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Lysosomal Nature of Hormonally Induced Enzymes in Wheat Aleurone Cells

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The subcellular distribution of the enzymes α-amylase, protease and ribonuclease in wheat aleurone layers after treatment with gibberellic acid was determined by differential centrifugation. Of the α-amylase 56% was precipitable from cell homogenates, indicating that it is a particulate enzyme. Similar results were recorded with protease. Particulate α-amylase showed distinct structural latency, and membrane-rupturing mechanical or chemical treatments were required to release the enzyme in an active form; the results were completely analogous to results with lysosomal enzymes found in animal tissues. The identification of the hormonally induced enzymes as lysosomal suggests that the hormonal mechanism may be more closely associated with extracellular enzyme synthesis rather than with nucleic acid metabolism.

One of the clearest and best understood hormonal responses in plants is the gibberellin-induced initiation and control of hydrolytic enzyme synthesis in the cereal aleurone layer during germination. After synthesis, which is observable about 6h after hormone treatment of the tissue, the acid hydrolases are secreted into the starchy endosperm, where they convert stored reserves into soluble and mobile metabolites utilized by the embryo for growth.

The response was first reported in 1960 (Paleg, 1960; Yomo, 1960) and in the intervening years a great deal of effort has gone into the elaboration of the sequential events. From the standpoint of the type of enzymes and the nature of the process, an explanation involving lysosomes (i.e. discrete membrane-enclosed organelles containing acid hydrolases) initially seemed completely appropriate. In fact, MacLeod & Millar (1962) suggested that the enzymes might be lysosomal. However, MacLeod *et al.* (1964) retracted their earlier suggestion when they found different enzymes developing at different times, and failed to isolate a particle that could be stimulated *in vitro* by the hormone to release the hydrolases.

At that time also, the lysosomal concept orientated thinking towards preformed, though inactive, enzymes, the release and activation of which was in some way effected by the hormone. The demise of the theory that the gibberellin-induced enzymes were lysosomal came with the demonstration that at least two of the enzymes, α-amylase (Filner & Varner, 1967) and protease (Jacobsen & Varner, 1967), and presumably others were newly synthesized and not released from a bound or inactive form.

Attention shifted to other types of control mechanisms, although any other mechanism raised serious questions. During germination the cells of the cereal aleurone layer produce and secrete almost all of the hydrolytic enzymes involved in the complete degradation of the starchy endosperm, a process requiring up to 6–7 days. In spite of this, the aleurone cells retain their viability and integrity until the endosperm has been entirely digested. Unless the acid hydrolases produced by the aleurone cells are separated from the rest of the cellular constituents by some sort of membrane, how do the aleurone cells escape autolysis?

With the aid of a technique developed for the rapid mass isolation of viable, reproducibly responsive aleurone tissue (Phillips & Paleg, 1972), this question has been reinvestigated. The present paper reports results that demonstrate that α-amylase and protease are located within a sedimentable and membrane-enclosed organelle.

Experimental

Cell fractions

Aleurone layers (consisting of aleurone cells together with non-functional pericarp, testa and nucellar epidermis) were prepared from sterilized half-seeds of wheat (var. Olympic) by using the roller-mill technique of Phillips & Paleg (1972). Embryo-less half-seeds (20g dry wt.) that had imbibed water for 26h at 30°C were rolled for 3min with 60ml of 15mM-lactic acid, and the liquid was decanted. The half-seeds were rolled for a further 3min with 80ml of 15mM-lactic acid and, after the liquid had been decanted, the process was repeated. The resulting aleurone layers were washed with 1 litre of water and allowed to drain on several layers of tissue paper. The aleurone layers were weighed and samples (1g fresh wt.) transferred to 250ml conical flasks. The tissue was preincubated for 12h in 10ml of 20mM-$Ca(NO_3)_2$ in a shaking water bath (30°C) moving at

50 oscillations/min. At the end of this period, the liquid was poured off and replaced with 8ml of 10mM-glucose–20mM-$Ca(NO_3)_2$, with or without gibberellic acid (compound GA_3) at a concentration of 10μg/ml. The flasks were then returned to the water bath and the tissue was incubated for 24h. All operations were carried out aseptically and all solutions were sterilized by autoclaving or by passage through a Millipore filter (0.25μm pore size).

