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α-AMYLASE IN GERMINATING, DECORTICATED BARLEY—III.

EFFECTS OF ADDING CCC AND OTHER CHEMICAL SUBSTANCES

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Abstract—Chlorocholine chloride, CCC, has been shown to inhibit growth and the formation of α-amylase in germinating barley. The addition of gibberellic acid, GA_3, did not fully reverse the inhibition of growth caused by CCC, but α-amylase synthesis increased to the level found in grains cultured with GA_3 only. Thus CCC had a toxic effect on the embryo in addition to suppressing the endogenous formation of gibberellins. In the presence of GA_3 and large quantities of CCC α-amylase synthesis in barley was slow relative to the rate of enzyme formation with GA_3 only, but the rate of decline in enzyme was also retarded. Choline gave results generally similar to those produced by CCC, when applied to germinating barley. High levels of potassium sulphate slowed enzyme degradation but not synthesis in grain germinated with or without GA_3. Some twenty-three compounds were added to germinating barley in the presence and absence of gibberellic acid, and the results were interpreted in terms of varying damaging effects towards the endogenous production of gibberellins, embryo growth, and the system synthesizing α-amylase in the aleurone layer. The results suggest that choline, hordenine and other naturally occurring basic substances may regulate the endogenous synthesis of gibberellins in germinating barley.

INTRODUCTION

THE total removal of embryos from barley about 3 days after starting germination, under malting conditions, but not before, does not appreciably slow the malting process[1] nor does checking the growth of the embryo at this stage by a range of other chemical or physical treatments. Thus either sufficient gibberellin has been formed or gibberellin formation is not impaired so that the gibberellin-dependent formation of enzymes can proceed, or still again sufficient enzymes have been formed at this stage to allow the process to continue. Such treatments applied during malting with or without additional treatment with gibberellic acid, GA_3, reduce the respiration of the embryo and its growth, in particular the production of rootlets, while allowing the production of enzymes to continue. Consequently malt is obtained in higher yield because losses of dry matter to roots and as carbon dioxide and water are reduced. For example acetic acid has been used as a growth retardant in experimental malting.[2]

Substances have been tested on barley to investigate the physiology of the grain as well as to reduce malting losses. However results are often difficult to explain as the observed effects may be due to a general toxic effect on the embryo and the aleurone, a toxic effect confined to the embryo, or a selective metabolic inhibition resulting in a reduction in the quantity of gibberellin synthesized by the embryo. Because as previously outlined,[3,4] α-amylase synthesis in barley is apparently entirely dependent on a supply of gibberellins or

[1] B. H. KIRSOP and J. R. A. POLLOCK, *J. Inst. Brewing* **64**, 227 (1958).
[2] M. H. SPILLANE and D. E. BRIGGS, *J. Inst. Brewing* **72**, 398 (1966).
[3] D. E. BRIGGS, *Phytochem.*, previous communication, **7**, 513 (1968).
[4] D. E. BRIGGS, *Phytochem.*, previous communication, **7**, 531 (1968).

gibberellin-like substances coming either from the embryo or from the exterior of the grain, the appearance of this enzyme indicates the presence of such a substance and a viable aleurone layer.

Ionized chemical substances seem only to penetrate undamaged barley caryopses near to the embryo, while un-ionized substances penetrate the entire surface of the grain. For example we find that in decorticated grain, as in entire,[5,6] iodine will penetrate the entire surface of the decorticated grain from solution as shown by the blue–black colour given with the starch in the endosperm. When the iodine is present in solution with an excess of potassium iodide it is predominantly present as the I_3^- ion and penetration occurs only slowly, and only at the embryo end of the grain or rapidly through faults in the testa, so providing a rapid test for defective grains. Indirect evidence indicates that gibberellic acid penetrates at the embryo.[7] It is assumed that most ionized toxic substances also enter the grain locally, in the micropylar region. The pattern of results reported here generally supports this theory. Once the coleorhiza has split the testa embryonic tissues are directly exposed to substances in the culture solution. The fact that, in the presence of GA_3 and a toxic agent, embryo growth may be reduced while α-amylase synthesis is unimpaired indicates that although GA_3 has reached the aleurone and triggered the formation of enzymes the toxic agent evidently has not passed the embryo.

Chlorocholine chloride (CCC, 2-chloroethyl trimethyl ammonium chloride) is of interest for its dwarfing effects on wheat and barley although commercial trials testing its ability to combat lodgings have been disappointing.[8,9] This substance is known to suppress gibberellin formation in some strains of *Gibberella fujikuroi*[10–12] but not others,[13] and in isolated barley embryos[14,15] as in embryos of *Avena fatua*.[16] This blockage caused by CCC may occur on the biosynthetic pathway between kaurene and GA_3.[17] When added to germinating barley grains it slows germination and the formation of α-amylase.[18,19] However, this substance may dwarf plants by other mechanisms besides that involving a reduction in the synthesis of endogenous gibberellins. For example, the application of CCC alters the pattern of indoles in wheat seedlings[20] and the effects of CCC on several test systems are not reversed by adding GA_3.[21] Some effects of indoles and coumarin on lettuce may be reversed by the addition of CCC.[22,23] None of these effects of CCC appear to be related to the formation of gibberellins. CCC is known to activate isolated plant choline kinase.[24] As well as being a constituent of

[5] E. J. COLLINS, *Ann. Botany London* **32**, 381 (1918).
[6] W. H. THARP, *Botan. Gaz.* **97**, 240 (1935).
[7] D. E. BRIGGS, *J. Inst. Brewing* **70**, 14 (1964).
[8] D. W. A. BARRETT, B. E. MEENS and G. C. MEES, *J. Agr. Sci.* **68**, 39 (1967).
[9] J. R. GOODIN, C. M. MCKELL and F. L. WEBB, *Agron. J.* **58**, 453 (1966).
[10] H. KENDE, H. NINNEMANN and A. LANG, *Naturwissenschaften* **18**, 599 (1963).
[11] H. NINNEMANN, J. A. D. ZEEVAART, H. KENDE and A. LANG, *Planta Berlin* **61**, 229 (1964).
[12] H. HARADA and A. LANG, *Plant Physiol.* **40**, 176 (1965).
[13] D. MERTZ, *Plant Physiol.* **41** (Suppl.), viii (1966).
[14] H. YOMO and H. IINUMA, *Planta Berlin* **71**, 113 (1966).
[15] M. RADLEY, *Planta Berlin* **75**, 164 (1967).
[16] G. M. SIMPSON, *Can. J. Botany* **44**, 115 (1966).
[17] D. J. DENNIS, C. D. UPPER and C. A. WEST, *Plant Physiol.* **40**, 948 (1965).
[18] N. PRENTICE, A. D. DICKSON, B. A. BURKHART and N. N. STANDRIDGE, *Cereal Chem.* **40**, 208 (1963).
[19] A. A. KHAN and M. A. FAUST, *Plant Physiol.* **41** (Suppl.), lxxviii (1966).
[20] R. F. NORRIS, *Can. J. Botany* **44**, 675 (1966).
[21] S. KURAISHI and R. M. MUIR, *Plant Physiol.* **38**, 19 (1963).
[22] A. A. KHAN and N. E. TOLBERT, *Plant Physiol.* **40** (Suppl.), vii (1965).
[23] A. A. KHAN and N. E. TOLBERT *Physiol. Plantarum* **19**, 81 (1966).
[24] K. TANAKA and N. E. TOLBERT, *Plant Physiol.* **41**, 313 (1966).

