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Inhibition of Gibberellic Acid-induced α-Amylase Formation by Polyethylene Glycol and Mannitol[1]

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Abstract. **Both polyethylene glycol (PEG) and mannitol inhibit gibberellic acid-induced α-amylase production in barley aleurone layers. The effect of the osmotic solution is on enzyme synthesis rather than α-amylase secretion. The inhibition of α-amylase synthesis does not appear to be mediated *via* an indirect effect on respiration or protein synthesis. Rather it seems that the osmotic solutions reduce the extent of proteolysis of the stored aleurone grain protein thus making available less substrate for new protein synthesis.**

Gibberellic acid (GA) has been shown to induce the synthesis of several hydrolytic enzymes in cereal aleurone cells (7, 8, 9, 12, 13). The GA induced synthesis of α-amylase and protease in barley aleurone cells has been shown to be dependent on protein and nucleic acid synthesis (1, 2, 5). Recent experiments (3, 5) provide unequivocal evidence that the synthesis of these enzymes is *de novo*.

A consequence of the proof of *de novo* synthesis by Filner and Varner (3) is that new amino acids must be formed from pre-existing proteins stored in the aleurone grains. Hydrolysis of the stored protein would occur according to equation I, the formation of each amino acid requiring 1 molecule of water

$$\text{Protein (nAA Residues)} + n\text{H}_2\text{O} \rightarrow n\text{RCH-NH}_2\text{-COOH} \qquad \text{I}$$

Treatment of barley aleurone layers with GA when water is limiting, *e.g.* under conditions of water stress, should therefore result in a reduced level of new enzyme synthesis. This inhibition would arise because of the reduced availability of substrate for *de novo* protein synthesis.

This communication reports the results of experiments designed to demonstrate the effects of osmotica on GA induced enzyme formation in barley aleurone cells.

Materials and Methods

The preparation of barley half seeds and the incubation of the half seeds or aleurone layers is similar to the methods described previously, (1, 6). Dry *Hordeum vulgare* (cv. Himalaya) seeds were cut in half and the embryoless halves sterilized in 5 % commercial bleach solution for 30 min. After washing in sterile water, the half seeds were placed on wet sterile sand for 3 days. Half seeds were incubated intact or the aleurone layer was removed for incubation. Incubation was carried out at 25° on a reciprocal shaker in a mixture of 0.001 M sodium acetate buffer (pH 4.8), 0.01 M calcium chloride and varying concentrations of GA, polyethylene glycol (PEG) or mannitol. Following the prescribed incubation period the half seeds or aleurones were removed, washed with 2 ml of buffer, and the washing combined with the incubate. α-Amylase was determined using the starch/iodine method and the results obtained were expressed as $\frac{\Delta OD \times TV}{t \times v}$ (6).

Respiration rates of control, GA and osmoticum treated aleurone layers were determined by Warburg manometry.

The incorporation of ^{14}C-leucine was measured as described by Chrispeels and Varner (2). Labeled amino acid (1 μc) was added to the aleurone layers and allowed to incubate for varying time periods. After washing with cold leucine, aleurone layers were ground in 5 ml of 0.001 M sodium acetate buffer (pH 4.8) and the extracts centrifuged at 1500*g* for 15 min. Total uptake of amino acid was determined on an aliquot from this supernatant. A further aliquot of supernatant was removed, mixed with an equal volume of 5 mM cold leucine and precipitated with 2 equal volumes of 15 % trichloroacetic acid. The mixture was subjected to millipore filtration to remove the precipitated protein. The filters were washed repeatedly with 5 % trichloroacetic acid and dried. The dried millipore filters were counted on aluminum planchettes in a gas-flow counter.

Results

Aleurone layers were imbibed on sterile sand for 3 days, and exposed to GA and varying concentrations of mannitol or polyethylene glycol ('200' and

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'400' molecular weight polymers from J. T. Baker, Philipsburg, New Jersey). Following an incubation period of 24 hr, the aleurone layers were removed from the incubate, washed with 2 ml of buffer and the washings combined with the original incubates. Gibberellic acid induced substantial quantities of α-amylase, however, in the presence of polyethylene glycol (PEG) or mannitol at concentrations of between 0.2 and 0.8 M, significant inhibition of enzyme formation occurred, (fig 1). Further experiments

