Phytochemistry, 1980, Vol. 19, pp. 1025–1033. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0601-1025 $02.00/0

EXTERNALLY APPLIED GIBBERELLIC ACID AND α-AMYLASE FORMATION IN GRAINS OF BARLEY (*HORDEUM DISTICHON*)

MICHAEL T. SMITH and DENNIS E. BRIGGS*

Department of Biochemistry, University of Birmingham, Birmingham, B15 2TT, U.K.

(*Received* 12 *June* 1979)

Key Word Index—*Hordeum distichon;* Gramineae; barley; α-amylase formation; gibberellic acid; gibberellin-like substances; gibberellin conjugates.

Abstract—During germination the aleurone layer of barley grains becomes progressively less able to form more α-amylase in response to a dose of gibberellic acid (GA_3). This decline appears to be linked to the presence of a growing embryo. In whole grains the embryo 'modulates' the response (α-amylase formation) to controlled external applications of GA_3 in a dose-dependent manner. Sugars, and some other metabolites, repress α-amylase formation in transected grains, apparently by reducing levels of endogenously produced gibberellins. This effect is partly, but not completely, reversed by additions of GA_3. External applications of GA_3 augment the levels of several gibberellin fractions within the grain. The nature of the gibberellin material remaining on the surface of the grains alters with time. Grains treated with GA_3 contain a conjugate of low biological activity, possibly a glycoside, that is hydrolysed by a mixed glycosidase preparation to release a biologically-active gibberellin resembling GA_3.

INTRODUCTION

In germinating barley grains α-amylase production in the aleurone layer is triggered by gibberellins originating in the embryo [1, 2]. The formation of the smaller amount of enzyme originating in the scutellum is probably also gibberellin-dependent [3–5]. Gibberellin release into the endosperm begins when the sugar content of the embryo has been reduced by the initial period of metabolism triggered by imbibition. Gibberellin output declines when the sugar levels in the embryo are restored either by supplies arriving from the endosperm, or by sugars fed *in vitro* [5, 6]. Since the sugars from the endosperm are produced by the enzyme-catalysed hydrolysis of the starch and cell walls [7], or from the conversion of lipids to sucrose in the aleurone layer [8], and since the synthesis, release and activation of the enzymes involved are partly or completely gibberellin-dependent, it seems probable that there is a feed-back loop regulating the supply of endogenous gibberellins: embryo sugars decline → gibberellins released → enzymes synthesized/released/activated → reserves mobilized → sugars produced → embryo sugar levels replenished → gibberellin supply cut off [1, 2].

GA_3 supplied to grains in the initial stages of germination stimulates α-amylase formation, since the supplies of endogenous gibberellins are insufficient to fully activate the enzyme-forming mechanisms. However, experience shows that additions of GA_3 at a late stage of germination in the malting process do not always enhance enzyme formation to a useful extent. Furthermore, late additions of GA_3 to grains growing on a wet substratum may actually accelerate enzyme degradation [9]. The pattern of enzyme formation in decorticated grains supplied externally with GA_3 suggests that the hormone gains entry through the micropyle, or the coleorhiza when this splits the testa [4, 10, 11]. The testa itself is impermeable to the hormone [10]. Furthermore, when the testa over the embryo is broken and the embryonic axis is destroyed, grains synthesize less α-amylase in response to a supply of GA_3 than when the embryo is removed [12]. Presumably the residual embryonic tissues (mainly scutellum) also limit the penetration of the hormone.

The work reported here is an attempt to clarify why, under different circumstances, grain responds differently to external applications of GA_3.

RESULTS

When 'steeped' grain was germinated in a moist atmosphere, i.e. 'malted', α-amylase accumulated. Reimmersion in water for 20 min periods, to re-wet the grain surfaces, usually checked α-amylase production (Fig. 1a). The cause of the exceptional case, in which the check was not seen, is not understood. The film of surface moisture probably caused a temporary drop in oxygen availability, which usually checked enzyme formation [10]. When GA_3 solution was used to re-wet the grain, enzyme formation was greatly enhanced by early applications, but the response to an application made after 72 hr was less, and responses to applications made after 96 and 120 hr germination were negligible (Fig. 1b). Thus, although late applications of GA_3 apparently offset the effects of re-wetting grain

* To whom all communications should be sent.

