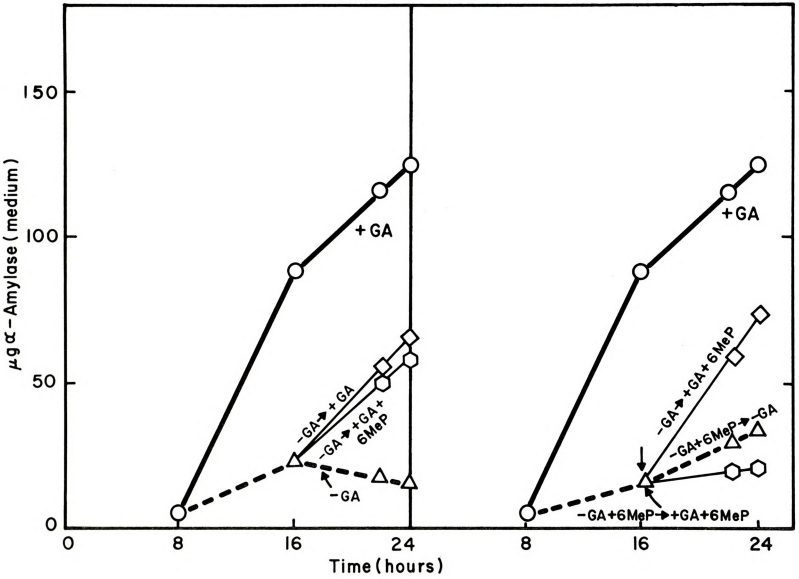
*The polyribosomal distribution [area of the polysome (P) region determined by planimetry divided by the sum of the areas of the polysome and monosome (M) regions--P/(P+M)] was determined following the removal and/or readdition of the hormone. All results are the average of 2 samples. Similar results were obtained in 3 experiments.

Figure 3. The effect of 6-methylpurine on α-amylase production upon removal and readdition of GA.

(a) GA was removed at 8 to 10 hours by washing and the aleurone layers were incubated without GA. 6-Methylpurine (1 m$\underline{M}$) was added at 16 hours.

(b) GA was removed at 8 to 10 hours by washing and the aleurone layers were incubated with 6-methylpurine. 6-Methylpurine was readded or removed at 16 hours.

Amylase assays were performed at 8, 16, 22, and 24 hours with duplicate samples. Similar results were obtained in 2 experiments.


150
100
50
0
μg α-Amylase (medium)
0
8
16
24
Time (hours)
+GA
-GA→+GA
-GA→+GA+6MeP
-GA
+GA
-GA→+GA+6MeP
-GA+6MeP→-GA
-GA+6MeP→+GA+6MeP

Different results are obtained when the aleurone cells are incubated with 1 mM 6-methylpurine after removal of GA (Figure 3b). If GA is added back and 6-methylpurine is removed, recovery of α-amylase synthesis occurs. The removal of 6-methylpurine allows a slight increase in α-amylase activity even without addition of the hormone. However, there is no recovery of α-amylase synthesis if GA is added back while 6-methylpurine is maintained.

The distribution of α-amylase between the medium and homogenate is shown in Table 3. Addition of 6-methylpurine at the start of the incubation period with GA inhibits more than 97% of the total α-amylase production.

Discussion

The goal of the work presented here was to establish the physiological significance of polysome formation as a prerequisite for the induction of enzyme synthesis by GA using the synthesis of α-amylase as an indicator of the latter. The incorporation of ^{32}P-ortho-phosphoric acid into polysomal RNA and of ^{14}C-amino acids into nascent polypeptides and the previous demonstration (Section 1) of RNAase sensitivity of the polysomes suggest that the polysomes are functional in vivo.

The results presented here demonstrate that ABA prevents GA-induced polysome formation. This hormone prevents α-amylase synthesis in the barley aleurone cells,

but does not alter the rate of respiration or total RNA or protein synthesis (Chrispeels and Varner, 1966), although it may inhibit specific RNA synthesis. Therefore, ABA inhibition presents a strong physiological argument that the polysomes studied are necessary for α-amylase synthesis.

The results of a mid-course addition of ABA suggest that continued synthesis and turnover of the polysomes are taking place. Removal of GA causes a similar decrease in the percent polysomes.

