Phytochemistry, 1968, Vol. 7, pp. 513 to 529. Pergamon Press. Printed in England

α-AMYLASE IN GERMINATING, DECORTICATED BARLEY—I.

α-AMYLASE, CONDITIONS OF GROWTH, AND GRAIN CONSTITUENTS

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(*Received* 11 *October* 1967)

Abstract—In germinating barley α-amylase is largely confined to the scutellum, starchy endosperm and aleurone. The levels attained are markedly altered by variations in the quantity of water available and the temperature. Added gibberellic acid, GA_3, accelerates enzyme formation and breakdown, and increases the maximum quantity of enzyme. Alterations in the level of α-amylase do not seem to be regulated directly by levels of endogenous amino acids or simple carbohydrates. Continuing proteolysis, the removal of starch and calcium ions and falling pH may all contribute to falling enzyme activity. A hypothesis is advanced relating the effects of GA_3 and the destruction of α-amylase.

INTRODUCTION

THE mobilization of the reserve substances, such as starch, in the caryopses of the *Gramineae* during germination is of direct importance in agriculture and to the maltster who must, by a limited germination process, convert barley into malt in the most economic way.[1,2] During germination the reserve substances of the starchy endosperm, notably proteins, cell-wall hemicelluloses, and starch, are progressively hydrolysed. These enzyme-catalysed hydrolyses produce materials of small molecular weight that support the growth of the embryo plant. In malting barley the germination process occurs while the grain is relatively dry and the lack of moisture limits the solution of enzymes, the movement of enzymes within the grain and the dissolution of the starchy endosperm. Consequently it is easy to show that the formation of hydrolytic enzymes, and hence the breakdown of the cell walls and starch granules in the starchy endosperm, begins adjacent to the embryo and moves backwards down the grain, progressing most rapidly at the periphery, under the dorsal aleurone layer.[3,4]

Among the enzymes that follow this pattern barley α-amylaze (EC 3.2.1.1.) has been studied[5,6] both because truly specific methods are available to estimate its activity, and because it is virtually absent from most samples of mature grain before germination, thus giving the experimental advantage of a uniform, zero base-line. In intact barley grains germinating in the absence of external supplies of gibberellins, the synthesis of α-amylase, and other enzymes, is entirely dependent on the presence of a viable embryo.[6–8] Dissected

[1] J. R. A. POLLOCK, in *Barley and Malt* (edited by A. H. COOK), p. 30. Academic Press, London (1962).
[2] D. E. BRIGGS, *J. Inst. Brewing* **69**, 244 (1963).
[3] H. T. BROWN and G. H. MORRIS, *J. Chem. Soc.* **57**, 458 (1890).
[4] A. D. DICKSON and H. L. SHANDS, *Proc. Am. Soc. Brewing Chem.* p. 1 (1941).
[5] F. V. GRAESSER and P. J. DAX, *Wallerstein Lab. Commun.* **9**, 26, 49 (1946).
[6] D. E. BRIGGS, *J. Inst. Brewing* **70**, 14 (1964).
[7] B. H. KIRSOP and J. R. A. POLLOCK, *J. Inst. Brewing* **64**, 227 (1958).
[8] H. YOMO, *Hakkô Kyôkaishi* **16**, 444 (1958).

embryos form small amounts of α-amylase when grown *in vitro* and contain small amounts when separated from whole, germinating grain.[6,9] It has been suggested that most of this *de novo* synthesis occurs in the associated small pieces of aleurone tissue, rather than the tissues of the embryo proper.[10] The greater part of the α-amylase in the grain is synthesized *de novo* in the living aleurone layer that invests the dead starchy endosperm. Enzymes are synthesized in response to materials originating in the growing embryo, whether they are true gibberellins, or gibberellin-like materials.[6,8,11–15] Barley embryos growing *in vitro* produce material with the properties of gibberellins, including GA_1 and GA_3.[15–17] The production of these materials is blocked by chlorocholine chloride, CCC. Gibberellic acid, GA_3, and sometimes a second gibberellin-like component have been found in immature barley.[18–20] Enzyme formation in the aleurone, or the release of soluble sugars and other substances from the associated starchy endosperm, when degraded by these enzymes, is elicited to varying extents by a range of gibberellins,[13,15,21–24] helminthosporol, helminthosporic acid,[25] and to a minor extent by various solvent residues.[26] Many other substances that in other test systems show hormonal effects on plants, including α-indole acetic acid and kinetin, have no effect on enzyme production by the aleurone.[15,26–28]

The addition of GA_3 to the whole grain augments the supply of endogenous gibberellins and increases the rate of enzyme formation,[29] a fact of commercial value in malting.[2] The added GA_3 accelerates the rate of formation of α-amylase first at the embryo,[6,28] suggesting that this substance is excluded by the testa and first penetrates at the basal end of the grain, through the micropyle as occurs with many other substances, or perhaps when the coleorhiza first breaks the seed-coats. From the large response elicited by added GA_3 it is evident that the endogenous gibberellins do not saturate the enzyme synthetic mechanisms of the aleurone. The formation of α-amylase in response to GA_3 is truly *de novo* synthesis as indicated by an absolute requirement for oxygen,[13] the effects of metabolic inhibitors,[6,15] and the incorporation of ^{14}C-amino-acids and of ^{18}O- as shown by equilibrium density gradient centrifugation.[15,30–34] GA_3, while causing the production and release of α-amylase, also causes an

[9] D. E. BRIGGS, *J. Inst. Brewing* **68**, 470 (1962).
[10] A. M. MACLEOD and G. PALMER, *J. Inst. Brewing* **72**, 580 (1966).
[11] H. YOMO, *Hakkô Kyôkaishi* **18**, 494 (1960).
[12] H. YOMO, *Hakkô Kyôkaishi* **18**, 500 (1960).
[13] H. YOMO, *Hakkô Kyôkaishi* **18**, 600 (1960).
[14] H. YOMO, *Hakkô Kyôkaishi* **18**, 603 (1960).
[15] D. E BRIGGS, *J. Inst. Brewing* **69**, 13 (1963).
[16] H. YOMO and H. I. IINUMA, *Planta* **71**, 113 (1966).
[17] M. RADLEY, *Planta* **75**, 164 (1967).
[18] M. RADLEY, *Chem. and Ind.* 87 (1959).
[19] D. F. JONES, J. MACMILLAN and M. RADLEY, *Phytochem.* **2**, 307 (1963).
[20] M. RADLEY, *Nature* **210**, 969 (1966).
[21] L. G. PALEG, *Plant Physiol.* **35**, 293 (1960).
[22] L. G. PALEG, *Plant Physiol.* **35**, 902 (1960).
[23] A. M. MACLEOD and A. S. MILLAR, *J. Inst. Brewing* **68**, 322 (1962).
[24] L. PALEG, D. ASPINALL, B. COOMBE and P. NICHOLLS, *Plant Physiol.* **39**, 286 (1964).
[25] D. E. BRIGGS, *Nature* **210**, 418 (1966).
[26] D. E. BRIGGS, *Nature* **210**, 419 (1966).
[27] R. CLELAND and N. MCCOMBS, *Science* **150**, 497 (1965).
[28] K. MORIMOTO and T. YOSHIDA, *Rept Res. Lab. Kirin Brewery Co.* **8**, 27 (1965).
[29] T. HAYASHI, *J. Agric. Chem. Soc. Tokyo* **16**, 531 (1940).
[30] G. R. CHANDRA and J. E. VARNER, *Plant Physiol.* **38**, (Suppl.), 10 (1963).
[31] J. E. VARNER, *Plant Physiol.* **39**, 413 (1964).
[32] J. E. VARNER and G. R. CHANDRA, *Proc. Natl Acad. Sci. U.S.* **52**, 100 (1964).
[33] P. FILNER and J. E. VARNER, *Plant Physiol.* **41** (Suppl.), 28 (1966).
[34] J. E. VARNER, G. R. CHANDRA and M. J. CHRISPEELS, *J. Cell Comp. Physiol.* **66**, 55 (1965).