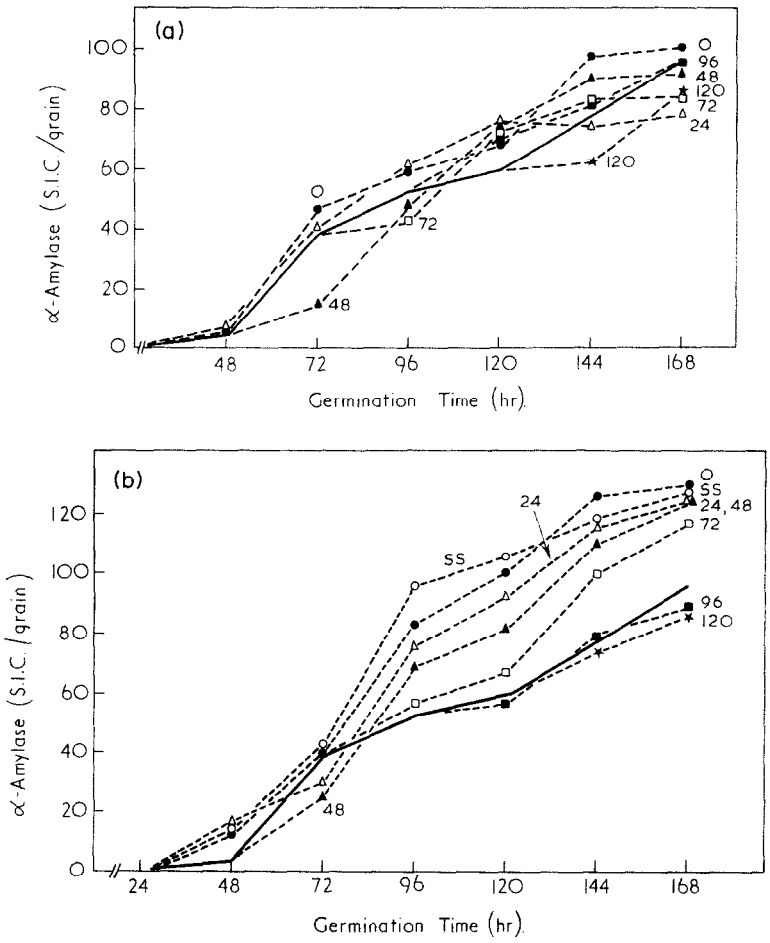

Fig. 1(a and b). α-Amylase formation in entire grains growing under malting conditions and re-wetted in water (1a) or GA_3 solution (1b) at various times. Grains were initially hydrated by two 24 hr immersions in water (heavy line, 1a and b) or GA_3 solution (1 mg/ml) in the second steep (1b, SS; ○---○). Samples were re-immersed (for 20 min periods) in water (1a) or GA_3 solution (1b, 1 mg/ml) at the end of the immersion (0; ●---●), or at the times indicated on the graphs (△---△, 24 hr; ▲---▲, 48 hr; □---□, 72 hr; ■---■, 96 hr; ★---★, 120 hr). Each sample contained 50 grains, and the points on the graphs are the means of duplicate analyses.

(cf. Figs. 1a and 1b), the grain was becoming progressively less responsive to the hormone. In a similar experiment with decorticated grain, embryo damage occurred, and so germination was poor and α-amylase production was unusually low. However, the response to external supplies of GA_3, which was not impaired, had not declined in 144 hr germination. It seemed that the decline in responsiveness to exogenous GA_3, seen in the first experiment, might have been a consequence of vigorous embryo growth.

Two experiments were carried out to test this hypothesis. In the first, decorticated barley was steeped in degassed water to minimize metabolic activity during imbibition. Grains were incubated, either intact or degermed, in a moist atmosphere. The degermed grains were individually treated with various solutions. α-Amylase was only formed in minimal amounts when degermed grains were treated with buffer alone. However, when the buffer contained GA_3, α-amylase was formed at a rate exceeding that which occurred in the whole grain (Fig. 2). Furthermore the response did not decline, but was as great after 144 hr incubation as it was in freshly degermed grain (Fig. 2). Thus in the absence of a growing embryo the enzyme-forming capacity of hydrated, degermed grain did not decline.

In the second experiment decorticated grain was hydrated and incubated as before, but samples were degermed and either left untreated or treated with buffer or buffer containing GA_3 at various times during the incubation period. In untreated grains early removal of the embryo, the source of endogenous gibberellins, practically prevented α-amylase formation, while late removal usually allowed the accumulation of nearly as much enzyme as was found in the intact grain (Fig. 3a). The exceptions are the enzyme contents of the 96 hr degermed grains, which appear to be anomalous. When the degermed grains were treated with buffer, subsequent enzyme production was checked, and in the 120 and 144 hr samples some

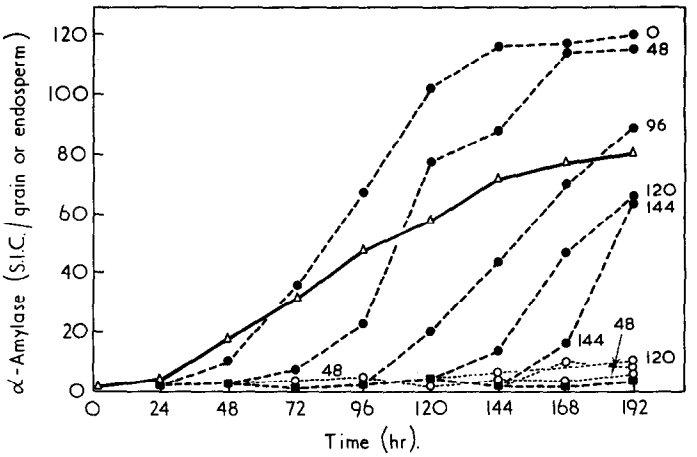

Fig. 2. α-Amylase development in sterile, decorticated grains growing under malting conditions either intact, or degermed and treated at different times. Intact, decorticated grains (△—△) or degermed (■----■), or degermed and treated, at the indicated times, with succinate buffer (1 μl; ○----○) or succinate buffer containing GA_3 (1 μl; 100 ng. GA_3; ●----●). Embryos were removed at the end of the steeping period. Each point represents the analysis of a group of 6 entire or degermed grains. Steeping was carried out continuously in degassed water for 48 hr.

enzyme destruction may have occurred (Fig. 3b). However when the buffer treatment contained GA_3, enzyme formation in the degermed grains was greatly enhanced by early treatments. But the response declined until, following treatments made after 120 and 144 hr, it was small (Fig. 3c), only being sufficient to offset the effects of adding liquid, as seen by comparing the results obtained by adding the buffer alone (Fig. 3b). Thus in the absence of the embryo the aleurone retained its gibberellin-triggered enzyme-forming capacity (Fig. 2), while in the presence of the growing embryo this capacity declined (Fig. 3), in the sense that 'extra' enzyme was not formed following embryo removal, and the application of GA_3.

