THE EFFECTS OF GIBBERELLINS AND OTHER PLANT GROWTH REGULATORS ON THE DEVELOPMENT OF ALPHA- AND BETA- AMYLASES AND PROTEASES DURING THE MALTING OF WHEAT

by

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INTRODUCTION AND REVIEW OF LITERATURE

The malting process is composed of three phases: steeping, germinating, and kilning, each of which must be carefully controlled in order to produce a satisfactory malt. Variations in the "environmental" conditions during each stage materially influence the biochemical and biophysical changes which take place during malting. Numerous studies concerning the effects of these conditions have been conducted and the conclusions reached have agreed well. Geddes et al. (22), Kneen, Miller, and Sandstedt (31), Dickson and Geddes (17) and Fleming, Johnson, and Miller (18) have shown that increases in steeping moisture, germination temperature, and germination time induce increases in enzyme production.

The use of chemicals in the steep water as a means of controlling the malting process has been primarily limited to the addition of lime to facilitate the removal of undesirable substances from the seed coat and the use of substances such as hydrogen peroxide or hypochlorites to control the microflora. Dickson, Shands, and Burkhart (16) tested the effect of several plant growth regulating substances, in the hope that they would stimulate enzyme production or modification of the endosperm during malting. These substances failed to induce the desired stimulation, but instead caused a reduction of growth. These results suggested that certain compounds might be useful in reducing malting losses through restricting the growth of rootlets and shoots. Similar responses to this group of compounds were obtained by Bawden (4), Bawden and co-workers (5), and Linko and Enari (36). Kirsop and Pollock (29) and Macey and Stowell (39) recently have suggested the use of coumarin and potassium bromate, respectively, for the same purpose. The foregoing treatments obviously were of little value to the current investigator whose primary aim was the increase of enzyme

activities of the malt.

A relatively new group of substances known as gibberellins has been shown to cause striking increases in the growth rates of higher plants. These substances, produced by a fungus, Gibberella fukijoroi, have only recently come to the attention of scientists in the "western world". Extensive studies by Japanese workers culminated in the preparation by Yabuta and Sumiki (71) of a crystalline material from a culture of the fungus which was capable of inducing effects on rice plants characteristics of the Bakanae (foolish seedling) disease. They termed the causative agent Gibberellin A. The original crude preparation has since been separated into several components which vary in their biological activity. They were called GA_1, GA_2, GA_3, and GA_4. The substance GA_3 is now universally known as gibberellic acid. Gibberellin A_1 has been found to be dehydrogibberellic acid and is produced by the reduction of the cyclohexene bond of gibberellic acid. It is the gibberellin which has been identified in tissues of higher plants. GA_2 and GA_4 are similar to GA_1 chemically but their structures have not been positively determined.

The effects induced by gibberellic acid (hereafter abbreviated GA) on higher plants include: reversal of dwarfism of genetic dwarf mutants (51), increase in leaf areas (23), elongation of stems of normal plants (7, 11), acceleration of "bolting" of biannuals (12), reversal of seed dormancy (52), replacement or partial reduction of light requirements of seeds for germination (26), breaking of dormancy (23, 37), stimulation of growth at low temperatures (45), stimulation of seed germination (20, 24, 26, 46), and the general stimulation of many other growth processes. Gibberellins differ from auxins in that they cause very pronounced increases in growth of intact plants, but very little increase in straight growth auxin assays with excised plant sections and no activity when curvature tests are used. Conversely, auxins cause very little

growth of intact plants but strongly stimulate section growth.