increase in the rate of respiration of the aleurone layer,[6, 35] and induces a characteristic series of alterations in the appearance of the cell contents, as seen with the electron microscope.[36]

The explanation for differences between the culture conditions needed for getting the most α-amylase from embryos or endosperms *in vitro* is not immediately apparent. For example the substantial need of the embryo for added amino acids and the suppression of enzyme formation by the addition of sugars[6] are effects that are scarcely or not at all evident with the endosperm.[6, 28, 37–39] One hypothesis is that the aleurone tissue associated with the embryo is the source of enzyme synthesis but its properties are altered by its close association with the growing tissue to which it loses nitrogenous and perhaps other constituents. Unless these are made good in the culture medium, amino acids are not available for enzyme synthesis. The effect of added sugar causing extra embryo growth and hence a greater drain on available nitrogen, would accentuate this effect. During germination, sugars accumulate in the starchy endosperm, as a result of the action of α-amylase and other enzymes, and it was previously suggested that these sugars might suppress α-amylase formation in the embryo and so form a "control loop".[6]

The quantities of α-amylase formed, or sugars released from de-embryonated grain *in vitro*, are greater from half-grains prehydrated for 24 hr and then treated with GA_3 rather than those placed directly in a GA_3 solution.[40–42] The quantities are greater still if the grain is soaked for 24 hr and then the embryo part of the grain is removed, and the remaining half-grain is incubated with gibberellic acid.[40, 41] Thus prehydration and a factor moving from the embryo during hydration appear to help regulate α-amylase synthesis in barley grain.

In whole grains the preferential formation of enzymes in the dorsal aleurone (when all parts of the aleurone incubated with GA_3 equally well induce the dissolution of the associated starchy endosperm *in vitro*)[6] has been attributed to the preferential delivery of endogenous gibberellins to this part of the grain. Gibberellins are supposed to move from the embryo node, the suggested site of synthesis, to the apex of the scutellum by vascular tissue that develops during germination.[10]

The investigations reported here, and in the subsequent communications, were made to gain more insight into the interplay of these with any other factors that might be discovered in controlling the levels of α-amylase in germinating grain. Duffus recently reported that when the peak of enzyme activity in the grain is approached, preparations made from the aleurone lose their ability to synthesize the enzyme,[43] so the question arose as to whether enzyme synthesis and breakdown proceeded concurrently.

RESULTS AND DISCUSSION

Methods of Culture

After a fixed period of growth, dehusked sterilized barley grains that had been germinated on petri dishes, with various quantities of water, were collected; the lengths of the coleoptiles

[35] L. PALEG, *Colloq. Intern. Centre Natl Rech. Sci. Paris* No. **123**, 303 (1964).
[36] L. G. PALEG and B. HYDE, *Plant Physiol.* **39**, 673 (1964).
[37] H. YOMO and H. IINUMA, *Proc. Am. Soc. Brewing Chemists* 97 (1964).
[38] H. YOMO and H. IINUMA, *Agr. Biol. Chem. Tokyo* **27**, 76 (1963).
[39] H. YOMO and H. IINUMA, *Agr. Biol. Chem. Tokyo* **28**, 273 (1964).
[40] C. PETRIDIS, R. VERBEEK and L. MASSART, *J. Inst. Brewing* **71**, 469 (1965).
[41] A. M. MACLEOD, J. H. DUFFUS and D. J. L. HORSFALL, *J. Inst. Brewing* **72**, 36 (1966).
[42] K-H. YUNG and J. D. MANN, *Plant Physiol.* **42**, 195 (1967).
[43] J. H. DUFFUS, *Biochem. J.* **103**, 215 (1967).

were measured, and the levels of α-amylase were determined. Coleoptile length and α-amylase activity in both the embryo and the endosperm were strongly influenced by the quantity of water present (Fig. 1). Frequency histograms of the lengths of coleoptiles showed that growth was most regular with 5 and 6 ml water/9-cm dish. In subsequent studies, 6-ml culture medium were usually used on 9-cm, and 10-ml on 11-cm petri dishes. A further trial, Fig. 2, in which the α-amylase present in grain was followed with time as the grain grew in the presence of two levels of moisture, with and without added gibberellic acid, shows how excess moisture suppresses the rate of formation of the enzyme, and results in a lower peak

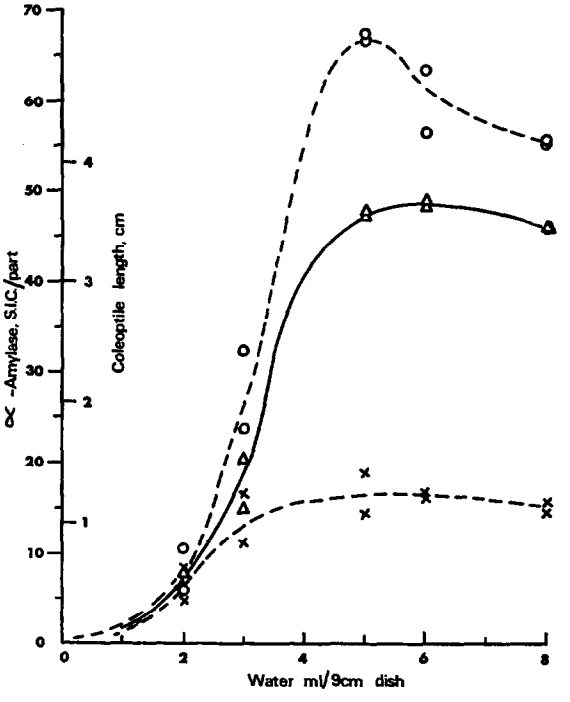

FIG. 1. COLEOPTILE LENGTH, AND α-AMYLASE, OF GRAIN GERMINATED 2¾ DAYS WITH VARYING VOLUMES OF LIQUID/DISH.

—△— Coleoptile length, cm.
- -×- - α-Amylase (S.I.C./embryo).
- -○- - α-Amylase (S.I.C./endosperm).

of enzyme activity. The addition of gibberellic acid to the culture medium as expected from malting experience[2, 29] results in a greater formation of enzyme, having a peak that occurs earlier than in the absence of the hormone. Coleoptile length and levels of α-amylase did not differ greatly between samples grown with fifteen or thirty corns/11-cm dish. In the interests of uniformity twenty-five corns were usually grown with 6·0 ml of culture medium on 9-cm petri dishes, and forty corns were grown with 10 ml of culture medium on 11-cm petri dishes, in each case the dishes contained two discs of Whatman No. 1 filter paper.

