

## Hormonal Control of Enzyme Synthesis: On the Mode of Action of Gibberellic Acid and Abscisin in Aleurone Layers of Barley<sup>1</sup>

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**Summary.** Gibberellic acid (GA) enhances the synthesis of  $\alpha$ -amylase and ribonuclease in isolated aleurone layers and this process is inhibited by abscisin. Removal of gibberellic acid in mid-course of  $\alpha$ -amylase production results in a slowing down of  $\alpha$ -amylase synthesis, suggesting a continued requirement of GA for enzyme synthesis. This is paralleled by a continuous requirement for RNA synthesis. Addition of 6-methylpurine or 8-azaguanine in mid-course results in an inhibition of  $\alpha$ -amylase synthesis within 3 to 4 hours. However, actinomycin D added in mid-course is almost without effect. This is not due to its failure to enter the cells, because it does inhibit <sup>14</sup>C-uridine incorporation at this stage. Addition of abscisin to aleurone layers which are synthesizing  $\alpha$ -amylase results in an inhibition of this synthesis within 2 to 3 hours. Cycloheximide on the other hand inhibits enzyme synthesis immediately upon its addition. These data are consistent with the hypothesis that the expression of the GA effect requires the synthesis of enzyme-specific RNA molecules. The similarity in the kinetics of inhibition between abscisin on the one hand and 8-azaguanine or 6-methylpurine on the other suggests that abscisin may exert its action by inhibiting the synthesis of these enzyme-specific RNA molecules or by preventing their incorporation into an active enzyme-synthesizing unit.

Gibberellic acid greatly enhances the synthesis of amylolytic enzymes (and several other hydrolytic enzymes) in aleurone cells of barley (20, 23, 27), wild oats (16), and rice (19). We have studied in detail the GA-enhanced synthesis and release of  $\alpha$ -amylase and ribonuclease (4) and of protease (Jacobsen and Varner, in preparation) by isolated aleurone layers. The manifestation of this action of gibberellic acid is inhibited by inhibitors of protein synthesis (*p*-fluorophenylalanine and cycloheximide) and of ribonucleic acid synthesis (actinomycin D and 8-azaguanine). Evidence obtained by a new method indicates that the appearance of  $\alpha$ -amylase (6) and protease (Jacobsen and Varner, in preparation) is due to the *de novo* synthesis of all the enzyme which is produced. This *de novo* synthesis is dependent on the synthesis of one or more species of ribonucleic acid.

Recently we reported that abscisin (abscisin II or dormin) inhibits the GA-enhanced synthesis of  $\alpha$ -amylase and the release of free amino acids by aleurone layers (3). The inhibition of enzyme

synthesis by abscisin can be partially overcome by the addition of a larger amount of GA. The kinetics of this interaction are neither competitive, nor non-competitive. The complexity of the system makes an interpretation of the kinetics data impossible. The inhibition of enzyme synthesis by abscisin is different from, and much more specific than, that obtained by metabolic inhibitors. Indeed, abscisin does not affect respiration or phosphorylation; neither does it inhibit the incorporation of radioactive precursors into protein (other than the hydrolases) and RNA (3).

An interaction between GA and abscisin has been reported in several systems. Abscisin inhibits the expansive growth of leaf sections of corn and this inhibition is reversed by GA (21). It also inhibits the GA-enhanced elongation of normal and dwarf peas (21). The elongation (normal and GA-enhanced) of lentil epicotyls is accompanied by DNA synthesis (16); both elongation and DNA synthesis are inhibited by abscisin and this inhibition can be partially reversed by the addition of GA (Chrispeels, unpublished).

We have studied in greater detail the effects of GA and abscisin on barley aleurone layers, as well as the effect of RNA-synthesis-inhibitors on  $\alpha$ -amylase synthesis in order to learn more about the mode of action of the 2 hormones.

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## Materials and Methods

Most of the materials and methods used in these experiments have been described in detail in our previous paper (4). Synthetic d,l-absicisn, a gift from Dr. J. van Overbeek (Shell), was used in all the experiments reported here. A stock solution of 0.2 mM absicisn in water was kept frozen or in the refrigerator.

The incorporation of  $^{14}\text{C}$ -leucine and  $^{14}\text{C}$ -uridine was measured in the following way: Aleurone layers were incubated as usual and 1  $\mu\text{C}$  of  $^{14}\text{C}$ -leucine (200 mc/mmole) or of  $^{14}\text{C}$ -uridine (25 mc/mmole) (both purchased from Schwarz Bio-research, Inc.) was added, and incorporation was allowed to proceed for the appropriate period of time. The aleurone layers were then rinsed 3 times with 1 mM cold, carrier leucine or uridine. When  $^{14}\text{C}$ -leucine was used the aleurone layers were homogenized in the cold in 5 ml of 0.2 N NaCl in the usual way, and the extracts were centrifuged for 10 minutes at  $2000 \times g$ . An aliquot of 0.2 ml of the supernatant was plated on a glass planchet to determine the total uptake. Another aliquot of 0.25 ml of the salt soluble proteins was mixed with an equal volume of 10 mM leucine and precipitated with 2 volumes of 15% trichloroacetic acid. The precipitated proteins were collected by filtration on Millipore filters and washed with 5% trichloroacetic acid. The filters were dried and counted in a gas flow counter. When  $^{14}\text{C}$ -uridine was used the aleurone layers were homogenized in the cold in 5 ml of 1 M NaCl with 5 mg of carrier RNA. Incorporation of the precursor into the salt-soluble nucleoproteins was determined as described above, except that carrier uridine was used. This method gives results similar to those obtained with more elaborate extraction procedures for nucleic acids.