Since their discovery in fungi, gibberellins have been found by many investigators (54, 55, 68) in tissues of higher plants. Radley (54) has reported the presence of a gibberellin-like material in barley, barley malt and germinated wheat. She stated that GA, when applied during malting, may enhance the action of endogenous gibberellin. Lazer, et al. (34) found that the amount of endogenous gibberellin increases appreciably during the early phase of barley seed germination and then levels off. The rate of appearance suggested that it was released from a bound form, and was not synthesized. These workers stated, however, that additional data would be necessary to positively confirm this supposition. Through the use of tritium-labeled GA they found that the GA which was added during malting remained unchanged but that a portion of it was translocated from the seed into the roots and shoots. The added GA was thus thought to stimulate the release of endogenous gibberellins. The latter suggestion is in agreement with that of Radley (55). The recent observations of Paleg (49, 50) indicate that gibberellins may be produced or released during germination and that they are the substances which are responsible for the release of amylases during germination.

Munekata and Kato (47) were the first to apply gibberellins to the malting of barley. They reported that acrospire growth was greatly stimulated and the alpha- and beta amylase, protease and catalase activities were much increased. Barley varieties were found to differ in their response to gibberellin. Sandegren and Beling (58) appear to have been the first "western" investigators to use GA during barley malting. They found that growth rate, enzyme activities, and extract yield were increased and malt properties in general were greatly improved. This study, and others by the same workers (59, 60) have indicated that the germination period could be materially reduced without losing enzyme

activity or otherwise imparing malt quality. GA-treated malts appeared to be darker after kilning than the controls. This doubtless reflects the presence of much greater amounts of free reducing sugars and amino acids which are available for the formation of "browning reaction" products. Worts from GA-treated malts were found by Krinstad, et al. (33) to be more easily fermented. This was due not only to higher extract yield but also to a more complete degradation of dextrins to simpler carbohydrates. Dahlstrom and Sfat (15) have shown that cellulase and transaminase activities were increased following appropriate treatment with GA in addition to the enzymes previously mentioned.

Kleber and co-workers (30) and Stadler, et al. (64) reported that the production of acceptable malts from barleys which normally were not satisfactory for malting purposes was possible through the use of GA. These observations apparently are manifestations of the fact that GA reverses certain types of dormancy, as reported earlier by Pollock (52).

The method of application and the concentrations specified vary appreciably. Most studies have indicated that the greatest response was achieved when GA was added at the start of the germination period. Kleber, et al. (30) and Stasko and Samoilova (65) reported satisfactory results when GA was present only in the final steep liquor. Sandegren and Beling (56, 60), Stadler, et al. (64), Kleber and co-workers (30) and Mastovsky, Karel, and Kahler (41) applied the GA in the form of a spray to the grain at the start of the germination period. This method elicited a satisfactory response and appears to be the one which would be the most feasible and economical for adoption by the malting industry since it requires less GA than would be required if it were added to the steep water.

The report of Fleming and Johnson (19) appears to be the only one to date concerning the use of GA during the malting of wheat. The responses obtained

were of approximately the same order as those reported for barley.

Tissue culture studies have clearly demonstrated that the control of growth is not mediated by any one substance, rather it is the result of the interaction of numerous regulatory substances. Kato (27) found that petiole elongation by gibberellic acid was contingent upon the presence either of an endogenous or an exogenously applied auxin. Several other investigators (9, 10, 63) have also shown that auxins must be present in tissues for GA to be effective. The nature of the interaction has not been completely elucidated. Galston (21) has suggested that gibberellin may react with some tissue component prior to interacting with the auxin, hence further complicating the relationship.

Skinner, et al. (62) have demonstrated a synergistic relationship between GA and kinetin and related compounds. Stowe (67) found that stem sections of pea plants reacted to an apparent synergistic combination of auxin, GA and alkyl lipids. Asen, Cathey, and Stuart (2) found that the aglycone of hydrangenol enhanced the growth promoting activity of endogenously applied gibberellin. Alvim (1) reported that treatments of growing plants with combinations of GA and urea or GA, urea, and sucrose were more effective than GA alone. This response probably may be attributed to the fact that GA treated plants grow more rapidly and, hence, need more nutrients in order to sustain the accelerated rate of growth. Wittwer and Bukovac (70) have found this to be the case.