Effects of Temperature

In grain germinated at 14·4° the maximum level of α-amylase exceeded the maximum found in grain germinated at 25°, and the phase during which enzyme breakdown occurred

began later, so that the peak of α-amylase activity at 14·4° occurred about 2 days later than at 25° (Fig. 3A and B). Such results were partly to be expected from experiments with de-embryonated barley and from malting experience.[23, 44, 45] In this trial it was shown that the addition of calcium sulphate (0·5 mM) to the culture medium did not result in an alteration in the pattern of enzyme production, probably because the calcium ions did not penetrate into the aleurone or starchy endosperm. In samples grown at 14·4° and 25° maximum enzyme activity occurred when the coleoptile had grown about 2 cm and, by the time the first leaves appeared, enzyme activity had fallen to about one-third of its maximum value.

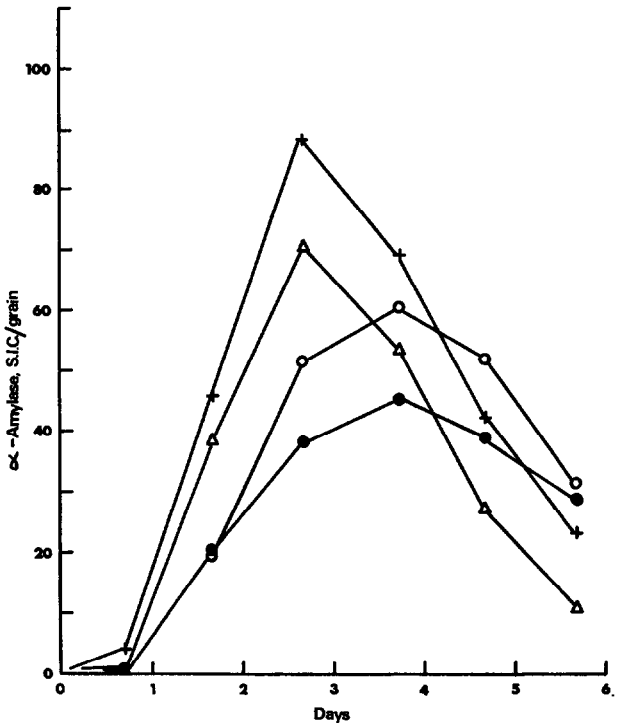

FIG. 2. α-AMYLASE IN BARLEY GERMINATED WITH TWO VOLUMES OF LIQUID/DISH, WITH OR WITHOUT GA_3 (50 μg/ml).

40 grains/11-cm dish. α-Amylase (S.I.C./grain):—

—○— 6·7 ml/dish —+— 6·7 ml/dish, with GA_3.
—●— 12 ml/dish —△— 12 ml/dish, with GA_3.

Distribution of α-Amylase in Germinating Barley

In this, and in the limited number of other trials in which separate measurements were made, the α-amylase activity associated with the embryo rose and fell roughly in parallel with the activity found in the endosperm. It was known that if germinated barley was rapidly dried in a current of warm air at less than 45° the α-amylase level remained unaltered and the dried "malt" and the enzyme in it would be stable and could be stored dry for several months.[46, 47] It seemed possible that during drying α-amylase might re-distribute itself

[44] L. G. PALEG, *Plant Physiol.* **36**, 829 (1961).
[45] H. L. SHANDS, D. DICKSON and B. A. BURKHART, *Cereal Chem.* **18**, 370 (1941).
[46] A. D. DICKSON and H. L. SHANDS, *Cereal Chem.* **19**, 411 (1942).
[47] D. E. BRIGGS, *J. Inst. Brewing* **73**, 361 (1967).

between the embryo and endosperm, but a comparative test between frozen and dried samples showed that this was not so (Table 1). Clean separations between the embryo and

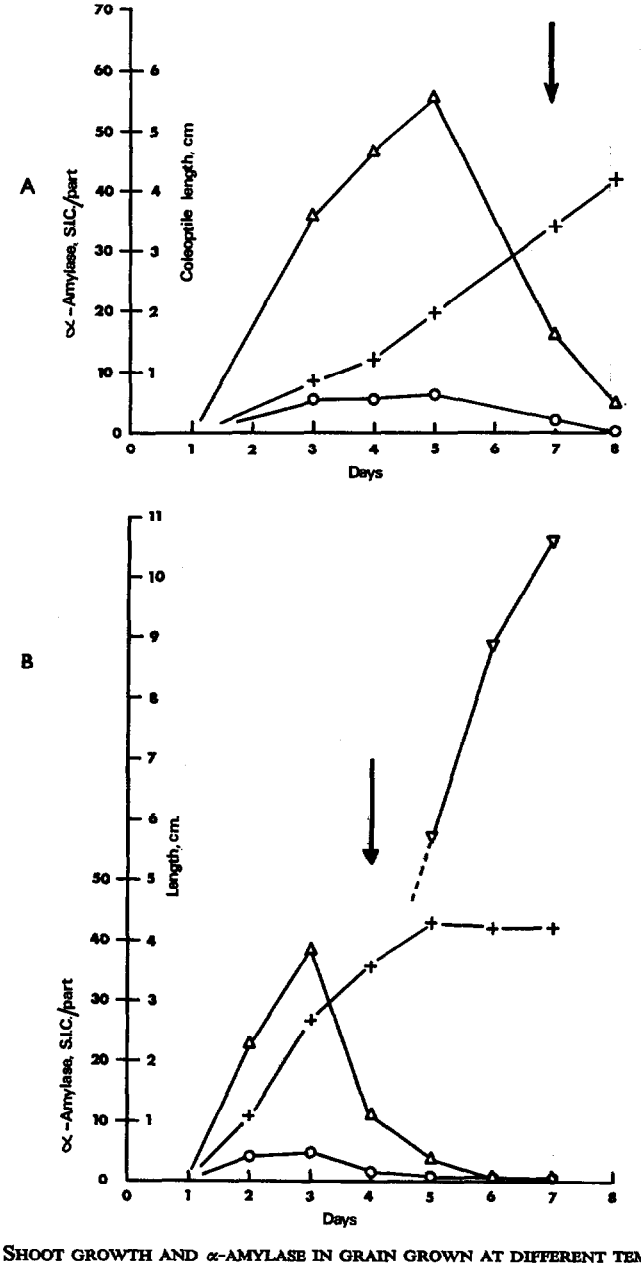

FIG. 3. SHOOT GROWTH AND α-AMYLASE IN GRAIN GROWN AT DIFFERENT TEMPERATURES.
A, 14·4°; B, 25·0°. Lengths, cm: —+—, coleoptile; —▽—, 1st leaf α-amylase (S.I.C./part); —△—, endosperm; —○—, embryo. The arrows mark the first appearance of the leaves.

the endosperm dissected from dry grain before enzyme analysis were not always achieved, and this may explain the variable proportion of the total activity associated with the embryo

in different trials. Tests with frozen and kilned grain divided in various ways showed that in the dissected embryo enzyme activity was usually confined to the scutellum (Tables 2 and 3). The exception in Table 3 may have been due to the failure to rinse away micro-organisms. This is consistent with the postulate that the enzyme is synthesized in the small fragment of