## Results

*Removal of Gibberellic Acid in Midcourse of  $\alpha$ -Amylase Production.* The addition of GA to isolated aleurone layers of barley results in a linear synthesis of  $\alpha$ -amylase after a lag-period of 7 to 9 hours. When aleurone layers are incubated in 0.05  $\mu\text{M}$  GA for 11 hours, frequent rinsing over a period of 1 hour to remove as much GA as possible results in a substantial reduction of the  $\alpha$ -amylase synthesized in the next 12 hour period (4). Gibberellic acid can therefore not be considered as a trigger because its presence is required during the lag-period as well as during the period of  $\alpha$ -amylase synthesis, if maximal enzyme synthesis is to be obtained. In the present experiments the removal of GA was improved by rinsing the aleurone layers 4 times at half-hour intervals with a medium containing 20 mM  $\text{CaCl}_2$  and 1 mM sodium acetate buffer pH 4.8. At the end of the rinsing period 1 set of aleurone layers was further incubated with GA and 1 without GA. The results of such an experiment

are shown in figure 1. It is apparent that  $\alpha$ -amylase synthesis is severely inhibited within a few hours, if GA is not added again after it has been removed.

When aleurone layers are incubated without GA for 10 hours, and 0.1  $\mu\text{M}$  GA is added, no  $\alpha$ -amylase is produced in the first 5 to 7 hours indicating that GA is required to overcome the lag-period. The lag-period can be shortened, but not abolished by first incubating the aleurone layers in a low concentration of GA (2.5  $\mu\text{M}$ ) and adding a higher amount of GA (0.5  $\mu\text{M}$ ) after 10 hours (fig 2). After such an increase in GA concentration there is a lag-period of 3 to 4 hours before the aleurone layers synthesize  $\alpha$ -amylase at a rate equivalent to the one obtained with the higher GA concentration.

Once the lag-period has been overcome it cannot be reintroduced by a prolonged incubation of the aleurone layers in the absence of GA. Aleurone layers were incubated for 8 hours in 0.05  $\mu\text{M}$  GA, and GA was removed as described above. The aleurone layers were further incubated without GA for 6 hours. The rate of enzyme synthesis had slowed down considerably at this time. Subsequent addition of GA resulted in an immediate resumption of  $\alpha$ -amylase synthesis at the control rate (fig 3).

*Inhibition of GA Enhanced Synthesis of  $\alpha$ -Amylase and Ribonuclease by Absicisn.* The GA-enhanced production of  $\alpha$ -amylase by aleurone layers is inhibited by absicisn (table I). When aleurone layers are incubated with 0.1  $\mu\text{M}$  GA and 1  $\mu\text{M}$  absicisn, very little enzyme is synthesized. Absicisn also inhibits the small amount of  $\alpha$ -amylase synthesis which occurs in the absence of GA. Addition of a larger amount of GA reverses this inhibition by absicisn, as shown earlier (3) and as demonstrated in a different way in table II. Aleurone layers were incubated with increasing amount of GA (from 0.1  $\mu\text{M}$  to 0.1 mM) and either without absicisn or with 0.05  $\mu\text{M}$  or 5  $\mu\text{M}$  absicisn. The inhibitory effect of 0.05  $\mu\text{M}$  absicisn is almost completely reversed by 1  $\mu\text{M}$  GA. When 5  $\mu\text{M}$  absicisn are used reversal by GA never exceeds 30% regardless of the amount of GA used. This demonstrates that reversal is not merely a matter of the molar ratio of GA and absicisn.

Absicisn inhibits the GA enhanced production of ribonuclease in a similar fashion (table III). The

Table I. *Inhibition of  $\alpha$ -Amylase Synthesis by Absicisn*

Ten aleurone layers were incubated with buffer, 20 mM of  $\text{CaCl}_2$  and with or without 0.1  $\mu\text{M}$  GA and the concentrations of absicisn indicated.