At the end of the incubation period, the solution was decanted, the tissue washed with 7ml of 5mM-calcium acetate and the combined washings (ambient solution) were made up to 15ml. The aleurone layers were then thoroughly washed with 100ml of water and allowed to drain. All subsequent operations were carried out at 3°C in a cold-room. The tissue was homogenized in 10ml of grinding medium in an Ultra-Turrax model TP 18/2 apparatus set at minimum speed for 30s, or in a mortar and pestle without sand for 1min. Unless otherwise stated, the grinding medium contained 0.4M-sucrose, 0.05M-tris–HCl (pH7.0), 0.1% bovine serum albumin, 0.01M-KCl, 1mM-EDTA and 0.1mM-$MgCl_2$ (Breidenbach & Beevers, 1967). The homogenate was filtered through two double layers of cheesecloth and the filtrate centrifuged at 1000$g_{av.}$ for 10min, and the resulting supernatant at 20000$g_{av.}$ for 20min. The subsequent supernatant was centrifuged at 105000$g_{av.}$ for 90min. All centrifugations were carried out in a Beckman–Spinco model L preparative centrifuge, in a 50 Ti rotor and 13ml capacity polyallomer tubes. The pellets obtained at each centrifugation, as well as the final supernatant, were assayed for enzymic activity as soon as possible. Pellets were suspended in 5ml of 5mM-calcium acetate for α-amylase assay, 2ml of 1mM-potassium acetate buffer, pH4.8, containing 20mM-2-mercaptoethanol for the protease assay and 2ml of 50mM-potassium acetate buffer, pH5.0, for ribonuclease assay. All pellets were thoroughly ground in a close-fitting glass homogenizer before assay.

Enzyme assays

α-Amylase activity was determined by a modification (Collins *et al.*, 1972*a*,*b*) of the method of Shuster & Gifford (1962), after inactivation of β-amylase activity by heating at 70°C for 20min (Paleg, 1960). Amylose (BDH Chemicals Ltd., Poole, Dorset, U.K.) was used as substrate. When EDTA was present care was taken to add excess of calcium acetate before inactivation of the β-amylase. The degradation of amylose was followed by measuring at 2min intervals the decreasing intensity of the colour of the blue amylose–iodine complex. All results are expressed in starch–iodine colour (S.I.C.) units as described by Briggs (1962).

Ribonuclease was assayed by the method of Wilson (1963) with yeast RNA (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as substrate. Enzyme activity is expressed in units, where 1 unit represents 0.1E_{260} generated in 1h at 37°C and pH5.0.

Protease activity was assayed by measuring amino acid release from the substrate gliadin (Sigma Chemical Co.) at 30°C and pH4.8 by the method of Jacobsen & Varner (1967).

Values given for α-amylase, protease and ribonuclease represent single determinations of enzyme activity. Boiled enzyme controls were included in all assays. Protein was measured by the method of Lowry *et al.* (1951).

Factors affecting latency of α-amylase

Initial examination of the properties of particles containing α-amylase was carried out on pellets prepared in the following way. Samples (1g) of gibberellic acid-treated aleurone tissue were homogenized as described above, the cheesecloth filtrates centrifuged at 1000$g_{av.}$ for 10min and the pellets discarded. The supernatant fractions were centrifuged at 60000$g_{av.}$ for 30min and the resulting pellets were resuspended in 1ml of a medium containing 0.4M-sucrose, 5mM-$Ca(NO_3)_2$, 0.1% (w/v) bovine serum albumin and 0.05M-tris–HCl at pH7.0. Solutions containing enzymes or detergents were introduced in a volume of 0.1ml and the mixtures were incubated for 30min at the temperature indicated.