RNA and protein synthesis inhibitors, including Act D, 5-FU, and cycloheximide, inhibit both α-amylase synthesis and polysome formation to roughly similar extents. Anaerobiosis also inhibits both processes.

Chrispeels and Varner (1967b) first reported that GA can be washed out with a concomitant arrest of α-amylase synthesis. The wash out experiments presented, performed with a higher GA concentration and more vigorous washing procedures than those previously used, clearly show the diminution of α-amylase synthesis following GA removal.

6-Methylpurine, an inhibitor of RNA synthesis, inhibits α-amylase synthesis even when added during the mid-course of α-amylase production (Chrispeels and Varner, 1967b), but does not inhibit the recovery of α-amylase synthesis when GA is added back subsequent to GA removal (Figure 3). If 6-methylpurine is present during both the period when GA is removed and the period when GA is added

back, no recovery occurs. This latter inhibition by 6-methylpurine is reversible with no reduction in α-amylase synthesis.

Removal of GA decreases the number of ribosomes present as polysomes. Readdition of the hormone causes a return to the maximal level of percent polysomes seen. The hormone was washed out just prior to attainment of the maximum level of percent polysomes. This is a further indication of the physiological correlation between both hormone-regulated polysome formation and the increase in the number of ribosomes present as polysomes and the hormone induction of enzymes.

Summary

Abscisic acid (ABA, 2.5×10^{-7} M) added at the start of the incubation period almost completely prevents the gibberellic acid (GA, 1 μM) induction of polysomes and α-amylase and causes a 10% decrease in the percent of the ribosomes present as polysomes in barley aleurone cells. Inhibitors of α-amylase synthesis prevent or reduce polysome formation to similar extents. The removal of GA by washing and the mid-course addition of ABA cause a 10 to 15% decrease in the percent polysomes within 2 hours, probably by arresting new polysome formation and aggregation while not affecting turnover. Anaerobiosis and actinomycin D added at the start of the incubation

period inhibit both α-amylase synthesis and polysome formation. Fluoro-uracil inhibits both GA responses to a lesser extent.

The polysomes are functional and show incorporation of both ^{32}P-*ortho*-phosphoric acid into acid insoluble, ribonuclease-sensitive polysomal material (RNA) and ^{14}C-amino acids into acid insoluble, puromycin-releaseable polysomal material (nascent polypeptides). 6-Methylpurine does not inhibit recovery of α-amylase synthesis when GA is added back to aleurone layers following GA removal. However, the administration of 6-methylpurine to aleurone layers 6 hours prior to the readdition of GA (following GA removal) completely inhibits any recovery of α-amylase synthesis. This inhibition is reversed if 6-methylpurine is removed when GA is added back.

III

HORMONE CONTROLLED ENDOPLASMIC RETICULUM SYNTHESIS IN BARLEY ALEURONE CELLS

Introduction

Isolated barley aleurone cells respond to exogenous gibberellic acid (GA) by the de novo synthesis of α-amylase and protease following an 8-10 hour lag period (Chrispeels and Varner, 1967a,b; Filner and Varner, 1967; Jacobsen and Varner, 1967). During the lag period, GA causes an increase in the number and proportion of ribosomes that can be isolated as polysomes in the hormone-treated cells, starting 3-4 hours after hormone addition (Section 1). The polysomes are, in vivo, probably bound to the endoplasmic reticulum (ER), because electron microscopy studies show extensive development of the rough ER in the aleurone cells of germinating barley (van der Eb and Nieuwdorp, 1967) as well as changes in the rough ER of isolated barley aleurone layers during both the lag and synthesis phases of α-amylase production (Jones, 1969a,b; Vigil and Ruddat, J. Cell. Biol., in press). However, the quantitative determination of

differences in hormone-treated and control tissues and the time of the onset of ER synthesis after hormone addition are difficult to establish by electron microscopy. I now report the estimation of the rate of ER synthesis by measuring ^{14}C-choline incorporation into a semi-purified ER fraction using the method of Nagley and Hallinan (1968). By this method of estimation, GA increases ER synthesis 4-8 fold. The increase in the rate of ER synthesis starts at about the same time as polysome formation.

