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ENZYME FORMATION AND RELEASE BY ISOLATED BARLEY ALEURONE LAYERS

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Key Word Index—*Hordeum distichon*; Gramineae; barley; α-amylase; β-amylase; α-glucosidase; hormones; gibberellic acid; aleurone.

Abstract—Aleurone layers, with testa attached, were prepared from degermed, decorticated barley with the aid of a fungal enzyme preparation. The preparations appeared intact under the scanning electron microscope. By using antibiotics only in an early stage preparations were obtained uncontaminated by micro-organisms and which, when incubated under optimal conditions with gibberellic acid, GA_3, produced near-maximal amounts of α-amylase. The enzyme accumulated in the tissue before it was released into the incubation medium. Daily replacement of the incubation medium, containing GA_3, depressed the quantity of α-amylase produced. α-Amylase was also produced in response to gibberellins GA_1, GA_4 and GA_7 and, to a much lesser extent, helminthosporol and helminthosporic acid. A range of other substances, reported elsewhere to induce α-amylase formation, failed to do so in these trials. At some concentrations, glutamine marginally enhanced the quantity of enzyme formed during prolonged incubations. It is confirmed that α-glucosidase occurs in the aleurone layer and embryo of ungerminated barley, and increases in amount during germination. GA_3 is shown to enhance this increase. When embryos are burnt, to prevent gibberellin formation, no rise in α-glucosidase levels occurs unless GA_3 is supplied to the grains. As the activity of α-glucosidase and other enzymes have been determined as 'α-amylase' by some assay methods, their alterations in activity in response to GA_3 necessitates a re-evaluation of the evidence for *de novo* synthesis of α-amylase in aleurone tissue.

INTRODUCTION

THE SEPARATION of sterile, viable aleurone layers from the dead starchy endosperm is a desirable step for metabolic studies. When degermed barley grains are hydrated in air it is possible, after three days, to peel the 'aleurone' from the starchy endosperm.[1] This technique has been used extensively to prepare aleurone layers from naked varieties of barley.[2,3] It is possible to prepare aleurone layers from decorticated British barley in this way, but the technique is laborious and slow, allowing the separation of only very limited quantities of material. A method has been developed that allows the preparation of aleurone tissue from decorticated barley in a rapid and reproducible manner.

There are reports that α-amylase occurs in the aleurone layers of resting cereal grains,[2] and that its activity, or the release of reducing sugars from degermed grains (which supposedly depends on its activity), increases in response to kinetin,[4] glutamine,[5] ornithine,[5]

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[2] M. J. CHRISPEELS and J. E. VARNER, *Plant Physiol.* **42**, 398 (1967).
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[5] A. G. GALSKY and J. A. LIPPINCOTT, *Plant Physiol.* **47**, 551 (1971).

glutamic acid,[5] aspartic acid,[5] RNA,[6] *ent*-kaurene,[7] phenobarbitone,[8] helminthosporol,[9–13] helminthosporic acid,[9–13] *cyclic*-3′,5′-AMP[14,15] mevalonic acid,[16] and residues from organic solvents[17] and other reports provide contradictory evidence.[17–20] If general metabolites such as glutamine or aspartate can evoke enzyme formation, then starch mobilization in germinating cereals must be under very poor control.[21,22] Experience with malting barley,[23] and direct experimentation,[24] makes this most unlikely. *A priori* it seemed likely that many of the assays used to measure 'α-amylase' in grain preparations would lack specificity. Classes of enzyme that occur in germinating barley and contribute to the breakdown of starch or its hydrolysis products include α-amylase,[25] β-amylase,[25] phosphorylase,[25] α-glucosidase (which has γ-amylase activity),[26–29] limit dextrinase,[25,30] *R*-enzyme,[25,30] and possibly specific transglucosylases. Many of the 'α-amylase' assays in use would be expected to be influenced by most of these enzymes since no attempt is made to separate or selectively inactivate them.[31]