To determine the effect of the embryo on the induction of α-amylase by exactly known quantities of externally applied GA_3, whole and degermed grains were individually treated with known volumes of buffer with or without GA_3. The amount chosen (40 ng GA_3/grain) does not induce maximal enzyme formation in degermed grains (see later; [13]), but greatly exceeds the maximal endogenous hormone content (1–2 ng GA_3 equivalents/grain) [9, 13, 14]. Enzyme formation was greatest in whole grains treated with GA_3, but the 'extra' enzyme formed by degermed grains in response to GA_3 eventually exceeded that found in intact grains (Table 1). Thus the presence of the embryo could be said to have enhanced or reduced the response to GA_3, depending on which response criterion was chosen. Embryo removal entails the removal of some existing enzyme, and the scutellum, which is also a source of small quantities of enzyme. As the pattern of enzyme formation changed in a complex manner both with time, and with the GA_3 dosage, it is impossible to draw firm conclusions from these results alone. However, when a comparable experiment was carried out in which embryo removal was carried out later in the germination period, and with a range of concentrations of GA_3, it was clear that degermed grains responded better by making more α-amylase than intact grains to low, 'physiological' concentrations of hormone (2 ng GA_3/corn), while at higher concentrations the situation was reversed (Table 2). Indeed the highest dose used (100 ng GA_3/grain) was supra-optimal in these conditions.

Thus the embryo also 'modulated' enzyme formation in grain in response to externally applied GA_3. One way it might do this would be to metabolize the GA_3 to substances with altered enzyme-inducing potencies. The fact that sucrose represses the release of active hormone from embryos [1, 5, 6] suggested that the effective internal concentration of externally applied GA_3 might be altered by conversion to a conjugate, e.g. a glycoside. Two types of experiments were performed; sugars, and some other compounds, were tested for ability to alter enzyme production and evidence for gibberellin conjugates was sought directly.

Sterile, decorticated grains were transected, and hydrated in buffer, with or without additions of GA_3 and/or sucrose. The parts were recombined. After 5 days germination, under malting conditions, the parts were again separated and their α-amylase contents determined. In every case the basal, embryo part contained more enzyme than the apical part, so that the embryo still had a 'promotive' effect on enzyme production even when both parts of the grain had been soaked in a solution of GA_3. The reason(s) for this is not certain, but it is conceivable that the scutellum was contributing a major part of the enzyme found. Sucrose repressed enzyme production, GA_3 enhanced it, and the hormone largely (but not completely) overcame the repressive effect of the sugar. Thus the sugar was more efficient in blocking the natural gibberellin supply to the enzyme-forming tissues, than blocking the response of these tissues to the hormone. These effects are similar to those observed previously, using different test conditions [5]. In this