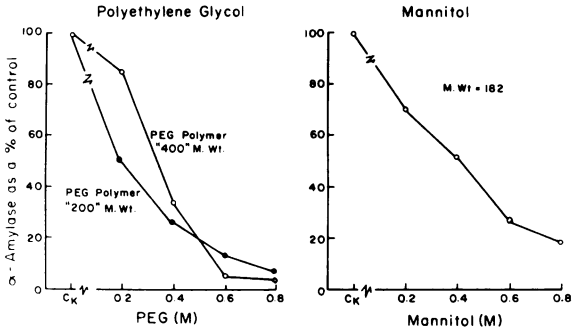

FIG. 1. Effect of polyethylene glycol (PEG) '200' and '400' molecular weight polymers and mannitol on GA_3-induced α-amylase formation in 10 barley half-seeds. GA_3 concentration 0.1 μg/ml.

showed that concentrations of PEG or mannitol as low as 0.05 M would bring about a significant inhibition of enzyme formation. This inhibition of α-amylase formation could not be overcome by the application of larger concentrations of GA, (fig 2). With

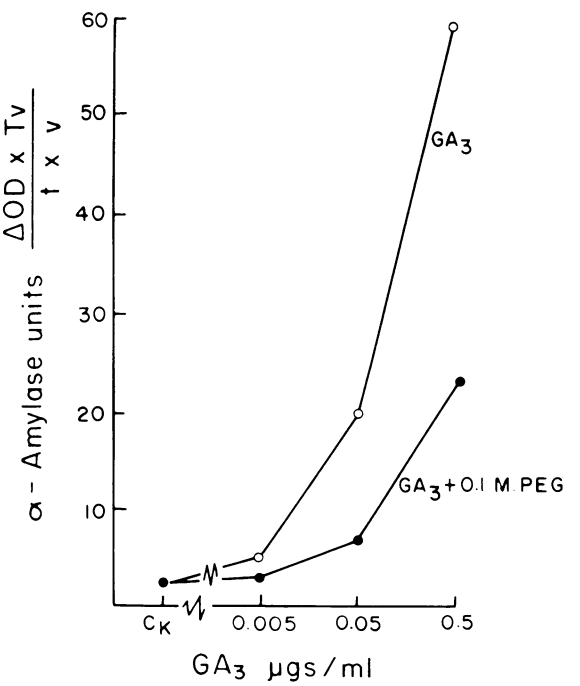

FIG. 2. Effect of increasing GA_3 concentrations on PEG inhibited α-amylase formation in 10 barley half-seeds.

an increase in GA concentration, the percentage inhibition by 0.1 M PEG remained the same, namely, 50 %, 60 %, and 60 %, (fig 2).

One obvious effect of the osmotica in reducing the amount of α-amylase appearing in the incubation medium would be an interference with the secretion of the enzyme. A result of this would be the accumulation within the cells of α-amylase. However, determination of α-amylase produced by cells in the presence of varying inhibitory concentrations of PEG indicate no significant effect on enzyme secretion, (fig 3). In fact, the data indicates that, with a decrease in medium α-amylase, a similar but less pronounced decrease in cellular α-amylase occurs, (fig 3).

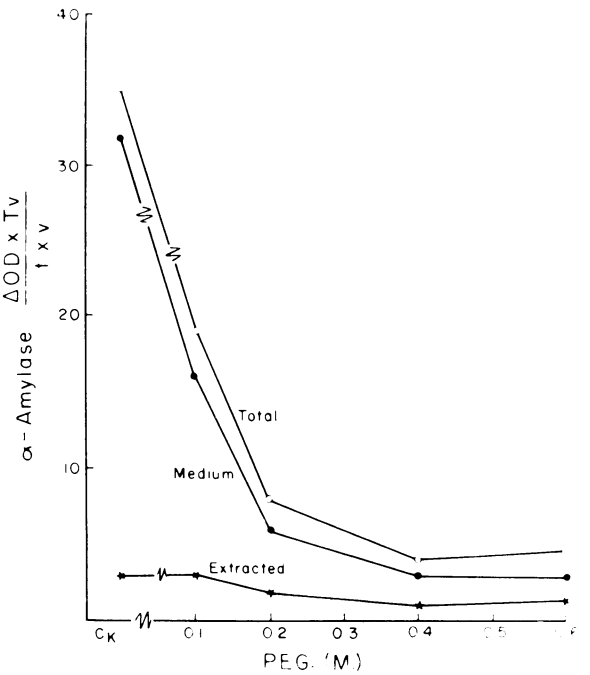

FIG. 3. Effect of PEG on GA_3-induced α-amylase synthesis and secretion in 8 barley half-seeds. Total indicates the sum of secreted α-amylase and that extracted from the aleurone layers after 22 hr of incubation. GA_3 concentration 0.1 μg/ml.