RESULTS

Aleurone layers were prepared from degermed, decorticated grain that had been split along the ventral furrow and preincubated with a crude preparation of *Aspergillus* 'pectinase'. The softened starchy endosperm material was displaced with a spatula. The 'pectinase' hydrolysed many polysaccharides besides pectin, which is virtually absent from the barley endosperm.[32] Many of the aleurone cells in layers made with crude enzyme lost their ability to stain with tetrazolium salts and the tissue tended to disintegrate. The state of aleurone layers prepared in different ways was routinely monitored by tetrazolium staining and by light and scanning electron microscopy (Fig. 1). Using purified pectinase, aleurones backed with testa were separated after three days incubation. Such tissues were essentially starch-free, the cell walls of the sub-aleurone layer having separated adjacent to the endosperm cells (Fig. 1). If the preliminary enzyme incubation was extended, the ability of the

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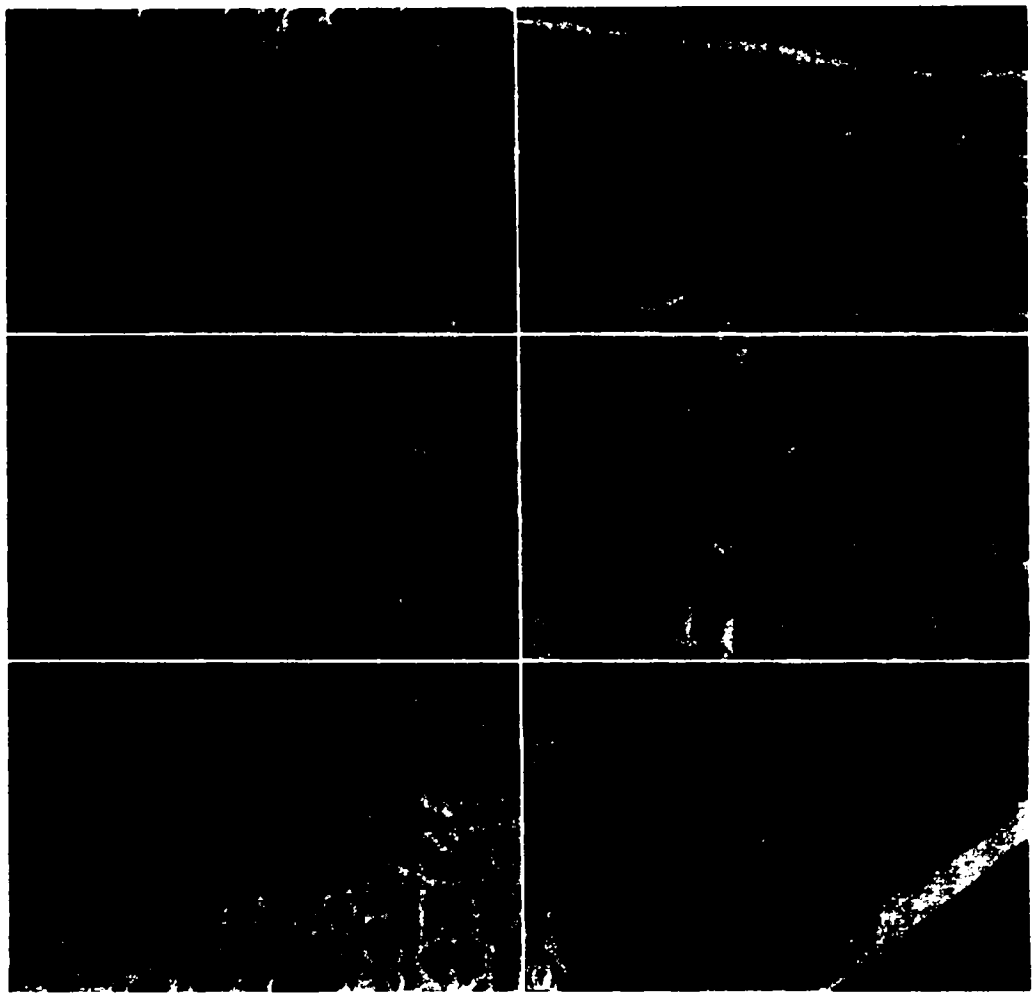

FIG. 1. SCANNING ELECTRON MICROGRAPHS OF PREPARATIONS OF THE BARLEY GRAIN. ON EACH THE LINE (—) REPRESENTS 20 μm.