At the end of the incubation period the suspensions were transferred to centrifuge tubes, made up to a volume of 10ml with resuspension medium and centrifuged for 30min at 60000$g_{av.}$. After the supernatant fractions had been decanted the pellets were treated for 30min with 0.1% sodium deoxycholate and the volume was made up to 5ml with 5mM-calcium acetate. Both the supernatant and particulate fractions were then heated for 20min at 70°C and assayed for α-amylase activity as described above.

Latency of α-amylase

The latency of α-amylase was established by two methods. The first involved centrifugation, the latent enzyme being that proportion of a suspension of a 60000$g_{av.}$ pellet preparation that was sedimented (by a further centrifugation) after incubation under various conditions. α-Amylase in the supernatant and pellet was determined by the methods already described. The second method, used commonly with animal lysosomes (Wattiaux & de Duve, 1956), involved assaying enzyme activity with and without a surface-active agent such as Triton X-100, after incubation of lysosomes under similar conditions. Equal volumes of a suspension of a 60000$g_{av.}$ pellet were assayed for α-amylase activity in substrate solutions containing 0.4M-sucrose. One sample, with-

out additive, was taken as the measure of free or available α-amylase and another, containing 0.1% Triton X-100, was assayed as a measure of total α-amylase activity. Both samples were preincubated at 30°C for 30min before assay. Latent α-amylase activity was calculated by subtracting the value obtained for free α-amylase activity from the value obtained for total α-amylase activity: i.e. latent enzyme = (activity with Triton X-100) − (activity without Triton X-100).

Results

Work carried out in this laboratory (Phillips & Paleg, 1972) on fractions obtained by differential centrifugation of wheat-aleurone homogenates prepared by using the grinding medium of Honda *et al.* (1966) indicated that although most of the detectable ribonuclease activity was contained in the supernatant fraction, a significant proportion (15%) of the total α-amylase could be sedimented. This suggested that α-amylase, at least, might be a particulate enzyme.

As the choice of grinding medium may not have been the most suitable for the isolation of particles containing hydrolytic enzymes, a search was initiated for a grinding medium giving a higher yet consistent percentage of sedimentable α-amylase. The results of this survey are summarized in Table 1. The grinding medium described by Breidenbach & Beevers (1967) consistently gave high activities of α-amylase in the pellets as well as high yields of the enzyme from the tissue. For these reasons, this medium was adopted in subsequent experiments.

Very high shear forces are required to break the massive cell walls surrounding the aleurone cells. To minimize the damage to organelles these forces might be causing, we examined a variety of homogenization techniques and compared the α-amylase activities in the pellets obtained. The results (Table 2) indicate that the highest yields of particulate α-amylase were obtained with a 30–60s treatment with the Ultra-Turrax apparatus, and that grinding for 1min in a mortar and pestle without sand also gave good results. Chopping the tissue with razor blades gave low yields of the enzyme from the tissue.

The possibility that the α-amylase activities in the pellets were due to artifacts caused by the grinding procedure or by non-specific binding of the enzyme to membranes was also investigated. A 1.0g sample of gibberellic acid-treated tissue was homogenized with the Ultra-Turrax apparatus for 1min in 7.5ml of water. After passage through two double layers of cheesecloth, the filtrate was centrifuged at 20000*g* for 30min and the supernatant fraction so obtained was mixed (1:1, v/v) with double-strength grinding medium. This mixture was then used to grind 1.0g of control tissue in the usual way. The results (Table 3) showed that the added α-amylase was associated with the supernatant fraction, strongly suggesting that the enzyme activities found in the pellets from gibberellic acid-treated tissue were not due to artifacts.