TABLE 1. DISTRIBUTION OF α-AMYLASE IN BARLEY GERMINATED FOR 2¾ DAYS

	α-Amylase (S.I.C./part)			
	Preparation			
	Frozen		Kilned	
	Separated regions			
	Embryo	Endosperm	Embryo	Endosperm
	6·4	75	5·0	60
	5·1	78	5·0	89
	9·9	59	5·7	72
	4·5	82	7·3	64
Mean	6·6	73	5·8	71

TABLE 2. DISTRIBUTION OF α-AMYLASE IN KILN-DRIED BARLEY PREVIOUSLY GERMINATED FOR 2¾ DAYS

Separated regions	α-Amylase (S.I.C./part)	α-Amylase (S.I.C./mg)
Shoot	0	0
Roots	0	0
Scutellum	14	0·31
Endosperm, Proximal	25	0·24
Distal	23	0·19

TABLE 3. DISTRIBUTION OF α-AMYLASE IN FROZEN BARLEY GERMINATED FOR 2¾ DAYS

Separated regions	α-Amylase (S.I.C./part)		
Shoot	0	0	0
Roots	1·6	0	0
Scutellum	0·5	0·8	1·7
Aleurone + testa	7·3	4·2	3·7
Endosperm starchy contents	32	41	42

aleurone that is associated with the embryo.[10] The α-amylase associated with the aleurone probably represents enzyme that has not yet been released into the starchy endosperm. The similarity in the enzyme levels in the proximal and distal halves of the starchy endosperm is in striking contrast to the well-known differential distribution of α-amylase in malting grain,[5,6] where the enzyme level is substantially higher in the proximal endosperm. This difference

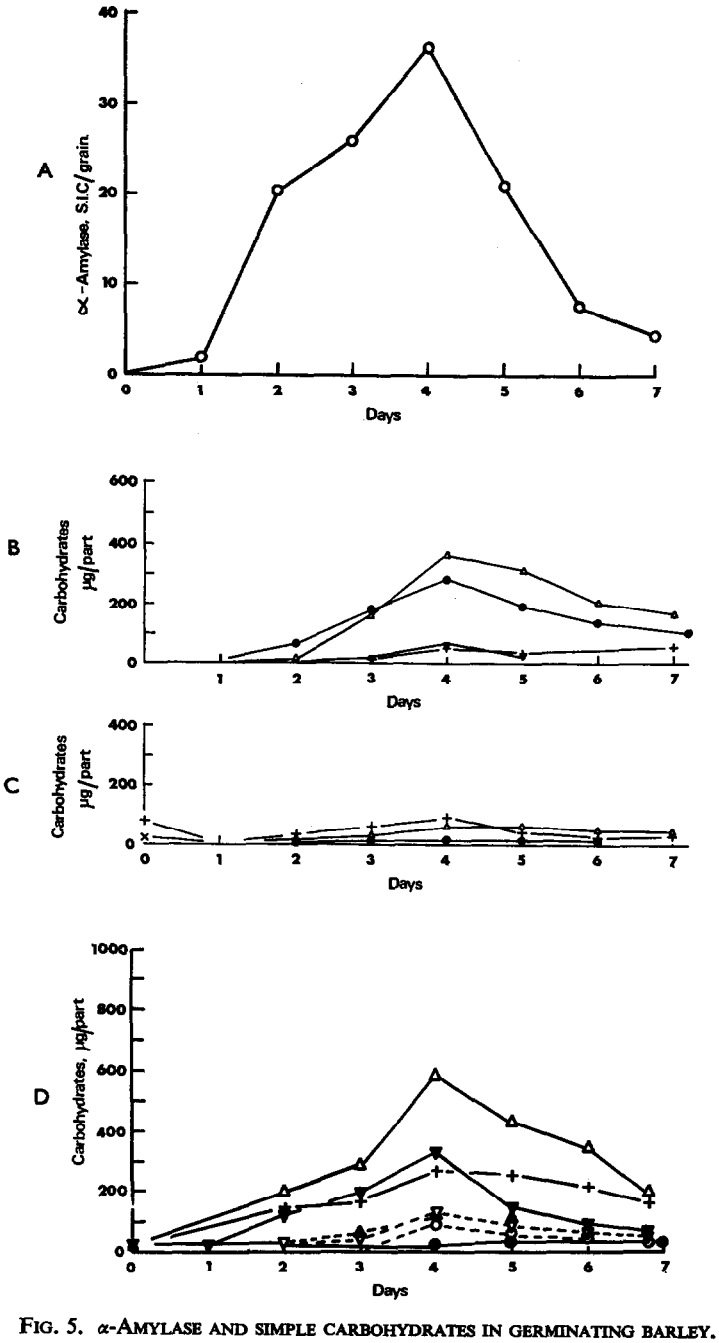

FIG. 5. α-AMYLASE AND SIMPLE CARBOHYDRATES IN GERMINATING BARLEY.

A, α-Amylase (S.I.C./grain). Simple carbohydrate; roots, B; scutella, C; endosperms, D; shoots (coleoptile+1st leaf), E. (over page)

—△— Glucose —×— Raffinose
—●— Fructose - -▽- - Disaccharide (?)
—▼— Maltose - -○- - Trisaccharide
—+— Sucrose - -▲- - Maltotriose.

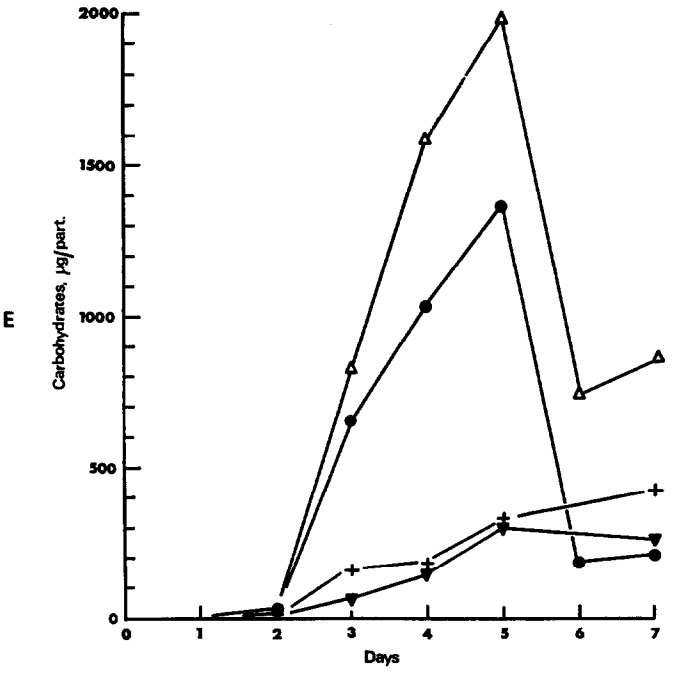

FIG. 5E. Caption on preceding page.

may have been due to the greater moisture content of the grain in the present experiments, which possibly allowed a more free diffusion of enzyme and gibberellin within the grain since it permitted the complete dissolution of the dorsal starchy endosperm (Fig. 4).