(*a*) Transverse section of the whole grain showing husk (h), pericarp (p), testa (t), aleurone layer (al) and starchy endosperm (en). (*b*) Transverse section of decorticated grain, sectioned while frozen. The aleurone layer retains its cellular contents. (*c*) Isolated intact aleurone layer, the inner surface. A few small starch grains are present. (*d*) Inner surface of barley aleurone layer, following treatment with excess enzyme. In most cases the end cell-walls are absent. (*e*) Transverse sections through two isolated aleurone layers. (*f*) Transverse section through an isolated aleurone layer after treatment with excess enzyme. The end cell walls are missing, and the fibrils of the other walls are separating and are clearly visible.

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separated aleurones to form α-amylase, in response to GA_3, was reduced. To prevent infection with micro-organisms the antibiotics benzyl penicillin, streptomycin, amphotericin B and nystatin were included in the preparative pectinase digest. However, in subsequent test incubations in which α-amylase was induced by GA_3, it was found that the enzyme yield was only 6–24% of that given by aleurones prepared and tested in the absence of antibiotics. Amino acids, vitamins, nucleosides, mineral salts and maltose all failed to overcome this inhibitory effect. Only benzyl penicillin was without effect when the antibiotics were tested individually. When half-grains, with the starchy endosperm still present, were incubated with GA_3, antibiotics again reduced the yield of α-amylase, by about 30%. Subsequently the use of antibiotics was confined to the pectinase digestion stage.

Incubation conditions were sought such that the yields of α-amylase from isolated aleurone layers, incubated with GA_3, would approach those of degermed grains. Aleurone layers from 'steely' high-nitrogen grains yielded up to 50% more enzyme than aleurone from 'mealy' grains, as occurs in whole endosperm treated with GA_3,[33] and so in the interest of uniformity 'mealy' grains were used subsequently. During the incubation of aleurone layers, or degermed grain, with GA_3 in succinate buffer the pH of the medium fell, as occurs in the whole grain.[34] As purified α-amylase is unstable below pH 4·9[25] the effect on enzyme yield of varying the initial pH of the medium was examined using succinate buffer (15 mM) containing calcium sulphate (15 mM). After 3 days incubation most enzyme was formed by preparations in media which were initially in the range pH 5·1–5·5. As enzyme determinations are made at the optimum, pH 5·3, and this value occurs in the starchy endosperm,[34] it was used in subsequent studies. To reduce the fall in pH that occurred, the succinate concentration was subsequently increased to 20 mM.

TABLE 1. α-AMYLASE FROM ISOLATED ALEURONE LAYERS PREPARED WITH PECTINASE CONTAINING THE LEVELS OF CALCIUM SULPHATE SHOWN, AND INCUBATED WITH GA_3 (2 mg/l.), IN SUCCINATE BUFFER, (15 mM, pH 5·3), AND THE SAME LEVELS OF CALCIUM SULPHATE. THE MEDIUM WAS CHANGED AFTER 48 hr INCUBATION

	α-Amylase (S.I.C./grain aleurone layer)			
Location of enzyme Incubation period (hr)	Medium 0–48	Medium 48–72	Aleurone 0–72	Total 0–72
$CaSO_4$ (mM) 15·0	29·2	11·6	39·3	80·1
9·0	42·4	29·8	18·6	90·8
4·5	39·0	33·6	20·6	93·2
2·3	44·9	29·4	15·6	89·9
1·5	49·2	21·0	14·0	84·2
0·75	51·8	23·0	15·4	90·2
0·0	0·0	0·0	0·9	0·9

Results are the means of triplicate determinations.

Calcium ions are essential for the activity and stability of α-amylase.[25] If calcium was omitted from the preparation and incubation stages little α-amylase was produced. However, in the range 0·75–15 mM calcium sulphate, the yield of α-amylase was approximately constant, although the ratio retained in the tissue to that released into the medium did alter (Table 1). This is in contrast to the results of Chrispeels and Varner.[2]

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With degermed grain the production of α-amylase follows the addition of GA_3 most rapidly when the tissue has been hydrated for a period before the hormone is applied.[35–37] It was of interest therefore to determine the rate at which α-amylase synthesis occurred in aleurones in response to added GA_3. Enzyme was just detectable in the aleurone after 9 hr incubation, but no enzyme could be found in the medium until 12–15 hr had passed, and the quantities present were very small. In the absence of added GA_3 no α-amylase was detected. Clearly the enzyme increased in amount in the aleurone tissue before release into the medium.