As the dithiothreitol in the grinding medium interfered with the α-amylase and ribonuclease assays,

Table 1. *Effect of grinding medium on distribution of α-amylase in cell fractions obtained from aleurone tissue treated with gibberellic acid for 24h*

Tissue was ground for 30sec (Ultra-Turrax apparatus) and the resulting homogenate subjected to differential centrifugation as described in the text. Composition of media: 1, glycerol (Yatsu & Jacks, 1968); 2, 0.25M-sucrose, 2.5% ficoll, 5% dextran, 0.01% bovine serum albumin, 0.025M-tris–HCl (Honda *et al.*, 1966); 3, 20% sucrose, 1mM-EDTA, 0.1% polyvinylpyrrolidone, 0.1M-tris–HCl (Balz, 1966); 4, 0.4M-sucrose, 0.05M-tris–HCl, 0.01M-dithiothreitol, 0.1% bovine serum albumin, 10mM-KCl, 1mM-EDTA, 0.1mM-$MgCl_2$ (Breidenbach & Beevers, 1967); 5, 0.35M-mannitol, 0.35M-sucrose, 10mM-K_2HPO_4, 0.1% bovine serum albumin, 1.0mM-EDTA, 2.0mM-$Na_2S_2O_5$ (Stokes *et al.*, 1968); 6, 0.5M-sorbitol, 0.05M-tris–HCl, 1.0mM-EDTA (Matile, 1968); 7, 0.4M-mannitol, 0.01M-tris–HCl (Spichiger, 1969). All media were at pH7.0.

Grinding medium ...	Distribution of α-amylase (%)						
Cell fraction	1	2	3	4	5	6	7
1000*g*	12.7	48.8	38.6	11.9*	6.2	3.7	3.5
20000*g*	16.4	2.4	7.4	18.8*	10.4	23.4	25.9
105000*g*	0	2.4	3.1	14.1*	39.1	24.4	15.7
Supernatant	70.9	46.4	50.9	55.2*	44.3	48.5	54.9
Total	100	100	100	100	100	100	100
	(13.4 units)	(37.9 units)	(32.4 units)	(60.1 units)	(19.2 units)	(42.7 units)	(40.1 units)

* Values represent the means of two experiments.

Table 2. *Effect of grinding technique on distribution of α-amylase in all fractions obtained from aleurone tissue treated with gibberellic acid for 24h*

The grinding medium used in all cases was that of Breidenbach & Beevers (1967). All other conditions were as described in the text.

	Distribution of α-amylase											
Technique ...	Ultra-Turrax apparatus								Mortar and pestle		Razor blade	
Procedure time ...	10s		20s		30s		60s		60sec		10min	
Cell fraction	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%
1000*g*	1.6	3.3	1.3	3.0	2.0	3.3	2.0	3.4	2.5	4.9	6.6	36.5
20000*g*	12.0	25.0	9.6	22.5	16.6	27.1	15.0	25.4	13.2	25.8	1.9	10.5
105000*g*	9.4	19.6	8.3	19.4	10.9	17.8	8.6	14.6	9.8	19.1	0.4	2.2
Supernatant	25.0	52.1	23.5	55.1	31.8	51.8	33.4	56.6	25.7	50.2	9.2	50.8
Total	48.0	100	42.7	100	61.3	100	59.0	100	51.2	100	18.1	100

Table 3. *Distribution of α-amylase in control aleurone tissue after homogenization with added α-amylase and subsequent differential centrifugation*

The grinding medium used was that described by Breidenbach & Beevers (1967).

	No gibberellic acid		No gibberellic acid +α-amylase		With gibberellic acid	
Cell fraction	Units	%	Units	%	Units	%
1000*g*	0	0	1.0	1.1	2.7	2.1
20000*g*	1.1	25.6	1.0	1.1	41.5	31.9
105000*g*	0.8	18.6	0.9	1.0	14.8	11.4
Supernatant	2.4	55.8	86.0	96.8	71.0	54.6
Total	4.3	100	88.9	100	130.0	100

the effect of excluding this compound from the medium was tested. Although the absolute amount of α-amylase extracted from the tissue was decreased slightly, the percentage of the enzyme in the various pellets was not greatly affected. Dithiothreitol was therefore excluded from the grinding medium in all subsequent experiments.

