Plant Physiol. (1976) 57, 175-178

Response of Barley Aleurone Layers to Abscisic Acid[1]

Received for publication July 9, 1975 and in revised form October 4, 1975

DAVID TUAN-HUA HO
Michigan State University/Energy Research and Development Administration Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

JOSEPH E. VARNER
Department of Biology, Washington University, St. Louis, Missouri 63130

ABSTRACT

Cordycepin, an inhibitor of RNA synthesis in barley (*Hordeum vulgare L.*) aleurone cells, does not inhibit the gibberellic acid-enhanced α-amylase (EC 3.2.1.1.) synthesis in barley aleurone layers if it is added 12 hours or more after the addition of the hormone. However, the accumulation of α-amylase activity after 12 hours of gibberellic acid can be decreased by abscisic acid. The accumulation of α-amylase activity is sustained or quickly restored when cordycepin is added simultaneously or some time after abscisic acid, indicating that the response of aleurone layers to abscisic acid depends on the continuous synthesis of a short lived RNA. By analysis of the newly synthesized proteins by gel electrophoresis with sodium dodecylsulfate, we observed that the synthesis of α-amylase is decreased in the presence of abscisic acid while the synthesis of most of the other proteins remains unchanged. From the rate of resumption of α-amylase production in the presence of cordycepin and abscisic acid, it appears that abscisic acid does not have a measurable effect on the stability of α-amylase mRNA.

Aleurone layers of barley seeds respond to GA_3 by synthesizing and secreting several hydrolases, begining 8 to 10 hr after the addition of GA_3 (22). During this lag period GA_3 enhances membrane proliferation as shown by direct electron microscopic observation (15) and by biochemical determination of phospholipid synthesis in membrane fractions (5, 17). Two enzymes, phosphorylcholine-cytidyl (EC 2.7.7.15) and phosphorylcholine-glyceride (EC 2.7.8.2) transferases, in the phosphatidyl choline pathway appear to be activated within minutes after GA_3 administration (1, 14). Membrane bound polysome formation (6) and poly(A)-RNA[2] synthesis (10, 13) are promoted in the presence of GA_3. All these GA_3 effects are reversed or prevented by ABA. ABA does not have any effect on general cellular metabolism as measured by O_2 consumption (2).

Although ABA can prevent the response to GA_3, no direct effects of ABA in aleurone cells have been observed. The failure of the aleurone cells to respond to GA_3 in the presence of ABA does not result from simple competition between these two hormones because a high concentration of GA_3 cannot completely overcome the ABA effect (2, 12).

Because most of the effects of GA_3 in aleurone cells depend on cellular metabolism, *e.g.* transcription and translation, it has been difficult to study the mode of action of ABA because application of metabolic inhibitors would inhibit GA_3 effects directly. We have demonstrated that α-amylase is translated from stable mRNA that is synthesized before the rapid increase of α-amylase activity (10). Since α-amylase synthesis after 12 hr of GA_3 is no longer sensitive to transcription inhibitors such as cordycepin (3'-deoxyadenosine) (10) and actinomycin D (4, 9), while ABA at this stage still effectively inhibits α-amylase production, it provides us a good opportunity to study the requirement of RNA synthesis for the effect of ABA as it prevents the tissue's response to GA_3.

MATERIALS AND METHOD

Sources of Chemicals and Seed. Cordycepin, GA_3, and ABA (mixture of equal amounts of *cis-trans* and *trans-trans* isomers; all concentrations used in this study refer to that of *cis-trans* isomer only) were purchased from Sigma Chemical Co. Potato starch for the α-amylase assay was obtained from Nutritional Biochemical Co. L-Leucine-4,5-^{3}H (5 Ci/mmole) and uridine-^{3}H (G) (5–15 Ci/mmole) were purchased from New England Nuclear. NCS tissue solubilizer was obtained from Amersham/Searle Co. Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1969 crop) were supplied by Department of Agronomy, Washington State University, Pullman, in 1972 and stored in the cold room since then.