lipids and occurring free,[25] choline also occurs in barley in substantial amounts as a sulphate and a phosphate.[26–28]

CCC is converted into choline in the barley plant.[29] The addition of choline to germinating barley also reduces growth.[30] Another quaternary ammonium compound that occurs naturally in barley is candicine, 2-(*p*-hydroxyphenyl)-ethyl trimethyl ammonium chloride.[31, 32] Candicine does not readily penetrate barley, but hordenine, which is readily methylated *in vivo* to form candicine, does.[32] Thus it was of interest to test the effects of adding choline, hordenine (which is another normal constituent of barley)[33] and other basic substances known to occur within the grain, to discover if they had the capacity to act as natural endogenous regulations of gibberellin synthesis.

N-Dimethylamino succinamic acid, B-995, is another material that reduces theg rowth of plants.[34] It is known to inhibit amine oxidases[35] but not to block the synthesis of GA_3 by *Gibberella*.[11]

Morphactins, derivatives of 9-hydroxyfluorene-9-carboxylic acid, when applied to plants check growth, and so result in dwarfs.[36–38] Morphactin IT-3233 (9-hydroxyfluorene-9-carboxylic acid *n*-butyl ester)[36] and morphactin IT-3456 (a mixture of isomers, mainly 2-chloro-9-hydroxyfluorene-9-carboxylic acid methyl ester)[36] were tested both because of their dwarfing effects and because of their superficial structural resemblance to gibberellins. Morphactins do not prevent the production of gibberellins by *Fusarium oxyformis*[39] and, despite conflicting claims in some systems at least, including the production of α-amylase by half-corns of barley, they do not act as competitive antagonists to GA_3,[40] Thus it was of interest to test their effects on germinating barley, and to discover if they alter gibberellin metabolism in the intact grain.

RESULTS AND DISCUSSION

Grain was grown for 2¾ days in the presence and absence of gibberellic acid (GA_3, 50 μg/ml), and in the presence of various concentrations of glucose or proline (0–100 mM). These substances were chosen for their respective abilities to suppress and support α-amylase formation in isolated barley embryos.[7] The results gave no indication of specific effects on the enzyme levels found in either the embryos or endosperms of the grain. However, glucose at 100 mM and, to a lesser extent, at 40 mM slightly inhibited coleoptile growth both in the presence and in the absence of GA_3. In similar tests a range of calcium chloride concentrations showed no effects on α-amylase levels that could be called specific (Fig. 1), even though the calcium ion forms an integral part of the α-amylase molecule. A previous test with a low

[25] H. T. Brown, *J. Inst. Brewing* **13**, 394 (1907).
[26] J. V. Maizel, A. A. Benson and N. E. Tolbert, *Plant Physiol.* **31**, 407 (1956).
[27] P. Nissen and A. A. Benson, *Science* **134**, 1759 (1961).
[28] P. Nissen and A. A. Benson, *Plant Physiol.* **39**, 586 (1964).
[29] E. F. Schneider, *Can. J. Biochem.* **45**, 395 (1967).
[30] A. A. Khan and M. A. Faust, *Nature* **211**, 1215 (1966).
[31] S. R. Lee, *Seoul Univ. J. Nat. Sci.* **7B**, 24 (1958).
[32] G. Rabitzsch, *Planta Med.* **7**, 268 (1959).
[33] E. Léger, *Compt. Rend.* **142**, 108 (1906).
[34] J. A. Riddell, H. A. Hageman, C. M. J'Anthony and W. L. Hubbard, *Science* **136**, 391, 1044 (1962).
[35] D. J. Reed, T. C. Moore and J. D. Anderson, *Science* **148**, 1469 (1965).
[36] E. Merck, A. G. Darmstadt, *Technical data sheets* (1965).
[37] G. Schneider, *Naturwissenschaften* **51**, 416 (1964).
[38] G. Schneider, D. Erdmann, S. Lust, G. Mohr and K. Niethammer, *Nature* **208**, 1013 (1965).
[39] F. J. Richards and R. G. Coleman, *Nature* **170**, 460 (1952).
[40] T. A. Smith and F. J. Richards, *Biochem. J.* **84**, 292 (1962).

concentration of calcium sulphate also gave a negative result.[3] At high concentrations of calcium chloride (40 mM and 100 mM) coleoptile growth was substantially reduced, and in grains grown in the additional presence of GA_3 the level of α-amylase was markedly increased. The effect of a non-calcium containing salt, potassium sulphate, was tested to see if calcium ions were specifically involved in stabilizing α-amylase, or stimulating its synthesis, or if the result obtained with high concentrations of calcium sulphate was due to a non-specific effect, such as high osmotic pressure.