With the optimum homogenization techniques already described, the various cell fractions obtained by differential centrifugation of homogenates from aleurone layers, incubated for 24h with and without gibberellic acid, were assayed for three hydrolytic enzymes, i.e. α-amylase, protease and ribonuclease. An average of 56% of the detectable α-amylase activity found in the three experiments was sedimentable and about half of this activity was located in the 20000*g* pellet (Table 4). Similar results were obtained for protease. Unlike α-amylase and protease, the ribonuclease activity was primarily located in the supernatant fraction with only about 20% of the enzyme activity detectable in the pellets.

To examine the properties of particles containing these hydrolytic enzymes, and as an aid to further purification, it was desirable to concentrate all the particulate enzyme activity into a single fraction. The usual homogenization technique was used, followed by a comparison of different centrifugal forces (Table 5). A centrifugal force of 60000*g* sedimented almost all of the enzymes that were previously distributed between the 20000*g* and 105000*g* pellets. As in the preceding experiments, only 20% of the ribonuclease activity appeared in the pellets, and most of this was located in the 60000*g* pellet. The total α-amylase activity values (160 and 161 units) shown for the two gibberellic acid treatments correspond to a 78% recovery of the total α-amylase activity in the tissue before differential centrifugation, as measured by a separate analysis. Thus the recovery of activity through the diverse operational techniques was good.

By measuring the protein content, the specific activity of the α-amylase in the various cell fractions was calculated (Table 6). The results show that although 56% of the total enzyme activity was located in the 60000*g* pellet, this fraction contained only

Table 4. *Distribution of α-amylase, protease and ribonuclease in cell fractions obtained from aleurone tissue treated with or without gibberellic acid for 24h*

The grinding medium used in this and all subsequent experiments was that described by Breidenbach & Beevers (1967) with dithiothreitol excluded from the medium.

	α-Amylase				Protease				Ribonuclease			
Gibberellic acid ...	−		+		−		+		−		+	
Cell fraction	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%
Expt. 1												
1000*g*	0.6	13.3	7.3	9.1	0	0	0.4	6.1	0.2	2.6	0.9	3.4
20000*g*	1.1	24.5	14.7	18.3	0.3	42.9	2.0	30.3	0.2	2.6	1.7	6.4
105000*g*	0.6	13.3	16.3	20.3	0.1	14.2	1.8	27.3	0.1	1.3	2.0	7.5
Supernatant	2.2	48.9	42.0	52.3	0.3	42.9	2.4	36.3	7.2	93.5	22.1	82.7
Total	4.5	100	80.3	100	0.7	100	6.6	100	7.7	100	26.7	100
Expt. 2												
1000*g*	0.8	15.7	11.1	11.3	0.3	37.5	0.9	20.5	1.0	10.2	2.9	11.4
20000*g*	0.8	15.7	37.0	37.5	0.1	12.5	1.4	31.8	0.2	2.0	2.2	8.6
105000*g*	0.4	7.8	14.5	14.7	0.1	12.5	1.4	31.8	0.1	1.0	1.8	7.0
Supernatant	3.1	60.8	36.0	36.5	0.3	37.5	0.7	15.9	8.5	86.8	18.6	73.0
Total	5.1	100	98.6	100	0.8	100	4.4	100	9.8	100	25.5	100
Expt. 3												
1000*g*	2.2	21.0	19.3	12.1	0.3	17.6	1.2	17.9	1.4	7.8	2.2	7.3
20000*g*	1.4	13.3	67.0	41.8	0.6	35.3	2.7	40.3	0.2	1.1	2.6	8.6
105000*g*	0.6	5.7	11.9	7.4	0.1	5.9	0.6	9.0	0.1	0.6	1.5	5.0
Supernatant	6.3	60.0	62.0	38.7	0.7	41.2	2.2	32.8	16.2	90.5	24.0	79.1
Total	10.5	100	160.2	100	1.7	100	6.7	100	17.9	100	30.3	100
					Average values							
1000*g*	1.2	17.9	12.6	11.1	0.2	20.0	0.8	13.6	0.9	7.6	2.0	7.2
20000*g*	1.1	16.4	39.6	35.0	0.3	30.0	2.0	33.9	0.2	1.7	2.2	8.0
105000*g*	0.5	7.5	14.2	12.6	0.1	10.0	1.3	22.0	0.1	0.8	1.8	6.5
Supernatant	3.9	58.2	46.7	41.3	0.4	40.0	1.8	30.5	10.6	89.9	21.6	78.3
Total	6.7	100	113.1	100	1.0	100	5.9	100	11.8	100	27.6	100