Analyses of Other Grain Constituents

Analyses were performed to see if alterations in the constituents of the grain could explain the fall in activity of α-amylase in the second phase of each experiment. Gas chromatography of the trimethylsilyl ethers of carbohydrates showed that in the roots, scutella and endosperm the greatest quantities of soluble sugars were present at time of the peak level of α-amylase (Fig. 5A–D). However, in the shoots (coleoptile + 1st leaf), the peak in total soluble carbohydrates occurred about a day later (Fig. 5E). These results do not support the idea that in the entire grain soluble carbohydrates in the starchy endosperm serve to inhibit the synthesis of the enzymes catalysing these processes, as in a feed-back loop.[6] In another trial, the levels of amino acids in the endosperm and the scutellum reached maxima on day 3 when the peak level of α-amylase occurred, but the levels in the roots and shoots continued to increase throughout the experiment (Fig. 6B). Throughout germination the protein level of the endosperm continued to decline, showing that proteolysis was proceeding (Fig. 6A). The weights of the shoots and roots increased, while that of the starchy endosperm declined (Fig. 6A). That this decline in weight was largely due to the hydrolysis of starch, which constitutes some 75 per cent of the dissected endosperm of decorticated barley, was confirmed by a limited number of direct analyses. So striking was the removal of the reserves constituting the starchy endosperm that towards the end of the experiments the dorsal side of endosperms became nearly transparent (Fig. 4) and, on drying, they contracted, shrank and shrivelled very markedly.

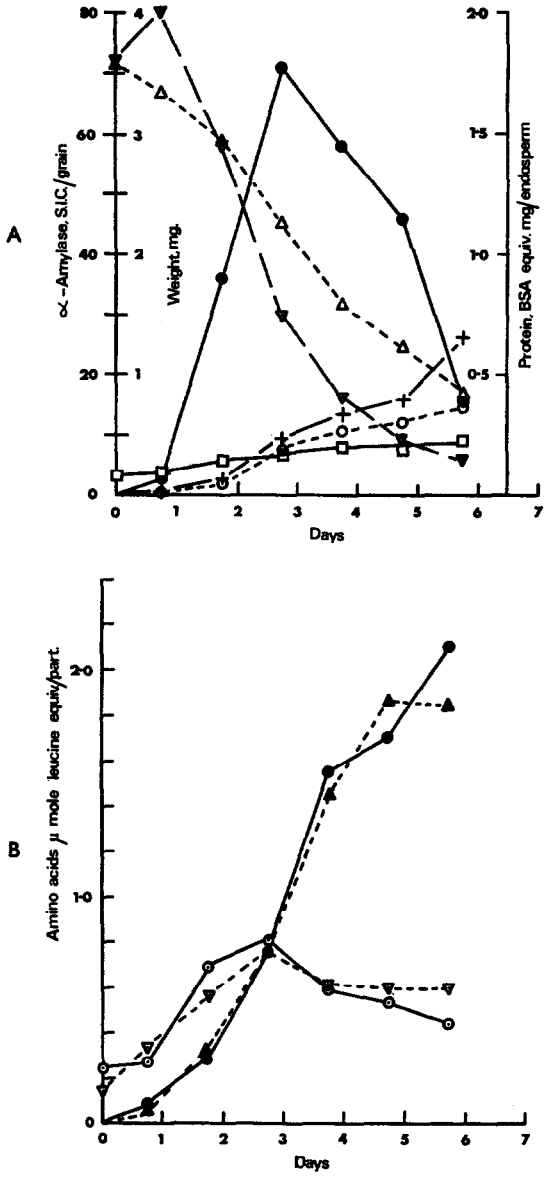

FIG. 6. α-AMYLASE, α-AMINO ACIDS AND OTHER CONSTITUENTS OF GERMINATING BARLEY.

A. —●—, α-Amylase (S.I.C./grain); —▽—, endosperm protein (mg. BSA equivalent/part). Weights of parts (mg): - -△- -, endosperm; - -○- -, shoot (coleoptile + 1st leaf); —+—, roots; —□—, scutellum.

B. α-Amino acids (μg leucine equivalent/part): —●—, roots; - -▲- - shoots (coleoptile + 1st leaf); —⊙—, endosperm; - -▽- -, scutellum.

The fall in the level of amino-acids in the parts of the grain where enzyme synthesis occur did not seem great enough to limit the synthesis of new enzyme. The experiments of Duffus[43] indicated that enzyme synthetic mechanisms in the aleurone failed just at the peak of enzyme activity, when in our experiments the level of α-amino acid was still high. Proteolysis was

still proceeding as the level in α-amylase declined, showing that enzymes capable of attacking the amylase were still active. The removal of the enzymes substrate, starch, might be expected to potentiate the enzyme instability. Malt α-amylase was known to be stabilized by the presence of calcium ions[48–52] which, as in α-amylase from other sources,[53, 54] probably forms an integral part of its structure. The binding of calcium ions by EDTA, citrate, phytic acid, polyphosphates or other chelating agents, greatly reduces the stability of solutions of barley α-amylase[53, 54] and enhance its susceptibility to heat inactivation and proteolytic attack.

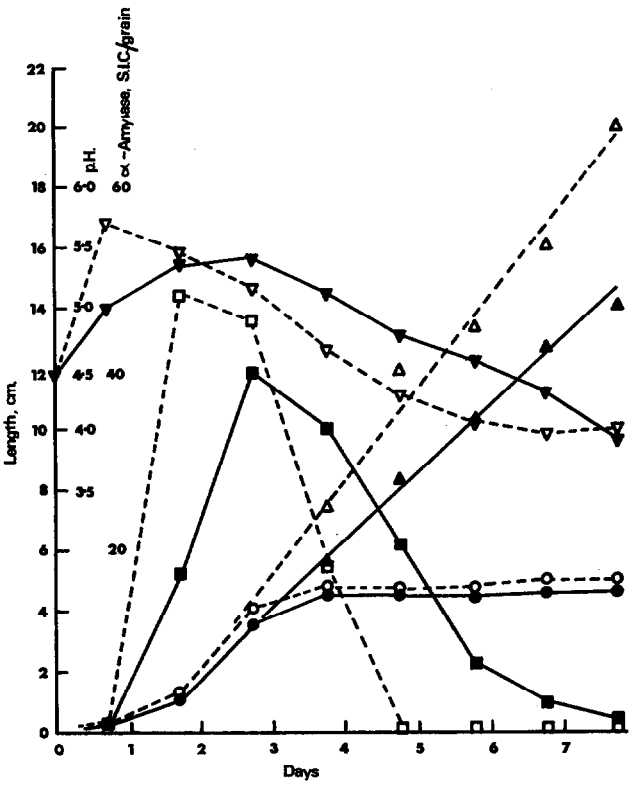

FIG. 7. α-AMYLASE, SHOOT GROWTH AND ENDOSPERM pH IN GERMINATING BARLEY.

—▼—, - -▽- -, Endosperm pH; —■—,- -□- -, α-Amylase (S.I.C./grain); —●—, - -○- -, length of coleoptile (cm); —▲—, - -△- -, length of 1st leaf (cm).

Hollow symbols and dotted lines: grain grown in the presence of GA_3 (100 μg/ml); solid symbols and continuous lines: grain grown without.