Comparatively few attempts have been made to apply combinations of auxins or other substances with GA during malting. Macey (38) has reported that combinations of GA and potassium bromate were effective in increasing extract, modification and enzyme activities, while curtailing malting losses. Linko and Enari (36) and Linko, et al. (35) used 2,4-dichlorophenoxyacetic acid, and 2 (3) benzoxazolone, respectively, in combinationwith GA in order to reduce malting loss, while retaining many of the beneficial effects derived from the

use of GA. Pollock (52) reported that some barley seeds germinated more rapidly with combinations of GA and hydrogen sulfide than with either agent alone. Mastovsky, et al. (41) found that spraying barley during malting with combinations of glucose and GA was more effective than treatment with GA alone.

Moffatt and Radley (44), Stowe and Yamaki (68), and Bukovac and Wittwer (13) have tested a number of derivatives of gibberellic acid. Acetylation of the hydroxyl groups was found to influence the biological activity only very slightly but esterification of the acid group often reduced the effectiveness of the compound. Moffatt and Radley (44) found that several metallic salts, notably those of copper, silver and manganese stimulated growth to a greater degree than GA when absorbed from nutrient solutions through the roots. Application of the same substances to the leaves was less effective than the parent substance.

Assay methods for gibberellin activity have been primarily of the "bio" type because of the general lack of specific chemical methods sensitive for the low concentrations normally encountered and the uncertainty concerning proper methods for extraction prior to chemical analysis. The ability of GA to overcome genetic dwarfism appears to be specific for this substance. It has been used as the basis of the method developed by Phinney (51), which makes use of a dwarf mutant corn. Radley (55) used the growth rate of Meteor pea seedlings as a means of detecting gibberellin or gibberellin-like activity. Many other selected cultivars have been similarly used by other workers. Baumgartner, et al. (3) has developed isotopic dilution methods for the detection of small amounts of gibberellins. Washburn, Scheske, and Schenck (69) qualitatively measured individual acids by means of infra-red spectroscopy. Kavanaugh and Kuzel (28) developed a quantitative fluorophotometric procedure which measures GA by detecting the amount of a fluorogen present which is formed by the

interaction of GA with sulfuric acid. The latter procedure was used in the current study.

MATERIALS AND METHODS

Malting Equipment

The laboratory scale malting equipment was modeled after that observed in several commercial malting laboratories. The steeping bath was an insulated, refrigerated stainless steel tank provided with facilities for the constant movement and exchange of water. Temperatures were maintained thermostatically within ± 1° F. The germination cabinet was a large commercial refrigerator which was modified to include additional cooling coils and heating, air circulation and humidification units. The desired temperature and a saturated atmosphere were maintained by means of thermostatic controls. A record was obtained using a Minneapolis-Honeywell temperature and humidity recorder. The sprouted wheats (green malts) were dried in a large forced-air convection-type oven with accurate temperature control.

Malting Procedure

An earlier investigation (18) concerning optimum malting conditions dictated those used in this study. Wheats (50 gram samples) were steeped in 60 ml. of the desired solutions at 50° F. until the moisture content of the grain reached 42 per cent. The steep liquor was changed at six hour intervals in order to prevent damage to the embryo by asphyxiation or through the accumulation of naturally occuring germination inhibitors. Germination was carried out at

62° F. for the times specified and kilning was performed at 104° F. for 24 hours.

The gibberellin used, unless otherwise stipulated, was potassium gibberellate (GA-K).[1]