23% of the total protein. This represents a specific activity of 2.5 times that found in the original cell homogenate.

Lysosomal enzymes exhibit structural latency; thus access to substrates is restricted by the membranes enclosing the enzymes. The enzymes may be released by a variety of physical and chemical treatments and the effect of such treatments is usually taken to be important evidence of the presence of lysosomes. An examination of the release of α-amylase from 60000*g* pellets was carried out by using the methods outlined (Table 7). At 5°C only 20% of the α-amylase leaked into the supernatant during the 30min incubation period. This value was increased by physical stresses imposed by tenfold dilution, grinding in an Ultra-Turrax apparatus for 10min or freezing and thawing four times. Treatment with the surface-active agents Triton X-100 and sodium deoxycholate at 5°C resulted in almost complete loss of the α-amylase from the pellet. At 30°C, leakage of α-amylase into the supernatant was increased to 60%, indicating a temperature-dependent autolysis of the α-amylase-containing particles. Phospholipase A at 30°C slightly enhanced this leakage but phospholipase C or D appeared to be without effect.

It should be noted that the techniques used in the experiments indicated in Table 7 are perhaps more rigorous than those usually employed with lysosomes of animal origin. In experiments with animal lysosomes, enzyme latency is usually determined as the difference in activity with and without the addition of a surface-active agent (e.g. deoxycholate, Triton X-100 etc.) to the incubation medium. In the experiments reported in Table 7, latency was estimated by the amount of enzyme that was sedimented during a second centrifugation at 60000*g*. When lysosomes comparable with those used in the experiments reported in Table 7 were tested for enzyme latency with

Table 5. *Effect of increasing centrifugal force from 20000g to 60000g on distribution of enzymes from homogenates of aleurone tissue treated with gibberellic acid for 24h*

Conditions were the same as described for Table 5.

	α-Amylase						Protease						Ribonuclease					
Gibberellic acid ...	−			+			−			+			−			+		
Cell fraction	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%	Units	%
1000*g*	2.2	21.0	19.3	12.1	19.3	12.0	0.3	18.8	1.2	17.9	1.2	17.4	1.9	10.3	2.2	7.1	2.2	7.4
20000*g*	1.4	13.3	67.0	41.8	—	—	0.6	37.5	2.7	40.3	—	—	0.2	1.1	2.6	8.4	—	—
60000*g*	—	—	—	—	76.0	47.2	—	—	—	—	3.3	47.8	—	—	—	—	4.0	13.4
105000*g*	0.6	5.7	11.9	7.4	1.8	1.1	0.1	6.2	6.0	0.9	0.2	2.9	0.1	0.5	1.5	5.0	0.2	0.7
Supernatant	6.3	60.0	62.0	38.7	64.0	39.7	0.6	37.5	2.2	32.8	2.2	31.9	16.2	88.1	24.5	79.5	23.5	78.5
Total	10.5	100	160.2	100	161.1	100	1.6	100	6.7	100	6.9	100	18.4	100	30.8	100	29.9	100

the technique established with animal lysosomes, completely comparable results (Table 8) were obtained.