Solutions of the crude enzyme are more stable than solutions of the pure enzyme, even in the presence of adequate quantities of calcium ions. The pure enzyme is known to be stabilized by crude preparations of barley proteins.[55] The addition of calcium salts to the medium in

[48] E. H. FISCHER and C. H. HASELBACH, *Helv. Chim. Acta* **34**, 325 (1951).
[49] I. A. PREECE, *J. Inst. Brewing* **53**, 154 (1947).
[50] I. A. PREECE, *J. Inst. Brewing* **54**, 141 (1948).
[51] W. J. OLSON, B. A. BURKHART and A. D. DICKSON, *Cereal Chem.* **20**, 126 (1943).
[52] E. KNEEN, R. M. SANDSTEDT and C. M. HOLLENBECK, *Cereal Chem.* **20**, 399 (1943).
[53] S. SCHWIMMER and A. K. BALLS, *J. Biol. Chem.* **179**, 1063 (1949).
[54] B. L. VALLEE, E. A. STEIN, W. N. SUMERWELL and E. H. FISCHER, *J. Biol. Chem.* **234**, 2901 (1959).
[55] C. T. GREENWOOD and A. W. MACGREGOR, *J. Inst. Brewing* **71**, 405 (1965).

which isolated aleurone layers are cultured increases the level of GA_3-induced α-amylase to that achieved by the aleurone in whole endosperms.[56] The pure enzyme is unstable when heated at 70°,[48] while crude preparations are stable unless they are prepared from highly kilned malt, or have boiled extracts of coloured malt added to them.[47] Below pH 4·9 the pure enzyme is unstable in solution, and is rapidly destroyed at pH 4·5 or below.[48] In our experience the impure α-amylase found in β-amylase concentrates is destroyed only if calcium ions are removed by dialysis against EDTA and the pH is reduced to 3·5. Because of these facts, the pH of the endosperm contents and its calcium status were investigated during germination.

Both in the presence and in the absence of gibberellic acid the pH of the endosperm contents were found to rise and then to fall during germination. In each case they fell below

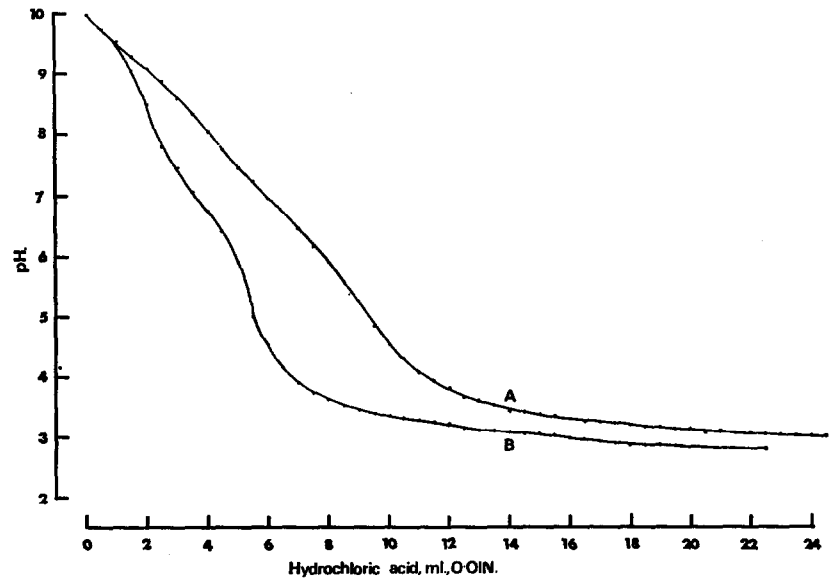

FIG. 8. TITRATION OF EXTRACTS OF ENDOSPERMS OF BARLEY GERMINATED IN THE PRESENCE OF GA_3 (100 μg/ml).

A, after 1 day's germination.

B, after 5 days' germination.

pH 4·9, when pure α-amylase in solution would not have remained stable[48] (Fig. 7). However, by the time this pH was reached the peak of α-amylase activity was past and the level had declined to an appreciable extent, while enzyme activity only disappeared when the pH had fallen to 4·3 and 4·0 respectively in the presence and absence of added gibberellic acid. In solution the pure enzyme would have been destroyed in 18 hr at pH 4·5.[48] It is possible that the pH of the fluid in the endosperm was marginally lower than the values measured as it was necessary to add a little water to the homogenate in order to measure its pH. Even so, in the endosperm the enzyme seems more resistant to low pH values than when pure, possibly because 1,4-α-glucosidic oligosaccharides in the starch series are present to stabilize the enzyme. Titrations were made, at first with alkali and subsequently with acid of aqueous extracts prepared of homogenized endosperms from grain germinated for 1 and 5 days in the

[56] M. J. CHRISPEELS and J. E. VARNER, *Plant Physiol.* **42**, 398 (1967).

presence of gibberellic acid (Fig. 8). Since the extracts were relatively dilute the initial pH values were higher than undiluted homogenates of endosperm. Only the graphs of altering pH with the titration from the alkaline to the acid end of the pH range are shown. The graphs of the other titrations were essentially similar. The most striking difference is the much greater buffering capacity of the extract from the endosperms of grain germinated 1 day (Fig. 5, curve A) as against 5 days (curve B), particularly in the range below pH 5. When de-

TABLE 4. α-AMYLASE FROM ENDOSPERMS CULTURED FOR 2¾ DAYS WITH GA_3 (50 μg/ml)

Succinate buffer, initial concentration (mM)	Final pH of culture medium			α-Amylase (S.I.C./ endosperm)		
0	3·01	3·58	4·00	0	4·2	14·2
3·4	3·40	3·10	3·08	3·0	1·6	1·2
6·7	4·60	4·44	6·62	51	44	60
10·0	5·52	7·08	5·52	65	66	105
20·0	5·72	5·74	7·12	56	93	64

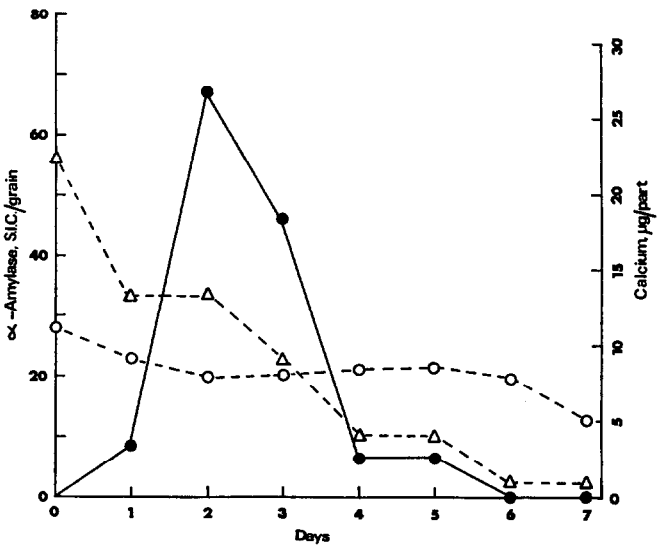

FIG. 9. α-AMYLASE AND CALCIUM LEVELS IN GERMINATING BARLEY, ($GA_3$50μg/ml).

—●— α-Amylase (S.I.C./grain):
Calcium (μg/part):
- -○- - Embryo
- -△- - Endosperm

embryonated grains were incubated with GA_3 it was found that the pH of the incubation medium fell sharply, and was only maintained over 3 days by strong (10 or 20 mM) succinate buffer (Table 4)—a point of interest when using this system as a bioassay for gibberellins.[57] The fall in pH should favour the activity of the proteolytic enzymes of the endosperm, as well as reducing the stability of α-amylase. There are no data available on the degradation of barley α-amylase by barley proteases.