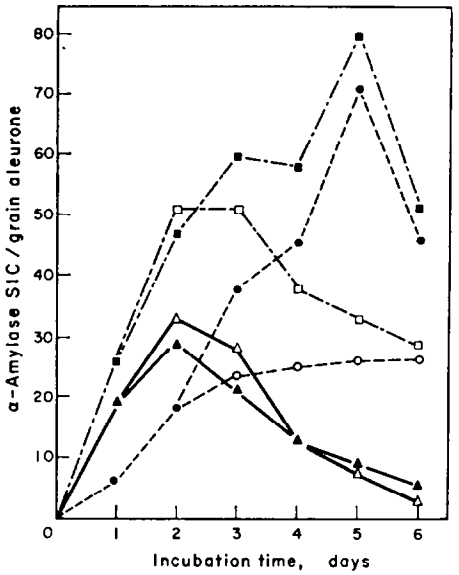

FIG. 2. α-AMYLASE CONTAINED AND RELEASED BY ALEURONE LAYERS INCUBATED WITH GA_3 (2 mg/l.) WITH AND WITHOUT CHANGING THE CULTURE MEDIUM EVERY 24 hr.
α-Amylase in the aleurone layers with △ and without ▲ changing the culture medium. The total α-amylase released into the culture media when these were changed ○, and when they were not ●. Total quantities of α-amylase (aleurone layers + media) produced when the media were changed □, and when they were not ■.

The effects of changing the incubation medium every 24 hr on the yields of α-amylase were determined. Changing the medium once had little effect but repeated changes depressed the total amount of enzyme released (Fig. 2). Thus some factor needed to stabilize or otherwise increase the level of enzyme was lost. Nevertheless, the small effect of one change of medium was thought to justify the practice of using one change in subsequent experiments. It should be noted that values shown in Fig. 2 are total amounts of enzyme and give no indication as to the relative concentrations of enzyme in the tissue and in the medium. Since the effective tissue volume is not known the data cannot be used to distinguish between the possibilities of active or passive release of the enzyme from the tissue into the medium.

In repeated trials α-amylase was not induced in isolated aleurone layers by kinetin, benzylaminopurine, hydroxylamine, glutamine, ornithine, *ent*-kaurene, *ent*-kaurenol, *ent*-kaurenoic acid, phorone, isophorone, phenobarbitone, ATP, ADP or *cyclic*-3′,5′-AMP.

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TABLE 2. α-AMYLASE PRODUCED BY ALEURONE LAYERS PREPARED NORMALLY, AND THEN INCUBATED WITH GA_3 (2 mg/l.) AND VARIOUS LEVELS OF KINETIN (a), OR WITH KINETIN PRESENT DURING THE PREPARATIVE PROCEDURE AND THE TEST INCUBATION (b)

	α-Amylase (S.I.C./grain aleurone layer)						
Location of enzyme Incubation period (hr)	Medium 0–24	Medium 24–72	Medium 24–72	Aleurone 0–48	Aleurone 0–72	Total 0–48	Total 0–72
Kinetin 0	13·2	57·0	—	7·0	—	77·2	—
(Control)	13·2	—	73·8	—	3·6	—	90·6
Kinetin 10^{-6} M	23·4	25·6	—	4·6	—	53·6	—
(a)	20·6	—	33·2	—	2·9	—	56·3
Kinetin 10^{-5} M	19·4	19·2	—	4·6	—	43·2	—
(a)	22·2	—	32·0	—	3·4	—	57·6
Kinetin 10^{-4} M	15·2	17·7	—	5·2	—	38·1	—
(a)	15·4	—	27·4	—	3·4	—	46·2
Kinetin 10^{-6} M	22·4	21·4	—	3·2	—	47·0	—
(b)	15·8	—	25·1	—	1·6	—	42·5
Kinetin 10^{-5} M	5·6	7·4	—	6·4	—	19·4	—
(b)	7·6	—	10·6	—	2·6	—	20·8
Kinetin 10^{-4} M	3·4	5·2	—	10·8	—	19·4	—
(b)	2·9	—	12·4	—	12·6	—	27·9

Results are the means of triplicate determinations.