Polyethylene glycol and mannitol could function to inhibit enzyme synthesis *via* indirect effects on cellular metabolism. Although it is recognized that ethylene glycol is an inhibitor of respiration, there is no evidence that its polymers affect these processes. Measurements of respiration by Warburg manometry supports this observation. Concentrations of mannitol and PEG which were effective in causing nearly complete inhibition of enzyme synthesis had no significant effect on respiration when added together with GA or alone (table I).

Similarly, it could be suggested that the effects of the osmotica are on protein synthesis, namely that PEG and mannitol function as non specific inhibitors of protein synthesis. Estimates of protein synthesis

Table I. *Rate of O_2 Uptake by Aleurone Layer Cells*

Aleurone layers were incubated in 0.001 M sodium acetate buffer, 0.01 M calcium chloride, 0.05 μg/ml GA_3 and PEG as indicated in table. Measurement of oxygen uptake were started 1 hr after addition of GA_3 and PEG. Readings were continued for 5 hr at 15 min intervals.

Treatment		μl Q_2/aleurone layer×hr
Expt. 1	GA_3	8.1
	H_2O	10.1
	GA_3 PEG(0.6 M)	7.8
Expt. 2	GA_3	13.2
	H_2O	11.0
	GA_3 PEG(0.6 M)	10.8

Table II. *^{14}C-Leucine Incorporation Into Trichloroacetic Acid Precipitable Aleurone Cell Protein*

Aleurone layers were incubated in 0.001 M sodium acetate buffer (pH 4.8), 0.01 M calcium chloride, 0.05 μg/ml GA_3, 1.0 μc ^{14}C-leucine and PEG as indicated above. ^{14}C-Leucine was added 2 hr after start of induction and continued for 3 hr.

Treatment	cpm/10 half aleurone layers
GA_3	7227
GA_3 PEG(0.2 M)	7347
GA_3 PEG(0.6 M)	8147

obtained by measuring ^{14}C-leucine incorporation, indicate that PEG has no effect on the amount of ^{14}C leucine incorporated into trichloroacetic acid precipitable protein (table II).

Evidence that the osmotic media were specifically affecting GA induced protein synthesis was obtained by the following experiments. It is well established that the process of GA-stimulated enzyme induction in barley is a 2 stepped phenomenon (1, 2). During the first 8 to 10 hr following treatment of seeds with GA a lag phase ensues—this is followed by a phase of active enzyme secretion. Addition of PEG to aleurone layers during the lag phase (first 12 hr) resulted in a more marked and significantly greater inhibition than when added during the secretory phase (second 12 hr) (fig 4). Other experiments indicated that the osmotica were most effective when added during the lag phase. Addition of PEG to imbibing half seeds during 24 or 48 hr of imbibition

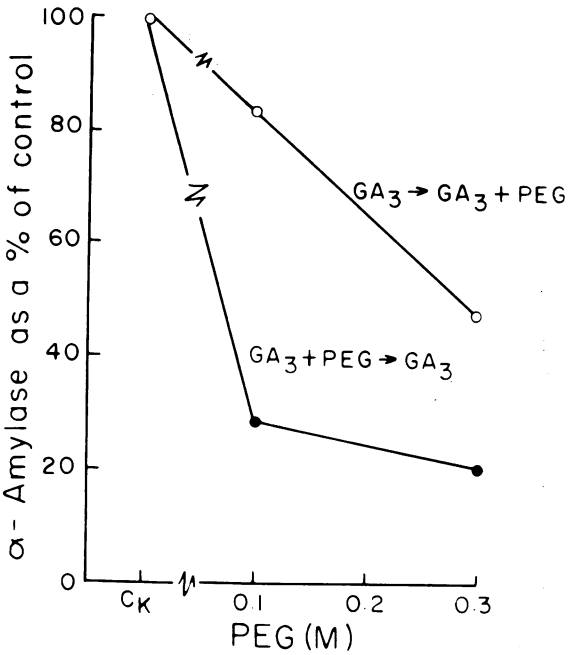

FIG. 4. Effect of PEG on GA_3 induced α-amylase formation. $GA_3 \rightarrow GA_3$+PEG, 10 half-seeds incubated for 12 hr in 0.1 μg/ml of GA_3 then transferred to a solution of 0.1 μg/ml GA_3 varying concentrations of PEG. GA_3+PEG→GA_3, 10 half-seeds incubated for 12 hr in a solution of 0.1 μg/ml GA_3 and varying concentrations of PEG then transferred to a solution of 0.1 μg/ml GA_3.

had a less marked effect on enzyme production than the same concentration of PEG added during the first 8 hr of enzyme induction, (table III).