Discussion

The lysosomal concept is well established for animal cells and has been the subject of many review articles (Dingle & Fell, 1969). Implicit in the concept is that, under normal conditions *in vivo*, the hydrolytic enzymes are enclosed within a sac-like structure, limiting the access of the enzymes to their substrates. Lysosomal enzymes possess three important characteristics: (1) they usually have acid pH optima; (2) they are particulate and sediment more or less together when tissue homogenates are fractionated by differential centrifugation; (3) the particulate enzymes exhibit structural latency, disruptive treatments being required to release the soluble enzymes in an active form.

The enzymes involved in this work, α-amylase, protease and ribonuclease, all have acid pH optima. Detailed analysis of the pellets obtained by differential centrifugation of homogenates of aleurone layers indicate that the 20000*g* and 105000*g* pellets are particularly rich in α-amylase and protease activity and contain some ribonuclease activity. Enzyme activity distributed between these fractions can be concentrated in a single fraction if a centrifugal force of 60000*g*, rather than 20000*g*, is imposed. The percentage yield obtained in these fractions is considered particularly significant in view of the high shear forces necessary to break aleurone cell walls. Since all of the enzyme activity was originally derived from within gibberellic acid-treated aleurone tissue, we conclude that the enzymes α-amylase and protease are particulate in nature in the cells of the aleurone layer.

The nature of ribonuclease is less clear, as most of this enzyme is found in the supernatant fraction. This reinforces the conclusion that sedimentation of α-amylase and protease is most unlikely to be a consequence of non-specific protein binding to membranes. The amount of ribonuclease present in the control tissue is large and the enzyme activity undergoes the smallest percentage increase in the time-interval (24h) employed. In this time the percentage of enzyme in either the 20000*g* and 105000*g* fractions or the 60000*g* fractions shows the greatest increase. If, as suggested by Chrispeels & Varner (1967), ribonuclease synthesis is initiated later than that of α-amylase and protease it seems possible that the proportion of sedimentable ribonuclease would increase if the incubation time were extended. Indeed, other workers (Jones & Price, 1970) have suggested that after gibberellic acid induction this enzyme in barley aleurone tissue may be sedimentable.

Many of the experimental treatments demonstrat-

Table 6. *Specific activity of α-amylase in cell fractions obtained from aleurone tissue treated with gibberellic acid for 24h*

Fraction	α-Amylase Units	α-Amylase %	Protein mg	Protein %	Sp. activity (units/mg of protein)
1000*g*	8.3	11.4	2.0	25.0	4.2
60000*g*	41.0	56.0	1.8	22.5	22.8
Supernatant	23.8	32.6	4.2	52.5	5.7
Total	73.1	100	8.0	100	
Original homogenate	76.0		8.2		9.3

Table 7. *Effect of various treatments on the latency of α-amylase in 60000g pellet preparations*

Conditions for this experiment are described in detail in the text. Values represent means of two experiments.

Treatment	α-Amylase (% of total) Supernatant (free)	Pellet (latent)
5°C		
Control	20	80
Dilution (×10)	42	58
Grinding	82	18
Freezing (four times)	55	45
Triton X-100	91	9
Sodium deoxycholate	98	2
30°C		
Control	63	37
Phospholipase A	79	21
Phospholipase C	61	39
Phospholipase D	61	39

ing the 'activation' of lysosomal enzymes were established by de Duve and his collaborators (Beaufay & de Duve, 1959). They include mechanical breakage in a homogenizer, breakage by freezing and thawing, rapid rupture in media of low osmoticity, treatment with surface-active agents such as sodium deoxycholate or Triton X-100, temperature-dependent autolytic activation and treatment with phospholipases and proteases. Beaufay & de Duve (1959) considered that these treatments act primarily by causing injury to the membrane, allowing the enzymes to leak out. Our results are consistent with those obtained by these workers. Homogenization, freezing and thawing, dilution, treatment with deoxycholate and Triton X-100, and temperature, all cause an increase in the amounts of α-amylase leaking into the supernatant fraction. The effect of phospholipases is less dramatic, as only phospholipase A appears to increase leakage of α-amylase. Our results are clearly consistent with the current lysosome concept.