Preparation and Treatment of Aleurone Layer. Embryoless half-seeds were surface-sterilized by sodium hypochlorite (5-fold dilution of commercial bleach) for 20 min and rinsed several times with sterile deionized H_2O, and further stirred in 0.01 M HCl for 10 min to destroy any remaining sodium hypochlorite. After thorough rinsing with H_2O and with 20 mM sodium succinate buffer, pH 5, containing 10 mM $CaCl_2$, the half-seeds were allowed to imbibe water under sterilized conditions on sand moistened with the same sodium succinate buffer. Aleurone layers were peeled from 3-day imbibed half-seeds and incubated in sodium succinate buffer containing different combinations of hormones and inhibitors, in a reciprocal metabolic shaker (120 oscillations/min) at 25 C. The concentration of GA_3 used in this work was 2.5 μM.

Extraction and Assay of Enzyme. α-Amylase was extracted according to the methods of Chrispeels and Varner (3), combined with enzyme in the medium, and assayed as described by Varner and Mense (21). The unit of α-amylase was defined as a change of 1 absorbance unit/min at 620 nm.

RNA Extraction and Fractionation. Total, RNA from aleurone layers was extracted and poly(A)-RNA was separated from the other RNA species by oligo(dT) cellulose chromatography as described before (10).

Sodium Dodecyl Sulfate Gel Electrophoresis. The procedures

[1] This work was supported by United States Atomic Energy Commission (Contract No. E(11-1)-1338) and by the National Science Foundation (GB-39944).

[2] Abbreviation: poly(A)-RNA: RNA species containing polyadenylic acid.

used were modification of those of Laemmli (18). Gels composed of 2.5 cm of stacking gel (4%), and 10.5 cm of separation gel (12%) were used. Samples for gel electrophoresis were prepared by grinding 30 to 40 aleurone layers with 0.5 ml 0.2 M NaCl, containing 10 mM $KBrO_3$, with a mortar and pestle. The mortar was rinsed with another 1 ml of NaCl-$KBrO_3$ solution and the solutions were combined. After centrifuging for 30 min at 12,000*g*, the supernatant was decanted and 1 ml of SDS reagent containing 0.125 M tris-HCl, pH 6.8 4% SDS, 10% mercaptoethanol was added. This supernatant is referred to as salt-soluble protein preparation. To the washed pellet 1 ml of SDS reagent was added. After storage at room temperature for several days, the pellet with SDS reagent was centrifuged. The resulting supernatant was diluted with equal volume of distilled H_2O and referred to as salt-insoluble protein preparation. A 100-μl sample was applied to each gel and electrophoresis was carried out at 80 v (constant) and an initial current of 40 mamp/12 gels.

RESULTS

Effect of Cordycepin on GA_3-enhanced α-Amylase Formation. Cordycepin is believed to act as a chain terminator during RNA synthesis and inhibits the formation of both poly(A)-RNA and RNA species not containing a poly(A) segment in barley aleurone layers (Fig. 1). Cordycepin is still effective as a transcription inhibitor when it is added 12 hr after GA_3 (Fig. 1). The inhibitory effect of cordycepin on GA_3-enhanced α-amylase synthesis is less and less as cordycepin is added later and later after the hormone, and no inhibitory effect is observed if cordycepin is added 10 hr or later after the addition of GA_3 (Table I). In fact, an enhancement of α-amylase formation by cordycepin added 12 hr after GA_3 was occasionally observed (Table I). Because α-amylase is synthesized at the time the increasing activity of this enzyme is observed, and cordycepin has no effect on the degradation of the enzyme (Ho and Varner, manuscript in preparation), it is concluded that α-amylase is translated from stable mRNA (10).

Effect of ABA on α-Amylase Formation. Abscisic acid (5 μM), inhibits α-amylase formation if it is added at the same time as GA_3. Higher concentrations of ABA (10–25 μM) are needed to reduce α-amylase synthesis when ABA is added 12 hr after GA_3. It has been reported that ABA, at a concentration of 38 μM forms a complex with fungal α-amylase, resulting in inhibition of the enzyme activity (19, 20). ABA at concentrations up to 50 μM has no significant effect on barley aleurone α-amylase activity in a cell-free enzyme preparation after 23 hr of incubation at 25 C (Table II). Since the concentration of ABA we used here was 25 μM (Figs. 2 and 3) and the time span of the experiment was no longer than 12 hr (from 12–24 hr after addition of GA_3), we conclude that the ABA effect on α-amylase production must be via a physiological process.