Treatment	$\alpha$ -Amylase per 10 aleurone layers
Control	40
Control + 0.5 $\mu\text{M}$ absicisn	8
GA	475
GA + 1 $\mu\text{M}$ absicisn	67

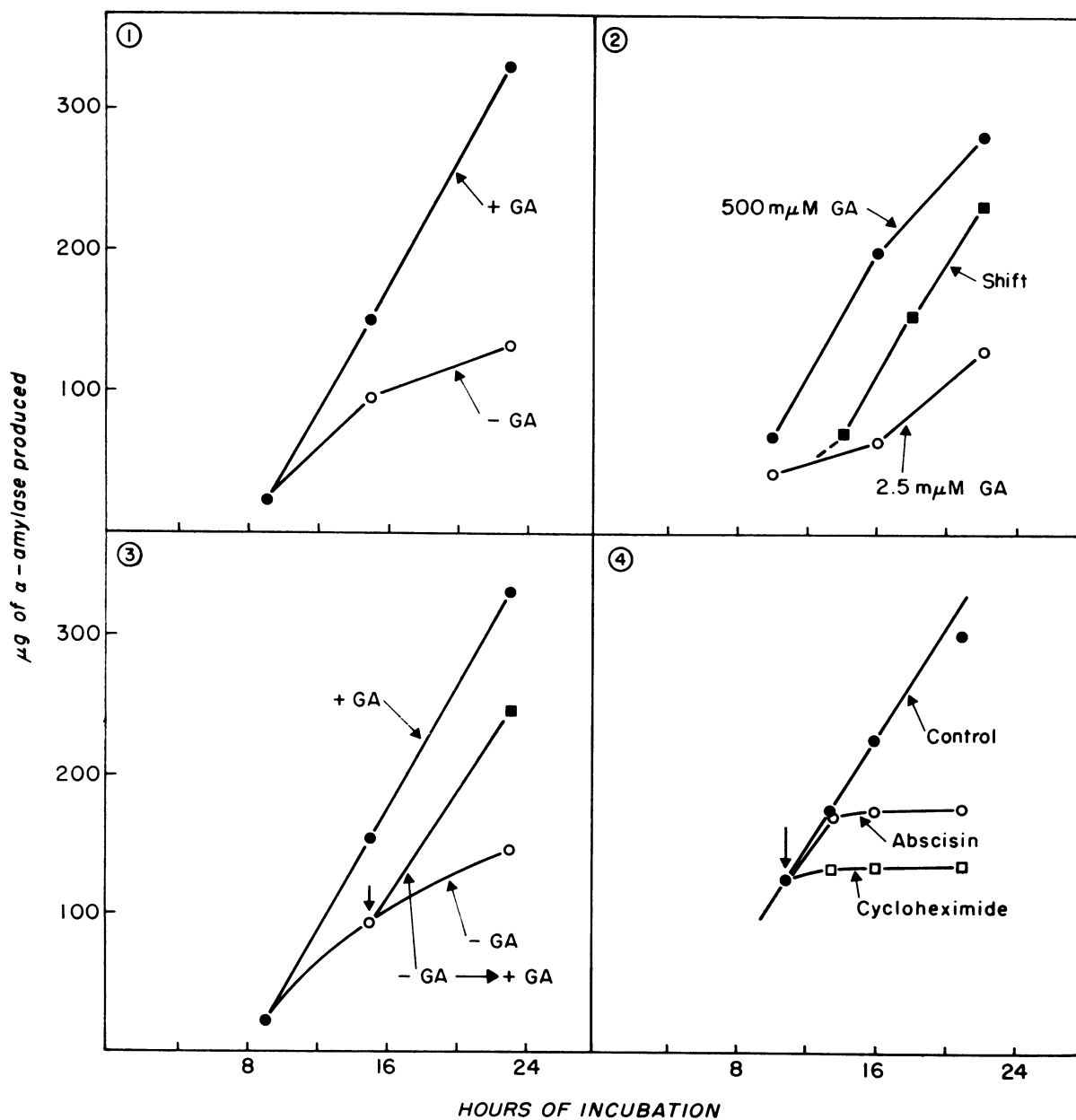


FIG. 1. Effect of removing GA at the end of the lag-period. Aleurone layers were incubated for 7 hours in  $0.5 \mu\text{M}$  GA. GA was then removed by 4 consecutive one-half-hour rinses. The aleurone layers were further incubated either with GA, or without GA, and  $\alpha$ -amylase synthesis was measured 6 and 14 hours later.

FIG. 2. Shift from a low to a high concentration of GA. Aleurone layers were incubated in  $2.5 \text{m}\mu\text{M}$  GA or in  $500 \text{m}\mu\text{M}$  GA and  $\alpha$ -amylase synthesis was measured after 10, 16 and 22 hours. To 1 set of flasks the GA concentration was increased to  $500 \text{m}\mu\text{M}$  after the aleurone layers had been in  $2.5 \text{m}\mu\text{M}$  GA for 10 hours (Shift).

FIG. 3. Effect of adding GA after it has been withheld for 6 hours. All conditions as in figure 1, except that GA was added again at 15 hours to aleurone layers from which it had been withheld at 9 hours. Total  $\alpha$ -amylase synthesis was measured 15 and 23 hours after the start of the incubation.