[57] R. L. JONES and J. E. VARNER, *Planta* 72, 155 (1967).

Satisfactory estimations of the levels of total calcium ions in the parts of the germinating grain proved difficult to obtain. A variety of methods were tried and discarded, as in each case the results showed an unacceptable scatter. Nevertheless the trends indicated a decline in the level of calcium ions in the endosperm during germination. It was known that in malting grain the roots contain calcium drawn from the rest of the grain.[58] Finally, using an oxalate precipitation method, the results shown in Fig. 9 were obtained. These showed that during germination a marked decline occurred in the quantity of calcium present in the endosperm, but showed no corresponding increase in the embryo. This result was in contrast to the results of some other experiments. However, losses of ions from roots are well known and minerals and other metabolites are known to be exuded in guttation fluid and to be readily leached from leaves.[59] Guttation occurred in most of our experiments. Thus the "missing" calcium was probably lost from the plants during this experiment. In other experiments we showed that calcium was gained by filter papers when grain was germinated in contact with them. However, exact loss–gain calcium balances between the seedlings and the filter

TABLE 5. EFFECTS OF ADDING H_2O OR GA_3 (100 μg/ml) TO GERMINATING BARLEY ON LEVELS OF α-AMYLASE

	α-Amylase (S.I.C./grain)						
	Time, hr						
0	18	43	67	91½	114	138½	162½
$+H_2O$	0	20	42	47	27	16	7·1
$+GA_3$	0	50	61	21	3·3	1·9	0
		$+GA_3$	61	27	26	6·1	0
			$+GA_3$	37	41	5·5	4·2
			$+H_2O$	42	30	17	7·7
				$+GA_3$	22	9·7	7·5
				$+H_2O$	36	14	2·0

papers were not achieved. Still, the losses in total calcium from the endosperm appear to be real. It is recognized that the proportion of calcium present as free ions, and not bound by phytate for example, is not known.

The inclusion of GA_3 in the culture medium not only accelerated the rate of formation of α-amylase in seedlings and increased the maximum level that was attained, but also caused a more rapid and more complete disappearance of the enzyme (Fig. 2). Thus it was of interest to investigate the effects of adding a solution of GA_3 to the petri dishes at various times in an attempt to separate these effects (Table 5, Fig. 10). The most notable results were that GA_3, added to the grain early in the culture period, accelerated α-amylase formation and degradation just as when it was initially present in the culture fluid (Table 5). The addition of GA_3 later in the experimental period when the maximum level of α-amylase had been attained resulted in an earlier onset to the decline of α-amylase, while still later additions apparently resulted in a marginal increase in levels, relative to controls (Table 5, Fig. 10). Thus GA_3 accelerated the onset of the degradative processes at a time when the synthetic processes in the aleurone may have ceased to function.[43] Other experiments [75, 76], in

[58] P. KOLBACH and W. RINKE, *Monatsschr. Brau.* **16**, 11 (1963).
[59] H. B. TUKEY, *Bull. Torrey Botan. Club* **93**, 385 (1966).

which embryo growth was restricted, suggested that this may be due to the accelerated growth of the embryonic plant and hence a faster withdrawal from the liquefied starchy endosperm of materials that stabilise α-amylase. Certainly the growth of embryonic barley plants is accelerated by gibberellins *independently* of the increased supply of nutrients that is available in grain treated,[60] as is also true for wheat.[61] For example, in barley, the mean lengths with GA_3 of coleoptiles of embryos detached from the endosperm and cultured *in vitro* for 2 days on the same nutrient medium, with or without added gibberellic acid (100 μg/ml), were 1·55 and 1·19 cm respectively.

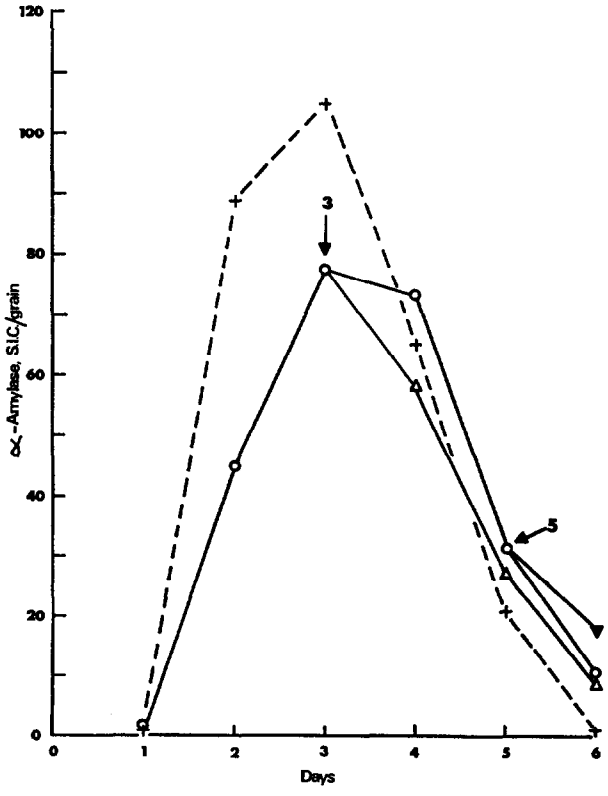

FIG. 10. EFFECT ON α-AMYLASE OF ADDING GA_3 (50 μg/ml) TO GERMINATING BARLEY.
—○—, No GA_3; - -+- -, GA_3 time 0; —△—, GA_3 added day 3; —▼—, GA_3 added day 5.

During trials, diffuse light reached some cultures causing slight greening of the first leaves. It has been reported that rice plants utilized their endosperm nutrient reserves more slowly in light than in darkness.[62] It was known that light alters the pattern of terpene formation in barley.[63] Light was also known to increase gibberellin synthesis by cultures of *Gibberella*.[64, 65] Consequently it seemed advisable to check the effects of light on the formation of

[60] A. B. SCHOOLER, *Agron. J.* **52**, 411 (1960).
[61] N. P. KEFFORD and A. H. G. C. RIJVEN, *Science* **151**, 104 (1966).
[62] T. M. DAS and J. M. CHOUDHURY, *Current Sci.* 64 (1960).
[63] T. M. GOODWIN, in *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), p. 57. Academic Press, London (1962).
[64] G. ZWEIG and J. E. DEVAY, *Mycologia* **51**, 877 (1959).
[65] D. Mertz and W. HENSON, *Nature* **214**, 844 (1967).

α-amylase in germinating grain in case gibberellin metabolism had been altered. When plants were strongly illumined with diffuse light the leaves became dark green. The growth of the leaves was enhanced and the growth of the coleoptiles was reduced, both in the presence and absence of exogenous gibberellic acid. There was however no significant difference between the weights of the endosperms and the levels of α-amylase of the control and illuminated plants (Table 6). Thus it seems unlikely that the diffuse illumination that was accidentally received by our samples altered the production of endogenous gibberellins.