In all cases control incubations were carried out with and without GA_3. However, when some of these compounds were tested together with GA_3 the rate of release of enzyme from the tissue was substantially altered. For example, when kinetin was included in the incubation medium, initially more α-amylase was released from the tissue although the total amount finally produced during the incubation period was reduced (Table 2a). If kinetin was included in the pectinase preparative digest, as well as the test incubations of the aleurones with GA_3, then this inhibitory effect was enhanced (Table 2b). Benzylaminopurine gave generally similar but less pronounced results. The incorporation of adenine nucleotides, particularly ATP, into the GA_3-containing incubation mixture, enhanced the rate of release of α-amylase from the tissue, although the total quantities finally produced tended to be reduced (Table 3). A similar trend was found in trials in which the aleurones were prepared in media with or without various nucleotides that were afterwards used in the GA_3-response incubation media. Hydroxylamine is reported to induce lipase and phytase in wheat bran,[38,39] but in the present trials it depressed the response of the aleurones to GA_3 (Table 4). However glutamine slightly but consistently enhanced enzyme production and the total amount formed (Table 4). This compound may act as an effective source of supplementary nutrient for the tissue. High concentrations of glutamine (0·1 M) depressed enzyme production, and ornithine (0·1 M) was totally inhibitory. In other trials glutamine also increased the overall production of enzyme by about 13%, but not until the incubation period had been extended to 92 hr.

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The failure of a number of compounds to induce α-amylase in our system, when they were previously reported to do so, led us to re-evaluate the biological activities of some other compounds. Small quantities of helminthosporol and helminthosporic acid were available and were known to cause substantial sugar release from degermed barley grains.[9] Isolated aleurone layers formed and released small, but significant quantities of α-amylase when incubated with high concentrations of these substances. Levels of 80 mg/l. after 68 hr incubation, α-amylase yields equal to 15% and 50% of those given by GA_3 were given by helminthosporol and helminthosporic acid respectively. The comparable figures for 92 hr incubations at doses of 40 mg/l. were 8 and 6%. Trials with GA_1, GA_3, GA_4, GA_7 and GA_8 at levels of 0·5 and 0·005 mg/l. indicated that degermed grains did not respond to GA_8 at any dose and only GA_1 and GA_7 at the lower dose level, but responded approximately equally well to GA_1, GA_3, GA_4 and GA_7 at the higher dose level. Isolated aleurones,tested in a similar fashion, did not respond to any of the gibberellins at the lower dose level. At the higher dose level, slightly more enzyme was found with GA_4 and GA_7 (about half of the maximum produced by saturating doses of GA_3) than with GA_1 and GA_3, and there was no response with GA_8. Such results agree in order with some biological responses obtained with isolated barley endosperms but not others.[40–44]

TABLE 3. α-AMYLASE PRODUCED BY ISOLATED ALEURONE LAYERS INCUBATED WITH GA_3 (2 mg/l.) AND VARIOUS NUCLEOTIDES

	α-Amylase (S.I.C./grain aleurone layer)						
Location of enzyme Incubation period (hr)	Medium 0–24	Medium 24–48	Medium 24–72	Aleurone 0–48	Aleurone 0–72	Total 0–48	Total 0–72
Control	0·7	10·2	—	52·0	—	62·9	—
(no nucleotides)	3·1	—	76·8	—	25·0	—	104·9
Cyclic-3′,5′-AMP	2·8	21·0	—	56·6	—	80·4	—
2×10^{-4} M	1·9	—	66·6	—	22·6	—	91·1
ADP	2·2	26·2	—	51·8	—	80·2	—
$2{\cdot}5 \times 10^{-3}$ M	1·9	—	36·8	—	33·2	—	71·9
ATP	2·7	36·1	—	53·8	—	92·6	—
$2{\cdot}5 \times 10^{-3}$ M	2·4	—	85·4	—	12·6	—	100·4

Results are the means of triplicate determinations.

Reasons were sought for the numerous discrepancies that were apparent between our work and the work of others. In most other studies α-amylase was estimated by the method of Shuster and Gifford,[45] or the modification of this method devised by Chrispeels and Varner,[2] hereafter called diastase determinations. The reasons for using our method, involving combined warm extraction and selective inactivation of other enzymes are given elsewhere.[31] It seemed that the diastatic determination methods would detect starch break-

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[45] L. SHUSTER and R. H. GIFFORD, *Archs. Biochem. Biophys.* **96**, 534 (1962).

