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α-AMYLASE IN GERMINATING, DECORTICATED BARLEY—II.

EFFECTS OF PHYSICALLY DAMAGING THE GRAIN

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Abstract—α-Amylase was measured in barley grains, incubated with or without gibberellic acid, after the grains had been burnt or punctured in different ways. Conditions were found such that α-amylase production, and by inference the production of endogenous gibberellins, was inhibited independently of the effect of treatments on coleoptile growth. Damage to a small area of the aleurone results in an unexpectedly large reduction in α-amylase synthesis, suggesting that to some extent the aleurone functions as one unit rather than as a collection of independent cells. α-Amylase, induced by exogenous GA_3 in grains where the embryo had been burnt, was destroyed more slowly than in grains where embryo growth was normal, favouring the hypothesis that withdrawal of substances by the growing embryo from the endosperm favours the breakdown of α-amylase. The pH of endosperm homogenates also favoured this view. The method by which the grains were sterilized before burning the embryos influenced the rate of formation of α-amylase relative to whole grains, each in the presence of GA_3.

INTRODUCTION

THE rise in the level of α-amylase in germinating barley is caused by *de novo* synthesis of the enzyme. This sysnthesis is triggered by endogenous gibberellins or gibberellin-like substances synthesized in the embryo and it is further stimulated by the addition of gibberellic acid, GA_3, to the culture medium as was previously outlined.[1] Enzyme formation is greatest in the aleurone on the dorsal side of the grain probably due to a high local concentration of endogenous gibberellin, because it has been shown that aleurone from different parts of the grain can equally well cause the breakdown of the adjacent starchy endosperm in the presence of excess GA_3.[2] MacLeod and Palmer[3] indicated that endogenous gibberellins may be synthesized in the embryonic node and be delivered to the aleurone on the dorsal side of the endosperm through vascular tissue that develops in the scutellum during germination. From ringing experiments, in which the effects of removing strips of tissue from the surface of the grain were studied, it has been concluded that gibberellins move along the grain through the aleurone and not through the starchy endosperm.[4,5] Physical interference with the embryo, as caused by X-rays for example[6,7] or by freezing and thawing,[8] will reduce embryo growth and apparently the production of endogenous gibberellins, as the synthesis of enzymes, such as α-amylase, that are dependent on the presence of gibberellins or gibberellin-like compounds, is also reduced. However, such treatments do not damage the aleurone which still

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[2] D. E. BRIGGS, *J. Inst. Brewing* **70**, 14 (1964).
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responds to added GA_3 by synthesizing enzymes, allowing one to follow α-amylase levels while embryo growth is impaired. Physical damage to the aleurone might be expected to interfere with the passage of the gibberellin and certainly to interfere with the *de novo* production of enzymes. In addition, significant microbiological contamination is very hard to prevent on cut or punctured grains when the testa, which excludes many chemicals from the interior of the grain and prevents the loss of materials from within the grain, is broken. Penetrating the coverings of grain, in the absence of added GA_3, is known to interfere with various aspects of the germination process.[9–11] We have tried to kill selected areas of the grain, using a hot wire in such a way as to locally disrupt metabolic processes. Further, we tried drilling holes into the grain to allow us to introduce substances into the interior, even though Tullin,[11] working with wheat, reported large reductions in embryo growth when the dorsal endosperm was punctured. The results obtained throw doubt on the validity of conclusions based on experiments involving ringing or drilling the endosperm. Experiments were also designed to test our hypothesis[1] that α-amylase should be more stable in grain where embryo growth, and hence endosperm depletion, is prevented.

RESULTS AND DISCUSSION

Parts of barley grains were selectively burnt with electrically heated wires and after a short germination period coleoptile length and α-amylase were measured. The results may be considered in two groups: (*a*) those in which the embryo was burnt and (*b*) those in which some part of the endosperm was burnt. Almost any substantial burn that involved the embryo checked the production of α-amylase. Thus burning the tip of the coleorhiza in dry grain prevented the production of α-amylase and reduced the growth of the coleoptile to 38 per cent of the controls. Other results of spot burns, as percentages of control values, were for α-amylase and coleoptile length respectively: burn tip of plumule, 0 per cent and 0 per cent; spot burn where the scutellum meets the aleurone, on the dorsal side of the grain, 13 per cent and 0 per cent; spot burn on the ventral side of the grain, at the join of the scutellum and the ventral furrow, 10 per cent and 35 per cent.