FIG. 4. Mid-course inhibition of  $\alpha$ -amylase synthesis by abscisin and cycloheximide. Aleurone layers were incubated in  $0.1 \mu\text{M}$  for 11 hours. At this time abscisin ( $5 \mu\text{M}$ ) or cycloheximide ( $10 \mu\text{g}/\text{ml}$ ) was added and  $\alpha$ -amylase synthesis was measured 2 and one-half, 5 and 10 hours later.

Table II. *Reversal by Gibberellic Acid of the Abscisin Inhibition of  $\alpha$ -Amylase Synthesis*

Ten half aleurone layers were incubated in buffer with 20 mM  $\text{CaCl}_2$  and the concentrations of GA and abscisin indicated. Activity of  $\alpha$ -amylase was measured in the medium and a tissue-extract after 24 hours.

GA concentration	$\alpha$ -Amylase per 10 aleurone layers		
	Abscisin concentration		
	0	0.05 $\mu\text{M}$	5 $\mu\text{M}$
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
0.1 m $\mu\text{M}$	73	10	...
1 m $\mu\text{M}$	248	71	...
10 m $\mu\text{M}$	522	280	22
0.1 $\mu\text{M}$	638	525	101
1 $\mu\text{M}$	662	584	189
10 $\mu\text{M}$	692	...	185
0.1 mM	580	...	113

increase in ribonuclease which takes place in isolated aleurone layers in the absence of added GA, or in half seeds preincubating on moist sand (4) is also inhibited by abscisin.

In the experiments discussed above the 2 hormones, GA and abscisin, were added simultaneously. However, abscisin is also effective as an inhibitor of  $\alpha$ -amylase synthesis if it is added in mid-course, while enzyme synthesis is in progress (fig 4). Aleurone layers were incubated for 11 hours in 0.1  $\mu\text{M}$  GA and 5  $\mu\text{M}$  abscisin was added at that time. Further synthesis of  $\alpha$ -amylase was inhibited completely after a delay of 2 to 3 hours. If both hormones had been added at these concentrations, at the same time, at the beginning of the incubation,  $\alpha$ -amylase synthesis would have been inhibited completely. Addition of cycloheximide (10  $\mu\text{g}/\text{ml}$ ) at this time results in an immediate inhibition of enzyme synthesis. It was also found that both the production and the release of ribonuclease can be inhibited by the addition of abscisin in mid-course (20–24 hrs after GA).

*Requirement for RNA Synthesis.* Actinomycin D and various base analogues which inhibit RNA

Table III. *Inhibition of Ribonuclease Production by Abscisin*

Ten aleurone layers were incubated in buffer, 20 mM of  $\text{CaCl}_2$  and with or without 2 m $\mu\text{M}$  GA and the concentrations of abscisin indicated. Ribonuclease activity was measured after 48 hours in the medium and an extract of the tissue. Initial refers to the amount of ribonuclease present in the aleurone layers at the start of incubation.

Treatment	Units of ribonuclease per 10 aleurone layers
Initial	29
Control	108
Control + 0.5 $\mu\text{M}$ abscisin	61
GA	256
GA + 5 $\mu\text{M}$ abscisin	53

synthesis prevent  $\alpha$ -amylase formation if they are added at the same time as the hormone (4, 23, 24). The synthesis of  $\alpha$ -amylase is inhibited to a much lesser extent if actinomycin D is added 4 hours after GA, and is almost unaffected if the antibiotic is added 8 hours after the hormone (table IV). A different effect is observed with 6-methyl-purine, a potent inhibitor of all RNA synthesis (11). When aleurone layers are incubated with 1  $\mu\text{M}$

Table IV. *Inhibition of  $\alpha$ -Amylase Synthesis and  $^{14}\text{C}$ -Uridine Incorporation by Actinomycin D Added 4 and 8 Hours After GA*

Ten aleurone layers were incubated in 0.1  $\mu\text{M}$  GA and after 4 or 8 hours actinomycin D (100  $\mu\text{g}/\text{ml}$ ) was added. Enzyme synthesis was measured at the end of the 24 hour incubation period.  $^{14}\text{C}$ -Uridine (1  $\mu\text{c}/\text{flask}$ ) was added 4 hours after actinomycin D and incorporation was allowed to proceed over a 4 hour period.

Treatment	$\alpha$ -Amylase per 10 aleurone layers	$^{14}\text{C}$ -Uridine incorporated
	$\mu\text{g}$	cpm
GA	359	1380
GA + Act. D after 4 hrs	212	
GA + Act. D after 8 hrs	325	466

Table V. *Inhibition of  $\alpha$ -Amylase Synthesis by 6-Methylpurine*

Ten aleurone layers were incubated in buffer, 20 mM,  $\text{CaCl}_2$  and 0.5  $\mu\text{M}$  GA. 6-Methylpurine was added at the same time as GA or 4 or 8 hours later and total  $\alpha$ -amylase production was measured after 24 hours incubation.