To determine the most effective concentration of GA-K on enzyme production when applied to the wheat during the steeping period, samples of Pawnee wheat were steeped to 42 per cent moisture content in GA-K solutions ranging from .00001 to .10 per cent. In order to determine the effect of GA-K on different varieties of wheat, eight varieties of wheat were steeped in .0005 per cent, .001 per cent and .005 per cent solutions. The effect of GA-K on enzyme development at varying stages in the germination period was studied by steeping samples of wheat in 0.001 per cent GA-K solution or water to 42 per cent moisture content and germinating for 12 to 192 hours. In order to determine the length of contact with GA-K solution during the steep period which would induce the maximum response, wheats were steeped in 0.0005 and 0.005 per cent solutions of the salt for 1 to 52 hours. The period of contact with GA-K in each instance was for the final portion of the steep time, the full steeping period being 52 hours. The effectiveness of treatment of wheat with GA-K during the germination period was investigated by spraying wheats which had received the normal steep treatment with 5 ml. of water or GA-K solutions after 0, 24, 48, and 72 hours of germination. The amount of GA-K applied is indicated in Table 6.

The second phase of the study involved an investigation of the effects of combinations of GA-K with growth regulating substances and other compounds on the production of alpha-amylase and protease. Treatments consisted of steeping the wheat in the substance other than GA-K for the final 12 hours of the steep period followed by the addition of GA-K for the final hour of the steep time. Preliminary trials had indicated that a longer period of contact with many of

[1]Potassium gibberellate supplied by Merck and Company, Rahway, New Jersey.

the substances used was required in order to induce appreciable responses. The purpose of this portion of the work was to devise combinations which would make possible (1) greater responses at relatively slight increases in cost over that of GA-K alone or (2) the use of lesser amounts of GA-K, thus reducing the cost of treatment.

Analytical Methods

Alpha-amylase activity was determined by the method of Sandstedt, Kneen, and Blish (61) as modified by Redfern (56). Protease activities were measured by the procedure of Miller (43). Beta-amylase was determined by the Kneen and Sandstedt method (32). All enzyme activities were reported on the dry weight basis. The amount of gibberellin observed by the wheat during steeping was determined by assaying the steep liquor for GA before and after the steep period by the Kavanaugh-Kusel method (28). Details of the above methods are given in the appendix. Malt yield was determined by comparing the dry weight of 1000 kernels of cleaned malt with that of 1000 kernels of the original wheat.

RESULTS AND DISCUSSION

Effect of Potassium Gibberellate (GA-K) Concentration and Mode of Application on Enzyme Activities

Seed germination, as reflected by the rate and extent of acrospire (coleoptile) growth, was appreciably increased following the steeping of wheats in GA-K solutions ranging from 0.00005 to 0.005 per cent. Rootlet growth was not stimulated at any concentration and was materially reduced when GA-K levels were

in excess of 0.001 per cent. Loss in dry weight during the malting process (malting loss), as expected, was slightly greater when wheats were treated with GA-K (Table 1).

Table 1. Effect of GA-K on malting loss (Pawnee wheat germinated for four days).

GA-K Concentration %	Malting Loss		
	Total %	Shoots & Roots %	Respiration %
0.0 (Control)	9.8	5.4	4.4
0.0001	10.9	5.9	5.0
0.001	11.4	6.3	5.1
0.005	11.8	6.6	5.2
0.01	10.3	5.8	4.5

The difference in loss between the total amount and that which can be attributed to the removal of roots and shoots was considered to be due to respiration. Additional evidence of a preliminary nature obtained through the use of a Warburg respirometer confirmed the above data which appeared to indicate that the respiration rate of treated wheats was greater than that of the control. The apparent stimulation of respiration noted in these trials is in agreement with results obtained by Nielsen and Bergquist (48) for barley seeds which had been treated with gibberellin.

The response to variations in the concentration of GA-K in the steep liquor on enzyme production are summarized in Table 2. Each value is the average for five replicate malts. The optimum concentration for the production of both alpha- and beta-amylase appeared to be approximately 0.005 per cent. The gain in alpha-amylase activity which was induced by increasing the concentration from

0.0005 to 0.001 per cent was very appreciable, while the further increase noted when the concentration was advanced to 0.005 per cent was relatively slight. Beta-amylase production was enhanced to a greater degree when the GA-K concentration was increased to the 0.0005 per cent level. The optimum concentration for the development of protease activity appeared to be in the range between 0.0005 and 0.001 per cent. Alpha-amylase activity was increased more than that of beta-amylase or protease. This response is in agreement with that for barley, as reported by Munekata and Kato (47) and Sandegren and Beling (58, 60). The extremely high concentrations (above 0.01 per cent were found to be detrimental, especially with respect to the production of proteolytic activity. This was also true for barley (47, 58, 60).