down by α-amylase, β-amylase, α-glucosidase and (since the starch digestions are made in phosphate buffer) also phosphorylase. It is well known that α-amylase and β-amylase co-operate to degrade starch,[46] and probably the other named enzymes, together with limit dextrinase and R-enzyme, will contribute to the observed rate of starch breakdown. The participation of β-amylase and α-glucosidase was tested directly, as their special properties could account for some of the results recorded in the literature. A commercial preparation of β-amylase caused the degradation of soluble starch to a β-limit dextrin, (which gave a reduced colour with iodine), maltose and glucose, as found by paper chromatography. The presence of an α-glucosidase contaminant was inferred from the appearance of glucose, and was confirmed by showing that maltose was hydrolysed to glucose. Enzyme activities were totally destroyed by a selective heat inactivation treatment, demonstrating the absence of α-amylase.[31] However, starch-degrading activity was detected in the unheated preparation by the method of Shuster and Gifford.[45] Similar results were obtained with saline extracts of degermed, decorticated perfect barley grains, in which α-amylase does not occur. No doubt β-amylase from the starchy endosperm is partly responsible for the 'α-amylase' values observed by workers who use unspecific diastase enzyme assays. Freshly isolated aleurone layers contained no α-amylase, but homogenates that had not received a heat treatment slowly degraded soluble starch, as shown by the decline in iodine-staining power, with the production of glucose. α-Glucosidase is known to occur in this tissue, and to degrade soluble starch.[29,47] The presence of the enzyme in our aleurone homogenates was confirmed by their ability to hydrolyse maltose and *p*-nitrophenyl-α-D-glucopyranoside. The greatly enhanced solubility of α-glucosidase[26,47] and β-amylase[25] in solutions with high salt concentrations probably explains the stimulation of 'α-amylase' (diastase) activity caused by calcium sulphate concentrations increasing in the range 1–100 mM, observed by Chrispeels and Varner.[2]

TABLE 4. α-AMYLASE PRODUCED BY ALEURONE LAYERS INCUBATED WITH GA_3 (2 mg/l.) AND VARIOUS ADDITIVES, WITH (b) OR WITHOUT (a) PRETREATMENT WITH THE ADDITIVES DURING THE TISSUE PREPARATION PROCEDURE

	α-Amylase (S.I.C./grain aleurone layer)						
Location of enzyme Incubation period (hr)	Medium 0–24	Medium 24–48	Medium 24–72	Aleurone 0–48	Aleurone 0–72	Total 0–48	Total 0–72
Control	3·4	34·4	—	62·2	—	100·0	—
(no additives)	3·5	—	98·8	—	16·8	—	119·1
Glutamine, (a)	2·6	26·2	—	82·4	—	111·2	—
10^{-3} M	3·9	—	103·8	—	30·6	—	138·3
Glutamine, 10^{-3} M	2·6	11·4	—	81·6	—	95·6	—
(also pretreated, b)	2·4	—	102·2	—	41·4	—	146·0
Hydroxylamine, 10^{-3} M	1·8	15·8	—	66·8	—	84·4	—
(a)	0·9	—	39·8	—	71·4	—	112·1
Hydroxylamine, 10^{-3} M	1·4	8·8	—	62·8	—	73·0	—
(also pretreated, b)	5·4	—	93·4	—	26·2	—	125·0

Results are the means of triplicate determinations.

Spectra were obtained of the coloured solutions given with iodine by solutions of starch degraded to different extents by preparations of barley β-amylase, partially purified α-amylase and diastase. As was expected the spectra differed significantly. Extracts were prepared

[46] R. M. SANDSTEDT, E. KNEEN and M. J. BLISH, *Cereal Chem.* **16**, 712 (1939).
[47] O. B. JØRGENSEN, *Acta Chem. Scand.* **19**, 1014 (1965).

from barley endosperms that had been incubated with GA_3 in two ways according to Chrispeels and Varner[2] and according to Briggs.[31] Both types of extract caused the degradation of soluble starch but the spectra of the colours given with iodine by the digests of different stages of breakdown were different, and resembled the spectra given by diastase[2] and α-amylase[31] respectively. Further in neither method was the decline of starch–iodine colour exactly linear with time, a fact that must be allowed for in working out enzyme activities.[31]

Numerous disagreements have occurred in the literature because the impression is that α-amylase is being specifically determined, whereas the determinations are actually for diastatic power.[21,22] The specific role of gibberellins in controlling enzyme formation in the endosperm appears to be secure.

