Enhancement of Polyribosome Formation and Induction of Tryptophan-Rich Proteins by Gibberellic Acid*

Warren H. Evins†

ABSTRACT: Gibberellic acid (GA) causes the formation of polysomes and an increase in the proportion of ribosomes present as polysomes during the 8- to 10-hr lag period of α-amylase induction in barley aleurone layers, and an almost twofold increase in total ribosomes. Polysome formation starts at 3–4 hr and reaches a maximal level at 12–15 hr after hormone addition. A linear increase in the per cent polysomes is seen between 3 or 4 hr, reaching a maximum of 76% polysomes. The per cent polysomes more than doubles following GA treatment, while polysome formation increases over 2.5-fold. A greater breakdown of rat liver polysomes is observed following the addition of polysomes isolated from hormone-treated aleurone cells (rather than untreated cells), indicating that the stimulation of polysome formation occurs despite an increase in the amount of ribonuclease present in GA-treated cells. The number of active ribosomes (ribosomes capable of synthesizing nascent polypeptides, measured by the formation of acid-insoluble [^{3}H]peptidylpuromycin) doubles at 12 hr following hormone treatment; the proportion of total ribosomes that are active is however not affected. The rate of protein synthesis (determined as incorporation of [^{14}C]amino acids into acid-insoluble material) shows a doubling within 8 hr of hormone treatment. The bulk of the GA-induced proteins are tryptophan-rich and have high tryptophan:tyrosine ratios. 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.

The aleurone layer of the barley grain is a highly specialized tissue consisting of nondividing cells. It forms the outermost layers of the endosperm. After imbibition of the grain, the aleurone produces and releases enzymes that degrade the food reserves of the inner, "starchy" part of the endosperm to supply the embryo with energy and metabolites necessary for the development of the young seedling. The enzymes include: α- and β-amylases (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1; α-1,4-glucan maltohydrolase, EC 3.2.1.2.), peptidases, protease, RNase (polyribonucleotide 2-oligonucleotide transferase, EC 2.7.7.16.) (Chrispeels and Varner, 1967a; Paleg, 1960; Yomo, 1960a–d). The appearance of the increase of these enzymes is dependent on the plant hormone gibberellin. Gibberellin is normally supplied by the embryo. In isolated aleurone layers, however, the same responses can be obtained with GA_3[1] supplied through the incubation medium. Abscisic acid counters the effect of GA while other plant hormones are without effect (Cleland and McCombs, 1965; J. E. Varner, unpublished observations). The increases in α-amylase and protease have been shown to be due to *de novo* synthesis (Filner and Varner, 1967; Jacobsen and Varner, 1967). They occur, however, only after an 8- to 10-hr lag period following GA_3 addition (Chrispeels and Varner, 1967a; Filner and Varner, 1967; Jacobsen and Varner, 1967).

The evidence that transcription of new RNAs is required for the hormone response is inconclusive. Although GA_3 induces enzyme synthesis, it was reported that there is no increase in the rate of total protein synthesis at 12 hr following hormone treatment (Varner *et al.*, 1965).

One approach to determine the mechanism of action of GA_3 is to find out what is happening during the 8- to 10-hr lag period before α-amylase production starts. Perhaps some fundamental biochemical parameter responds to GA_3 earlier than α-amylase synthesis.

In addition, the earlier measurements of protein synthesis were subject to error because of isotope dilution and proteolysis. The level of protein synthesis can be inferred from (1) measurement of the levels of polysomes after various treatments and (2) by determination of the number of active ribosomes.

I now report that polysome formation starts 3 to 4 hr after hormone treatment. The rate of protein synthesis (incorporation of [^{14}C]amino acids) doubles toward the end of the lag period, and the number of ribosomes increases. The polysomes are probably bound to the endoplasmic reticulum. The hormone has no effect on the proportion of ribosomes active in protein synthesis. α-Amylase and other GA_3-induced proteins are rich in tryptophan (Fischer and Stein, 1960; J. E. Varner, unpublished observations), thus the tryptophan:tyrosine ratio of the nascent peptides was used as a characteristic identification tag to show that the polysomes isolated were responsible for the synthesis of the GA_3-induced proteins. The Results section is divided into two parts: increase in polyribosomes and synthesis of specific proteins.

Methods

Preparation and Incubation of Aleurone Layers. Half-seeds of barley (*Hordeum vulgare* L. cv. Himalaya, supplied by

* From the MSU–AEC Plant Research Laboratory and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received May 20, 1971.* This work was supported by the U. S. Atomic Energy Commission (Contract AT(11-1)-1338) and a National Science Foundation grant to Professor J. E. Varner (GB-8774). Michigan Agricultural Experiment Station Journal Article 5493.

† Present address: The Rockefeller University, New York, N. Y. 10021.

[1] Abbreviations used are: GA_3, gibberellic acid (gibberellin A_3); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; ER, endoplasmic reticulum.