The gibberellin-dependent formation of α-amylase is very sensitive to such growth conditions as temperature, and availability of water, so that these variables must be closely controlled. The failure of the α-amylase levels to go on rising may be due to a failure or exhaustion of the synthetic mechanisms of the aleurone cells or less probably to the rate of breakdown exceeding the rate of synthesis. The breakdown of the enzyme may provisionally be explained by postulating that the removal of starch proteins and calcium ions will decrease its inherent stability, and make it more susceptible to proteolytic attack. Further, as the pH of the endosperm drops the stability of the enzyme will decline and the activity of the proteases, which in

TABLE 6. EFFECTS OF LIGHT ON BARLEY α-AMYLASE AND GROWTH WITH AND WITHOUT GA_3 (100 μg/ml)

Day sampled	Condition	Coleoptile (cm)	1st leaf (cm)	Endosperm + scutellum (mg)	α-Amylase (S.I.C./ grain)
3	Dark control	3·6	—	27·2	52
	Dark + GA_3	4·6	—	22·9	60
	Light	2·8	3·9	26·3	53
	Light + GA_3	3·2	5·5	25·0	71
6	Dark control	4·0	11·5	11·8	11
	Dark + GA_3	5·0	15·2	8·6	0
	Light	2·9	12·8	11·1	10
	Light + GA_3	3·3	16·6	9·4	0

any case are increasing in amount, will rise. All these factors may contribute to the destruction of the α-amylase. To test the effects of reducing embryo growth further experiments were initiated (following communications). [75, 76]

EXPERIMENTAL

Barley Culture

Two-row barley Proctor, *Hordeum distichon* L., of the 1965 and 1966 harvests was decorticated with 50 per cent H_2SO_4[66] at 25° for a minimum period of time which varied from 70 to 90 min for different samples. The grain was subsequently washed and air-dried as previously described.[9, 15] Mellow grains were selected for all experiments.[67] Decorticated grains were disinfected by immersion for 1 hr in commercial sodium hypochlorite, 10–14 per cent (w/v), available chlorine, diluted 1/10 before use, then rinsed well × 8 with sterile, distilled water. Between the second and third rinses they were momentarily covered with a very dilute solution of I_2 in strong KI. Under these conditions the I_2 only penetrated the grain at faults in the testa, producing an easily detected blue-black colour with the starch in the endosperm, so allowing the rejection of damaged grain. Usually twenty-five corns were grown with 6 ml liquid in a 9-cm petri dish or forty corns with 10 ml liquid in an 11-cm petri dish, always with two × Whatman No. 1 filter paper discs. The pH of the culture medium containing any addition adjusted and was usually buffered to pH 5·3 with phosphate + succinate (each 1 mM) or phosphate (0·1 mM). Petri dishes, pipettes and solutions were sterilized before use.

[66] J. R. A. POLLOCK, R. E. ESSERY and B. H. KIRSOP, *J. Inst. Brewing* **61**, 295 (1955).
[67] R. E. ESSERY, B. H. KIRSOP and J. R. A. POLLOCK, *J. Inst. Brewing* **62**, 150 (1956).

Cultures involving many grains, as used when following changes in calcium levels, were carried out in washing-up bowls, covered with aluminium foil. Usually experiments were made at 25°, in an atmosphere saturated with water vapour. Samples were taken over a period of time, or after 2 days 18 hr germination, and excluded ungerminated grain, or grain obviously infected with micro-organisms. Samples were rinsed with cold distilled water and either frozen at −18° or dried in a flow of warm air, 35–45°, before extracting and estimating α-amylase, or any other component. Coleoptile length was measured on fresh samples that were often pre-chilled to 4° to slow alterations in their composition.

α-Amylase was extracted into a solution of calcium acetate (0·2 per cent, pH 6·0) at 70°/20 min, using 1·0 ml solution/grain or part of grain. This extraction inactivated interfering enzymes. Estimation involved following the decline in the power of solutions of soluble starch to give colour with an I_2 solution when incubated with diluted samples of these extracts. Results were calculated using standard graph principles, and were expressed in starch–iodine colour units (S.I.C.).[47, 68]

Sugars and amino acids were extracted from samples with three changes of hot, 90 per cent alcohol. The residue remaining after evaporation was washed with $CHCl_3$ and ether, to remove lipids, redissolved in water and analysed. Amino-acids were measured as leucine equivalents, with ninhydrin.[69] With sugar analyses erythritol and mannitol were added to the samples, during extraction, as internal standards. Dry aliquot portions were dissolved in pyridine and converted to the trimethylsilyl ethers with hexamethyldisilazane and trimethylchlorosilane.[70] The derivatives were analysed on a Pye 104 single-column gas chromatograph with a flame ionization detector using 1·5-m columns of 3 per cent SE-30 on siliconized 100/120 gas-chrom Z or 1·5-m column of 5 per cent SE-52 on siliconized celite using argon carrier gas and temperatures rising from 150° to 300 or 320° respectively. The results were calculated from the weights of the paper cut from the recorded peak areas, a method found valid for known standard mixtures. Starch, and protein, were measured in the endosperm following an alcohol extraction. Starch was precipitated with acetone from 33 per cent chloral extracts of finely milled endosperm, and dried to constant weight.[71] Protein was estimated with the Folin–Ciocalteau reagent following Jennings procedure.[72] pH values were determined on frozen, dissected endosperms, homogenized with water (0·1 ml/endosperm). Titrations were carried out on 10-ml samples of extracts made from frozen endosperms (100), homogenized with distilled water (50 ml).

A variety of methods were used in attempts to estimate total calcium. The method finally adopted was as follows: Samples were ashed in acid- and EDTA-washed crucibles in an electric muffle-furnace, at dull red heat, with an addition of HNO_3 before the end.[73] The ash was dissolved in 2 N HCl and the HCl was evaporated to dryness on a boiling water-bath, to precipitate silica. The calcium was dissolved in distilled water and absorbed by a short column of Amberlite IR-120 (H^+). The calcium was eluted with 1/3 conc. HNO_3 and the solution was evaporated to dryness. The calcium salts were redissolved in distilled water (5 ml) and the calcium was estimated spectrophotometrically by measuring the quantity of ceric sulphate used to oxidize the calcium oxalate precipitate from a 2·0-ml sample.[74]

Acknowledgements—I wish to thank Dr. J. S. Hough for reading the manuscript, Janet Rostance, Janet Phillips and Susan Candy for their expert technical assistance; the Maltsters' Association of Great Britain for a grant, and Dr. A. Macey of Associated British Maltsters (Newark) for supplies of barley.

[68] D. E. Briggs, *Enzymologia* **24**, 97 (1962).
[69] H. Rosen, *Arch. Biochem. Biophys.* **67**, 10 (1957).
[70] C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Am. Chem. Soc.* **85**, 2497 (1963).
[71] I. C. MacWilliam, R. D. Hall and G. Harris, *J. Inst. Brewing* **62**, 226 (1956).
[72] A. C. Jennings, *Cereal Chem.* **38**, 467 (1961).
[73] G. Middleton and R. E. Stuckey, *Analyst.* **79**, 138 (1954).
[74] A. H. Cornfield and A. G. Pollard, *J. Sci. Food Agri.* **2**, 135 (1951).
[75] D. E. Briggs, *Phytochem.* **7**, 531 (1968). This issue.
[76] D. E. Briggs. *Phytochem.* **7**, 539 (1968). This issue.