The finding that in wheat aleurone cells, and presumably in barley aleurone cells as well, α-amylase and protease are lysosomal and thus are separated from their substrates within the aleurone cells by a limiting membrane, may have important implications for an understanding of the mechanism of action of gibberellic acid in the cells of the cereal aleurone layer.

The widely accepted explanation of gibberellic acid action, in which activation of specific genes or replication of specific messengers or both is invoked, largely rests on the fact that until recently there was no other experimental basis for differentiating between extracellular enzyme synthesis and intracellular or cytoplasmic enzyme synthesis. However, the work of Bulova & Burka (1970) demonstrated that free ribosomes synthesize different types of protein from those produced by ribosomes attached to the endoplasmic reticulum. In fact, several workers have shown that free ribosomes synthesize intracellular protein whereas extracellular protein is chiefly synthesized on membrane-bound ribosomes of the rough endoplasmic reticulum (Ganoza & Williams, 1969; Redman, 1969).

These findings, in addition to the present demonstration that α-amylase and protease are lysosomal, prompt us to propose an alternative explanation of the action of gibberellic acid in aleurone cells. Instead of an effect on nucleic acid synthesis, the stimulation of lysosomal enzyme synthesis could be achieved by a hormone (gibberellic acid) effect that influences, at an early stage, either the synthesis of endoplasmic reticulum or the association of ribosomes with pre-existing endoplasmic reticulum. Either type of response (and possibly others) could explain a hormone effect that was specifically associated with the synthesis of extracellular enzymes. It is unlikely that an effect such as stimulated endoplasmic reticulum synthesis, or the association of ribosomes with pre-existing endoplasmic reticulum, would be the mechanism of gibberellic acid action, although little evidence is yet available on this point.

Table 8. *Latency of α-amylase in 60000g pellet preparations*

Conditions for this experiment are described in detail in the text.

Incubation conditions	Method of determining latency	Fraction	α-Amylase (% of total)
5°C, 30min	Centrifugation	Supernatant (free)	20
		Pellet (latent)	80
30°C, 30min	Centrifugation	Supernatant (free)	59
		Pellet (latent)	41
30°C, 30min	Triton X-100	Free	63
		Latent	37

Finally, the new proposals tend to suggest that gibberellic acid acts at a site in the extranuclear portion of the cell.

Some other experimental evidence supports the proposals outlined above. The finding (Collins *et al.*, 1972*a*,*b*) of a specific effect of gibberellic acid on the turnover of CTP in the aleurone between 30 and 90min after application of the hormone implies that an early manifestation of the hormone may, in fact, be related to endoplasmic reticulum synthesis. In addition, the identification of the hormone-induced enzyme complement as lysosomal implies that, concomitant with enzyme synthesis, there must be a synthesis of the lysosomal membranes surrounding the enzymes. In this connexion the report (Evins & Varner, 1971) of a gibberellic acid-dependent increase in membrane synthesis, occurring at about the time (3–5h after hormone application) that enzyme synthesis is initiated, is pertinent and also supports the proposals.

Two other implications of our findings require comment. Although previous reports have indicated the presence of lysosomes in plant tissues (Yatsu & Jacks, 1968; Matile, 1968), no other work has demonstrated the complete comparability between lysosomes of plant and animal origin. Finally, and perhaps most importantly, the synthesis of lysosomal enzymes is clearly under direct hormonal control. Hormones have been implicated in the synthesis of lysosomes in animal tissue, but the response is a much delayed one. Tadpole tail explants showed increased lysosomal enzyme complement 3–4 days after the addition of thyroxine (Eeckhout, 1966; Weber, 1969) and isolated bone cells showed similar responses to additions of parathyroid hormone 1–2 days after application (Vaes, 1968). By contrast, the lag period of only 4–6h after application of gibberellic acid to aleurone cells makes this tissue ideal for elucidating the exact relationship between the hormone and induced lysosomes. In addition, it indicates that the hormonal control of lysosomal enzyme synthesis may be a much more common phenomenon than presently appreciated.

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