Drs. R. A. Nilan, B. V. Conger, and the Agronomy Club at Washington State University, Pullman) were prepared by making two 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 min, the solution was stirred, and rinsed six 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 incubated in a 50-ml erlenmeyer flask with 5 ml of incubation medium containing 1 mM sodium acetate (pH 4.8)–20 mM $CaCl_2$, and where required, 1 μM K^+ gibberellic acid by shaking, on a Dubnoff shaker (approximately 80–100 oscillations/min, 25°). At the end of the incubation period, the layers were rinsed six 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 ribonuclease which might cause degradation of polysomes.

Polyribosome Isolation. The procedures of Wettstein, Staehelin, and Noll (1963; Staehelin *et al.*, 1964) as modified using ideas of Leaver and Key (1967) were used for polysome isolation from barley aleurone layers. 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 milliliters of "GB" (grinding buffer, containing: 450 mM sucrose, 100 mM Hepes, pH 7.55, with 50 mM K^+, 2 mM magnesium acetate, and 7 mM 2-mercaptoethanol (0.5 μl/ml of total volume added just prior to use)) was then added to the homogenizer. The powder was allowed to thaw for 5 min and then ground with two to three strokes and three to five quarter turns per stroke. The homogenate was decanted into a cold centrifuge tube. The homogenizer and pestle were rinsed twice with 3 ml (total) of GB.

The homogenate was centrifuged at 0° three times. The first two centrifugations were in the SS-34 rotor of the Sorvall centrifuge. The supernatant of the first centrifugation at 2 kg for 10 min was decanted and spun at 10 kg for 15 min. This supernatant was then centrifuged 2 hr 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 M sucrose buffer (RNase-free sucrose, Mann Research Laboratories, New York, N. Y., containing: 50 mM Hepes (pH 7.55), potassium salt added (25 mM), 2 mM magnesium acetate, and 7 mM 2-mercaptoethanol (0.5 μl/ml of total volume)), a middle layer of 3.0 ml of 0.6 M sucrose buffer, and the top layer consisting of the 10-kg supernatant in 0.45 M sucrose. The pellet produced will be referred to as the polysomal pellet.

The polysomal pellet was resuspended in 0.3 ml of ribosomal buffer (same as above sucrose buffers, but without any sucrose) with a ground-glass pestle (Pyrex 7725, supplied by Sargent-Welch Scientific Supply Co., Chicago).

Rat liver polysomes were isolated as described (Wettstein *et al.*, 1963), concentrated, and stored in small aliquots at −70°.

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 min in the SW65 rotor, while 40 min was 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, Waukegan, Ill. 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 cm^2. 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} unit 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 A unit as the full-scale chart deflection (approximately 0.0025 A_{260} unit/area unit).

The polysomal distribution or the "per cent 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 $[(P/P + M) \times 100]$.

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 of RNA per 100 aleurone layers.

Incorporation of Amino Acids. Incorporation of [^{14}C]amino acids was performed with 10 aleurone layers and 0.5 μCi 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, Calif.). Carrier-free amino acid was added to the aleurone layers at the start of the incubation period. The aleurone layers were ground and the homogenates 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 three times with 1 ml of 70% ethanol and combining the washings. Carrier bovine serum albumin (crystalline, A grade, Calbiochem, Los Angeles, Calif.) was added to each fraction (25 μg). The homogenate, medium, and extract were brought to "10%" trichloroacetic acid by the addition of 50% (w/v) trichloroacetic acid and allowed to sit overnight in the cold room. The precipitate was col-

lected on Millipore filters (0.45 μ, 25 mm diameter, Millipore Filter Corp., Bedford, Mass.), washed with at least 30 ml of 5% trichloroacetic acid containing carrier amino acids (neutralized casein acid hydrolysate, Calbiochem), dried for several hours at 70°, and counted with 10 ml of scintillation fluid "A" (4 g of PPO + 100 mg of POPOP per l. 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.

Determination of Tryptophan:Tyrosine Ratios. L-[^{14}C]-Tyrosine (1 μCi, uniform label, New England Nuclear Corp., Boston, specific activity 362 mCi/mmole) and 5 μCi of L-[^{3}H]tryptophan (general label, New England Nuclear Corp., specific activity 5.4 Ci/mmole) were added to the aleurone layers for 2 hr, beginning 8 hr after the start of incubation. In some experiments, the labels were present for all 10 hr. Cell fractions were prepared as described above (see incorporation of amino acids).

In another series of experiments, a 16-min pulse of labeled tryptophan and tyrosine was given in order to label nascent peptides that are attached to the polysomes. [^{3}H]Tryptophan and [^{14}C]tyrosine were used to determine the tryptophan:tyrosine ratio. The experiment was also performed using L-[3-^{14}C]tryptophan (side-chain label, New England Nuclear Corp., specific activity 22.8 Ci/mmole) and L-[3,5-^{3}H]tyrosine (New England Nuclear Corp., specific activity 16.5 Ci/mmole). The pulse was given to the aleurone layers so that the aleurone layers were incubated a total of 10 hr. At 9.75 hr after the start of incubation, the aleurone layers were transferred to a clean, sterile 25-ml erlenmeyer flask after rinsing three times with sterile H_2O and blotting on sterile paper towels under a hood equipped with an ultraviolet light. A mixture (100 μl) containing the carrier-free tryptophan and tyrosine labels and incubation medium $\pm GA_3$ was spread evenly over the 40 aleurone layers. All transfer operations were performed in the sterile transfer hood. The flask was returned to the Dubnoff metabolic shaker at 25°. It was removed from the shaker after the labeling period, rinsed six 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 ribosomal buffer and 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 min 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 hr. 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 tube of the SW56 rotor, and 2.5 ml in the tube of the SW65 rotor; the total volumes were 3.8 and 4.8 ml, respectively.