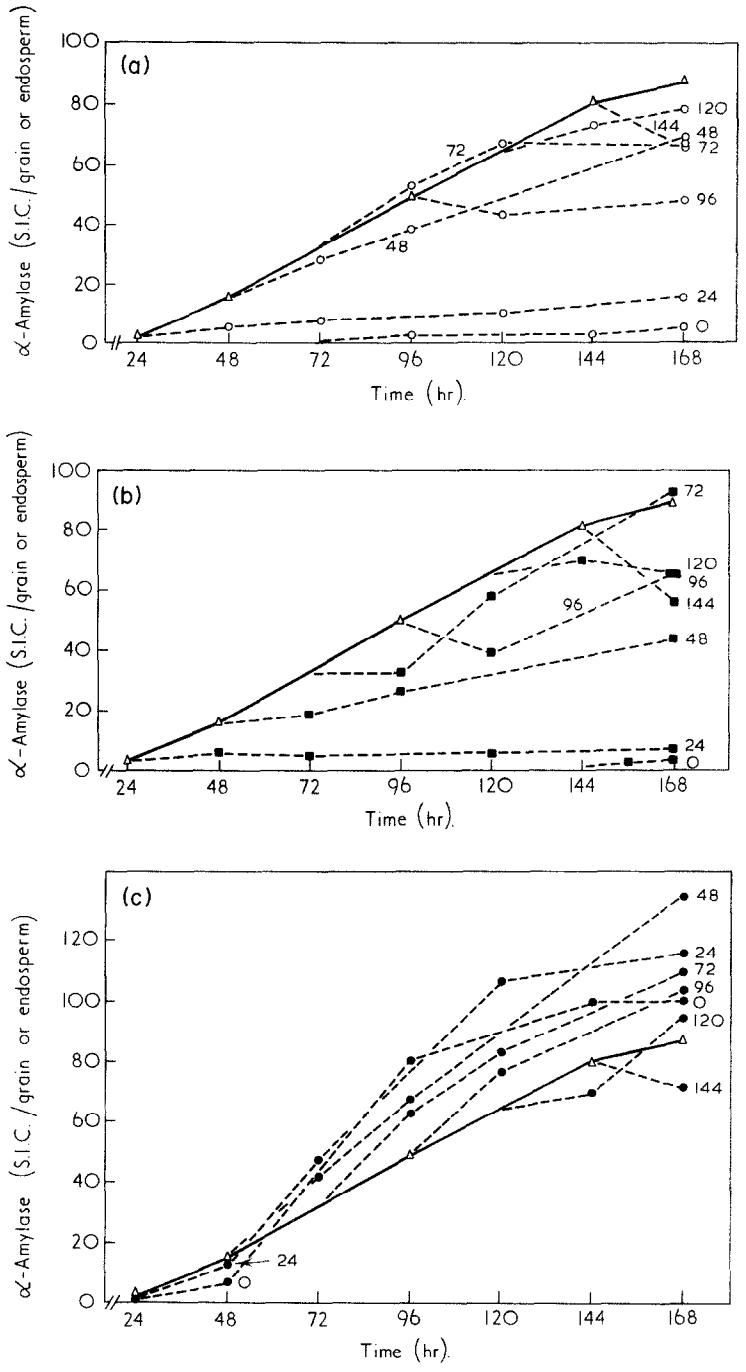

Fig. 3(a–c). α-Amylase levels in sterile, decorticated grains incubated under malting conditions. Intact grains (△——△). Grains degermed at the times indicated (Fig. 3a; ○---○). Grains degermed at times indicated and treated with succinate buffer (1 μl; Fig. 3b; ■---■). Grains degermed at times indicated and treated with GA_3 in succinate buffer (100 ng in 1 μl; Fig. 3c; ●---●). Each point is the mean for the analysis of six grains, or grain parts, analysed separately.

type of experiment each transected grain is treated with unknown quantities of solutes taken up during imbibition. To create more controlled conditions, transected grains were incubated, with shaking, in small volumes of buffered solutions containing the substances under test (Table 3). A casein hydrolysate preparation (casamino acids) had little influence on enzyme production. All the sugars, *meso*-inositol and glycerol reduced enzyme production to varying extents. Mannose was toxic, in that it prevented embryo growth. Inhibition of α-amylase production was reduced to varying extents by including GA_3 in the medium, but in no case was the reversal of the inhibitory effect complete (Table 3). Thus it seems that

Table 1. α-Amylase levels in intact or degermed, decorticated grains incubated under malting conditions either without additions, or treated with buffer, or buffer containing GA_3

	Doses applied after 24 hr incubation (1 μl)		α-Amylase activity (S.I.C. units/grain or endosperm) after various periods			
			48 hr	72 hr	96 hr	120 hr
Intact grain	None	(a)	7.7	64.7	85.2	103.6
	Succinate buffer (20 mM, pH 6)	(b)	10.4	55.5	79.1	98.0
	Succinate buffer +GA_3 (40 ng)	(c)	20.6	118.3	131.8	152.9
	'Extra enzyme'	(c−b)	10.2	62.8	52.7	54.9
Grain degermed after 24 hr incubation	None	(d)	4.4	9.5	7.5	9.2
	Succinate buffer (20 mM, pH 6)	(e)	2.6	3.5	6.6	7.9
	Succinate buffer +GA_3 (40 ng)	(f)	10.1	48.8	81.7	105.1
	'Extra enzyme'	(f−e)	7.5	45.3	75.1	97.2

Germs (with α-amylase contents of 0.18 S.I.C. units/embryo) were removed from grains after 24 hr germination. Results are the means of duplicate determinations, each made on 15 grains.

sugars, and some other substances inhibit the release of gibberellin from the embryo, since α-amylase formation is proportionately more depressed in their presence if GA_3 is omitted from the culture medium. Since the effects were not proportional to the molar concentrations of the sugars the results cannot be due merely to osmotic effects [5].

The formation of α-amylase in embryo-less barley half-grains incubated on segments of paper chromatograms used to separate the solubility functions of grain extracts, was chosen as an appropriate bioassay for gibberellins. The method finally adopted, which has some novel characteristics, gave a roughly linear response over a satisfactory dose range, and an adequate recovery of biological activity from chromatograms of standard samples (Fig. 4).

A considerable amount of gibberellin activity remained on the surfaces of decorticated grains steeped in solutions of GA_3, and this could be removed by washing with a solution of sodium bicarbonate. The GA_3 that penetrated the grains was of most interest, but that remaining on the surface may have acted as a 'reservoir', continuously draining to the grains interior. Washings of untreated grains did not contain detectable gibberellin-like activity. Ethyl acetate extracts of such washings, adjusted to pH 2.5, made from grains germinated for 26 hr, were chromatographed. Biological activity chromatographed mainly in the R_f 0.4–0.6 region, consistent with it being chiefly unchanged GA_3. However, chromatography of material washed from grain that had been germinated for 96 hr moved in a broader zone, R_f 0.4–0.8, suggesting that new

Table 2. α-Amylase in whole and degermed grains, individually dosed with gibberellic acid

	Additions	α-Amylase (S.I.C./grain or endosperm)						Mean of A+B
			Set A			Set B		
Intact Grain	None		37.5	39.3		38.7	41.6	39.3
	Buffer	39.5	38.1	40.3		36.7	39.5	38.8
	Buffer+GA_3 (2 ng)		48.9	45.5		52.0	47.1	48.4
	Buffer+GA_3 (40 ng)		99.0	98.3	90.8	98.0	90.2	95.3
	Buffer+GA_3 (100 ng)	92.9	90.0	85.3		79.7	87.9	87.2
Grain degermed after 30 hr incubation	None		33.1	34.1		33.8	32.7	33.4
	Buffer	14.2	5.0	3.9	2.3	9.8	4.4	6.6
	Buffer+GA_3 (2 ng)		70.2	71.9	66.8	70.2	68.5	69.5
	Buffer+GA_3 (40 ng)		77.5	80.1		73.4	71.6	75.7
	Buffer+GA_3 (100 ng)		62.3	61.3	55.8	55.1	64.0	59.7

Decorticated grain was hydrated, then set in PTFE racks in Petri dishes. After 30 hr half of the grains were degermed, and grains were individually treated (1 μl) with succinate buffer (20 mM) or buffer containing the indicated doses of GA_3. Samples were incubated for a further 72 hr. α-Amylase was determined on homogenates of 10 grains. In set A the root-tips of the intact grains were removed after 24 hr. In set B the roots remained intact, and the Petri dishes stayed closed.