Treatment	$\alpha$ -Amylase per 10 aleurone layers	
Time of addition of 6-methylpurine	0.1 mM Of 6-methylpurine	1.0 mM Of 6-methylpurine
	$\mu\text{g}$	$\mu\text{g}$
0 hrs	38	9
4 hrs	115	55
8 hrs	208	140
Control	384	426

GA and 0.1 mM 6-methylpurine  $\alpha$ -amylase synthesis is inhibited by 90%. However, if the analogue is added 4 or 8 hours after the hormone enzyme synthesis is inhibited by only 70% and 45% respectively (table V). Larger inhibitions are obtained with 1 mM 6-methylpurine, and  $\alpha$ -amylase synthesis is still inhibited by 66% if this concentration of the analogue is added 8 hours after GA.

The failure of actinomycin D to inhibit  $\alpha$ -amylase synthesis if the antibiotic is added 8 hours after the hormone could be due to its inability to inhibit RNA synthesis in general at this stage or to its inability to inhibit the RNA fraction which

is required for  $\alpha$ -amylase synthesis. To test the first possibility aleurone layers were incubated in 0.1  $\mu\text{M}$  GA, and 100  $\mu\text{g}/\text{ml}$  of actinomycin D were added after 8 hours. After 4 more hours 1  $\mu\text{C}$  of  $^{14}\text{C}$ -uridine was added to each flask containing 10 aleurone layers and incorporation was allowed to proceed for 4 hours. During this period actinomycin D inhibited the incorporation of  $^{14}\text{C}$ -uridine by 66% (table IV). We can assume that the inhibition of RNA synthesis was even greater because some of the incorporation represents turnover of the terminal ends of the transfer RNA molecules. (It was shown by Chandra and Varner (2) that there is a rapid conversion of uridine to cytidine in this tissue). It appears then that actinomycin D is a good inhibitor of  $^{14}\text{C}$ -uridine incorporation whether it is added at the same time as GA (4) or 8 hours after the hormone. This leads to the conclusion that the inability of actinomycin D to inhibit  $\alpha$ -amylase synthesis must be due to its inability to prevent the synthesis of the specific RNA fraction which is required for continued  $\alpha$ -amylase synthesis.

Table VI. *Sensitivity of  $\alpha$ -Amylase Synthesis to Actinomycin D and 6-Methylpurine After Removal of GA*

Ten aleurone layers were incubated in buffer, 20 mM  $\text{CaCl}_2$  and 0.5  $\mu\text{M}$  GA for 9 hours. GA was removed by 4 subsequent one-half-hour rinsings in a medium without GA. The aleurone layers were further incubated for 11 hours without GA or with 0.5  $\mu\text{M}$  GA and actinomycin D (100  $\mu\text{g}/\text{ml}$ ) or 6-methylpurine (0.2 mM). Initial refers to the amount of  $\alpha$ -amylase present in the aleurone layers after the removal of GA (at the end of the washing out procedure).

Treatment	$\alpha$ -Amylase per 10 aleurone layers
	$\mu\text{g}$
Initial	61
—GA	128
+GA	285
+GA + actinomycin D	221
+GA + 6-methylpurine	153

We have shown above that GA is not a trigger, but is required continuously for maximal  $\alpha$ -amylase synthesis. The following experiment was done to test whether the  $\alpha$ -amylase synthesis which is dependent on the second addition of GA also requires continued RNA synthesis. Aleurone layers were incubated for 10 hours in 0.05  $\mu\text{M}$  GA and the GA was removed by 4 successive one-half-hour rinses. The aleurone layers were then further incubated without GA, or with GA and with actinomycin D (100  $\mu\text{g}/\text{ml}$ ) or 6-methylpurine (0.2 mM). The results show (table VI) that the  $\alpha$ -amylase synthesis which is dependent on the second addition of GA is not very sensitive to actinomycin D but is very strongly inhibited by 6-methylpurine. Synthesis of  $\alpha$ -amylase under these circumstances proceeds without delay upon the addition of GA, even if GA has been withheld for many hours (fig 3). This indicates that the aleurone layers are capable of a rapid synthesis of the RNA fraction necessary for  $\alpha$ -amylase synthesis. When aleurone layers are incubated with GA there is normally a 7 to 9 hour lag before  $\alpha$ -amylase synthesis begins.

The observations discussed above suggest that this lag is not necessitated by the requirement for the synthesis of the metabolically unstable RNA fraction, the synthesis of which must accompany  $\alpha$ -amylase synthesis and can be inhibited by 6-methylpurine. It seems likely that other biochemical processes are associated with the lag-period and must occur before  $\alpha$ -amylase synthesis can start. The possibility that a more stable RNA needs to be synthesized during the lag-period can, of course, not be ruled out.