Effect of Cordycepin on Action of ABA. ABA added 12 hr after GA_3 gradually inhibits further accumulation of α-amylase (Fig. 2). Because the accumulation of α-amylase activity in response to GA_3, both before and after 12 hr, is due to *de novo* synthesis (*i.e.*, there is no evidence for accumulation of an inactive precursor of α-amylase, Ho and Varner, manuscript in preparation) and because α-amylase accumulation after 12 hr of GA_3 is not inhibited by cordycepin, it is clear that ABA inhbits the synthesis of α-amylase posttranscriptionally. When ABA is added 12 hr after GA_3 and cordycepin is added at the same time or later, the accumulation of α-amylase either does not stop, or is quickly resumed (Figs. 2 and 3). The effect of cordycepin cannot be due to a generally toxic effect because α-amylase production remains normal in the presence of cordycepin without ABA. The effect of ABA seems to depend on the continuous synthesis of a short lived RNA which is inhibited by cordycepin. Because the rate of α-amylase accumulation after cordycepin addition is close to that of tissue treated with GA_3 alone (Figs. 2 and 3), it appears that the amount of α-amylase mRNA is not limiting in the presence of ABA and cordycepin, *i.e.* ABA does not reduce the stability of α-amylase mRNA.

In order to determine whether ABA specifically prevents the

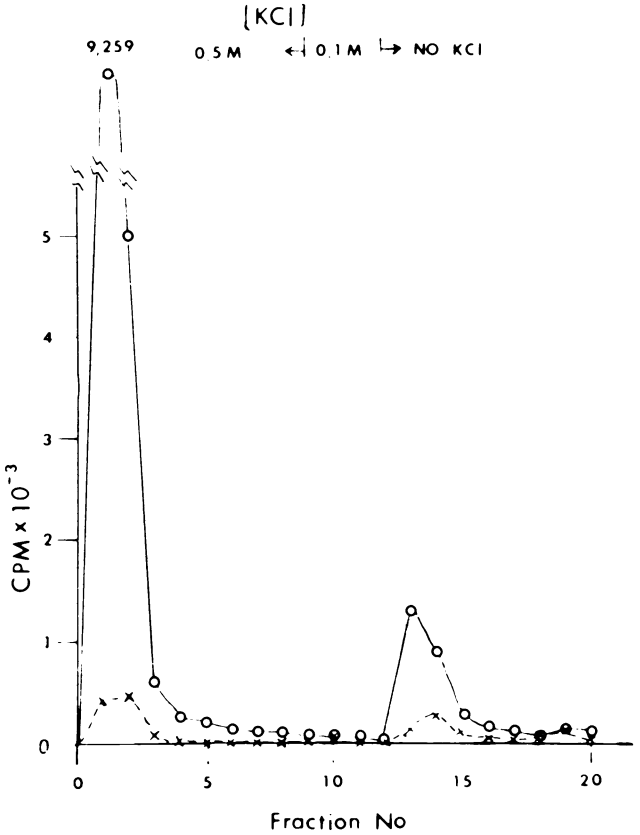

FIG. 1. Effect of cordycepin on RNA synthesis. Two samples, 80 aleurone layers each, were pretreated with GA_3 for 12 hr and cordycepin was then added to one of the samples. Label (^{3}H-uridine, 5 μCi/ml) was introduced between 13.5 and 18 hr after GA_3. RNA was extracted and fractionated by oligo(dT) cellulose chromatography as described previously (8). The first peak (fractions 1–4) eluated by 0.5 M KCl consists of RNA species containing poly(A) segment. The peak (fractions 13–15) eluted in the absence of KCl contains poly(A)-RNA. Sometimes a tiny peak was eluted by 0.1 M KCl. Control (+ GA_3) only (O——O); cordycepin (0.1 mM) treated (×- - -×).

Table I. *Effect of Cordycepin on GA_3-enhanced Synthesis of α-Amylase*

Cordycepin (0.1 mM) was added at different times as indicated, and aleurone layers were further incubated until 24 hr after GA_3 when α-amylase was extracted and assayed along with enzyme secreted to the medium.