TABLE 5. α-GLUCOSIDASE IN DECORTICATED GRAINS, WITH OR WITHOUT BURNT EMBRYOS, CULTIVATED AT 25° WITH OR WITHOUT GA_3 (2 mg/l.)

Culture conditions		α-Glucosidase (*p*-nitrophenyl α-D-glucopyranoside, μmol hydrolysed/hr/grain part)					
		Whole grain				Burnt embryo	
		Embryo	Endosperm	Aleurone	Endosperm +aleurone	Embryo	Endosperm +aleurone
Ungerminated		1·2	—	—	1·1	—	—
Day 1	Control	2·2	—	—	1·6	0·7	1·9
	GA_3	2·3	—	—	1·6	1·0	1·7
Day 2	Control	3·9	—	—	1·8	0·7	1·5
	GA_3	3·9	—	—	3·0	1·1	2·0
Day 3	Control	6·9	2·7	2·5	5·2*	0·9	1·1
	GA_3	7·0	3·1	3·6	6·7*	1·8	4·0
Day 4	Control	7·6	3·3	3·0	6·3*	1·2	1·4
	GA_3	7·3	2·3	4·4	6·7*	3·2	5·4
Day 5	Control	11·4	2·4	4·5	6·9*	0·9	1·3
	GA_3	11·8	5·4	10·6	16·0*	2·8	5·4
Day 6	Control	10·8	2·4	4·5	6·9*	1·0	1·3
	GA_3	10·4	3·4	8·6	12·0*	3·4	5·9

* In these samples the aleurone layers and endosperms were separated, and their enzyme contents were estimated separately.

Results are the means of duplicate determinations.

It was of interest to determine whether α-glucosidase activity increased in grains treated with GA_3. When appropriate extraction techniques are used it has been shown that the enzyme increases in germinating grain.[47] Direct assays of dissected grains and histochemical investigations of sections of frozen grains confirmed[47] that α-glucosidase occurred in the embryo and aleurone layer before germination. Activity increased in germinating grains, and α-glucosidase appeared in the starchy endosperm (Table 5). In whole grain with GA_3, the enzyme levels in the endosperm part of the grain exceeded those found in controls after several days germination. The embryos of some decorticated grains were burnt to prevent the production of the endogenous gibberellins,[33] and the grains were placed on wet filter paper with or without GA_3. In the absence of GA_3 the α-glucosidase activity remained unchanged, while in the presence of the hormone the enzyme level increased (Table 5). Evidently, as

with α-amylase,[33] this exogenous level of GA_3 did not fully activate the α-glucosidase forming mechanisms of the burnt grain.

DISCUSSION

It is possible to prepare aleurone layers from barley that retain most of their capacity for synthesizing α-amylase in response to GA_3 demonstrating the minimal importance of the adjacent starchy endosperm in this connection. The reports of substances, other than gibberellins, that are capable of inducing α-amylase in degermed barley seem to be largely incorrect. Confusion is due to the use of assays that are not specific for α-amylase but which will also determine starch breakdown by other members of the diastase complex of enzymes, for example, β-amylase and α-glucosidase. The use of such assays is unacceptable for critical work. In particular, the enhanced solubility of β-amylase[25] and α-glucosidase[26,47] in the presence of salt solutions and the increased accessability of α-glucosidase in autolysed cells[48,49] may explain many of the results in which diastatic activity, (called 'α-amylase' in the original publications), is enhanced by agents as improbable as phenobarbitone.[8] Possibly the synthesis of α-glucosidase occurs *de novo* in response to GA_3. In this case the change in buoyant density of 'α-amylase' observed when aleurones are treated with GA_3 in the presence of $H_2{}^{18}O$,[50] cannot be regarded as unequivocal evidence for the *de novo* synthesis of α-amylase, as the assay used will determine the change in density of any of the diastatic enzymes, any of which might account for the observed results.