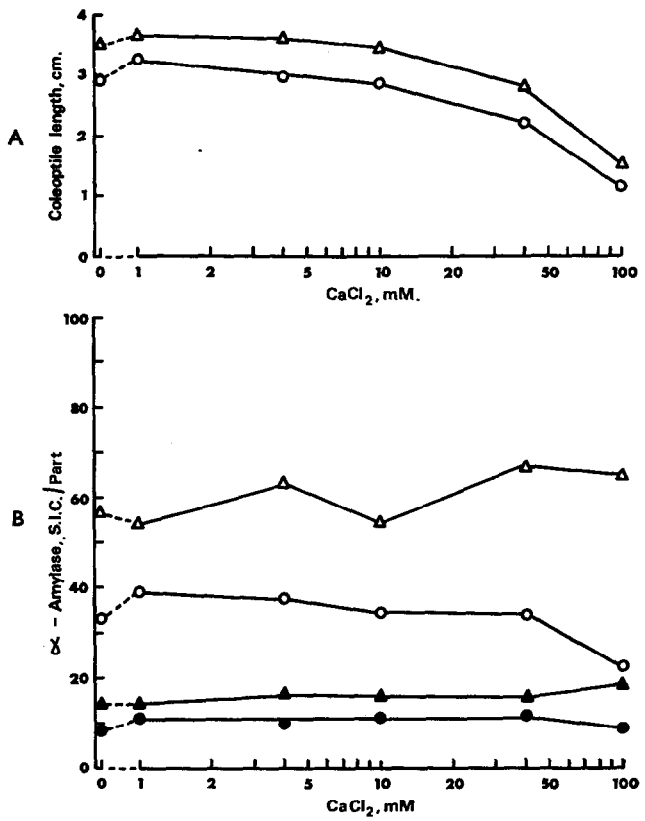

FIG. 1. EFFECT OF VARIOUS CONCENTRATIONS OF CALCIUM CHLORIDE ON COLEOPTILE LENGTH AND α-AMYLASE IN BARLEY GERMINATED WITH OR WITHOUT GA_3 (50 μg/ml).

A. Coleoptile length. —△—, —○—, with and without GA_3.

B. α-Amylase. —○—, —●—, endosperm, embryo.
—△—, —▲—, endosperm, embryo, with GA_3.

The higher concentrations of potassium sulphate that were tested markedly restricted coleoptile growth, both in the presence and absence of gibberellic acid, and they also suppressed α-amylase formation in barley grown in the absence of GA_3 (Fig. 2). In the presence of GA_3 and moderate amounts of potassium sulphate (50 mM) unusually high levels of α-amylase were found, while in grain grown in the absence of GA_3 but with this concentration of potassium sulphate (50 mM) enzyme levels were only slightly suppressed, relative to controls. These samples were taken at a time when peak activity in α-amylase was known to occur in GA_3-treated grains and it was possible that experimental applications of salts

altered the time at which peak enzyme activities occurred. To test this time-course experiments were run with and without GA_3 and with and without potassium sulphate. The results illustrate in a striking fashion that potassium sulphate allowed a greater maximum level of α-amylase in barley corns, both with and without exogenous GA_3, and caused a delay in the onset of the breakdown processes (Fig. 3). Potassium sulphate evidently did not inhibit the

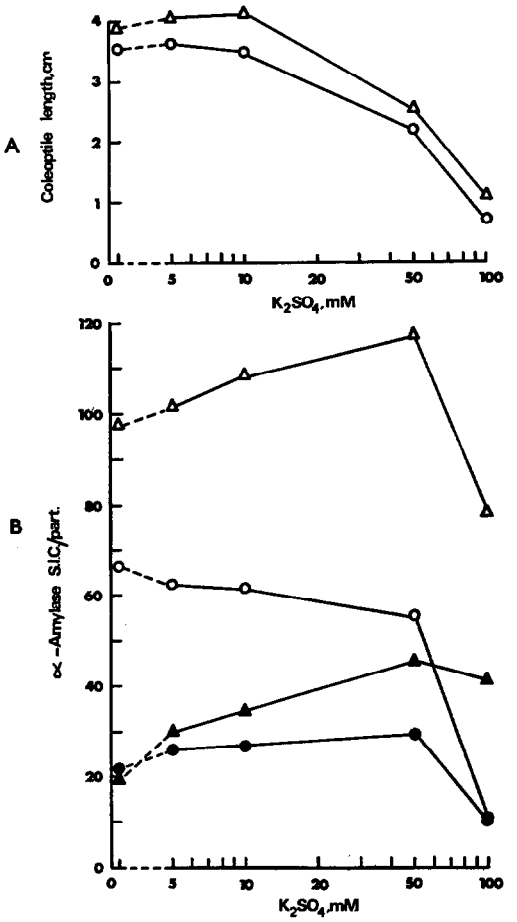

FIG. 2. EFFECT OF POTASSIUM SULPHATE ON COLEOPTILE LENGTH, AND α-AMYLASE IN BARLEY GERMINATED WITH AND WITHOUT GA_3 (50 μg/ml).

A. Coleoptile length. —△—, with GA_3; —○—, without GA_3.

B. α-Amylase. —○—, —●—, endosperm, embryo.
—△—, —▲—, endosperm, embryo, with GA_3.

formation of endogenous gibberellins and may even have enhanced their formation to a slight extent. The greater quantity of enzyme found in the presence of potassium sulphate might be explained on the basis of the hypothesis previously advanced[3, 4] that the salt, by restricting embryo growth, limited the withdrawal of calcium and other α-amylase-stabilizing substances from the starchy endosperm and so indirectly slowed enzyme breakdown. The greater maximum level of enzyme attained indicates that enzyme synthesis and degradation were proceeding simultaneously at this time. In the light of these and other results given

below it is interesting that putrescine and agmatine accumulate in barley plants that are deficient in potassium.[39, 40]

The effects of various concentrations of chlorocholine chloride, CCC, were tested on germinating grain to discover if this substance, which was expected to inhibit both embryo growth and the formation of endogenous gibberellins, would give results parallel to those obtained with grains in which embryos were burnt[4] (Fig. 4). GA_3 did not reverse the CCC-induced inhibition of coleoptile growth. It entirely overcame the inhibition of α-amylase

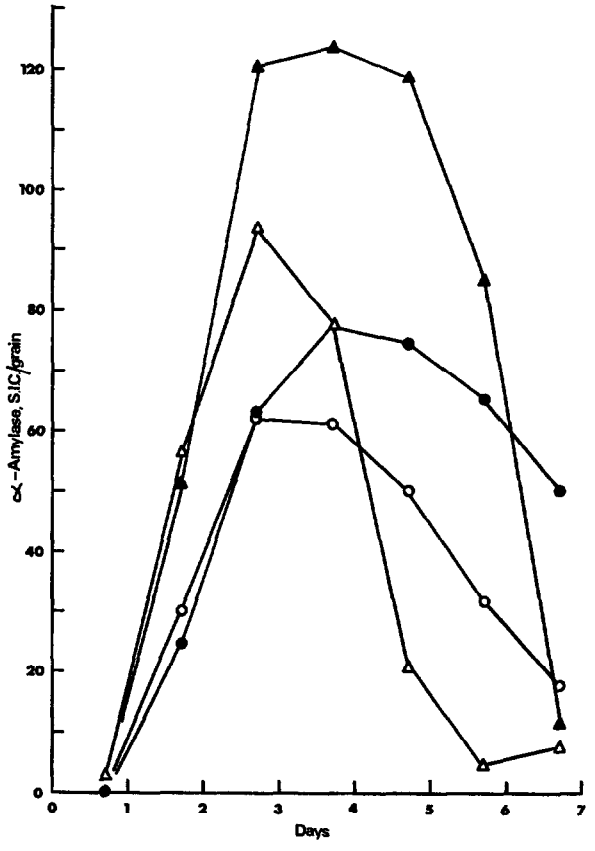

FIG. 3. α-AMYLASE IN BARLEY GERMINATED WITH AND WITHOUT POTASSIUM SULPHATE (40 mM), WITH AND WITHOUT GA_3 (50 μg/ml).