Table 3. The influence of sugars, and other substances, on the development of α-amylase in transected grains incubated with or without GA_3 at 25°, for 65 hr in a minimal volume of culture medium

Additions to the culture medium	α-Amylase activity (S.I.C./transected grain) 0	GA_3 (1 ng/ml)	(% relevant control) 0	GA_3 (1 ng/ml)
None (controls)	49	145	(100)	(100)
Maltose (50 mM)	32	134	65	93
Glucose (100 mM)	0.8	97	1.6	67
Fructose (100 mM)	2.1	112	4.3	78
Sucrose (50 mM)	0.9	90	1.8	62
Galactose (100 mM)	2.2	91	4.5	63
Arabinose (100 mM)	5.9	76	12	53
Raffinose (33.3 mM)	6.3	122	13	84
Mannose (100 mM)	0	94	0	65
Xylose (100 mM)	0.5	97	1.0	67
meso-Inositol (100 mM)	17	132	34	92
Glycerol (100 mM)	30	126	62	87
Casamino acids (0.15% w/v)	45	140	91	97

Results mean of 2 or 3 incubations, each with 8 transected grains. Controls—mean of 4 incubations.

gibberellins were being formed from the GA_3, possibly by the microbial flora. In malting grain, in which the husk was present and no attempt had been made to control the microbial population, surface GA_3 disappeared in *ca* 48 hr [11].

Analyses of extracts from grains germinated for various periods showed that, besides the major acidic and ethyl acetate-soluble fractions, significant traces of biological activity occurred in the hexane-soluble material, the residual aqueous phase, or butanol extracts of the residual aqueous phase, and the amounts present in these fractions altered with time. The 'peak' of gibberellin activity occurred after *ca* 30 hr germination. In acidic, ethyl acetate-soluble extracts of grains germinated for 26 hr, all prewashed with bicarbonate solution, the contents of gibberellin-like materials were estimated as 0.75 ng GA_3-equivalents/untreated grain (range ±0.11) in the controls and 2.3 ng GA_3-equivalents/grain (range ±0.5) in samples originally steeped in a solution of GA_3 (0.5 μg/ml). At this time little α-amylase could be found in the grains. In comparable grain samples germinated for 96 hr, by which time the aleurone has become largely insensitive, the gibberellin contents were 0.44 ng GA_3-equivalents/untreated grain (range ±0.25) and 1.27 ng GA_3-equivalents/GA_3-dosed grain (range ±0.41), respectively. Thus externally applied GA_3 enhanced the endogenous levels of 'free' gibberellins. Further, like the endogenous hormone, this 'extra' hormone was inactivated or otherwise altered during subsequent germination [11, 13].

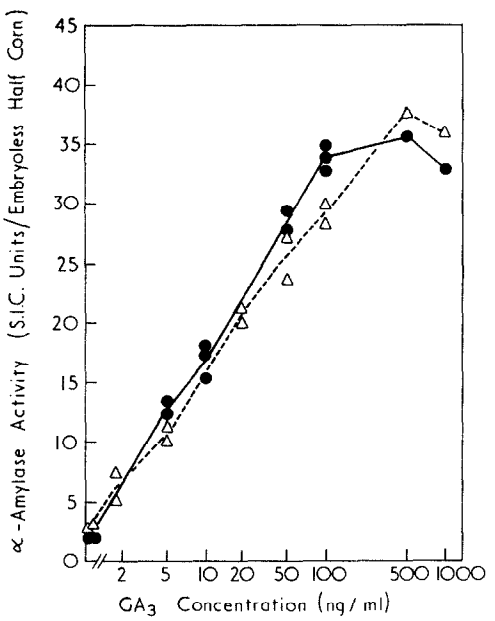

Fig. 4. Recovery of GA_3 in the bioassay. Levels of α-amylase induced by different quantities of GA_3 either added directly to paper in the Petri dish wells (●——●), or chromatographed, and assayed on segments of the chromatograms (△ - - - △; see Experimental).

In another experiment germinated grains (untreated and treated with GA_3) were washed with bicarbonate solution, then extracted. Some samples of the extracts were incubated with a mixture of hydrolytic enzymes. The extracts were then partitioned with organic solvents, and bioassayed (Fig. 5). Treatment with hydrolytic enzymes had no influence on the yields of gibberellin-like substances found in control grains. However, enzyme treatment greatly increased the acidic, ethyl acetate-soluble material found in extracts of grains dosed with GA_3. This 'freed' material chromatographed in the region occupied by GA_3. Thus, in grains 'dosed' with GA_3, extractable conjugates having little or no biological activity are found, and can be converted to free, active gibberellin(s) by a mixture of hydrolytic enzymes.

DISCUSSION

The growing embryo reduces the ability of the remainder of the grain to make more α-amylase in