Treatment	α-Amylase Activity		
	Total	Increase over control	
	units/layer		%
GA_3 only	27.7	26.6	100
Control (No GA_3)	1.1		
GA_3 + cordycepin			
Added 0 time	6.0	4.9	18.4
Added 4 hr after GA_3	10.9	9.8	36.8
Added 8 hr after GA_3	25.0	23.9	85.4
Added 10 hr after GA_3	27.6	26.5	99.7
Added 12 hr after GA_3	31.2	30.1	113.2

synthesis of α-amylase (and perhaps other GA_3-enhanced hydrolases as well) or whether ABA slows down protein synthesis in general, the profile of newly synthesized proteins was examined by SDS gel electrophoresis. α-Amylase is detected as the predominant radioactive band on SDS gel with a mol wt of about 50,000 daltons. As shown in Figure 4 for salt-soluble proteins, the synthesis of α-amylase is substantially decreased in the presence of ABA, while the amount of radioactivity in most of the minor bands remains essentially the same as in control tissue (+GA_3 only).

Table II. *Lack of Direct Effect of Abscisic Acid on α-Amylase Activity*

α-Amylase secreted from 24 hr GA_3-treated aleurone layers was used. The unit of α-amylase is A_{620}/min·ml.

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

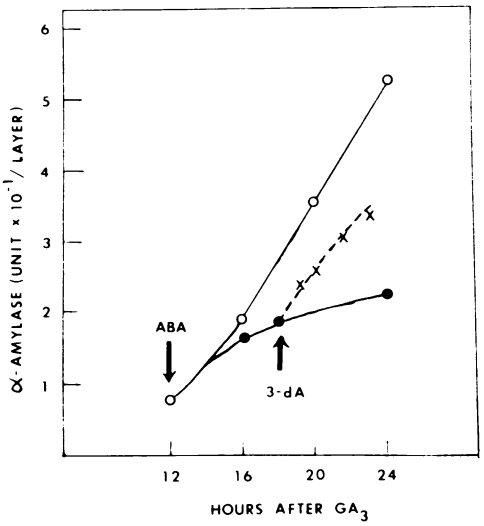

FIG. 2. Effect of midcourse addition of (25 mM) ABA and 0.1 mM cordycepin on the synthesis of α-amylase. GA_3 only (O——O); GA_3 and ABA (●——●); GA_3, ABA, and cordycepin (3-dA) (×– – –×).

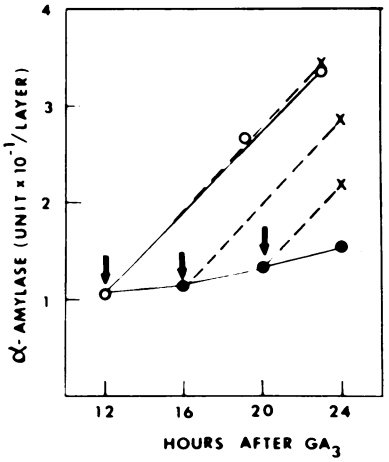

FIG. 3. Effect of cordycepin added at different times on the synthesis of α-amylase in the presence of both GA_3 and ABA. ABA was added 12 hr after GA_3. Arrows indicate the time of cordycepin addition. GA_3 only (O——O); GA_3 and ABA (●——●); GA_3, ABA, and cordycepin (×– –×).