To test whether the requirement for RNA synthesis can be satisfied by incubating for a long time with a small amount of GA, half seeds were preincubated for 3 days on sterile sand moistened with GA (0.01 or 0.1  $\mu\text{M}$ ) instead of water. The aleurone layers were removed from the starchy endosperm and further incubated with GA (1  $\mu\text{M}$ ) or with GA and actinomycin D (50  $\mu\text{g}/\text{ml}$ ). The data show (table VII) that actinomycin D inhibits enzyme synthesis to approximately the same extent, whether the half seeds were preincubated in water

Table VII. *Inhibition of  $\alpha$ -Amylase Synthesis by Actinomycin D After Preincubation of Half Seeds in Gibberellic Acid*

Half seeds were preincubated in water or in GA (0.01  $\mu\text{M}$  or 0.1  $\mu\text{M}$ ) for 3 days. The aleurone layers were removed and further incubated in buffer, 20 mM,  $\text{CaCl}_2$  and 1  $\mu\text{M}$  GA, and with or without actinomycin D (50  $\mu\text{g}/\text{ml}$ ). 0 hours refers to the amount of  $\alpha$ -amylase present in the aleurone layers at the start of the incubation.

Treatment and time	$\alpha$ -Amylase per 10 aleurone layers		
	Preincubation in water	Preincubation in 0.01 $\mu\text{M}$ GA	Preincubation in 0.1 $\mu\text{M}$ GA
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$
0 hrs GA	11	22	64
8 hrs GA	35	64	136
24 hrs GA	408	445	505
24 hrs GA + actinomycin D	240	270	278

or in GA. As a result of preincubation in  $0.1 \mu\text{M}$  GA  $\alpha$ -amylase synthesis is initiated at a slow rate (compare  $\alpha$ -amylase synthesis at 0 and 8 hrs after preincubation in  $0.1 \mu\text{M}$  GA with preincubation in water), but this does not obviate the necessity for the initiation of more RNA synthesis if a higher concentration of GA is added.

*Comparison of the Effect of Abscisin and Inhibitors of RNA Synthesis.* We have shown that the addition of cycloheximide, a potent inhibitor of protein synthesis at the level of translation, to aleurone layers results in an immediate cessation of  $\alpha$ -amylase synthesis and also of the incorporation of  $^{14}\text{C}$ -leucine into the cellular proteins of aleurone layers (Varner, unpublished). Abscisin, on the other hand, inhibits enzyme synthesis after a lag of 2 and one-half to 3 hours. Experiments with 6-methylpurine show a continuous requirement for RNA synthesis during the period of  $\alpha$ -amylase synthesis. It seemed, therefore, of interest to study the mid-course inhibition of  $\alpha$ -amylase synthesis by abscisin and base analogues and compare their kinetics.

Aleurone layers were incubated in  $0.5 \mu\text{M}$  GA for 11 hours to induce the GA enhanced synthesis of  $\alpha$ -amylase. At this stage the medium was withdrawn, the aleurone layers were rinsed and further incubated for 12 hours in a medium containing GA ( $0.5 \mu\text{M}$ ) or GA and various inhibitors ( $10 \mu\text{M}$  abscisin,  $0.5$  or  $5 \text{ mM}$  6-methylpurine,  $5 \text{ mM}$  8-azaguanine and  $5 \text{ mM}$  5-azacytidine). The results in table VIII indicate that abscisin and  $5 \text{ mM}$  6-methylpurine give the same amount of inhibition ( $74\%$ ) while  $0.5 \text{ mM}$  6-methylpurine and  $5 \text{ mM}$  8-azaguanine or 5-azacytidine are less inhibitory ( $58$ – $39\%$ ). The kinetics of inhibition of  $\alpha$ -amylase synthesis by abscisin and the base analogues are very similar (fig 6). Abscisin inhibits  $\alpha$ -amylase synthesis completely within 2 and one-half to 3 hours, as shown earlier (fig 4). The base analogues also

Table VIII. *Mid-course Inhibition of  $\alpha$ -Amylase by Abscisin and Base Analogues.*

Aleurone layers were incubated in buffer,  $20 \text{ mM}$   $\text{CaCl}_2$  and  $0.5 \mu\text{M}$  GA for 11 hours. The medium was drawn off and the aleurone layers were rinsed. The aleurone layers were further incubated for 12 hours in  $0.5 \mu\text{M}$  GA together with the inhibitors, as indicated. The figures represent the amount of  $\alpha$ -amylase produced in the 12 hour incubation.