After centrifugation, the top two layers were removed with a disposable Pasteur pipet, the bottom layer was decanted, and the pellet was taken up in 5% trichloroacetic acid. 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% trichloroacetic acid, dried for several hours at 70°, and counted with 10 ml of scintillation fluid "A."

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

Preparation of Polysomal RNA. The polysomal pellet was resuspended with a ground-glass homogenizer in five-tenths volume of 0.1 M Na_2EDTA and five-tenths volume of ribosomal buffer (total volume 0.3 ml) and was incubated for 30 min 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 Na_2EDTA–sucrose buffer gradients and spun in the SW65 or SW56 rotors of the Beckman ultracentrifuge at full speed. A spin of 3.75 hr was used to separate the heavy and light ribosomal subunits from 5S RNA and tRNA. In order to separate the mRNA region, the heavy ribosomal subunit was pelleted during 9- to 13-hr runs. The sucrose gradients were prepared by the Noll (1967) method using 15 and 37% sucrose buffer–EDTA solutions.

The tube was punctured as described above (see Polysome Sedimentation) and scanned in the Gilford spectrophotometer. Fractions were collected with an LKB fraction collector or by hand. Carrier DNA (25 μg) was added to each fraction, followed by 50% (w/v) trichloroacetic acid 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% trichloroacetic acid with 0.1 M carrier sodium phosphate, dried at 70°, and counted.

Formation of [^{3}H]Peptidylpuromycin. 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 (Wool and Kurihara, 1967). 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 potassium salt added), 50 μM magnesium acetate, 7.3 mM 2-mercaptoethanol (0.5 μl/ml of total volume), 5 mM ATP, 50 μM GTP, 1 mM phosphoenolpyruvate, and 10 μg of pyruvate kinase. In most experiments 5 μCi of puromycin ([*methoxy*-^{3}H]puromycin dihydrochloride, specific activity 1.11 Ci/mmole) was used. The reaction was terminated by the addition of 5 ml of 10% trichloroacetic acid, filtered on a Millipore filter, and washed with 50 ml of 5% trichloroacetic acid 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 (1967) with rat muscle ribosomes were repeated with aleurone layer ribosomes.

The amount of RNA present in each of the resuspended polysomal pellets being 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 counts per minute of [^{3}H]peptidylpuromycin formed per microgram of RNA.

The following parameters were determined or calculated: (a) total number of ribosomes, (b) total molecules of peptidylpuromycin formed, and (c) the ratio b:a to obtain the molecules [^{3}H]peptidylpuromycin per ribosome from which the number or per cent active ribosomes is easily obtained.

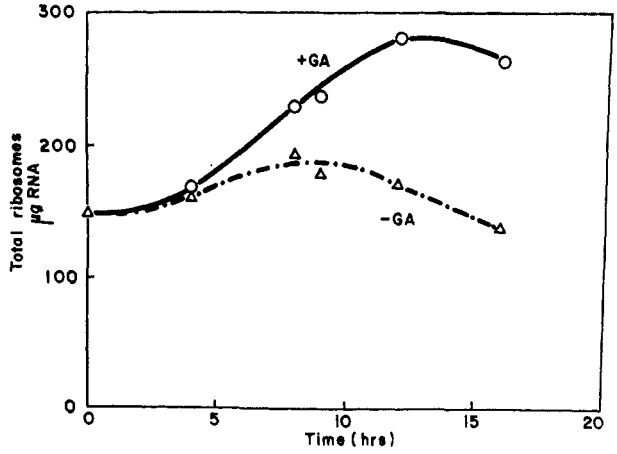

FIGURE 1: The effect of GA_3 on the total number of ribosomes present in the polysomal pellet. (The µg of RNA/100 aleurone layers was determined spectrophotometrically using the method of Warburg and Christian (1942) by measuring the $A_{260}:A_{280}$ ratios of ribonuclease-sensitive material in the polysomal pellet.)

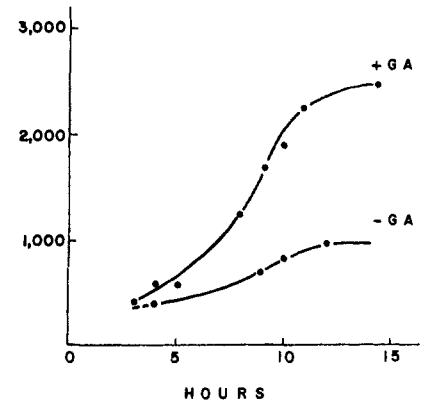

FIGURE 3: The effect of GA_3 on polysome formation. (The amount of polysomes/100 barley aleurone layers was determined by centrifuging ribosomes in 0.3–1.0 M isokinetic sucrose gradients. Forty aleurone layers were incubated at 25° for various times in 1 mM acetate buffer pH 4.8 with 20 mM $CaCl_2$ and 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.)