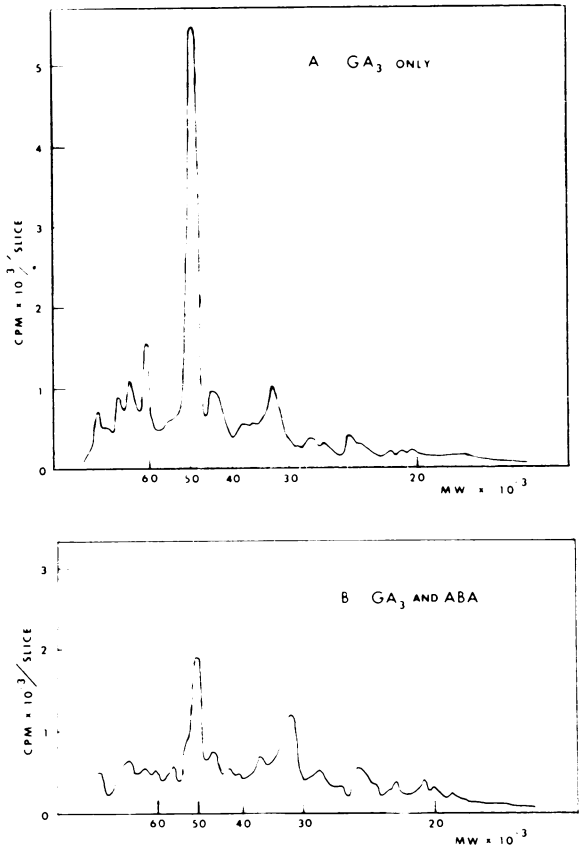

FIG. 4. Profile of newly synthesized salt-soluble proteins on SDS gel. Aleurone layers (30–40) were labeled with ^{3}H-leucine (15 μCi/ml) for 2 hr (18–20 hr after GA_3). Salt-soluble proteins were extracted as described under "Materials and Methods." One mm thick gel pieces were sliced and digested in 0.5 ml of NCS solubilizer (9 parts of full strength NCS solubilizer and 1 part of distilled H_2O) at 50 C for 2 hr. Ten ml of toluene-based scintillation fluid (6 g of PPO and 75 mg of POPOP) were used in each sample.

DISCUSSION

Although the synthesis of α-amylase after 12 hr of GA_3 treatment is no longer subject to transcriptional control, the inhibitory effect of ABA on α-amylase production at this same stage apparently depends on the continuous synthesis of a short-lived RNA (regulator RNA in Fig. 5). Apparently, this regulator RNA, or its translation product, can decrease the rate of synthesis of α-amylase without influencing protein synthesis in general. The mRNA of α-amylase is stable, at least after 12 hr of exposure of the tissue to GA_3, and its stability is maintained in the presence of ABA.

Ihle and Dure (11), working with precociously germinating cotton embryos, obtained evidence that the translation of carboxypeptidase mRNA was inhibited by ABA. Because they found that actinomycin D prevented the ABA inhibition, they proposed that a suppressor molecule had to be formed to bring about the ABA inhibition (11).

The action of ABA thus appears to be similar in the two systems, the precociously germinating cotton embryos where gibberellins probably have no regulatory role, and the mobilization of reserve nutrient in the germinating barley seed where ABA prevents the GA_3-enhanced α-amylase production in the aleurone cells. It seems reasonable to suggest that many ABA effects depend on transcription, although there is no evidence

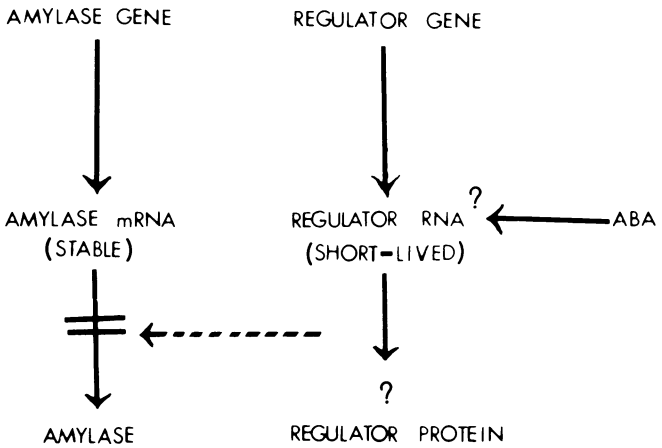

FIG. 5. Postulated mechanism of ABA action.

for a requirement of transcription in some fast effects of ABA like that of preventing the activation of phosphorylcholine glyceride transferase (1) and that of stomatal closure (16).

There are two alternative sites of action for ABA (Fig. 5): (*a*) ABA might derepress the regulator gene and cause the synthesis of regulator RNA and perhaps regulator protein, or (*b*) the regulator RNA is under continuous turnover; ABA activates it or works with it to prevent the translation of α-amylase mRNA. In human reticulocytes a new species of low mol wt RNA has been reported to be able to preferentially stimulate the synthesis of one of the globin chain (7, 8).

Because of the many convenient properties of barley aleurone tissue and because of the ability of ABA to modify the tissue's response to GA_3, as reported in this paper, we feel that aleurone tissue is an important system for the further study of how ABA modifies a tissue's response to GA_3.