Data concerning the effect of GA-K on enzyme activities after varying periods of germination are presented in Table 3. The response was most pronounced during the early stages of growth. While the activity levels continued to increase for the full germination period, the greatest response to GA-K treatments occurred during the first 24 to 36 hours. The GA-K induced stimulation diminished gradually thereafter. The enzyme activities of the GA-K treated malts, in most instances, were equal to those of control malts which had been germinated for one to three additional days. This type of response makes use of GA attractive to commercial maltsters. The rapid increase in enzyme production during early stages of germination has been reported previously (19, 47, 58). Wittwer and Bukovac (70) reported that the effect induced by GA on the growth rate of plants was temporary and that applications must be repeated in order to maintain the stimulatory effect. The same workers indicated that the slow-down could also be due to a depletion of readily available nutrients in the plant. The abnormally high rate of growth depletes the nutrient supply more rapidly than usual as shown by the fact that GA-treated plants

Table 2. Effect of GA-K on Enzyme Production (fifty grams of Pawnee wheat, steeped to 42 per cent moisture in the presence of 50 ml. of GA-K solution and germinated at 62° F. for four days).

GA-K in Steep Liquor %	α-Amylase Activity[a] SKB Units[b] g.	Std. Dev.	Response to GA-K %	β-Amylase Activity[a] KS Units[c] g.	Std. Dev.	Response to GA-K %	Protease Activity[a] HUd g.	Std. Dev.	Response to GA-K %
0	158	4.4	---	23.9	1.2	---	70	3.6	---
0.00001	163	3.8	+ 3	25.0	2.3	+ 5	73	4.3	+ 4
0.00005	169	5.5	+ 6	26.5	1.8	+11	80	4.4	+14
0.0001	179	4.4	+13	28.4	1.1	+19	83	2.7	+19
0.0005	199	6.9	+26	30.3	1.2	+27	87	5.0	+24
0.001	227	5.4	+45	31.1	1.5	+30	92	3.3	+31
0.005	238	8.8	+50	33.7	2.1	+41	86	3.3	+21
0.01	205	5.0	+29	30.4	1.7	+27	72	4.1	+ 3
0.05	175	4.8	+10	24.1	2.0	+ 1	64	3.6	- 9
0.10	130	4.5	-18	17.8	2.0	-25	57	3.0	-19

a. Average for five replicate malts prepared on different days.
b. Sandstedt-Kneen-Blish units (14).
c. Kneen-Sandstedt units (5).
d. Hemoglobin units (7).

Table 3. Effect of GA-K on enzyme activities after varying germination periods. Fifty grams of Pawnee wheat, steeped in 50 ml. of water (or 0.001 per cent GA-K solution) to 42 per cent moisture, and germinated for the period indicated at 62° F.

Germination Period Hours	α-Amylase Activity			β-Amylase Activity			Protease Activity		
	Control SKB Units: g.	GA-K treated SKB Units: g.	Response to GA-K: %	Control KS Units: g.	GA-K treated KS Units: g.	Response to GA-K: %	Control HU: g.	GA-K treated HU: g.	Response to GA-K: %
12	---	---	---	10.4	13.0	+25	14	20	+41
24	12	20	+66	11.3	15.1	+34	29	43	+47
36	32	54	+70	12.5	16.0	+30	38	55	+44
48	63	98	+56	13.4	19.4	+45	52	72	+38
72	113	168	+49	19.6	24.5	+25	61	82	+35
96	163	235	+44	24.2	30.5	+33	73	96	+31
120	186	251	+35	27.0	34.9	+29	80	102	+27
144	204	271	+33	30.1	37.8	+26	81	101	+25
168	234	304	+30	33.6	39.3	+17	83	104	+25
192	267	338	+25	34.3	41.7	+22	88	108	+23

require a higher level of fertility in soil (or nutrient medium) than untreated plants. Its possibility that the slow-down in growth of seedlings observed in this study could be due to lack of necessary nutrients.