Results

Increase in Polyribosomes. TOTAL RIBOSOMES. A number of short-term-labeling experiments were performed with carrier-free [^{32}P]orthophosphoric acid in order to measure the rate of ribosome synthesis. [^{32}P]Orthophosphoric acid (1.5–2.5 mCi) in very small volumes of incubation medium (±GA_3) was evenly distributed over the aleurone layers and was present during the last 30- or 60-min incubation. The incorporation of label was measured following incubation periods of different lengths. Polysomal RNA was prepared as described. Incorporation of ^{32}P occurs into RNase-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 (W. H. Evins, unpublished observations). 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 1. 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 after about 12-hr incubation with GA_3. The effect of ribosome turnover increases after 12 hr as a decrease in the amount of ribosomes is seen in the presence of the hormone after 12 hr.

The polysome formation data which follow, and both the spectrophotometric data and the incorporation of isotopically labeled precursor indicate that one effect of hormone addition is ribosome synthesis and an increase in the total number of ribosomes present.

POLYSOME FORMATION. Typical polysome profiles are obtained after 8-hr incubation with 10^{-6} M GA_3 (Figure 2a) or in the absence of GA_3 (Figure 2b).

After 5-min incubation with 0.1 µg of pancreatic RNase at 37°, the polysomes from the +GA tissue are entirely degraded (Figure 2a). Similar degradation occurs in control samples.

More polysomes can be isolated from GA-treated aleurone layers than from the control layers (Figure 3). Polysome formation starts at 3- to 4-hr incubation and continues until 12–15 hr both in the presence and absence of GA_3. Over 2.5 times more polysomes were found after 12-hr hormone treatment, as compared to control tissue. At longer times of incubation, the amount of polysomes found decreases from the maximal levels seen at 12–15 hr in both the control and hormone-treated tissues.

POLYSOMAL DISTRIBUTION. Not only does GA_3 increase polysome formation, but it also causes an increase in the distribution of ribosomes in polysomes—the per cent polysomes (Figure 4). 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-

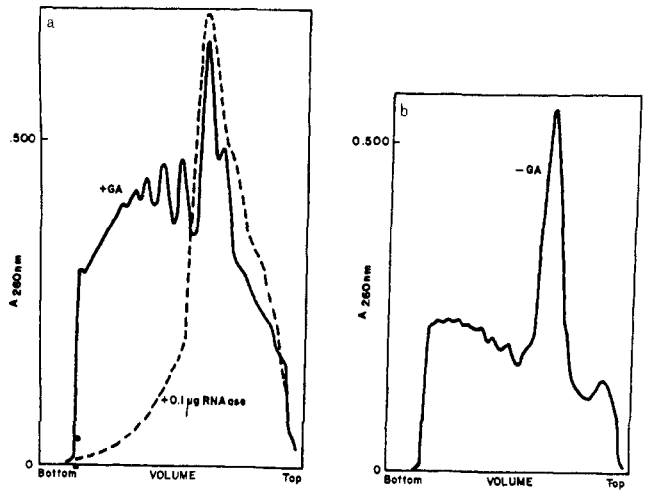

FIGURE 2: Typical polysome profiles and the effect of RNase. (Polysomes were isolated from 40 barley aleurone layers incubated for 8 hr at 25°. The polysomal profiles were determined by pelleting the polysomes through 0.3–1.0 M isokinetic sucrose gradients: (a) in the presence of 1 µM GA_3 alone = +GA, and following 5-min incubation with 0.1 µg of RNase at 37° = +RNase, or (b) without GA_3 = −GA.)

TABLE I: Sedimentation Coefficient of Polysomes.[a]

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

[a] 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 three experiments.

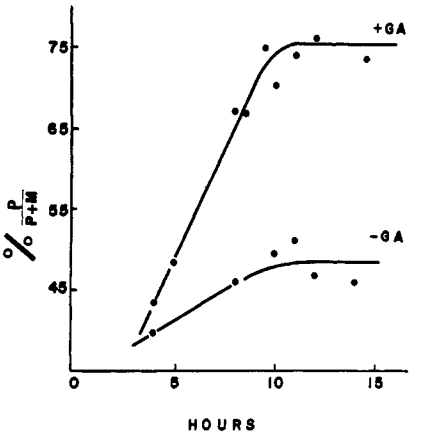

FIGURE 4: The distribution of ribosomes in polyribosomes (per cent polysomes). (The distribution of ribosomes in polyribosomes was determined in the presence or absence of 1 μM GA_3. The polysomal distribution, $\%(P/P + M)$, was calculated by determining the area of the polyribosome (P) and monosome (M) regions of the A_{280} profile with a planimeter. Each point represents the average of duplicate samples.)

treated tissue more than doubles by 10 hr after the start of incubation. The per cent polysomes increases linearly starting at 3–4 hr, *i.e.*, at the same time that polysome formation starts (Figure 3). A maximum level of about 76% polysomes is reached at approximately 10 hr after the start of incubation. At longer times of incubation, the per cent polysomes decreases as does the amount of polysomes found.