LITERATURE CITED

1. BEN-TAL, Y. AND J. E. VARNER. 1974. An early response to gibberellic acid not requiring protein synthesis. Plant Physiol. 54: 813–816.
2. CHRISPEELS, M. J. AND J. E. VARNER. 1966. Inhibition of gibberellic acid induced formation of α-amylase by abscisin II. Nature 212: 1066–1067.
3. CHRISPEELS, M. J. AND J. E. VARNER. 1967. Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42: 398–406.
4. CHRISPEELS, M. J. AND J. E. VARNER. 1967. Hormonal control of enzyme synthesis: on the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Physiol. 42: 1008–1016.
5. EVINS, W. H. AND J. E. VARNER. 1971. Hormone-controlled synthesis of endoplasmic reticulum in barley aleurone cells. Proc. Natl. Acad. Sci. U.S.A. 68: 1631–1633.
6. EVINS, W. H. AND J. E. VARNER. 1972. Hormonal control of polyribosome formation in barley aleurone layers. Plant Physiol. 49: 348–352.
7. FUHR, J. E. AND C. NATTA. 1972. Translational control of α and β globin chain synthesis. Nat. New Biol. 240: 274–276.
8. FUHR, J. E. AND M. OVERTON. 1975. Translational control of globin synthesis by low molecular weight RNA. Biochem. Biophys. Res. Commun. 63: 742–747.
9. GOODWIN, P. B. AND D. J. CARR. 1972. Actinomycin D and the hormonal induction of amylase synthesis in barley aleurone layers. Planta 106: 1–12.
10. HO, D. T-H. AND J. E. VARNER. 1974. Hormonal control of messenger ribonucleic acid metabolism in barley aleurone layers. Proc. Nat. Acad. Sci. U.S.A. 71: 4783–4786.
11. IHLE, J. N. AND L. DURE, III. 1970. Hormonal regulation of translation inhibition requiring RNA synthesis. Biochem. Biophys. Res. Commun. 38: 995–1001.
12. JACOBSEN, J. V. 1973. Interactions between gibberellic acid, ethylene, and abscisic acid in control of amylase synthesis in barley aleurone layers. Plant Physiol. 51: 198–202.
13. JACOBSEN, J. V. AND J. A. ZWAR. 1974. Gibberellic acid causes increased synthesis of RNA which contains poly(A) in barley aleurone tissue. Proc. Nat. Acad. Sci. U.S.A. 71: 3290–3293.
14. JOHNSON, D. K. AND H. KENDE. 1971. Hormonal control of lecithin synthesis in barley aleurone cells: Regulation of CDP-choline pathway by gibberellin. Proc. Nat. Acad. Sci. U.S.A. 68: 2674–2677.
15. JONES, R. L. 1969. Gibberellic acid and the fine structure of barley aleurone cells. I. Changes during the lag phase of α-amylase synthesis. Planta 87: 119–133.
16. RASCHKE, K. 1975. Stomatal action. Annu. Rev. Plant Physiol. 26: 309–340.
17. KOEHLER, D. E. AND J. E. VARNER. 1973. Hormonal control of orthophosphate incorporation into phospholipids of barley aleurone layers. Plant Physiol. 52: 208–214.
18. LEAMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685.
19. MILBORROW, B. V. 1974. The chemistry and physiology of abscisic acid. Annu. Rev. Plant Physiol. 25: 259–307.
20. MOUSSERON-CANET, M., J-C. MANI, B. DURAND, J. NITSCH, J. DORNAND, AND J-C. BONNAFOUS. 1970. Analogues de l'acide abscisique (±) hormone de dormance. Relations structure-activite. C. R. Acad. Sci. Ser. D 270: 1936–1939.
21. VARNER, J. E. AND R. MENSE. 1972. Characteristics of the process of enzyme release from secretory plant cells. Plant Physiol. 49: 187–189.
22. YOMO, H. AND J. E. VARNER. 1971. Hormonal control of a secretory tissue. *In*: A. A. Moscona and A. Monroy, eds., Current Topics in Developmental Biology, Vol. 6. Academic Press, New York. pp. 111–144.