Since an exogenous supply of gibberellic acid allowed the formation of α-amylase, the effect of burning the embryo may be attributed to a resultant cessation in the endogenous production of gibberellins since the production of this enzyme is entirely dependent on a supply of gibberellin-like substances (Tables 1 and 2). "Dry-sterilized" grain suffered less general damage than "wet-sterilized" grain in this type of experiment—as is to be expected from the greater susceptibility of wetter grain to heat in commercial drying plant.[12] Limited coleoptile growth followed some burning treatments (e.g. of the tip of the coleorhiza or where the scutellum joined the ventral furrow) when no α-amylase production occurred, while conversely a burn on the dorsal side where the scutellum reaches the endosperm prevented coleoptile growth but allowed a limited production of α-amylase. The greater production of α-amylase by steely than mealy grains, with or without GA_3, justified the use of only mealy grains in all our experiments (Table 2).

Burning the testa and aleurone layer on the surface of the endosperm in various ways, particularly when using dry-sterilized grain, had little effect on coleoptile growth in the short term, even when the burning was so extreme that the distal part of the ventral furrow was

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[10] D. H. B. SPARROW, *J. Inst. Brewing* **71**, 523 (1965).
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hollowed out or the distal half of the aleurone was totally burnt away (Table 3). These treatments resulted in an α-amylase level only about one-fifth of that of the controls. The treatment (i) (Table 3) may have reduced growth more than (d) (Table 3) because it involved an attempt to burn very near to the embryo. Thus, in the short term, embryo growth provides a very poor index of the mobilization of the reserves of the endosperm. In the long term, leakage of materials from the burns that puncture the surface of the grain and so allow microbiological contamination make meaningful long-term experiments of this kind exceptionally difficult to perform. Hence the results reported here are of short experiments and

TABLE 1. α-AMYLASE AND COLEOPTILE LENGTH IN WHOLE AND DRY BURNT BARLEY GRAINS

	Entire			Embryos burnt dry		
		α-Amylase (S.I.C./part)			α-Amylase (S.I.C./part)	
GA_3 additions to medium	Coleoptile length, cm	Embryo	Endosperm	Coleoptile growth	Embryo	Endosperm
None	3·5 3·5	9 12	62 64	−	0·3 0	0·7 0
				+	0·3	0
1 μg/ml	4·0 4·2	13 18	80 85	−	1·3 1·6	30 33
				+	1·8	30
50 μg/ml	4·0 4·6	20 24	101 101	−	4·6 4·0	87 91
				+	6·5	80

Grains frozen and dissected and enzyme extracted from frozen parts.

TABLE 2. α-AMYLASE IN WHOLE AND WET BURNT BARLEY GRAINS

	α-Amylase (S.I.C. part)									
Type of grain:	Mellow				Mellow				Steely	
Treatment of embryo:	None				Burnt				None	
Part of grain:	Embryo		Endosperm		Embryo		Endosperm		Embryo	Endosperm
GA_3 in medium										
None	12	12	53	54	0	0	0	—	20	72
1 μg/ml	19	17	70	61	6	6	34	42		
100 μg/ml	16	20	64	77	9	11	64	77	24	82

Grains kiln-dried before dissection.

where the surface of the grain was punctured this is stated. Thin burns made across the dorsal or ventral parts of the broadest part of the endosperm, about one-third of the way round the circumference, caused roughly equal reductions in the quantity of α-amylase produced (e.g. Table 3, d and e). Similarly, thin burns along the dorsal aleurone could be more damaging than thin burns across it (Table 3, h and l). Burning seemed to produce a different percentage of reductions in different trials. Similar results were obtained by making one large "spot burn" on the dorsal side of the endosperm or a ring of small "spot-burns" around the endosperm, or a thin burn made along the dorsal side of the aleurone (Table 3, f, j, i). Making one large burn on the apex of the grain, away from the embryo, reduced the production of α-amylase to only a limited extent.

Although not more than 10 per cent of the aluerone on the surface of the grain was damaged by these treatments and embryo growth was little altered, the quantity of α-amylase produced was only 50–60 per cent of controls. Thus it seemed that burning the surface of the endosperm tended to damage the enzyme-producing mechanisms of the whole aleurone as one tissue, rather than just locally killing some few cells and impeding the passage of gibberellins. This concept is supported by the finding that a thin burn on the dorsal side of the grain still reduces the production of α-amylase very markedly when the control and test samples are germinated in the presence of large concentrations of GA_3 which would have been expected to trigger all the enzyme-forming mechanisms within the grain (Table 3, l and m).