The trends indicated by the data in Tables 2 and 3 were found to apply when additional varieties of wheat were studied (Fig. 1). The 0.005 per cent level of GA appeared to be the most effective for stimulating production of the amylases but it was less effective than the 0.001 per cent concentration when protease production was considered. Production of alpha-amylase after four days of germination of Pawnee, Thatcher, Vernum and Genesee wheats was increased by 50 per cent or more when the steep liquor concentration of GA-K was 0.005 per cent. The 0.001 per cent level was but slightly less effective, while the increase induced in these varieties by use of 0.0005 per cent GA-K was approximately 40 per cent in all cases except for Thatcher which was 26 per cent. RedChief, Triumph, and Elmar varieties were effected to a somewhat lesser degree, while the response of Lee wheat to these treatments was the least of the varieties tested. Munekata and Kato (47) and Stadler, et al. (64) found that barley varieties differ in their responsiveness to GA. The latter workers (64) have stated that some varieties which normally were poor malting varieties could be induced to produce satisfactory malts when treated with GA in the proper manner. The response to GA of barley was thought to be correlated with its normal malting potential.

The percentage increase in enzyme activities induced by GA-K treatments in six varieties of wheat after two and four days of germination are given in Table 4. The data again indicate that the GA-K levels previously given are applicable in this case. Relative responses to GA-K treatment after two days of germination, as indicated previously for Pawnee wheat (Table 3) were greater than those elicited after four days of growth. The ratio of alpha-amylase to

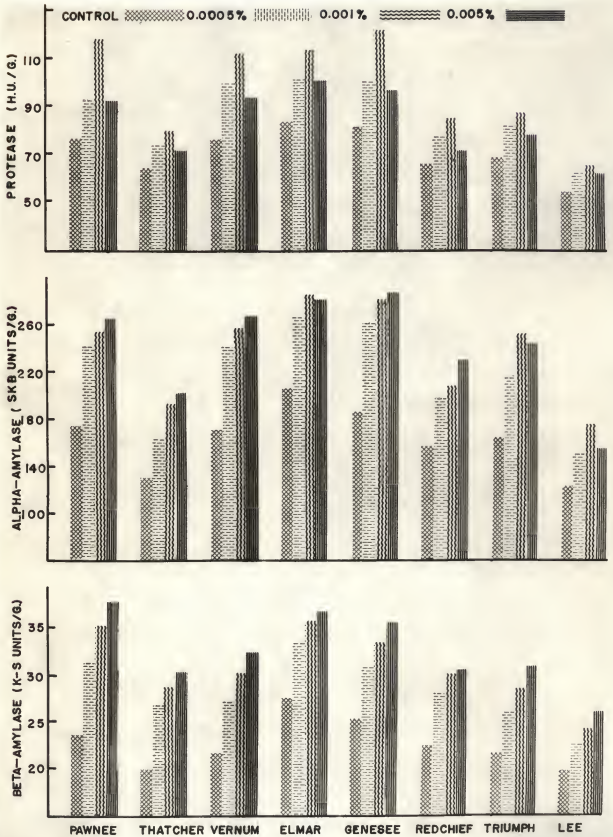

Figure 1. Effect of potassium gibberellate concentration in steep water on enzyme activities of various varieties of malted wheat.

protease activity was found to be readily alterable, through the use of varying amounts of GA-K. The effect on this relationship was relatively slight when low levels of GA were employed but alpha-amylase production was favored as the concentration was increased. The effects on the relationship between beta-amylase and protease, while not shown, were similar to that between alpha-amylase and protease. However, the effect on the difference between alpha- and beta-amylase was not consistent among varieties. Thus, it is evident that the commercial operator would have to consider not only the amount of enzyme required when choosing the treatment to employ but also the effect of the treatment on the balance between enzymes.