Discussion

The data presented above indicate that concentrations of PEG or mannitol in the range of 0.05 to 0.8 M reduced the level of GA-induced α-amylase synthesis in barley aleurone cells. This inhibition of enzyme synthesis is non-competitive; increasing concentrations of gibberellic acid do not overcome the inhibitory effect of the osmotica (fig 2). Because of the relatively high molar concentrations of the osmotic solutions used and since their effect is one of inhibition, it seemed prudent to eliminate the possibility that inhibition of enzyme synthesis is by

Table III. *Effect of Preincubation and Incubation in PEG on α-Amylase Synthesis*

Treatment[1]	α-Amylase as % control
Incubated in GA_3, Preincubated water 3 days	100
Incubated in GA_3, Preincubated water 1 day, 0.3 M PEG 2 day	69
Incubated in GA_3, Preincubated water 2 day, 0.3 M PEG 1 day	71
Incubated in GA_3 + PEG 0.3 M - Preincubated water 3 days	25

[1] Incubation medium as in table I.

[2] Incubated in PEG 0.3 M for lag phase only. α-Amylase determinations made on incubate from 8 to 24 hr after addition of GA_3 in all samples.

a non-specific mechanism. Determination of respiration rates indicated that neither PEG nor mannitol affected oxygen uptake even when added at concentrations which markedly inhibit α-amylase synthesis (table I). Similarly, inhibitory concentrations of osmotica had no effect on ^{14}C-leucine incorporation into trichloroacetic acid precipitable protein (table II). These findings are in agreement with the observations of Thimann *et al.* (11) who showed, in addition, that solutions of mannitol did not enter the cells of potato.

Increased water stress appears therefore to affect GA-induced protein synthesis or secretion. Since α-amylase appearance in the incubation medium is dependent on active enzyme secretion (1), PEG or mannitol could reduce the total amount of enzyme obtained without directly affecting synthesis. One result of inhibition of secretion would be the accumulation within aleurone cells of newly synthesized protein (1). This was not found to occur; in fact, the data obtained tended to support the hypothesis that enzyme synthesis was affected (fig 3).

Further support for a direct effect of PEG or mannitol on GA-induced enzyme synthesis was obtained. Treatment of aleurone layers with PEG or mannitol during the lag phase (first 12 hr following GA treatment) resulted in a more marked inhibition of enzyme synthesis than that obtained when cells were treated with osmotica during the secretory phase (second 12 hr period following GA treatment) (fig 4). Also, treatment with PEG or mannitol during imbibition for 24 and 48 hr does not inhibit enzyme synthesis as markedly as the application of PEG or mannitol for 12 hr of the lag phase (table III).

Additional evidence that the effect of osmotically active solutions is on GA-induced responses is given by the experiments of Ferrari (personal communication). Ferrari has shown that nitrate reductase induction in barley aleurone layers by nitrate is unaffected by concentrations of PEG which inhibit α-amylase production by as much as 50 %.

Several processes occur during the lag phase of GA-induced enzyme synthesis, one of which must be the proteolysis of stored protein (3). It is suggested that the effect of osmotically active solutions in reducing the level of GA-induced α-amylase synthesis is *via* an effect on the proteolysis of the stored aleurone-grain protein.

This apparent effect of osmotic media on α-amylase synthesis in barley may explain some of the observed inhibitory effects of osmotic media on germination in other species. Scheibe and Lang (10) suggested that germination in light-sensitive lettuce seed was a function of an osmotic force generated within the seed. This argument was based on the observation that germination was inhibited by mannitol solutions. Ikuma and Thimann (4) on the other hand proposed that lettuce seed germination was a function of hydrolytic enzyme induction in the cotyledons; the hydrolytic enzymes attack the endosperm wall releasing the seed from inhibition. In light of the data presented above, it is possible that both workers were observing the same phenomenon, the inhibition of germination by mannitol observed by Scheibe and Lang (10) could be a reflection of an inhibition of hydrolytic enzyme synthesis as proposed by Ikuma and Thimann (4). Likewise, other examples of inhibition of various phases of development by osmotic solutions, particularly in tissues rich in stored reserves, could be a result of the above described effect.

Acknowledgments

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