—○— No additions —●— K_2SO_4
—△— GA_3 —▲— K_2SO_4 and GA_3.

synthesis, however, so the effect of CCC on growth is not merely due to an inhibition of endogenous gibberellin formation, but also to some other toxic effect on the embryo. CCC was known not to alter the response of the barley aleurone to GA_3.[41] CCC did not interfere with any "potentiating factor" moving from the embryo to the endosperm during the hydration process, or with other hydration effects,[42–44] since the endosperm level of α-amylase in

[41] L. G. PALEG, H. KENDE, H. NINNEMANN and A. LANG, *Plant Physiol.* **40**, 165 (1965).
[42] C. PETRIDIS, R. VERBEEK and L. MASSART, *J. Inst. Brewing* **71**, 469 (1965).
[43] A. M. MACLEOD, J. H. DUFFUS and D. J. L. HORSFALL, *J. Inst. Brewing* **72**, 36 (1966).
[44] K-H. YUNG and J. D. MANN, *Plant Physiol.* **42**, 195 (1967).

grain grown in the presence of exogenous GA_3 was the same at all the tested concentrations of CCC. In the absence of GA_3 increasing concentrations of CCC inhibited the production of α-amylase proportionately more in the endosperm than the embryo. This result was to be expected since the embryo is the source of endogenous gibberellins, and therefore a reduced quantity of gibberellin would have been only locally available to trigger enzyme synthesis.

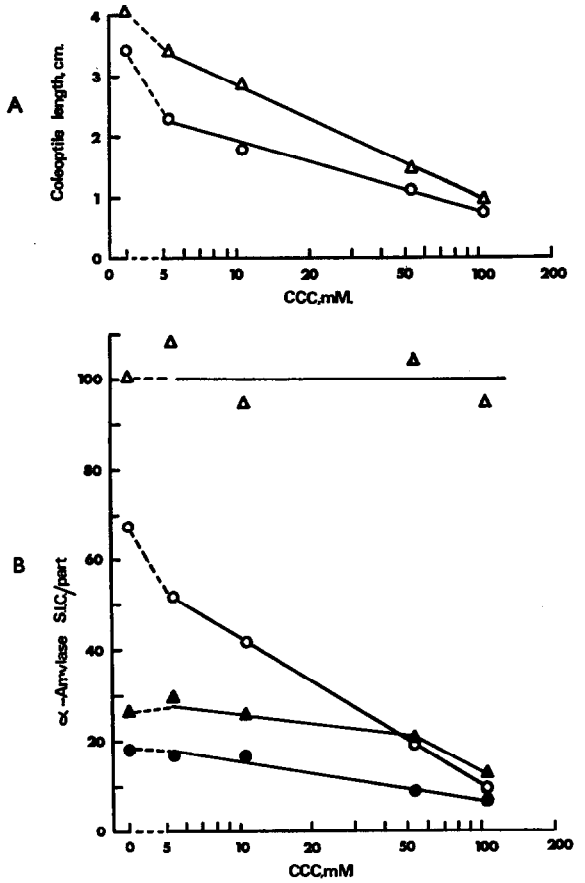

FIG. 4. COLEOPTILE LENGTH AND α-AMYLASE IN BARLEY CULTURED WITH CCC, WITH AND WITHOUT GA_3 (10 μg/ml).

A. Coleoptile length. —○— no GA_3; —△— with GA_3.

B. α-Amylase. —●—, —○—, embryo, endosperm.
—▲—, —△—, embryo, endosperm, with GA_3.

The addition of CCC together with GA_3 slightly retarded the initial rate of enzyme formation in the grain (Fig. 5) compared to GA_3 alone. The maximum amount of enzyme formed was greater and the onset of the decline in the quantity of enzyme was retarded. CCC added to germinating grain at different times during the growth period partly separated the effects associated with inhibiting enzyme formation, and reducing the rate of enzyme breakdown, presumably through reducing the synthesis of endogenous gibberellins, and inhibiting embryo growth respectively (Figs. 5 and 6). Since the pH of the endosperm homogenates was the same whether or not CCC had been in the culture medium, pH cannot have been a

significant factor. Thus adding CCC to the cultures on day 0 and on day 1 (50 mM or 100 mM) sharply reduced the quantity of enzyme formed. Addition of CCC on days 2 and 3 prevented the maximum amount of enzyme being formed, and marginally retarded the onset of enzyme degradation, at least at the higher dose level (Figs. 5 and 6). The addition of CCC on days 4 and 5 checked the decline in enzyme activity for about a day (Fig. 6.)

The results of an experimental attempt to totally stop plant growth and enzyme synthesis in the whole grain growing with GA_3 by the addition of the toxic uncoupling agent 2,4-dinitro-

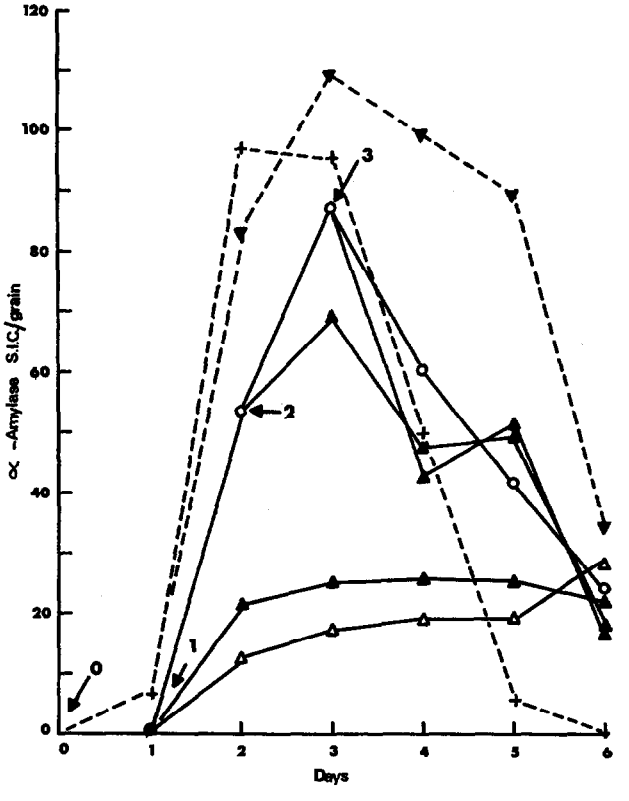

FIG. 5. α-AMYLASE IN BARLEY GERMINATING WITH AND WITHOUT GA_3 (50 μg/ml), WITH CCC (50 mM) ADDED AT DIFFERENT TIMES.

