Short Communication

Differential Effects of Mannitol on Gibberellin-Regulated Phospholipid Synthesis and Enzyme Activities of the CDP-Choline Pathway in Barley Aleurone Cells

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Summary. When barley aleurone layers are treated with gibberellic acid (GA₃) in the presence of increasing concentrations (0.2–0.8 M) of mannitol, the rate of ³²P incorporation into phospholipids becomes progressively inhibited. Mannitol does not affect this process in aleurone layers not treated with GA₃, nor does it appreciably inhibit GA₃-effected increases of ³²P incorporation into organic phosphates or the activities of the particulate enzymes of the CDP-choline pathway. These results suggest that some of the early events controlled by GA₃ can be separated from later activities regulated by the hormone, including α-amylase synthesis.

Gibberellic acid (GA₃)-effected α-amylase synthesis and secretion in barley aleurone cells commences after an initial lag period of 8–10 h (Varner and Chandra, 1964). There are, however, earlier events in barley aleurone cells which are controlled by the hormone and which may be required for the ultimate production and secretion of hydrolases. Jones (1969a) showed that proliferation of rough endoplasmic reticulum had occurred after 10 h of GA₃ treatment. Choline incorporation into a partially purified endoplasmic reticulum fraction (Evins and Varner, 1971) and ³²P incorporation into phospholipids (Koehler and Varner, 1971) are enhanced by GA₃ after a 4-h lag period. Furthermore, increases in the activities of two key enzymes involved in lecithin biosynthesis are evident after only 2 h of GA₃ treatment (Johnson and Kende, 1971). Metabolic antagonists such as cycloheximide and actinomycin D, and abscisic acid inhibit these GA₃ responses to similar extents (Chrispeels and Varner, 1967b; Evins and Varner, 1971; Johnson and Kende, 1971; Koehler and Varner, 1971).
When aleurone layers are subjected to an osmotic stress by agents such as mannitol or polyethylene glycol (0.2–0.8 M), GA$_3$-enhanced $\alpha$-amylase synthesis is markedly depressed (Jones, 1969b). Osmotically active solutes such as glucose and maltose (0.1–0.4 M) have a similar effect and may represent an in vivo control mechanism for GA$_3$-enhanced hydrolase synthesis (Jones and Armstrong, 1971). The results of experiments reported here show that although high concentrations of mannitol severely inhibit GA$_3$-enhanced phospholipid synthesis, mannitol does not affect the hormonal enhancement of $^{32}$P incorporation into other organic phosphate compounds, nor does it appreciably inhibit the promotion by GA$_3$ of the activities of phosphorylcholine-cytidyl transferase or phosphorylcholine-glyceride transferase.

Aleurone layers from barley seeds (Hordeum vulgare L. cv. Himalaya) were prepared according to Chrispeels and Varner (1967a). Ten layers per treatment were incubated in a 25 ml Erlenmeyer flask containing 2 ml of "treatment buffer" (1 mM acetate buffer, pH 5.0, 20 mM CaCl$_2$, 20 $\mu$g/ml chloramphenicol, mannitol at the various concentrations, and with or without 1 $\mu$M GA$_3$) at 25°C on a Dubnoff metabolic shaker. After 8.5 h, 100 $\mu$C carrier-free $^{32}$P were added to each flask. After a 45-min pulse, the layers were rinsed with 0.05 M KH$_2$PO$_4$ and incubated an additional 30 min in that solution. All manipulations were carried out under sterile conditions. The layers were then ground in a mortar in 4 ml of 0.1 M HEPES buffer, pH 7.55 containing 0.45 M sucrose; the homogenate was first centrifuged at 4000 $\times$ g for 10 min and then at 10000 $\times$ g for 15 min. The resulting supernatant was used for the following determinations.

Radioactivity in phosphate-containing organic compounds was determined by partitioning $^{32}$P as a phosphomolybdate complex into n-butanol-benzene (1:1) and counting an aliquot of the remaining aqueous phase (Saha and Good, 1970). This procedure measures all organic $^{32}$P including PPI except $^{32}$P in macromolecules and phospholipids. Phospholipids were extracted with 5 volumes of chloroform:methanol (2:1); the chloroform phase was washed 3 times with chloroform:methanol:water (3:48:47) containing 0.8% NaCl and 0.2% MgCl$_2$ (Folch et al., 1957). The chloroform phase was dried in a scintillation vial and counted in toluene:Triton X-100 (2:1) scintillation fluid containing 4 g PPO and 0.1 g POPOP per liter of toluene (Patterson and Greene, 1965). Uptake of $^{32}$P was measured by counting an aliquot of the 10000 $\times$ g supernatant.

The enzymes of the CDP-choline pathway were prepared from aleurone layers as described by Johnson and Kende (1971). Fifty half-seeds per sample were incubated for 8 h at 25°C on a reciprocal shaker. Aleurone layers were then isolated, homogenized, and the homogenate centrifuged at 500 $\times$ g. The resultant supernatant was centrifuged at 44000 $\times$ g and the pellet was used for the enzyme assays. Phosphorylcholine-cytidyl transferase was assayed by the method of Borkhagen and Kennedy (1957); phosphorylcholine-glyceride transferase was monitored by a modification (Johnson and Kende, 1971) of the method of Weiss et al. (1958). The reaction mixture for phosphorylcholine-cytidyl transferase contained in $\mu$moles: Tris-HCl (pH 7.0), 50; MgCl$_2$, 10; CTP, 1; 0.3 $\mu$C $^{14}$C-phosphorylcholine (23.3 mC/mmole), and enzyme (0.6 mg protein) in a total volume of 0.5 ml. The reaction mixture for phosphorylcholine-glyceride transferase was the same except that CTP was deleted and 0.25 $\mu$C of $^{14}$C-CDP-choline (12 mC/mmole) was used as substrate. Protein determinations were made according to Lowry et al. (1951).