Methods

For each sample 40 barley aleurone layers were prepared by the methods of Chrispeels and Varner (1967a) and incubated for the times specified in 5 ml of 1 m$\underline{M}$ Na acetate buffer pH 4.8, 20 m$\underline{M}$ $CaCl_2$, and 10^{-6} $\underline{M}$ gibberellic acid (GA_3) on a Dubnoff metabolic shaker at 25°.

The methods of Nagley and Hallinan (1968) were used to measure the rate of synthesis of endoplasmic reticulum (ER). During the last 30 minutes of incubation the aleurone layers were transferred to a medium containing 5 μc of (^{14}C-methyl)-choline chloride (New England Nuclear Corp., sp. act. 8.25 mc/mmole) and 10 mg of neutralized casein acid hydrolysate. Trichloroacetic acid (TCA)-precipitable membrane material in a semi-purified ER fraction was collected on a Millipore filter, dried at 70°, and counted with 10 ml of scintillation fluid "A".

The ER fraction was prepared by homogenizing the cells following a modified procedure of Wettstein et al. (1963, 1964) (Section 1). Following the homogenization, a 10 minute centrifugation at 4,000 x g, and a 15 minute centrifugation at 10,000 x g, the supernatant was layered onto a discontinuous sucrose density gradient and centrifuged for 12 hours at 50,000 rpm in a Beckman 65 or Ti50 rotor. The discontinuous gradient was composed of a bottom layer of 3.5 ml of 1.6 M sucrose buffer, containing ribonuclease-free sucrose, 50 mM HEPES pH 7.55, with 25 mM K^+, 2 mM Mg acetate, and 7 mM 2-mercaptoethanol (0.5 µl/ml total volume), a middle layer of 0.6 M sucrose buffer, and in the top layer, the 10,000 x g supernatant, in 0.45 M sucrose. The pellet was resuspended in 10% TCA, filtered on a Millipore filter, and washed with 50 ml of 5% TCA containing carrier choline chloride.

Phospholipids were extracted by the methods of Bieber et al. (1961) modified as follows. The resuspended pellet was extracted twice with 15 volumes of chloroform: methanol (1:1) with 1 mg of butylhydroxytoluene (an antioxidant), and the organic layer was collected with a disposable Pasteur pipet following centrifugation. The extraction was repeated twice with chloroform:methanol (2:1). The 4 extracts were combined, washed twice with 0.2 volumes of a 0.1% $MgCl_2$ and 8% NaCl solution, and counted with toluene scintillation fluid after air

evaporation. Protein was measured by the method of Lowry et al. (1951). Where necessary, 2-mercaptoethanol was removed by dialysis.

Results

Following a 30 minute labeling period, at different times after GA treatment, the specific radioactivity of the TCA insoluble portion of the microsomal fraction was 10 times greater than other subcellular fractions (Table 1). Longer periods of labeling lead to a greater proportion of activity in other subcellular fractions; this is also the case in rat liver (Hallinan et al., 1966). The counts in the microsomal fraction are soluble in lipid solvents and not removable by extraction procedures for nucleic acid (Table 2).

More ^{14}C-choline is incorporated into the microsomal fraction isolated from GA-treated aleurone layers than that from control layers (Figure 1). The ratio between the slopes of the two curves varied in different experiments between 3 and 8 (+GA/-GA), as did the ratios of the amount of α-amylase produced later. The increase in counts of ^{14}C-choline incorporated into ER isolated from GA-treated cells starts between 2 and 4 hours after hormone addition. In contrast, the increase in ^{14}C-choline incorporation in control tissues begins only after 4 or more hours.

TABLE 1

^{14}C-Choline Incorporation (Acid Insoluble) in Various Cell Fractions*

	Specific Activity cpm/mg protein	Relative Amounts % of Microsomal Fraction
Microsomal fraction	2506	100.0
S 105	234	9.5
5 Kg pellet	65	2.6
24 Kg pellet	305	12.2

*Forty aleurone layers were labeled for 30 minutes with ^{14}C-methyl choline following an 8 hour incubation period at 25°. The results are the averages of triplicate samples.