EXPERIMENTAL

Grain. Two-rowed barley, *Hordeum distichon* L., cv. Proctor, of malting quality, screened through slots 2·8 mm wide but retained on slots 2·5 mm wide was decorticated, surface-sterilized and selected by a standard technique.[24]

The preparation of aleurone layers. Manipulations were performed using sterilized solutions and equipment. Embryos were removed by inclined cuts adjacent and parallel to the scutellum. The degermed grains were bisected by vertical cuts along the ventral furrow and 10 of the 'half endosperms' were incubated for 3 days at 25°, with gentle shaking to soften the starchy endosperm. The incubation medium (5 ml) contained sodium succinate (20 mM) and calcium sulphate (15 mM) pH 4·7, purified pectinase and a mixture (165 mg/100 ml) containing benzyl penicillin, streptomycin, nystatin, and amphotericin B (8:16:8:1, by wt). Technical pectinase from *Aspergillus niger* (Koch-Light) was purified by dissolving in a minimum vol. of phosphate buffer (5 mM, pH 4·7), adding 2 vol. of a saturated solution of $(NH_4)_2SO_4$ and storing at 4°. After 2 days the precipitated protein was redissolved in buffer, dialysed and freeze-dried. Different batches varied in activity, so the freeze-dried material was used at a concentration selected by trial and error, about 0·1 g/100 ml digestion medium. At the end of the incubation period the softened starchy endosperm was removed mechanically.

Scanning electron microscopy. Samples were attached to circular aluminium stubs (1 cm dia.), with an emulsion of deflocculated Acheson graphite (D.A.G.) and coated with Au–Pd (3:2; 10–30 nm). Stubs were viewed at 45° to an electron beam (30 kV) in a Stereo-scanning Electron Microscope S.4 (Cambridge Scientific Instruments Co.). Photographs were taken on Ilford FP4 film.

Tetrazolium staining. Aleurone layers were incubated at 25° under vacuum, in a solution (0·3%) of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl) 5-phenyltetrazolium chloride. 'Intact' cells stained red.

Test incubation conditions. Groups of 5 degermed grains, or more usually 10 'half aleurone' layers, were incubated aseptically in the dark at 25° in 25 ml conical flasks plugged with cotton wool agitated on a slowly moving orbital shaker. The incubation medium was sodium succinate buffer (5 ml, 20 mM, pH 5·3) containing $CaSO_4$ (15 mM). Unless stated otherwise the concentration of GA_3 was 2 mg/l.

Determinations of α-amylase. α-Amylase and 'diastase' were determined by the methods of Briggs[31] and Shuster and Gifford[41] respectively. α-Amylase activities were expressed as starch–iodine colour units (S.I.C.) as defined previously.[31]

[48] W. N. Davis, *Biochem. J.* **10**, 31 (1916).
[49] A. J. Daish, *Biochem. J.* **10**, 56 (1916).
[50] P. Filner and J. E. Varner, *Proc. Natn. Scad. Sci., U.S.* **58**, 1520 (1967).

PC of sugars. This was performed on Whatman No. 4 paper irrigated with *n*-PrOH–EtOAc–H_2O (7:1:2) Sugars were located using silver nitrate–alkali dips.

Germination of whole grains. Surface-sterilized decorticated grains, with or without burnt embryos, were germinated on filter paper with or without GA_3 (2 mg/l.).[33,34]

α-Glucosidase, histochemical localization. Freeze-dried sections cut on a freezing microtome, were used with or without fixation in buffered glutaraldehyde. The sections were incubated overnight with 6-bromo-2-naphthyl-α-D-glucopyranoside and hexazotized *para*-rosaniline (HPR).[51] Enzyme location was indicated by a strong red colouration. Incubation with HPR alone produced a general yellowing of the sections.

α-Glucosidase estimation. Grain was frozen at −15° and dissected into the parts to be analysed. These parts (10) were finely ground with sand and polyvinyl pyrrolidone in an all-glass pestle and mortar, with a medium (10 ml) containing Na_2SO_4 (3%), cysteine hydrochloride (2 mM), adjusted to pH 4·5. The homogenate was adjusted to pH 4·5 with ammonia, and incubated at 37° for 1 hr. The reaction was started by adding 10 mM *p*-nitrophenyl-α-D-glucopyranoside (10 ml; citrate, 40 mM; acetate, 10 mM; pH 4·5) at 37° and mixing well. Incubation, with shaking, was continued at 37°. At intervals samples (2·0 ml) were added to Tris buffer (0·5 ml; 2·0 M, pH 9·0) at 0° to stop the reaction. The *p*-nitrophenol liberated was estimated in the solution, clarified by centrifugation, at 410 nm.

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[51] A. G. E. PEARSE, *Histochemistry, Theoretical and Applied*, 3rd Edn, Vol. 1, p. 563, Churchill, London (1968).