TABLE 3. EFFECTS OF VARIOUS BURNING TREATMENTS APPLIED TO THE ENDOSPERM

Treatment	Coleoptile length (cm)	α-Amylase (S.I.C./grain)			α-Amylase (%controls)
Trial A					
(a) None (controls)	4·0	55		57	100
(b) Burn out distal half of ventral furrow	4·0	12		10	20
(c) Burn away the aleurone over the distal half of the endosperm	3·9	3·1		2·4	5
(d) Thin burn across the aleurone on the dorsal side of endosperm	3·8	14		19	30
(e) Thin burn across the aleurone on the ventral side of endosperm	3·8	14		14	25
(f) Thick spot burn on the aleurone on the dorsal side of endosperm	4·0	24		23	42
Trial B					
(g) None (controls)	3·9	45	45	43	100
(h) Thin burn across the aleurone on the dorsal endosperm	3·3	33	24		63
(i) Thin burn along the aleurone on the dorsal endosperm	3·6	12	15		30
(j) Ring of five small spot-burns on the aleurone round the endosperm	3·5	20	19		43
(k) Spot burn at apex of grain	3·8	35	47	45	94
(l) None, $+GA_3$ (100 μg/ml)	4·4	66	88	74	170
(m) Thin burn across aleurone on the dorsal endosperm $+GA_3$ (100 μg/ml)	4·2	29	37	49	85

Drilling the coleoptile region of the embryo to a depth of about 1 mm prevented coleoptile growth or the production of α-amylase. Drilling where the apex of the scutellum joined the dorsal aleurone prevented coleoptile growth but allowed a 41 per cent production of enzyme, so agreeing with some of the burning experiments in indicating that coleoptile growth is not essential for the endogenous production of gibberellins. Cutting away the tip of the coleorhiza reduced coleoptile length and α-amylase to 53 per cent and 27 per cent of the controls respectively. Lightly drilling or burning the surface of the embryo allowed some, often distorted, growth of the coleoptiles (Tables 4 and 5).

Grains germinated with or without GA_3, after having holes drilled through the testa and aleurone into the middle of the dorsal side of the endosperm, produced 40–50 per cent less α-amylase than controls (Table 4). Since only a tiny fraction of the total aleurone was damaged directly by this treatment the result supports the hypothesis that this tissue synthesizes

the enzyme as an integrated unit rather than as a series of autonomous cells. Embryo growth was only marginally reduced by this treatment (Table 4), in contrast with Tullin's experience with wheat.[11] Experiments with drilled grains all had to be of short duration since in prolonged experiments the endosperm contents liquefied and exuded from the hole, and no suitable way of preventing this was found. Introducing 0·2 μl of solutions of calcium chloride or glucose into the interior of the grain, through drill holes, did not significantly alter the quantity of α-amylase found in the grain, although calcium ions are known to stabilize the enzyme[13, 14] and glucose reduces the formation of the enzyme by isolated barley embryos.[2] The concept of the aleurone layer acting as an integrated unit agrees with the finding that

TABLE 4. COLEOPTILE GROWTH AND α-AMYLASE IN WHOLE AND PUNCTURED GRAINS TREATED WHILE DRY

	Coleoptile length (cm)				α-Amylase (S.I.C./grain)			
GA_3 (μg/ml)	0		100		0		100	
Treatment								
(a) None	3·9	3·7	4·4	4·3	58	57	79	89
(b) Surface of embryo lightly drilled	2·2	1·9	1·8	1·9	20	22	67	68
(c) Small hole in dorsal side of endosperm	4·1	3·3	4·1	3·9	21	20	41	41

TABLE 5. α-AMYLASE IN VARIOUSLY TREATED GRAIN

	α-Amylase (S.I.C./part)											
	Embryo						Endosperm					
GA_3 (μg/ml)	0			100			0			100		
Treatment (grain dry)												
None		16	16	18	20		48	55		103		110
Spot burn on surface of embryo	8·2	5·5	3·0	16	12	10	14	11	7	67	60	54
		8·4*		13*			21*				73*	
Small hole in surface of embryo	11*	18*	19*	23*	33*	31*	23*	28*	22*	64*	59*	68*

* In these samples the shoot grew slightly.

endosperm flinders respond less well than whole, embryo-free endosperm to added GA_3.[15] However, comparisons between α-amylase production by the aleurone in whole and cut half grains are complicated by the various hydration effects[16–18] and the movement of a stimulating factor from the embryo to the endosperm during the hydration of the grain.[16, 17] The restriction of penetration of GA_3 into the whole grain is another complicating factor.[2, 10] It is not clear therefore that the damage incurred by the aleurone when grains are cut to

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remove the embryo, reduces its ability to produce α-amylase in response to added GA_3, in comparison to its performance in the whole grain.