TABLE 2

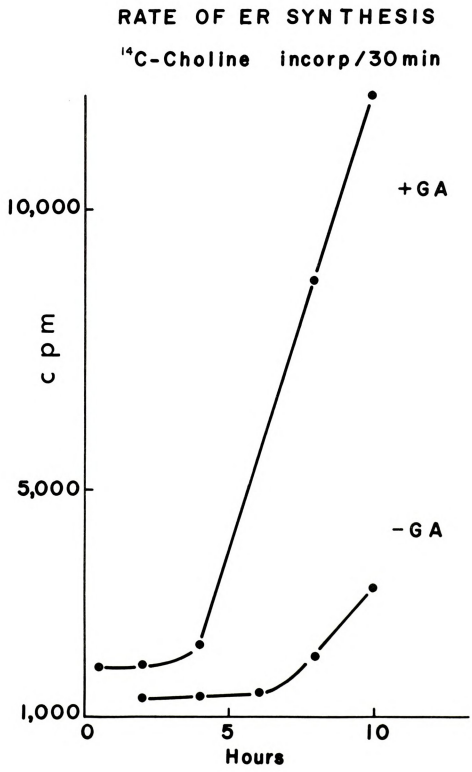
Removal of Acid-Insoluble Radioactivity from the Choline-Labeled Microsomal Fraction by Lipid and Nucleic Acid Extraction Procedures†

Treatment	cpm Remaining	% Remaining	% Removed	% Removed
Microsomes	2892	100.0	. .	. .
In Lipid Extract	2716	93.9	176	6.1
Microsomes, After Nucleic Acid Extraction*	2623	90.7	269	9.3

*5% TCA, 95°, 30 min.

†Forty aleurone layers were incubated for 5 hours with 10^{-6} M GA at 25°. The results are the averages of triplicate samples. Similar results were obtained in 2 experiments.

Figure 1.--The effect of GA on the rate of endoplasmic reticulum synthesis in barley aleurone layers. [The rate of endoplasmic reticulum (ER) synthesis in barley aleurone layers was determined by incubating 40 layers at 25° for various times in 1 m$\underline{M}$ acetate buffer pH 4.8 with 20 m$\underline{M}$ $CaCl_2$ and either +GA = 10^{-6} $\underline{M}$ gibberellic acid ($G\underline{A}_3$) or -GA = without GA. During the last 30 minutes of incubation, the aleurone layers were transferred to a medium containing 5 µc of (^{14}C-methyl)-choline and 10 mg of neutralized casein acid hydrolysate. Trichloroacetic acid precipitable membrane material in a semi-purified ER fraction was collected on a Millipore filter and counted. Each point is the average of duplicate samples. Similar results were obtained in 3 experiments.]


RATE OF ER SYNTHESIS
^{14}C-Choline incorp/30 min
+GA
-GA
10,000
5,000
1,000
cpm
0
5
10
Hours

Discussion

In general, secreted proteins are synthesized on membrane-bound polysomes. Many secretory cells, such as aleurone cells, endocrine gland cells (anterior pituitary, pancreatic islets, and secretory neurones), and exocrine gland cells (salivary, Brunner's gland cells, pancreatic acinar cells, goblet cells of the intestinal mucosa, mucous and chief cells of the gastric glands) have large amounts of rough ER (Fawcett, 1967), Proliferation of ER frequently accompanies hormone-induced changes in growth and development (Tata, 1968 and personal communication).

ER synthesis starts between 2-4 hours after the addition of GA, preceding polysome formation.

These results suggest a biological role for GA in the regulation of ER synthesis and the attachment of polysomes to rough ER. How this occurs is presently being studied. To the best of my knowledge, this is the first time that choline incorporation has been used to give a clear indication of the effect of a hormone in ER synthesis.

Summary

The rate of endoplasmic reticulum (ER) synthesis following hormone treatment in barley aleurone cells was determined by measuring ^{14}C-choline incorporation into

acid insoluble material in a semi-purified ER fraction. Ninety-four percent of the choline counts incorporated are lipid extractable and only 9% are removed by nucleic acid extraction procedures. GA increases ER synthesis 4 to 8 fold, starting 2 to 4 hours after hormone addition (at about the same time as polysome formation).

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