The per cent polysomes in a preparation 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 yielded a maximum of 86% polysomes. The samples plotted in Figures 2, 3, and 4 were prepared with the same homogenizer.

The previous two sections have shown that, in addition to ribosome synthesis, the hormone causes the formation of polysomes and an increase in the per cent polysomes. The polysomes appear to be typical, are degraded by exogenous RNase, and show typical profiles in sucrose gradients. The next section presents additional evidence that the polysomes are typical of eukaryotic polysomes in that the sedimentation coefficients of the aleurone polysome are similar to those of rat liver polysomes.

SEDIMENTATION COEFFICIENTS. In order to characterize further 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 I) were determined by comparison with the known sedimentation values of the rat liver polysomes, monosome, and large ribosomal subunit (Noll, 1967). Calibration of the isokinetic gradients used with rat liver polysomes and mixing experiments were performed using the method of Stutz and Rawson (1968). Polysome species from both rat liver and aleurone layer sources have similar sedimentation properties. Therefore, it seems justified to conclude that the polysome profile represents the separation of polysomal units containing two, three, four, or move ribosomes.

The next section switches from a description of the GA_3 effect on polysome formation to attempts to explain this effect of the hormone, or rather to eliminate a reasonable explanation for the effect on polysomes.

ENDOGENOUS RNase. Is the lack of polysomes in the absence of GA_3 due to a greater degree of polysome degradation when the hormone is not present, *i.e.*, is there more endogenous RNase in the control tissue? In order to answer this question, mixing experiments were performed using polysomes isolated from rat liver, and from aleurone layers incubated for 8 hr in the presence or absence of 10^{-6} M GA_3.

Degradation occurs when polysomes isolated from aleurone layers and rat livers are mixed and allowed to sit for short times at 0° (Table II). Fewer polysomes are recovered when +GA polysomes are mixed with rat liver polysomes than when −GA polysomes are used. Thus, polysome preparations from aleurone cells treated with GA_3 seem to contain more endogenous RNase activity than those from control layers. This agrees with the known fact that RNase is among the GA-induced enzymes (Chrispeels and Varner, 1967b).

More ribosomes are found in the polysome structures after mixing polysomes isolated from rat livers and nontreated aleurone cells compared to the distribution in the control aleurone tissue alone. 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 breakdown of polysomes and the consequent increase in monosomes.

These experiments show that there is greater polysome breakdown in polysomes from hormone-treated aleurone tissue. Therefore, the lack of polysomes in the control samples is not due to greater polysome degradation.

Synthesis of Specific Proteins. PROTEINS SYNTHESIS. The incorporation of a carrier-free mixture of [^{14}C]amino acids was measured in order to estimate the rate of overall protein synthesis (Table III). 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-hr incubation 2.0 times more label is incorporated into acid-precipitable material in the GA-treated tissue. At 16 hr, there is more incorporation of acid-precipitable [^{14}C]amino acid mixture into the control tissue presumably because of increasing protein turnover and an expanding amino acid pool in the hormone-treated samples. The incorporation of amino acids continues to increase at

TABLE II: Recovery of Polysomes from Mixtures of Rat Liver and Aleurone Layer Ribosomes.[a]

	Polysome Area[b]	% Polysomes/ Polysomes and Monosomes	Change in Polysome Distribution Due to Rat Liver Polysomes	Area of Rat Liver Polysomes[c]	% of Rat Liver Polysomes Recovd[c]
Rat liver polysomes	1244	84.5		1244	100.0
8h + GA + RLP	986	59.0	−4.0	1074	86.3
8h − GA + RLP	880	61.0	+6.8	1178	94.7
8h + GA	897	63.0			
8h − GA	582	54.2			

[a] Duplicate samples of 40 barley aleurone layers (±GA), of 20 barley aleurone layers + rat liver polysomes (+ GA + RLP), or of twice as much rat liver polysomes (RLP) were incubated at 25° in the presence and absence of 1 μM GA_3. [b] The polysome and monosome areas were determined by planimetry (see Methods). [c] Calculated by subtracting one-half the area of the unmixed barley aleurone layer polysomes (±GA) from the area of the mixed polysomes (±GA) then doubling the result, *e.g.*, + GA + RLP: 986–897/2 = 537 × 2 = 1074.

TABLE III: Effect of GA_3 on [^{14}C]Amino Acid Incorporation by Barley Aleurone Layers.[a]

		Cpm (× 10^{-3}) [^{14}C]Amino Acid Incorporated into Trichloroacetic Acid Precipitable Material			
Time of Incubn (hr)	Treatment	Total	Medium	Homogenate	Extract
8	+GA	261.1	194.8	66.3	
	−GA	129.3	17.3	112.0	
16	+GA	141.6	72.2	69.5	
	−GA	157.7	51.8	105.8	
24	+GA	132.0	91.4	40.6	
	−GA	198.4	46.2	152.2	
With carrier amino acids, 8	+GA	168.7	13.0	91.1	64.6
	−GA	140.2	11.8	70.2	58.1

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

24 hr in the absence of the hormone, but decreases further with GA.

It seems that the increase in protein synthesis due to GA_3 can be seen only during short incubations with the hormone.