- - - + - - -, GA_3; - - - ▼ - - -, GA_3 and CCC, day 0.
—○—, no additions; —△—, CCC, day 0.
—▲—, CCC added days 1, 2, 3, as indicated by the arrows.

phenol, DNP, did not work in the anticipated way, but the results were interesting (Table 1). DNP was known to completely inhibit enzyme formation in separated parts of the grain.[7, 45] The irregularity of the present results arose from the ability of some few grains to grow at once in the presence of DNP and of others to begin growing after an initial lag. Some of the seedlings used in this trial became coloured orange. The addition of DNP after 47 hr growth only marginally slowed the final decline of α-amylase, but additions made at 69 hr and 93 hr slowed the enzyme breakdown process to a noticeable extent.

[45] D. E. BRIGGS, *J. Inst. Brewing* **69**, 13 (1963).

A survey was made of the effects of adding a variety of substances to the culture media of germinating grains to pick out other toxic agents useful in these studies. It was found that choline, as indicated later, showed properties similar to CCC, but that betaine did not, although at 5 mg/ml it began to reduce coleoptile growth but not α-amylase levels. Hydroxy-

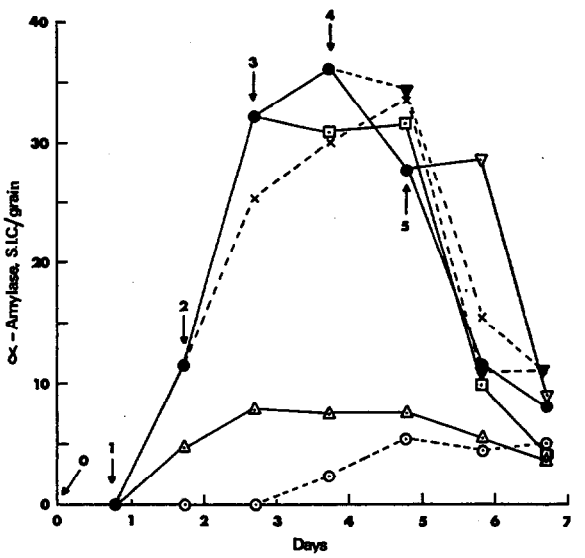

FIG. 6. α-AMYLASE IN GERMINATING BARLEY TO WHICH CCC (100 mM) WAS ADDED AT VARIOUS TIMES.
—●—, no additions.
CCC added: - - ○ - -, day 0; —△—, day 1; - - × - -, day 2; —□—, day 3; —▼—, day 4; —▽—, day 5.

TABLE 1. EFFECTS ON α-AMYLASE OF ADDING 2,4-DINITROPHENOL (DNP 24 mM) TO BARLEY GRAINS GROWING IN THE PRESENCE OF GA_3 (100 μg/ml)

	α-Amylase (S.I.C./grain)							
	Time (hr)							
	0	21	47	69	93	117	140	164
Additions	H_2O	9·4	83	72	24	21	2·7	0
	DNP	4·4	0	4·9	5·5	0	2·0	0
			H_2O	66	36	16	0	0
			DNP	70	42	11	7·4	2·7
				DNP	50	32	12	0
					DNP	22	15	3·1

proline (10 mM), which has been reported to inhibit the growth of isolated *Avena* coleoptiles,[46] slightly inhibited α-amylase formation unless GA_3 was present. Histamine (40 mM), which is said to occur in small quantities in barley,[47] caused a sharp reduction in α-amylase formation unless GA_3 was also present. The details of studies with hordenine (N-dimethyl

[46] R. CLELAND, *Plant Physiol.* **39**, (Suppl.), iv (1964).
[47] U. VON HAARTMANN, G. KAHLSON and C. STEINHARDT, *Life Sci.* **5**, 1 (1966).

tyramine) are given later. The related compound candicine, into which hordenine is converted in the barley grain,[32] was less toxic, on an equal weight basis, as is expected from its relative inability to penetrate the grain. The results of several other preliminary trials are summarized in Table 2, where the degree of damage caused by the levels of the compounds tested is of interest. The fact that most compounds, especially those that are ionized, may only have penetrated the grain at the embryo, probably accounted for several features; thus, where the toxicity was selective towards the embryo, in that coleoptile growth was reduced and gibberellin production was reduced, as inferred from a reduction in the quantity of α-amylase present, and the enzyme-synthesizing capacity of the aleurone layer was not destroyed, as shown by the production of enzyme in response to added GA_3.

GA_3 and some toxic substances acting together produced levels of α-amylase equal to or greater than that obtained with GA_3 only, but with minimal coleoptile growth. In these cases

TABLE 2. EFFECT OF SUBSTANCES ON COLEOPTILE GROWTH AND α-AMYLASE FORMATION IN BARLEY GRAINS, WITH OR WITHOUT GA_3 (50 OR 100 μg/ml)

Additions	α-Amylase (% controls)		Coleoptile length (%)	
	0	GA_3	0	GA_3
Tyrosine, 40 mM	72	98	98	71
Tyramine, 40 mM	18	69	43	42
Tryptophan, 40 mM*	49	88	31	38
Tryptamine, 40 mM	10	114	0	13
Gramine, 40 mM	31	96	37	38
Adrenaline, 40 mM*	0·5	64	34	38
Ephedrine, 40 mM*	40	107	63	63
Agmantine, 40 mM	53	60	63	65
Putrescine, 40 mM	53	95	77	66
Spermine, 40 mM	1	118	0	0
6-Mercaptopurine, 20 mM (suspension)	97	115	90	83
Copper sulphate, 2 mM	37	120	40	42
DL-*p*-Fluorophenyl-alanine, 5 mM	26	151	32	34
N-Ethylmaleimide, 5 mM	0	0	3	5

* Treated grains had a scorched appearance.

embryo growth was prevented without the leakage associated with burning or drilling experiments.[4] Of these substances tryptamine and spermine, which is known to occur in barley,[48] were extreme cases and were chosen for further study.