The procedures considered, thus far, have consisted of steeping the wheat in GA-K for the full normal steep period. Data given in Table 5 was obtained by steeping for lesser periods of time in solutions of GA-K. The maximum stimulation of alpha-amylase and protease production occurred following contact with GA-K during the final 18 to 24 hours of steeping. The optimum period of contact appeared to be essentially equal for both concentrations of GA-K tested in this study. Very significant increases in enzyme production were achieved, however, as a result of much shorter periods of contact. The full steep period (52 hours) appeared to be necessary for maximum stimulation of beta-amylase activity. In this instance, too, there were very pronounced increases when shorter periods of steeping in GA-K were employed. Treatments with the 0.005 per cent solution was generally more effective than with the 0.0005 per cent level. It would appear that it would be expedient from a commercial standpoint to shorten the period of contact with GA in order to reduce costs.

The amounts of GA-K which is absorbed by 50 gram lots of wheat during the final portions of the steep time are given in Table 6. The quantity absorbed was found to increase as the period of contact or the concentration of the

solution was increased. The amounts which were absorbed were found to be relatively minor, however, when the quantity present in the steep water was taken into consideration. These data and those presented in Table 5 seem to indicate that long steep periods are needlessly wasteful of gibberellin.

Table 4. Effect of gibberellic acid treatments on enzyme activities.

Variety	Treatment GA Conc. %	Increase due to GA Treatment: α-amylase 2 day malt %	α-amylase 4 day malt %	β-amylase 2 day malt %	β-amylase 4 day malt %	Protease 2 day malt %	Protease 4 day malt %	α-amylase/Protease Ratio 2 day malt	α-amylase/Protease Ratio 4 day malt
Pawnee	0.0							1.46	2.25
	0.0005	76	40	47	43	44	23	1.80	2.54
	0.001	88	48	80	50	76	54	1.55	2.16
	0.005	104	56	92	63	26	21	2.36	2.90
Thatcher	0.0							1.34	2.02
	0.0005	38	35	47	35	29	17	1.46	2.16
	0.001	46	44	60	44	40	26	1.40	2.33
	0.005	82	49	66	49	22	12	2.00	2.75
Vernum	0.0							1.46	2.26
	0.0005	76	40	38	28	44	23	1.80	2.57
	0.001	88	48	56	42	76	54	1.55	2.18
	0.005	104	55	69	50	26	22	2.36	2.87
Elmar	0.0							1.48	2.43
	0.0005	67	29	45	28	30	24	2.00	2.54
	0.001	75	43	54	36	45	35	1.77	2.58
	0.005	85	40	60	42	21	19	2.00	2.86
Triumph	0.0							1.43	2.30
	0.0005	70	33	44	19	22	17	1.84	2.54
	0.001	100	55	66	32	50	24	2.00	2.87
	0.005	82	47	75	43	32	11	2.14	3.00
Lee	0.0							1.38	2.24
	0.0005	50	23	58	16	27	14	1.60	2.40
	0.001	84	41	74	27	32	20	1.92	2.63
	0.005	60	26	84	34	17	13	1.86	2.50

Table 5. Effect of duration of treatment with GA-K on enzyme production (fifty grams of Pawnee wheat, steeped to 42 per cent moisture in the presence of 50 ml. of GA-K solution and germinated for two days at 62° F.).