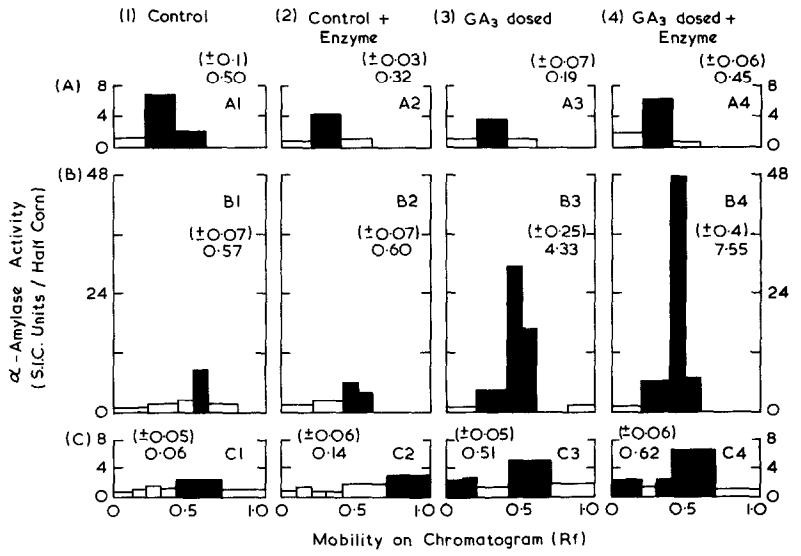

Fig. 5. Gibberellin-like activity in extracts of grain either untreated, or treated, with GA_3 before germination (96 hr), in which some of the primary extracts were treated with a mixture of hydrolytic enzymes before fractionation. Grains were washed with sodium bicarbonate solution before extraction. Extracts were fractionated by solvent partition, and then chromatographed before bioassay. A, hexane (pH 7) extracts; B, ethyl acetate (pH 2.5) extracts; C, residual aqueous phase (pH 7). 1. Fractions from control grain, primary extract treated with an inactivated mixture of enzymes. 2. Fractions from control grain, primary extract treated with active enzymes. 3. Fractions from grains germinated after an initial application of GA_3. 4. Fractions from grains germinated after an initial application of GA_3; primary extract treated with a mixture of hydrolytic enzymes. The figures are the gibbérellin-like materials (GA_3 equivalents, ng/grain) calculated from the blackened bars. The values in parentheses represent the range of the results.

response to a dose of GA_3. At least 3 mechanisms may be involved. Firstly, as the embryo accumulates various substances the aleurone layer may have been depleted of some necessary component needed for making more enzyme, as is suggested by electron-microscopic studies [15]. Secondly, the balance of conditions in the starchy endosperm may have altered to favour enzyme degradation [9, 12, 16]. Thirdly, the ability of the aleurone to degrade added gibberellins or convert them into inactive conjugates may have been enhanced [17–19].

The metabolism of free gibberellins, including exogenous GA_3, to biologically less active conjugates, such as glycosides, is well known [20–28] and since sucrose impeded the release of gibberellins from isolated embryos [5, 6], this suggested that the levels of active gibberellins, and hence α-amylase synthesis, might be controlled by conjugation of hormone in the embryo. In a variety of plant tissues conjugates probably serve as readily mobilized reserves of active gibberellins (e.g. [29]). The demonstration that a range of sugars, and some other substances, impede enzyme formation in transected grains, shows that this effect is not specific to sucrose. The inhibitory effects could be largely, but not completely overcome by added GA_3. A consideration of the concentrations of the inhibitory substances and their effectiveness makes it unlikely that they repress gibberellin release from embryos through increasing osmotic pressure as has been suggested [30]. Rather, they probably exert their effect through some metabolic mechanism [5]. However there is some doubt about whether all the substances tested would be converted to sugars in the embryo [31, 32].

The presence of an enzyme-hydrolysable gibberellin conjugate of low biological activity in grain dosed with GA_3, but not in untreated controls, indicates that the embryo may indeed use conjugation as a means of regulating the quantity of active hormone reaching the enzyme-forming tissues.

EXPERIMENTAL

Barley grain (*Hordeum distichon*), cv Proctor, 2.5–2.8 mm width, 1974 crop, was stored cool (97% viability). Grain was decorticated, when applicable, with 50% H_2SO_4 [9, 13]. Surface-sterilization was either (a) by immersion in Na hypochlorite soln (0.8% available Cl, 4°/20 min), followed by washing with H_2O and H_2O_2 (0.5%; 4°/15 min) to destroy residual hypochlorite, followed by the location of faulted grains using I_2/KI solution (0.025% I_2–0.4% KI w/v), which were rejected; or (b) by immersion in Ca hypochlorite (1% suspension, filtered; 4°/20 min), followed by washing and sorting as for (a). Where antibiotics were used, the mixture was streptomycin sulphate (0.08%), mycostatin (0.04%), benzyl penicillin (0.04%) and amphotericin B (0.04%) [33, 34].

Germination and incubation conditions. (a) Grains (50 and 100), germinated using 'micromalting' techniques, were sterilized, placed in sterile boiling tubes (50 ml) secured with muslin, then imbibed by immersion (steeped) for 2 × 24 hr periods at 16.5° in sterile H_2O (or various solns), which on occasion was degassed under vacuum before use. Between steeps and at the end of steeping the tubes were inverted and

drained for 1 hr. Tubes were tapped to arrange the grain into a single layer on the side, at the base, then arranged sloping slightly downwards (to allow drainage) on perforated shelves in a humid chamber, held at 16.5°. Dishes containing NaOH (10%) were placed on the shelves to absorb CO_2. The grain was mixed by shaking the tubes twice/day, to prevent the roots tangling. Growth was terminated by drying in a rapid airflow (43°/24 hr) or by freezing (−18°). Where gibberellins were to be extracted 20–50 g samples of grain were treated in essentially the same way, but were contained in bottles. (b) For experiments involving individual treatment of intact or degermed sterile, decorticated grains the second steep was in antibiotic soln, and after treatment corns were arranged embryo-ends uppermost, in holes (5 mm dia., 2.5 mm deep) in sterile PTFE blocks, held in Petri-dishes lined with wet paper at the base. Embryos (each with covering testa) were removed by gentle pressure at the base of the scutellum [13] leaving a shallow depression at the end of the exposed endosperm. Grains, entire or degermed, were treated with microdrops (1 μl) of sterile Na succinate buffer (20 mM, containing $CaSO_4$, 0.5 mM; pH 6), with or without dissolved GA_3, delivered from disposable PP30 Portex tubing placed on to the needle of a Hamilton microsyringe (5 μl). Aseptic conditions were maintained throughout. Incubations were in a moist atmosphere at 16.5°. Samples were stored frozen (−18°). Where microbial contamination was detected, in isolates from grains incubated in nutrient broth or on a solid medium, they were rejected. (c) For investigating the effects of sugars, sterile, decorticated grains, treated with antibiotics in the second steep, were bisected transversely, 'transected', and incubated with Na succinate buffer (20 mM; $CaSO_4$, 0.5 mM; pH 6; 8 transected grains/ml) in 25 ml conical flasks, plugged with non-absorbant cotton wool and loosely capped with aluminium foil, on an orbital shaker (100 rpm, at 25°) [5]. Asepsis was maintained. Before use sugars were partially deionized by passing solns down an ion-exchange column (Amberlite IR-120, H^+; 0°). Samples were stored at −18°. Those contaminated with microbes were rejected.