Hordenine was shown to be usefully toxic towards barley grains and was known to be methylated *in vitro* to candicine, which may be regarded as an analogue of CCC.[32] Candicine was known to penetrate barley only with difficulty[32] so it seemed logical to test chloroethyldimethylammonium chloride, CDC, in the hope that it might penetrate barley more readily than CCC and be converted into it by methylation *in vivo*. In the presence of GA_3 and low concentrations of CDC the level of α-amylase in treated grain was increased at a dose that was without effect on coleoptile length (Fig. 7). Unlike CCC, moderate concentrations of CDC did not interfere with the endogenous formation of gibberellins since α-amylase formation was not suppressed (Fig. 7), and so CDC probably was not converted into CCC in substantial amounts.

[48] G. MORUZZI and C. M. CALDERERA, *Arch. Biochem. Biophys.* **105**, 209 (1964).

Choline has not shown the biological effects of CCC in some tests[49] although CCC is converted into choline in plant tissues.[29] With our test conditions, choline gave results qualitatively similar to those given by CCC (Fig. 8). When the choline dose–α-amylase response graph was compared with that given by CCC (Fig. 4), it was seen that the decline in the

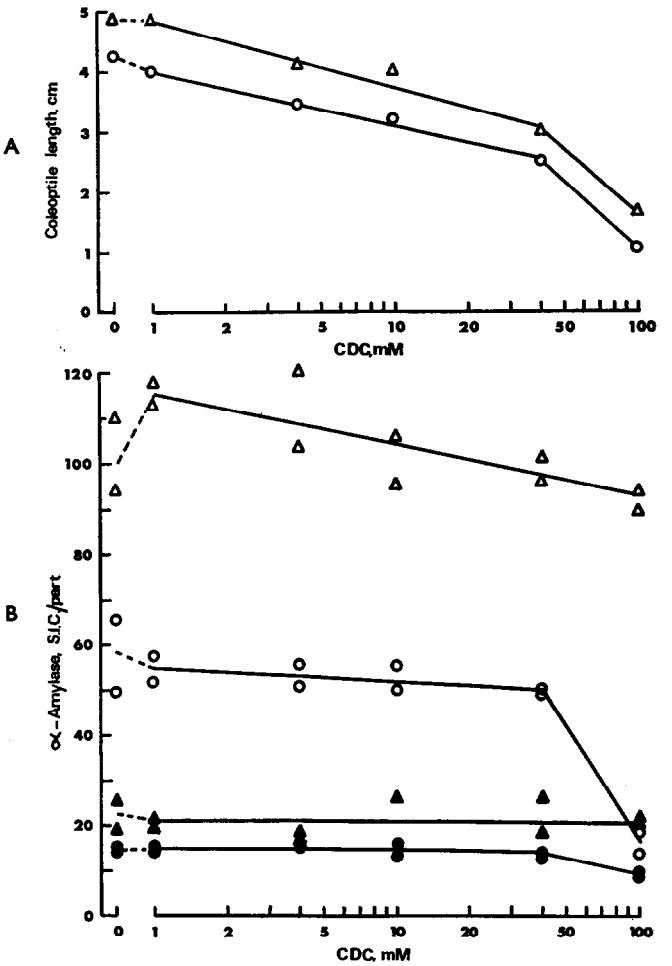

FIG. 7. EFFECTS OF CHLOROETHYL DIMETHYL AMMONIUM CHLORIDE, CDC, ON COLEOPTILE LENGTH AND α-AMYLASE LEVELS IN BARLEY GERMINATED WITH AND WITHOUT GA_3 (50 μg/ml).

A. Coleoptile length. —Δ—, with GA_3; —○—, without GA_3.

B. α-Amylase. —○—, —●—, endosperm, embryo.
—Δ—, —▲—, endosperm, embryo, with GA_3.

quantity of α-amylase present in the endosperm and the decline in the length of the coleoptiles were not directly proportional to the logarithm of the dose of choline. The rate of formation of α-amylase in the presence of gibberellic acid was retarded by increasing concentrations of choline, but the onset of the decline in enzyme activity was postponed (Fig. 9) so that the area of the peak on the graph was increased.

[49] N. E. TOLBERT, *J. Biol. Chem.* **235**, 475 (1960).

Trials with spermine and tryptamine (Figs. 10 and 11) gave quantitatively different results. In each case in the absence of an external supply of gibberellin α-amylase formation was sharply reduced, indicating that the production of endogenous gibberellins, as well as growth, was greatly inhibited. In the presence of an external supply of GA_3 the synthesis of enzyme

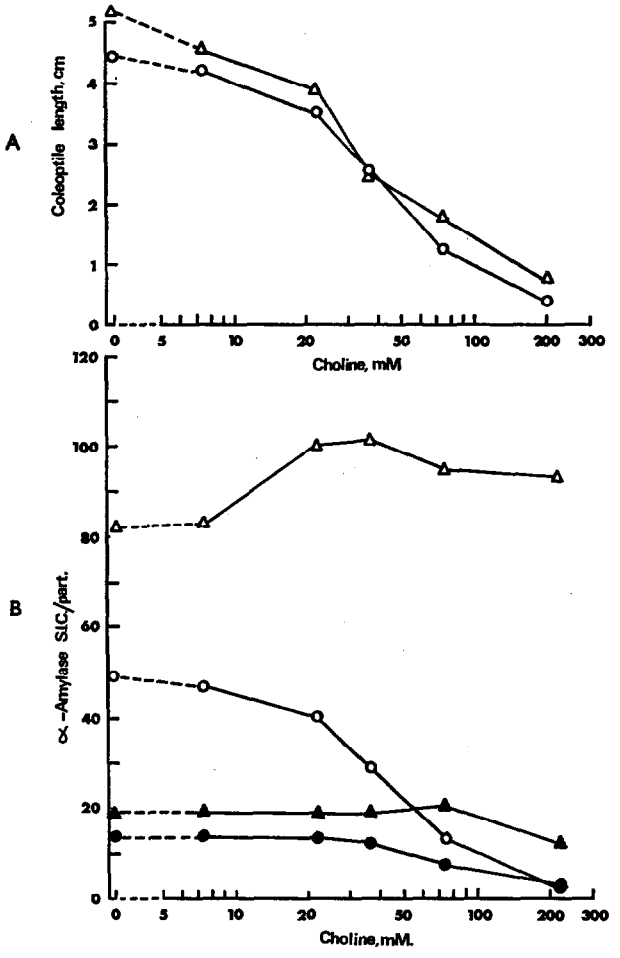

FIG. 8. EFFECTS OF CHOLINE ON COLEOPTILE LENGTH AND α-AMYLASE IN BARLEY GERMINATED WITH AND WITHOUT GA_3 (50 μg/ml).