When the embryonic coleoptile of dry grain was lightly burnt or drilled, enzyme production and hence endogenous gibberellin production was reduced but not entirely prevented. However, when cultured in the presence of GA_3 the endosperm of treated grains often contained less enzyme than the intact control grains. The embryo of the drilled grains contained substantially more enzyme than controls, a surprising result possibly due to enzyme from the endosperm leaking forward into the damaged area (Table 5). In experiments in which the embryos of dry grains were burned, GA_3 in the culture solution did not raise the level of α-amylase in the endosperm and certainly not in the embryo, up to the control level, even when partial growth of the coleoptile occurred (Tables 1 and 5). Other results obtained with

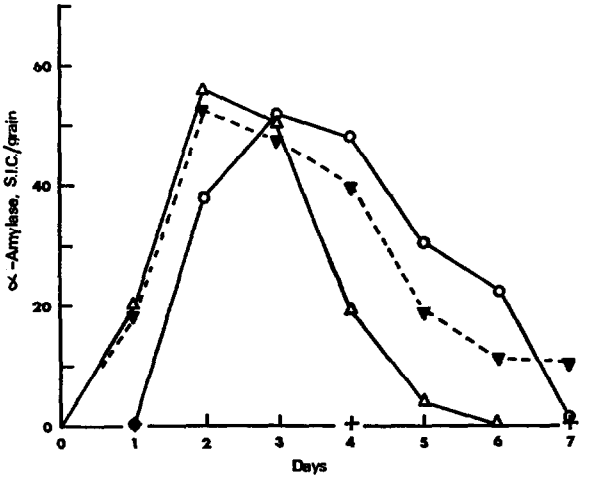

FIG. 1. TIME COURSE OF α-AMYLASE DEVELOPMENT IN BARLEY GRAINS WITH NORMAL AND BURNT EMBRYOS.

Embryos were burnt 4–6 hr after wetting. GA_3 solution (100 μg/ml), pH not adjusted.

—○— Normal grains - -▼- - Burnt grains + GA_3
—△— Normal grains + GA_3 —+— Burnt grains.

grain in which the embryo was severely burned while wet and cultured with GA_3 showed that the α-amylase level in the endosperms but not the embryos equalled the levels found in the controls (Table 2). Thus burning the embryo wet disrupted the gibberellin-synthesizing mechanisms and partly damaged the enzyme-forming centre of the embryo, without altering the ability of the aleurone to respond to "saturating" doses of gibberellic acid supplied in the culture medium.

Time-course studies were made with whole grain and grains with burnt embryos incubated in the presence and absence of GA_3. In one type of trial, burning was carried out on wet-sterilized, and therefore partly hydrated, grains and the GA_3 solution was applied in water at its natural pH (Fig. 1). Embryo growth in the whole grain was partly restricted, probably because of the acidity of the GA_3 solution. In the presence of GA_3 the synthesis of α-amylase in the burnt grain was as rapid as in the whole grain in the presence of GA_3 but the rate of enzyme destruction proceeded more slowly. In the absence of GA_3 no enzyme was found in the burnt grain (Fig. 1). Towards the end of the culture period the high osmotic

pressure of the solution of hydrolysis products formed in the endosperm was so great that in some few cases the embryos were split from the endosperm and the contents escaped. Also glutinous matter exuded from the burnt areas of nearly all the embryos, and so some enzyme may well have been lost.

To overcome this difficulty, the embryos of dry-sterilized grains were burnt and the burns were sealed with clear nail-varnish. These and entire grains were incubated with and without GA_3 in buffered solution. Glutinous matter again exuded from beneath the varnish on some burns but the losses of materials from within the grain seemed to be less. No α-amylase was found in the burnt grain in the absence of GA_3. In the presence of GA_3 enzyme synthesis in