Treatment	$\alpha$ -Amylase per 10 aleurone layers
Control	$\mu\text{g}$ 317
Abscisin ( $10 \mu\text{M}$ )	82
6-Methylpurine ( $0.5 \text{ mM}$ )	156
6-Methylpurine ( $5 \text{ mM}$ )	84
5-Azacytidine ( $5 \text{ mM}$ )	194
8-Azaguanine ( $5 \text{ mM}$ )	132

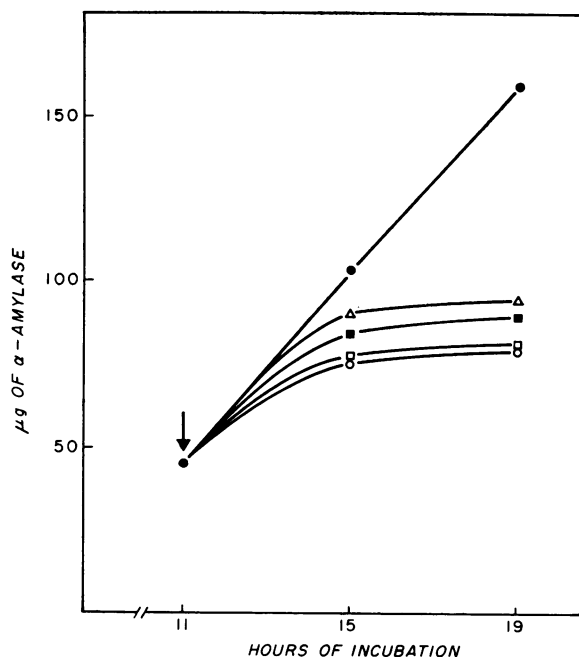


FIG. 5. Mid-course inhibition of  $\alpha$ -amylase synthesis by abscisin, 6-methylpurine and 8-azaguanine. Aleurone layers were incubated in  $0.05 \mu\text{M}$  GA for 11 hours. At this time the medium was removed, the aleurone layers were rinsed, and they were further incubated with  $0.05 \mu\text{M}$  GA ( $\bullet$ — $\bullet$ ), or with GA and  $10 \mu\text{M}$  abscisin ( $\circ$ — $\circ$ ),  $5 \text{ mM}$  6-methylpurine ( $\square$ — $\square$ ),  $0.5 \mu\text{M}$  6-methylpurine ( $\blacksquare$ — $\blacksquare$ ), or  $5 \text{ mM}$  8-azaguanine ( $\triangle$ — $\triangle$ ).

inhibit  $\alpha$ -amylase synthesis completely within 2 and one-half to 4 hours depending on the concentration used. The incorporation of  $^{14}\text{C}$ -uridine by the aleurone layers is inhibited by  $1 \text{ mM}$  6-methylpurine in the following way:  $30$  to  $35\%$  within 2 and one-half hours of its addition and  $65$  to  $70\%$  after 4 hours of its addition. However, if the 6-methylpurine concentration is increased to  $5 \text{ mM}$  the inhibition of  $^{14}\text{C}$ -uridine incorporation is  $65$  to  $70\%$  within 2 and one-half hours. This may account for the shorter lag time in the inhibition of  $\alpha$ -amylase synthesis observed with the higher concentration of 6-methylpurine. The incorporation of  $^{14}\text{C}$ -leucine by the aleurone layers into the cellular proteins is inhibited by only  $30\%$ , 4 hours after the addition of  $1 \text{ mM}$  6-methylpurine. This is probably an indirect effect mediated through its inhibition of RNA synthesis. We therefore conclude that the rapid inhibition of  $\alpha$ -amylase synthesis caused by 6-methylpurine is not due to a direct inhibition of protein synthesis.

## Discussion

It is not yet possible to decide whether these hormones work at the level of transcription or at

the level of translation. The data presented here, as well as the earlier results obtained by Varner and coworkers (4, 22, 23, 24), are consistent with the hypothesis that GA exerts its control at the level of the gene, to bring about the synthesis of an RNA fraction specific for the proteins being synthesized. The similarity in kinetics for the inhibition of  $\alpha$ -amylase synthesis by abscisin and 6-methylpurine or 8-azaguanine suggests that abscisin exerts its control by inhibiting the synthesis of such a specific RNA fraction. However, much caution must be used in basing a model on the mode of action of the hormones on a study of the effect of inhibitors alone. The data are equally consistent with a control mechanism at the level of translation with a requirement for continued RNA synthesis.

Many hormonal effects are inhibited by actinomycin D (1, 9, 13, 17, 20, 25), and this has often been interpreted as evidence that the hormones affect the synthesis of messenger RNA in a direct way while all that can be concluded with certainty is, that some kind of DNA directed RNA synthesis must be allowed to go on before the hormonal effect can express itself at the level of protein synthesis or of morphogenesis. Hybridization experiments with bacterial nucleic acids have shown regions of complementarity between DNA and messenger RNA, ribosomal RNA, and transfer RNA suggesting that the synthesis of all 3 major classes of cytoplasmic RNA is DNA directed. The synthesis of any or all of these 3 may be required for the hormonal effect to become evident.

A second reason for caution is that the hormone-enhanced enzyme synthesis may result in a more rapid turnover of one of the RNA's involved in the synthesis of these enzymes (e.g. messenger RNA or a particular transfer RNA). This would result in a requirement for RNA synthesis, and such a process would be inhibited by actinomycin D or by base analogues, even though the initial effect of the hormone is not at the level of transcription.