An experiment was performed with carrier amino acids present in order to observe the effect of isotope dilution (Table III, last lines). 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-hr incubation, there was more incorporation of acid-precipitable [^{14}C]amino acids in the presence of GA_3. However, there was significantly less difference in incorporation between the hormone-treated and control tissue than was seen when the carrier-free amino acids label was added.

Incorporation of amino acids has shown that another effect of the hormone is an increase in overall protein synthesis. The *de novo* synthesis of α-amylase and protease indicates that new proteins are being synthesized as a consequence of GA_3 addition. Other hydrolytic enzyme activities appear or increase in response to GA_3. The hormone-controlled formation and aggregation of polysomes and synthesis of ribosomes has been demonstrated in earlier sections. The next section will demonstrate that the polysomes isolated are responsible for the synthesis of specific proteins.

IDENTIFICATION OF GA-INDUCED ENZYMES BY TRYPTOPHAN: TYROSINE RATIOS. α-Amylases from various sources have proved to be rich in tryptophan. This usually results in a high tryptophan:tyrosine ratio (Fischer and Stein, 1960). Barley α-amylase is no exception: it has a high tryptophan content and a high tryptophan:tyrosine ratio. Many other hydrolytic enzymes, including some of those present in GA-treated aleurone cells are also high in tryptophan, resulting in high tryptophan:tyrosine ratios (J. E. Varner, personal communication).

The distribution between medium and homogenate of [^{3}H]tryptophan incorporated into trichloroacetic acid precipitable protein of barley aleurone cells is shown in Table IV. Proteins formed in the presence of labeled tryptophan and tyrosine have 36% higher tryptophan:tyrosine ratios in the

TABLE IV: Location of Acid-Precipitable [^{3}H]Tryptophan in Barley Aleurone.[a]

Treatment	Homogenate		Medium	
	Cpm	Trp:Tyr Ratio	Cpm	Trp:Tyr Ratio
+GA	48,000	0.87	9600	4.78
−GA	32,400	0.64	5200	1.35

[a] Triplicate samples of ten aleurone layers were incubated at 25° in the presence and absence of 1 μM GA_3. Between 8 and 10 hr 5 μCi of [^{3}H]tryptophan and 1 μCi of [^{14}C]tyrosine were added. Cell fractions were prepared (see Methods), and the trichloroacetic acid precipitates were collected on Millipore filters and washed with 5% trichloracetic acid containing carrier amino acids. The samples were counted in a Beckman three-channel liquid scintillation counter and the tryptophan:tyrosine ratios were calculated using specially prepared ^{3}H and ^{14}C standards.

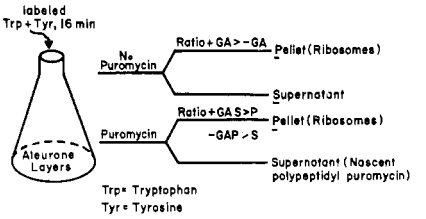

FIGURE 5: Diagram of expected tryptophan:tyrosine ratio results following hormone treatment. (Aleurone layers were incubated during the last 16 min of a 10-hr incubation period with [^{3}H]tryptophan (Trp) and [^{14}C]tyrosine (Tyr) or [^{14}C]tryptophan and [^{3}H]-tyrosine (reverse experiment) in the presence or absence of GA_3. The polyribosomal pellet was isolated. The ribosomes should have a greater tryptophan:tyrosine 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 peptidylpuromycin remained in the supernatant (S). The expected results are shown. The actual results agree with the predictions.)

homogenate, 350% higher tryptophan:tyrosine ratios in the medium, and more tryptophan incorporated in hormone-treated aleurone layers. The GA-induced hydrolytic enzymes are secreted into the endosperm of the barley grain or into the medium when isolated aleurone layers are incubated in sterile flasks. Likewise, the medium contains proteins with high tryptophan:tyrosine ratios. These results suggest that the bulk of the GA-induced proteins are tryptophan-rich and have high tryptophan:tyrosine ratios. The results demonstrate that the tryptophan:tyrosine 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 5). Growing polypeptide chains of the GA-induced hydrolases should have a higher tryptophan:tyrosine 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 tryptophan:tyrosine ratios than nascent peptides of the control polysomes.

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

The ^{14}C:^{3}H experiments shown in Table V give the same results. The ratios in the supernatant are lower, primarily because of the reduction of contaminating label by the addition of carrier tryptophan and tyrosine. All important differences are significant at $P < 0.1$.

The high tryptophan content of some of the GA-induced enzymes has proven to be a useful handle. It has been possible to demonstrate that the hormone causes an increase in those polysomes capable of synthesizing the specific proteins characteristic of the hormone response. In the next section, one possible explanation for the ability of the cell to increase both the rate of overall protein synthesis and the synthesis of specific proteins is explored.

