EARLY EFFECTS OF GIBBERELLIC ACID ON BARLEY ALEURONE LAYERS

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The most obvious effects resulting from the application of the hormone gibberellic acid (GA) to cereal aleurone layers are the secretion of metabolites and the de novo synthesis and secretion into the medium of enzymes, mostly hydrolases (Varner, 1964; Varner et al., 1965; Chrispeels and Varner, 1967 a,b; Jacobsen and Varner, 1967). For that reason research on the mode of action of GA has focused on the elucidation of the roles of protein synthesis and RNA synthesis in the responses. The present report follows observations that the effects of GA occur sequentially (Pollard, 1968). Furthermore, we have found that GA enhances the secretion of more than 20 metabolites and enzymes. It is desirable to identify the earliest of these responses since they are most likely to be near the primary action of the hormone.

We wish to report (1) the effect of GA on the secretion of soluble carbohydrates and several phosphatases, the earliest effects it has been possible to detect and (2) data relating the effects of the hormone to other aspects of metabolism, especially protein synthesis, RNA synthesis and respiration.

Results and Discussion

GA exerted a very small effect on the incorporation of leucine into protein (Table I). Note, however, that in this experiment a four fold increase in soluble carbohydrates was found in the medium at the 8th hour.

Table I. Influence of GA on the Incorporation of Labelled Precursors into Protein and RNA and on Respiration of Aleurone Layers

	Hours of Incubation					
	2		4		8	
	Control	Treated	Control	Treated	Control	Treated
Leucine-1-^{14}C						
Cmp in total Protein	15,565	17,145	109,810	114,655	265,300	275,690
Soluble Carbohydrate	.10	.09	.12	.18	.22	.95
Adenine-8-^{14}C						
Cmp in RNA	-	-	250	273	527	537
Soluble Carbohydrate	-	-	.27	.34	.34	.72
Uracil-5-^{3}H						
Cmp in RNA	812	584	2,415	1,530	2,623	2,253
Soluble Carbohydrate	.27	.34	.34	.72	-	-
Oxygen Consumption						
Microliters/hr/30 layers	61	62	97	103	128	143

Betzes variety barley seeds were dehusked with 50% sulfuric acid for 1 hour, washed with distilled water, sterilized with 1% hypochlorite, rewashed and allowed to imbibe water at 3°C for 3-7 days. Layers were dissected from the endosperm ends with a razor blade, and sterilized as above. All incubation media contained 250 micrograms streptomycin sulfate and 200 micromoles calcium chloride per ml. Treated samples contained 1 microgram GA per ml. Results from separate experiments. In labelling experiments 45 layers were incubated in 22.5 ml. of media. Samples of 7.5 ml. and 15 layers removed at intervals and assayed. In experiments with leucine, adenine and uracil, 10, 3, and 6 microcuries, respectively used. Specific activities of precursors were: adenine-8-^{14}C, 48.2 Mc/mM; DL leucine-1-^{14}C, 48.2 mc/mM and uracil-5-^{3}H, 27 c/mM. Radioactivity in RNA and protein determined in a scintillation counter as cold trichloroacetic acid precipitable material after layers homogenized and washed 6 times with cold acid solutions. Values for soluble carbohydrates are absorbancies (620 μ) of anthrone reactions (Umbreit et al., 1964) of 0.2 ml. centrifuged aliquots. Absorbancy of 0.16 equals 20 micrograms glucose. Values for oxygen uptake are averages from 2 Warburg manometers (25°C).

The growth regulator did not affect the incorporation of adenine over the 8 hours and lesser amounts of uracil were incorporated in treated tissues than in controls. Even after prolonged incubation (12-24 hours) we have observed this phenomenon. We therefore conclude from this and other data that in these tissues GA causes a slight increase in protein synthesis but has no stimulatory effect on RNA synthesis during the first 8 hours of incubation.

Increased oxygen uptake appears to occur after that of increased sugar secretion and was first evident at the 4th hour; maximum rates of oxygen uptake for both tissues were noted near the 10th hour (145 and 161 microliters, in control and treated tissues in the experiment in Table I). Respiration in both tissues declines at the same rate so that after 40 hours the difference was still observed.

Within 2 to 4 hours of incubation an increased secretion of soluble carbohydrates occurred in treated layers; Tables I (uracil experiment) and II. This response was followed by increased amounts of ATPase. GTPase activity increased at about the 5th hour while phytase activity appeared still later.