α-Amylase was extracted and assayed using the starch–I_2 colour (SIC) method, but colour was measured semi-automatically, and calculations were made on a programmed electronic calculator [35, 36].

Extraction and partition of gibberellins. Frozen (−18°) grain samples (20–50 g) were usually washed with $NaHCO_3$ (1% w/v, pH 8.2; 4°, 2 min) and rinsed with H_2O before extraction. Free surface gibberellins were recovered from the combined washings, following evapn (to 10 ml, 40°), acidification (pH 2.5, 6 N HCl), and extraction into EtOAc (6× 50 ml). Extracts were concd (10 ml; 25°) prior to chromatography and bioassay. Grains (300–500) were homogenized in MeOH (70%, 300 ml, 4°) with 10 sec bursts in a Willems Polytron mechanical ultrasonicator (Type PT10). The homogenate was stirred (18 hr; 4°) and filtered. The residue was re-extracted (4 hr, 20°). Extracts were combined and concd (15–25 ml; 25°), H_2O (25 ml) was added and concn was continued to 25 ml. This soln, 'primary grain extract' was sometimes stored (−18°). The gibberellins were usually partitioned before chromatography. (a) The aq. soln was extracted with hexane (3×30 ml). The hexane fraction was washed with K_2HPO_4 (1 mM, pH 8, 30 ml), and the washings added to the aq. phase. The hexane was concd (10 ml, 25°). (b) The combined aq. extracts were acidified (2 N HCl; pH 2.5) and the 'free acidic gibberellins' were extracted (EtOAc, 6×50 ml). The EtOAc was washed (H_2SO_4, 1%) and concd (10 ml, 25°). (c) Occasionally the residual aq. phase was extracted with *n*-BuOH (3×70 ml) to obtain a 'bound gibberellin' fraction. The organic phase was concd (10 ml). (d) The residual aq. phase from (b) or (c), was neutralized (pH 7, 18 M NH_4OH), concd (10 ml) and stored (−18°). Complete separations of aq. droplets from organic phases were achieved using Whatman IPS separators. Using these, recoveries of standard gibberellin solns were excellent (Fig. 4).

Enzymic hydrolysis of gibberellin conjugates. Crude enzyme prepns (a) a mixture of 5 g each of *Aspergillus niger* cellulase (Sigma Pract. grade II), *Trichoderma viride* cellulase, (BDH), Onozuka SS enzyme (Japan Biochemicals), and *Aspergillus niger* pectinase (Koch–Light), or (b) almond β-D-glucoside glucanohydrolase (50 mg, Sigma G. 8625) were purified by similar procedures. The mixture, a, was suspended in buffer (350 ml; pH 6, KH_2PO_4/K_2HPO_4, 10 mM; NaN_3; $CaCl_2$, 1 mM; β-mercaptoethanol, 0.1 mM), the suspension was clarified by centrifugation and enzymes precipitated from the supernatant plus washings (360 ml) with $(NH_4)_2SO_4$ (satd, 1.2 l., 18 hr, 4°). The ppt, collected by centrifugation, was dissolved in buffer and the proteins separated from NH_4^+ ions and some other contaminants by passage down a column of Bio-Gel P-4 (45×2 cm; 1 ml/min) equilibrated with buffer. The first UV absorbing protein peak was collected and freeze-dried. Prepns a and b contained *p*-nitrophenyl-β-D-glucopyranosidase activity. Prepn b also contained some *p*-nitrophenyl phosphatase. To hydrolyse gibberellin conjugates purified enzymes (40 mg a + 40 mg b) were dissolved in buffer (50 ml, Na succinate, 50 mM, pH 6). The soln was clarified by centrifugation and half was autoclaved 1.5 kg/cm^2, 120°/30 min) to destroy enzyme activity. Active and inactive solns were sterilised by membrane filtration (Millipore, 0.22 μm). Aliquots (10 ml) were incubated (2 hr, 30°, pH 6) with samples (10 ml) of primary grain extract, with gentle continuous stirring, with 2 drops $CHCl_3$ as antiseptic, and were then frozen (−18°). Samples which contained viable microbes were rejected. Sterile samples were fractionated and bioassayed for gibberellin-like substances as before.