DETERMINATION OF ACTIVE RIBOSOMES. Does GA_3 cause an activation of the ribosomes? To answer this question, the capacity of the cells to produce nascent peptidylpuromycin

TABLE V: Location and Puromycin Release of Tryptophan-Rich Nascent Polypeptides.[a]

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

[a] $P < 0.1$ for significant differences. Forty aleurone layers were incubated during the last 16 min of a 10-hr incubation period with 25 μCi of [^{3}H]tryptophan and 5 μCi of [^{14}C]-tyrosine or in the reverse experiment with 4 μCi of [^{14}C]-tryptophan and 20 μCi of [^{3}H]tyrosine at 25° in the presence and absence of 1 μM GA_3. 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 ^{14}C:^{3}H experiment, 5×10^{-4} M L-tryptophan and 5×10^{-4} M L-tyrosine were added to the first discontinuous gradients to reduce background counts. The samples were counted on a Beckman three-channel liquid scintillation counter (the pellet was precipitated with 10% trichloroacetic acid, collected on a Millipore filter, and washed with 5% trichloroacetic acid containing carrier amino acids—see Methods, and the supernatant was counted directly with Bray's scintillation fluid) and the tryptophan:tyrosine ratios were calculated using specially prepared ^{3}H and ^{14}C standards.

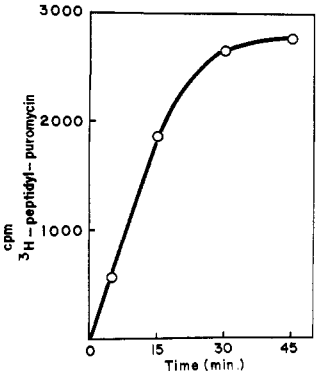

FIGURE 6: The time course of [^{3}H]peptidylpuromycin formation by barley aleurone layer ribosomes. (The ribosomes (from ten layers) were incubated with 5 μCi of [^{3}H]puromycin (see Methods) and the [^{3}H]puromycin peptides were precipitated and washed with trichloroacetic acid and collected on Millipore filters. Each point represents the average of four replicate samples. Similar results were obtained in two experiments.)

was determined using the method of Wool and Kurihara (1967). An active ribosome is a ribosome that is synthesizing nascent protein. It consequently has nascent peptidyl-tRNA as a structural part of the ribosome.

Barley aleurone ribosomes incubated with [^{3}H]puromycin formed radioactive [^{3}H]peptidylpuromycin rapidly (Figure 6). The formation of [^{3}H]peptidylpuromycin is essentially completed after a reaction period of 30 min. This was therefore used as the standard incubation time for all of the assays. The incubation temperature was 37°. Puromycin added *in vitro* inhibits protein synthesis, but allows an active ribosome to make one peptide bond releasing the nascent puromycin peptide. The estimation of the number of active ribosomes would be too high if a ribosome could form more than one molecule of peptidylpuromycin.

Ribosomes were isolated from aleurone cells that had been incubated for various times in the presence or absence of GA_3. The amount of RNA present in duplicate aliquots was determined. The sample was then divided into four equal parts: two assays and two blanks. Two duplicates were assayed for the amount of [^{3}H]peptidylpuromycin formed during a 30-min incubation period. The peptidylpuromycin formation reaction in the other two duplicates was immediately terminated with the addition of 5 ml of 10% (w/v) trichloroacetic acid 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 incorporation in the control samples without ribosomes and at zero time were subtracted from the counts incorporated during the 30-min incubation period.

The effect of GA_3 on the change in the number of active ribosomes in the barley aleurone cells with time was measured (Table VI). A peak of ribosome activity occurs about 4 hr after the start of incubation. This increase in ribosome activity is, however, not due to GA_3 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 hr, the ribosome activity decreases in both the control and hormone-treated aleurone cells. Although there 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 hr the control tissue has 55% more activity per ribosome than the hormone-treated tissue.

TABLE VI: Formation of Peptidylpuromycin by Ribosomes from Barley Aleurone: Effect of GA_3 and Time of Incubation.[a]

Treatment	Incubn Time (hr)	Molecules of [^{3}H]Peptidyl-puromycin Formed	μg of RNA	% Active Ribosomes
−GA	0	0.40×10^{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	0.40×10^{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

[a] The number of molecules of [^{3}H]peptidylpuromycin formed and the per cent active ribosomes were calculated from previous data (see text and Methods). All results are the average of two duplicates. Similar results were obtained in two experiments.

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, the *per cent* of active ribosomes is not affected by the hormone; that is, the hormone does not cause ribosome activation.

Discussion

An increase in polysomes is one of the earliest events to be seen after the application of GA. A silent period of 3 to 4 hr precedes polysome formation. During this period the synthesis of the ER to which the polysomes are attached, occurs (Evins and Varner, 1971a). The actual synthesis of α-amylase begins at 8–10 hr. The lag period between increased 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 12–15 hr after hormone addition. This amount of polysome formation induced by GA is most probably due to the fact that the aleurone system consists of a single cell type and that every cell responds to the added hormone. The increase in control tissues may be due to endogenous gibberellins.

Polysome formation has been demonstrated in a number of growing systems, after fertilization in sea urchin eggs (Humphreys, 1969) or the eggs of several animals (Monroy, 1970), and during germination in castor bean endosperm (Sturani *et al.*, 1968), pea (Barker and Rieber, 1967), pollen (Mascarenhas and Bell, 1969), or wheat (Marcus *et al.*, 1966). But hormone-induced polysome formation generally occurs long after addition of the hormone. During these times growth and development of the cells continue (reviewed in Tata, 1970).