The carbohydrates appear to be hexose oligosaccharides having few reducing groups and to arise from a soluble pool during the periods observed since their appearance in the medium parallels the disappearance of soluble carbohydrates from homogenates of the layers. Actinomycin D has no effect on sugar secretion until after 8 hours of incubation. The secretion of sugars is reduced under anerobic conditions, gives a log-linear response to GA concentrations between 10^{-9} and 10^{-6} grams/liter, responds optimally at 25-30°C and shows a pH optimum around 5.2 in 0.05M citrate buffer. There are several enzymes in the medium acting on each of the substrates studied here; this was shown by pH dependency studies; however, the enzymes showed negligible activity toward 12 other common phosphate esters tested.

Our evidence suggests that the secretion of soluble carbohydrate and of ATPase does not involve the synthesis of protein. The evidence is: (a)

Table II. Soluble Carbohydrate and Phosphatase Activities in the Medium of Control and GA Treated Aleurone Layers.

	Amount or Activity							
	Hours of Incubation							
	4		5		6		7	
	-GA	+GA	-GA	+GA	-GA	+GA	-GA	+GA
Soluble Carbohydrate.	.26	.31	.25	.38	.26	.50	.31	.76
ATPase.	.39	.36	.42	.53	.47	.70	.49	1.04
GTPase.	.37	.37	.44	.46	.67	.86	.40	.80
Phytase	.11	.14	.15	.13	.20	.25	.15	.48

Sixty layers were shaken at room temperature in 15 ml. solutions whose composition was described in Table I. Aliquots of 3.2 ml. and 12 layers removed at intervals. Anthrone reactions done on 0.1 ml. portions after centrifugation. Enzyme assays done with 0.3 ml. aliquots incubated with 0.1 micromole of substrates and 0.2 ml. 0.05 M citrate buffer pH 4.8 for 1 hour at 37°C and analyzed for release of inorganic phosphate (Chen et al., 1956). Controls where substrates added at end of incubation period utilized. Values are readings at 820 mu minus controls. Under the conditions used 0.02 micromoles of inorganic phosphate give absorbance of 0.13.

chloroamphenicol and p-flourophenylalanine have very little effect on the secretion of sugar (b) cycloheximide is a potent inhibitor if applied at the beginning of the incubation; it is without effect for at least 10 hours if applied in mid-course (at the 5th or 6th hour). Cycloheximide inhibits the rise in respiration in control and treated tissues; since respiration is required for secretion its effect may be secondary and therefore not useful in assessing the role of protein synthesis in the secretion. (c) There is no increase in the combined amounts of ATPase in the medium plus layers but there is a two-fold rise in the total phytase activity under the same conditions by the 6th hour. Therefore, phytase may be synthesized or activated. (d) In an experiment to determine when newly formed protein is secreted, 30 control and treated layers were incubated with 15 microcuries of leucine. At the end of the 3rd, 4th, and 5th hours, the ratio of control to treated of secreted soluble carbohydrate was 1.3, 1.9, and 2.5, respectively. Negligible ATPase was present in the control medium but that from treated medium was .07, .15, and .29. Significantly, no radioactivity appeared in the secreted proteins (precipitated with trichloroacetic acid with egg albumin as carrier) at the 3rd hour; 40 and 60 cpm appeared on the secreted proteins of both control and treated samples at the 4th and 5th hours and finally at the 6th hour the control sample showed 70 cpm and the treated 120 cpm in the protein. Thus, it appears that the ATPase precedes newly formed labelled protein in the medium and was therefore probably not radioactive.

Although the points of references of this work are the extensive studies with Himalaya variety of barley by Varner and associates, especially on amylase, the studies on the secretion of ribonuclease (Chrispeels and Varner, 1967a) appear to be most akin to those reported here. Aside from the difference in the varieties used the pretreatment of the seeds by these workers (imbibition of water by embryoless half seeds at room temperature for 3 days) is also different. Similar effects on secretion of sugar by layers prepared from Traill barley have also been seen. GA enhanced secretion of amylase

is not seen in our studies until after 7 hours of incubation; it appears to be one of the last enzymes affected.

Aside from the convenience of the availability of rapid responses, these studies provide another avenue for further studies on the mode of action of GA. Additionally, they pose the problem of the relation of secretion to the other responses induced by the hormone. The results suggest that the actions of the hormone studied here are not mediated through RNA; the involvement of protein synthesis is doubtful. To our knowledge these are the most rapid *in vivo* metabolic responses of the hormone reported.

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