A. Coleoptile length. —△—, with GA_3; —○—, without GA_3.

B. α-Amylase. —●—, —○—, embryo, endosperm.
—▲—, —△—, embryo, endosperm, with GA_3.

proceeded at a reduced rate, so the aleurone had remained at least partly viable. These substances also greatly reduced the rate of enzyme inactivation.

Varying the quantity of hordenine, with or without GA_3, showed that the coleoptile length and α-amylase, in the absence of GA_3, were not altered by 5 mM hordenine, but in the presence of hordenine and GA_3 the quantity of α-amylase produced was increased (Fig. 12), as was found with CDC, another substituted N-dimethyl ethylamine. As in many

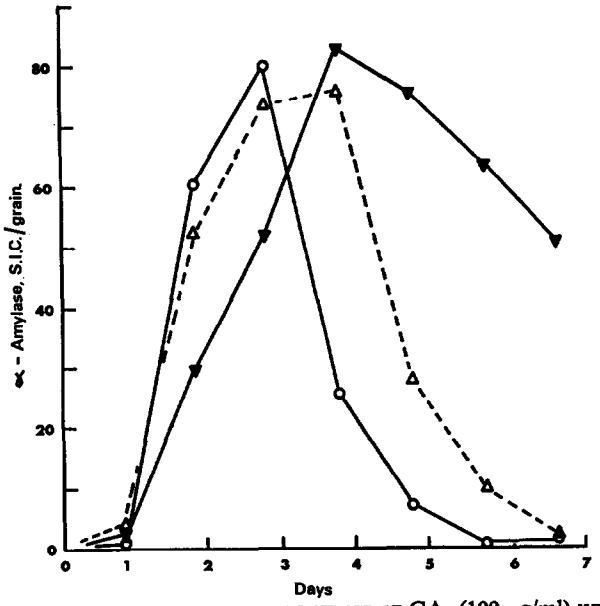

FIG. 9. α-AMYLASE IN BARLEY GROWING IN THE PRESENCE OF GA_3 (100 μg/ml) WITH AND WITHOUT CHOLINE.

—○—, GA_3.
- - △ - -, GA_3 and choline (40 mM).
—▼—, GA_3 and choline (200 mM).

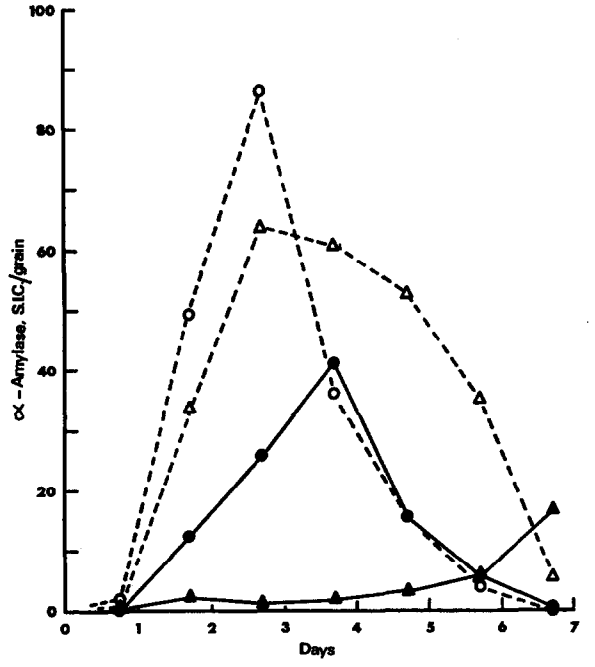

FIG. 10. α-AMYLASE IN BARLEY GERMINATING IN THE PRESENCE AND ABSENCE OF SPERMINE (40 mM) AND GA_3 (100 μg/ml).

—●— No additions —▲— Spermine
- -○- - GA_3 - -△- - GA_3 and spermine.

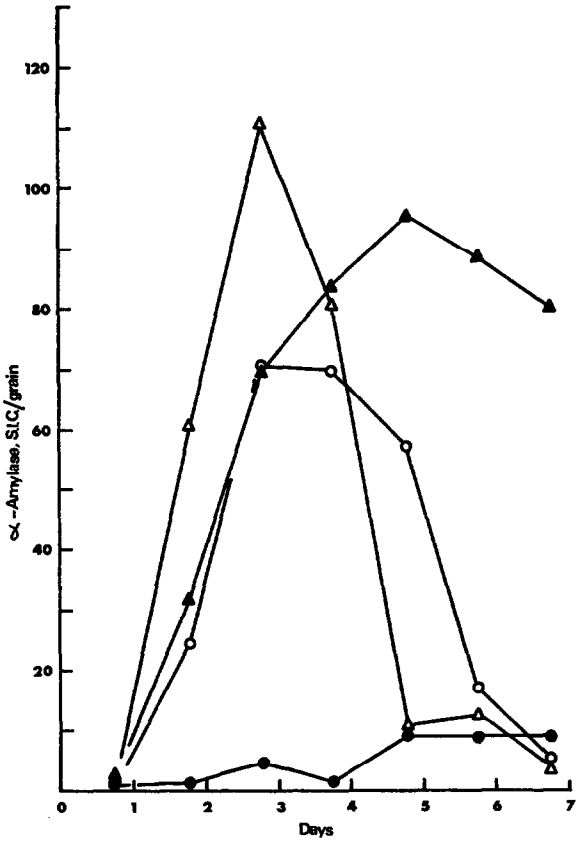

FIG. 11. α-AMYLASE IN BARLEY, GERMINATING IN THE PRESENCE AND ABSENCE OF TRYPTAMINE (40 mM) AND GA_3 (100 μg/ml).

—○— No additions —●— Tryptamine
—△— GA_3 —▲— GA_3 and tryptamine.