The increase in the number of ribosomes isolated as poly-

somes in GA-treated aleurone cells is due both to ribosome synthesis and aggregation (increase in the per cent 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 RNase in the hormone-treated cells. No increase in recovery was seen with different isolation methods, including the use of various RNase inhibitors, detergents, and different methods of isolation, centrifugation, and homogenization (unpublished results).

Polysome recovery increases in GA-treated cells following the addition of 0.5% sodium deoxycholate to the postmitochondrial supernatant 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 is not affected by the addition of detergent; although polysome recovery decreases in both hormone-treated and control samples.

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, 1970). Secreted proteins are thought to be synthesized on membrane-bound polysomes (Siekevitz and Palade, 1960, 1966; Andrews and Tata, 1968; Campbell, 1970). 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 one-half 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. The number of active ribosomes also doubles. The determination of the number of active ribosomes may be a measure of the rate of protein synthesis that is unaffected by isotope dilution. The increase in protein synthesis is consistent with the observed increase in polysomes.

Several different types of experiments have established that the polysomes isolated were those responsible for GA-induced enzyme synthesis. The high tryptophan:tyrosine 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 (W. H. Evins, unpublished observations). In addition, anaerobic conditions inhibiting α-amylase synthesis inhibit polysome formation, washing out GA reduces the number of ribosomes in polysomes, abscisic acid, a plant hormone that prevents α-amylase synthesis but does not generally inhibit RNA synthesis, prevents polysome formation, and inhibitors of α-amylase synthesis inhibit polysome formation (Evins and Varner, 1971b).

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 understood.

Acknowledgment

I thank Professor J. E. Varner for many helpful discussions and Professors Varner and A. Lang for review of the manuscript.

References

Andrews, T. M., and Tata, J. R. (1968), *Biochem. Biophys. Res. Commun. 32*, 1050.
Barker, G. R., and Rieber, M. (1967), *Biochem. J. 105*, 1195.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Campbell, P. N. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 1.
Chrispeels, M. J., and Varner, J. E. (1967a), *Plant Physiol. 42*, 398.
Chrispeels, M. J., and Varner, J. E. (1967b), *Plant Physiol. 42*, 1008.
Cleland, R., and McCombs, N. (1965), *Science 150*, 497.
Evins, W. H., and Varner, J. E. (1971a), *Proc. Nat. Acad. Sci. U. S. 68*, 1631.
Evins, W. H., and Varner, J. E. (1971b), *Plant Physiol. 48* (in press).
Filner, P., and Varner, J. E. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1520.
Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.
Humphreys, T. (1969), *Develop. Biol. 20*, 435.
Jacobsen, J. V., and Varner, J. E. (1967), *Plant Physiol. 42*, 1596.
Jones, R. L. (1969a), *Planta 87*, 119.
Jones, R. L. (1969b), *Planta 88*, 73.
Leaver, C. J., and Key, J. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1338.
Marcus, A., Feeley, J., and Volcani, T. (1966), *Plant Physiol. 41*, 1167.
Mascarenhas, J. P., and Bell, E. (1969), *Biochim. Biophys. Acta 179*, 199.
Monroy, A. (1970), *Biochem. J. 117*, 57.
Noll, H. (1967), *Nature (London) 215*, 360.
Noll, H. (1969), *Anal. Biochem. 27*, 130.
Paleg, L. (1960), *Plant Physiol. 35*, 293.
Redman, C. M. (1967), *J. Biol. Chem. 242*, 761.
Redman, C. M., and Sabatini, D. D. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 608.
Siekevitz, P., and Palade, G. E. (1960), *J. Biophys. Biochem. Cytol. 7*, 619.
Siekevitz, P., and Palade, G. E. (1966), *J. Cell Biol. 30*, 519.
Staehelin, T., Wettstein, F. O., Oura, H., and Noll, H. (1964), *Nature (London) 201*, 264.
Sturani, E., Cocucci, S., and Marré, E. (1968), *Plant Cell Physiol. 9*, 783.
Stutz, E., and Rawson, J. R. (1968), *Biochim. Biophys. Acta 161*, 564.
Tata, J. R. (1970), *in* Biochemical Actions of Hormones, Vol. 1, Litwack, G., Ed., New York, N. Y., Academic Press, p 89.
van der Eb, A. A., and Nieuwdorp, P. J. (1967), *Acta Bot. Neer. 15*, 690.
Varner, J. E., Chandra, G. Ram, and Chrispeels, M. J. (1965), *J. Cell. Comp. Physiol. 66, Suppl. 1*, 55.
Vigil, E. L., and Ruddat, M. (1971), *Plant Physiol.* (in press).
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Wettstein, F. O., Staehelin, T., and Noll, H. (1963), *Nature (London) 197*, 430.
Wool, I. G., and Kurihara, K. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2401.
Yomo, H. (1960a), *Hakko Kyokaishi 18*, 293.
Yomo, H. (1960b), *Hakko Kyokaishi 18*, 500.
Yomo, H. (1960c), *Hakko Kyokaishi 18*, 600.
Yomo, H. (1960d), *Hakko Kyokaishi 18*, 603.