The principal, easily observed effect of GA on the aleurone layers is the enhancement of the synthesis of several hydrolytic enzymes. RNA synthesis must be allowed to occur in order to obtain this effect. A control mechanism at either transcription or translation would probably involve the synthesis of a new RNA. There does not appear to exist a great reserve of this critical RNA fraction. Indeed, base analogues inhibit  $\alpha$ -amylase synthesis within 2 and one-half to 4 hours and continued RNA synthesis must accompany enzyme synthesis. We can also conclude that this RNA fraction is synthesized very rapidly since addition of GA to aleurone layers from which this hormone has been withheld for several hours results in an immediate resumption of  $\alpha$ -amylase synthesis which is completely dependent on continued RNA synthesis.

The principal, easily observed effect of abscisin

is the inhibition of hydrolase synthesis (both normal and GA-enhanced). The kinetics of this inhibition resemble those obtained with the base analogues 6-methylpurine and 8-azaguanine. In the presence of 10  $\mu$ M abscisin or 5 mM 6-methylpurine, at this concentration the base analogue inhibits  $^{14}$ C-uridine incorporation by 70% within 2 and one-half hours,  $\alpha$ -amylase synthesis is completely inhibited within 2 to 3 hours. With 0.5 mM 6-methylpurine or 5 mM 8-azaguanine it takes somewhat longer before inhibition is complete. The incorporation of  $^{14}$ C-leucine is inhibited to a much smaller extent (30%) than the incorporation of  $^{14}$ C-uridine (70%) when aleurone layers are incubated for 4 hours in 1 mM 6-methylpurine. This suggests that the inhibition of protein synthesis is a result of the inhibition of RNA synthesis, brought about by the decay of short-lived messengers. This was confirmed by A. Morris (private communication) who showed that 10 mM 6-methylpurine has no effect on the incorporation of  $^{14}$ C-valine into the proteins of rabbit reticulocytes, indicating that the analogue has no direct effect on protein synthesis in this system. (Reticulocytes have no nucleus and protein synthesis is independent of continued RNA synthesis). The 2 and one-half to 3 hour delay in the inhibition of  $\alpha$ -amylase synthesis which is observed with abscisin as well as with the base analogues could be due to the presence of a sufficient amount of the critical RNA fraction necessary to sustain enzyme synthesis for this period of time.

However, similar kinetics might be expected if abscisin exerted its action at the level of translation by inhibiting the incorporation of this RNA into an active enzyme-synthesizing unit. In this case the delay would reflect the half-life of this active complex.

The inhibition of  $\alpha$ -amylase synthesis by abscisin can be overcome, at least partially, by the addition of a larger amount of GA. However, if too high a concentration of abscisin is used, no reversal is obtained. Similar observations were made by J. van Overbeek (personal communication) who studied the inhibition of growth of *Lemna minor* by abscisin and the reversal of this inhibition by benzyladenine. Reversal by benzyladenine only takes place if growth has not been completely inhibited by abscisin.

A large number of studies indicate that plant hormones increase the rate of protein and RNA synthesis in the tissues to which they are applied. In some systems this can be measured by an increase in the total amount of protein and RNA, while in others there is only an increase in the rate of incorporation of radioactive precursors into these macromolecules. Hormone application always results in a general derepression of RNA synthesis and in no instance has a system been found in which the hormone preferentially increases the synthesis of 1 particular RNA fraction.

Gibberellic acid does not enhance the incorporation of  $^{14}\text{C}$ -uridine into the RNA<sup>3</sup> or of  $^{14}\text{C}$ -leucine into the cellular proteins of isolated aleurone layers, dissected from the half seeds after the customary 3-day preincubation period (Chandra, Chrispeels and Varner, unpublished); neither does abscisic acid inhibit these processes (3). Although the hormones appear to have no effect on protein synthesis as measured by precursor incorporation, they do affect the synthesis of specific enzymes. This observation has not been matched at the RNA level, and we have been unable to find a specific RNA fraction (after extraction of the RNA and chromatography on methylated albumen kieselguhr columns), the synthesis of which is evoked by GA and inhibited by abscisic acid. Recent improvements in the methods used to extract nucleic acids from animal tissues (12) and to separate the various RNA's (5,14) will be applied in our search for such an RNA fraction. The possibility that GA and abscisic acid exert their control at the level of translation can be checked by studying the effect which these hormones have on the *in vitro* synthesis of  $\alpha$ -amylase by a subcellular fraction isolated from aleurone layers.

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<sup>3</sup> Chandra and Varner (2) and Varner et al. (24) reported a GA-enhanced incorporation of radioactive precursors into the RNA of aleurone layers 8 to 24 hours after the start of imbibition of half seeds. Naylor (15) observed a GA-enhanced incorporation of  $^3\text{H}$ -cytidine into the nuclei of aleurone cells from wild oats, 24 hours after the start of imbibition. It seems likely that in these cases GA is accelerating processes which are associated with imbibition or with biochemical changes which follow it. It is exactly for this reason, to avoid all biochemical changes associated with imbibition, that half seeds are preincubated for 3 days before we study the effects of GA and abscisic acid on aleurone layers.



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