TABLE 3. EFFECT OF ADDITIONS OF N-DIMETHYLAMINO-SUCCINAMIC ACID (B-995) TO BARLEY GROWN FOR 2¾ DAYS WITH AND WITHOUT GA_3

	Additions of GA_3			
	0		100 μg/ml	
Final concentration of B-995 (mM)	Coleoptile length (cm)	α-Amylase (S.I.C./grain)	Coleoptile length (cm)	α-Amylase (S.I.C./grain)
0	3·5	47	4·5	86
1	3·3	34	3·9	94
4	3·6	47	4·2	82
10	3·2	46	3·6	97
40	0·8	9·1	2·3	82
100	0·4	2·4	0·9	60

of the trials reported here, the growth of the roots had been restricted, although the coleoptile length had not, and so the total quantity of material lost from the grain may still have been less than in controls when hordenine was present.

Trials with N-dimethylamino succinamic acid, B-995, did not reveal exceptional properties in our test system as its effects were explained as being due to a general toxic action on the embryo, so reducing growth and gibberellin production, and hence the production of α-amylase (Table 3).

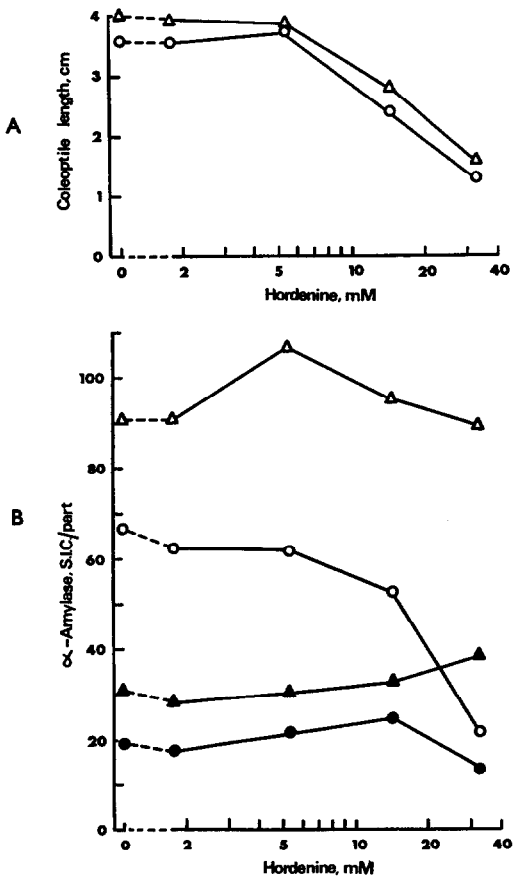

FIG. 12. EFFECTS OF HORDENINE ON COLEOPTILE LENGTH AND α-AMYLASE IN BARLEY GERMINATED WITH AND WITHOUT GA_3 (50 μg/ml).

A. Coleoptiles. —△—, —○—, with and without GA_3.

B. α-Amylase. —●—, —○—, embryo, endosperm.
—▲—, —△—, embryo, endosperm, with GA_3.

Saturated solutions of morphactin IT-3233, and morphactin IT-3456, both reduced coleoptile growth and α-amylase synthesis. In the presence of GA_3, however, α-amylase synthesis was restored while coleoptile growth was inhibited to a slightly greater extent. A more comprehensive trial with morphactin IT-3233, the pure substance, fully confirmed this unexpected result (Table 4). Thus morphactins act in a different way to any other of the substances tested since a strength of solution may be found that suppresses coleoptile growth and not α-amylase synthesis. Yet in the added presence of GA_3 coleoptile length was reduced still

further. Thus morphactins do not interfere with the formation or utilization of barley gibberellins, a result in agreement with tests on orange seedlings.[50]

The results presented here are consistent with our hypothesis advanced to explain the varying rates of breakdown of α-amylase in barley cultured under different conditions.[34] Thus substances such as potassium sulphate did not alter the formation of α-amylase, or by inference the endogenous gibberellins, but reduced embryo growth and so slowed the removal of materials from the starchy endosperm, including those that stabilized α-amylase. Thus the degradation of the enzyme was retarded. Materials, such as CCC, B-995 and copper sulphate, that reduce embryo growth and the formation of α-amylase in the absence, but not the presence, of GA_3, also prevent the production of endogenous gibberellins and so give results less extreme, but still similar, to those obtained by burning the embryonic axis.[4] Still other compounds, including choline, spermine and tryptamine, gave similar results, but were also toxic towards the aleurone to a greater or lesser extent as judged by a reduced rate of formation of α-amylase by grains in the presence of GA_3. At the extreme, DNP and N-ethylmaleimide were so toxic at the doses used that they usually killed all the living tissues.

TABLE 4. THE EFFECTS OF ADDITIONS OF 9-HYDROXYFLUORENE-9-CARBOXYLIC ACID n-BUTYL ESTER MORPHACTIN IT-3233 TO GROWING BARLEY WITH AND WITHOUT GA_3

	GA_3 (μg/ml)					
	0		1		100	
IT-3233 (μM)	Coleoptile length (cm)	α-Amylase (S.I.C./grain)	Coleoptile length (cm)	α-Amylase (S.I.C./grain)	Coleoptile length (cm)	α-Amylase (S.I.C./grain)
0	3·1	28	3·8	42	3·8	50
4	2·7	29	3·3	41	3·0	44
8	2·8	27	2·9	34	2·6	32
42	2·1	30	1·9	43	1·9	45
83	2·1	18	2·2	35	1·9	49

Further work would probably assign most toxic compounds to one of these groups, on the basis of their effects on barley. Clearly, results depend on the doses, the exact cultural conditions, and perhaps the varieties and samples of barley used. However, we have encountered no report of the action of a toxic substance on germinating barley that does not fit this classification.

The results lend much support to the current concepts on the regulation of the synthesis of hydrolytic enzymes, specifically α-amylase, in the germinating grain and to our suggestions concerning the mechanisms by which the destruction of α-amylase is regulated. The results also suggest that the endogenous choline hordenine, putrescine, and other basic substances might regulate the endogenous synthesis of gibberellins.

EXPERIMENTAL

Experiments were performed as previously described.[3,4] Before use the pHs of all buffered solutions were adjusted. Solutions were sterilized by passage through millipore filters. Experiments, terminated by taking all samples at the end of a fixed period, ran for 2½ or 2¾ days.

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[50] J. D. MANN, H. HIELD, K-H. YUNG and D. JOHNSON, *Plant Physiol.* **41**, 1751 (1966).