Chromatography and bioassay of gibberellins. All fractions (10 applications × 10 μl) were separated by descending PC on strips of Whatman No. 1 paper (19 mm) using *iso*PrOH–NH_4OH–H_2O, (10:1:1) [28]. In this system the 'inhibitor-β' complex runs near the front. Following 8 hr equilibration, chromatograms were developed for 40 cm, air dried and divided into sections for bioassay. Blank chromatograms, solvent residues and standards of GA_3 (recrystallized EtOH–hexane; R_f 0.4–0.6; 1–1000 ng/ml) were chromatographed in parallel with unknowns. Four bioassay chromatogram strips (19×400 mm) were cut into 19 mm squares, equivalent to intervals of 0.1 R_f, and were placed in the wells (2×2 cm) of Sterilin divided Petri dishes (10×10 cm). Medium (0.5 ml; Na succinate, 20 mM; $CaSO_4$, 1 mM; pH 6, containing streptomycin sulphate, 0.02%; mycostatin, 0.01; benzyl penicillin, 0.01% and amphotericin B, 0.01%) was added to each well. Embryo-less half-grains (4), cut from sterile, decorticated grains hydrated in antibiotic mixture, were placed cut-ends down on the paper. Petri dishes, wrapped in damp paper, were incubated at 25° for 48 hr. Samples were frozen (−18°) and subsequently assayed for α-amylase activity.

Acknowledgements—We thank Mr. C. R. Townshend of Wolverhampton and Dudley Breweries for grain, Dr. P. C. Morrall for a gift of carbohydrases, and Professor J. S. Hough for reading the manuscript. M. T. S. thanks the Brewers' Society of Great Britain for financial support.

REFERENCES

1. Briggs, D. E. (1973) in *Biosynthesis and its Control in Plants* (Milborrow, B. V. ed.) p. 219. Academic Press, London.
2. Briggs, D. E. (1978) *Barley*. Chapman & Hall, London.
3. Briggs, D. E. (1963) *J. Inst. Brew. (London)* **69,** 13.
4. Briggs, D. E. (1964) *J. Inst. Brew. (London)* **70,** 14.
5. Briggs, D. E. and Clutterbuck, V. J. (1973) *Phytochemistry* **12,** 1047.
6. Radley, M. (1969) *Planta* **86,** 218.
7. Morrall, P. and Briggs, D. E. (1978) *Phytochemistry* **17,** 1495.
8. Newman, J. C. and Briggs, D. E. (1976) *Phytochemistry* **15,** 1453.
9. Briggs, D. E. (1968) *Phytochemistry* **7,** 513.
10. Smith, M. T. and Briggs, D. E. (1979) *J. Inst. Brew. (London)* **85,** 160.
11. Brookes, P. A. and Martin, P. A. (1975) *J. Inst. Brew. (London)* **81,** 357.
12. Briggs, D. E. (1968) *Phytochemistry* **7,** 531.
13. Groat, J. I. and Briggs, D. E. (1969) *Phytochemistry* **8,** 1615.
14. Murphy, G. J. P. and Briggs, D. E. (1973) *Phytochemistry* **12,** 1299.
15. Jones, R. L. and Price, J. M. (1970) *Planta* **94,** 191.
16. Briggs, D. E. (1968) *Phytochemistry* **7,** 539.
17. Musgrave, A., Kays, S. E. and Kende, H. (1972) *Planta* **102,** 1.
18. Nadeau, R., Rappaport, L. and Stolp, C. F. (1972) *Planta* **107,** 315.
19. Stolp, C. F., Nadeau, R. and Rappaport, L. (1973) *Plant Physiol.* **52,** 546.
20. Davies, L. J. and Rappaport, L. (1974) *Plant Physiol.* **55,** 620.
21. Davies, L. J. and Rappaport, L. (1975) *Plant Physiol.* **56,** 60.
22. Hedden, P., MacMillan, J. and Phinney, B. O. (1978) *Annu. Rev. Plant. Physiol.* **29,** 149.
23. Reeve, D. R. and Crozier, A. (1975) in *Gibberellins and Plant Growth* (Krishnamoorthy, H. N. ed.) p. 35. Wiley Eastern, New Delhi.
24. Sembdner, G., Adam, G., Lischewski, M., Sych, F.-J., Schulze, C., Knöfel, D., Müller, P., Schneider, G., Liebisch, H.-W. and Schreiber, K. (1974) in *Plant Growth Substances* 1973, *Proc. 8th Int. Conf.*, Tokyo p. 349. Hirokawa, Tokyo.
25. Sembdner, G., Borgmann, E., Schneider, G., Liebisch, H. W., Miersch, O., Adam, G., Lischewski, M. and Schreiber, K. (1976) *Planta* **132,** 249.
26. Yokota, T., Murofushi, N., Takahashi, N. and Tamura, S. (1971) *Agric. Biol. Chem. (Tokyo)* **35,** 583.
27. Barendse, G. W. M. and de Klerk, G. J. M. (1975) *Planta* **126,** 25.
28. Murakami, Y. (1961) *Bot. Mag. (Tokyo)* **74,** 424.
29. Halevy, A. H., Simchon, S. and Shillo, R. (1974) in *Plant Growth Substances* 1973, *Proc. 8th Int. Conf.*, Tokyo p. 64. Hirokawa, Tokyo.
30. Jones, R. L. and Armstrong, J. E. (1971) *Plant Physiol.* **48,** 137.
31. Edelman, J., Shibko, S. I. and Keys, A. J. (1959) *J. Exp. Botany* **10,** 178.
32. McCready, R. M. and Hassid, W. Z. (1941) *Plant Physiol.* **16,** 599.
33. Gaber, S. D. and Roberts, E. H. (1969) *J. Inst. Brew. (London)* **75,** 303.
34. Clutterbuck, V. J. and Briggs, D. E. (1973) *Phytochemistry* **12,** 537.
35. Briggs, D. E. (1967) *J. Inst. Brew. (London)* **73,** 361.
36. Smith, M. T., Cornish-Bowden, A. and Briggs, D. E. (1979) *J. Inst. Brew. (London)* **85,** 157.