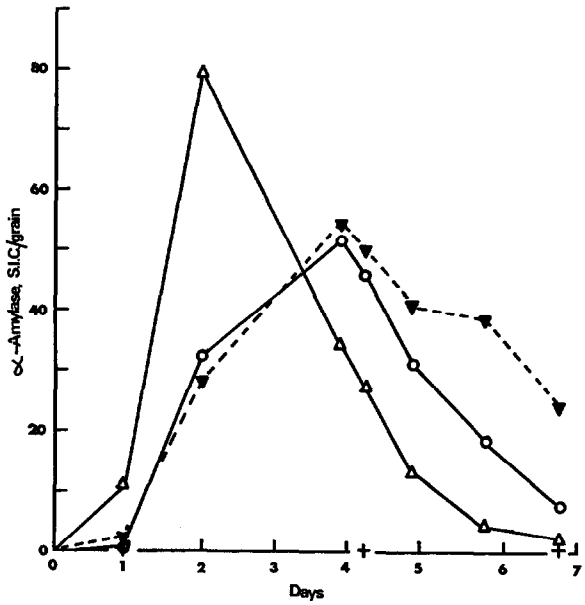

FIG. 2. TIME COURSE OF α-AMYLASE DEVELOPMENT IN BARLEY GRAINS WITH NORMAL AND BURNT EMBRYOS.

Grains were briefly sterilized with acetone. The embryos were burnt dry, and the burns were sealed with varnish. GA_3 (100 μg/ml) was neutralized and applied in a buffered solution.

—○— Normal grains
—△— Normal grains + GA_3
- -▼- - Burnt grains + GA_3.
—+— Burnt grains.

the burnt grain occurred more slowly, and reached a lesser maximum than in the whole grain with GA_3 (Fig. 2). The rate of decline in enzyme level was slower in the burnt grain (Fig. 2). Thus preventing embryo growth by burning resulted in a slower onset in the breakdown of α-amylase. Where growth occurred, especially in the presence of GA_3, the pH of homogenized endosperm fell (Table 6). With burnt grains the pH of the endosperm homogenates only declined if the grains were incubated in the presence of GA_3. Thus the endosperm homogenates of grains with burnt embryos had higher pH values than the endosperm homogenates from whole grains, cultured with and without GA_3, so favouring enzyme survival in the burnt grains (Table 6).[1]

A significant difference in α-amylase production was noticed between different groups of grains with burnt embryos cultured with GA_3, depending on whether before burning the grains had been dry-sterilized by brief immersion in acetone or wet-sterilized with a sodium

hypochlorite solution followed with water washes. After dry sterilization burnt grains, with GA_3, produced α-amylase more slowly than whole grains with GA_3 (Tables 1 and 5; Fig. 2). After wet sterilization the burnt grains, with GA_3, produced α-amylase at the same rate as whole grains with GA_3 (Table 2; Fig. 1).

To investigate whether burning grains after dry sterilization destroyed an embryo-factor needed for maximum α-amylase production[16, 17] some grains were burnt dry, hydrated for 24 hr with distilled water, blotted and incubated with GA_3, while others were hydrated first for 24 hr, burnt then blotted, and incubated with GA_3. α-Amylase levels were the same in both sets. Thus the observed differences must have been due to some unrecognized factor.

Thus the experiments in which the aleurone over the endosperm was damaged throw doubt on "ringing" experiments designed to locate the path by which endogenous gibberellins move in the barley grain. However, they do show that direct damage to a small area of aleurone has a very large effect on this tissue's ability to make α-amylase.

TABLE 6. pH OF HOMOGENATES OF ENDOSPERMS FROM WHOLE GRAINS AND GRAINS IN WHICH THE EMBRYOS WERE BURNT DRY, CULTURED WITH AND WITHOUT GA_3

			GA_3 (100 μg/ml)	
Sampled on	Whole	Burnt	Whole	Burnt
4th day	5·03*	5·01*	4·63*	4·56†
6th day	4·69*	5·06*	4·07*	4·30†

* Mean of duplicates.
† Mean of triplicates.

Despite the unexplained discrepancy between results obtained from grains sterilized in different ways, and the leaking of orange gum from the burnt embryos, it is clear that preventing embryo growth by burning the embryonic axis substantially slowed the rate of degradation of GA_3-induced α-amylase relative to unburnt controls. These findings led on logically to studies in which toxic agents were used to regulate embryo growth and the endogenous production of gibberellins.[19]

MATERIALS AND METHODS

Most cultural conditions were as previously described.[1] Dry sterilization was performed by immersing grain in acetone for a few seconds, rapidly draining and allowing the surface film to evaporate inside a sterile chamber.

Grains were punctured with a number 80 drill, 0·0135 in. (0·34 mm) dia., held in a pin-vice. Burning was carried out with electrically heated cautery wires.

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