GA-K in Steep Liquor %	Duration of Contact Hours	Time of Application Hours	α-Amylase Activity SKB Units /g.	Response to GA-K %	β-Amylase Activity KS Units /g.	Response to GA-K %	Protease Activity HU /g.	Response to GA-K %
0.0005	0	.....	73	....	15.8	...	52	...
	1	51-52	101	+ 38	16.4	+ 4	63	+21
	3	49-52	109	+ 49	16.4	+ 4	63	+21
	6	46-52	112	+ 53	17.1	+ 8	68	+31
	12	40-52	119	+ 63	18.8	+19	73	+40
	18	34-52	126	+ 72	20.4	+29	74	+40
	24	28-52	129	+ 76	21.1	+33	77	+48
	36	16-52	129	+ 76	22.0	+39	73	+40
	52	0-52	134	+ 83	23.8	+50	74	+42
0.005	0	.....	73	....	15.8	...	52	...
	1	51-52	126	+ 72	18.8	+19	57	+10
	3	49-52	133	+ 82	20.8	+32	59	+13
	6	46-52	138	+ 89	22.2	+41	63	+21
	12	40-52	146	+100	25.6	+62	67	+29
	18	34-52	154	+109	25.6	+62	68	+31
	24	28-52	148	+103	27.5	+74	73	+40
	36	16-52	151	+107	29.5	+86	70	+35
	52	0-52	152	+108	28.8	+82	72	+38

Table 6. Effect of time and concentration on absorption of GA-K from steep liquor.

Contact Time Hours	Original GA-K Concentration µg./50 ml	GA-K Absorbed[a]	Standard Deviation	GA-K Absorbed %
1	250	61	13.6	24.4
	500	76	12.4	15.2
	1000	131	19.4	13.1
	2500	230	23.6	9.1
6	250	80	12.4	32.0
	500	110	13.4	22.0
	1000	166	21.6	16.6
	2500	345	27.7	13.8

a. Average of 15 determinations.

The effects of treating wheat with GA-K during the germination period are given in Table 7. Measurable increases in activity were produced when wheats were sprayed with water during the germination period. That this type of treatment was effective was evident by the increase in growth of shoots and rootlets. This process was comparable to that referred to by the malting industry as "sprinkling" and is practiced commercially when grains which are difficult to malt are used. This practice can be detrimental, however, if the growth which is induced is too rampant. In this case, the malt yield may be curtailed. Spraying with GA-K induced appreciable increases in enzyme activities. Alpha-amylase production was most significantly influenced when so treated during the early portion of the germination period. Proteolytic and beta-amylolytic activities were increased following treatment at all stages of germination studied. The increase in enzyme activities caused by spray treatments was not as great as that observed following application of GA-K in the steep liquor. This method, however, obviously is the most economical from the standpoint of the gibberellin and appears to have been found satisfactory by several investigators who studied

Table 7. Effect of GA-K applied during wheat germination on enzyme production (fifty grams of Pawnee wheat, steeped to 42 per cent moisture in 50 ml. of water, and germinated at 62° F. for four days).

Mg. of GA-K/50 Grams of Wht.	Time of Treatment with GA-K	α-Amylase Activity SKB Units /g.	α-Amylase Total Response %	α-Amylase Response due to GA-K %	β-Amylase Activity KS Units /g.	β-Amylase Total Response %	β-Amylase Response due to GA-K %	Protease Activity HU /g.	Protease Total Response %	Protease Response due to GA-K %
Control										
0	Immediately after steeping	163	---	---	22.5	---	---	71	---	---
2.5		166	+ 2	---	23.3	+ 4	---	73	+ 3	---
1.5		196	+20	+18	26.7	+19	+15	79	+11	+ 8
1.0		204	+25	+22	27.3	+21	+17	83	+31	+28
0.5		209	+28	+26	25.8	+15	+11	87	+23	+20
		190	+16	+14	24.9	+11	+ 7	83	+17	+14
0	After germinating one day	171	+ 5	---	24.1	+ 7	---	73	+ 3	---
2.5		186	+14	+ 9	26.9	+20	+13	83	+17	+14
1.5		199	+22	+ 7	27.9	+24	+17	90	+27	+23
1.0		203	+25	+20	26.5	+18	+11	90	+27	+23
0.5		194	+19	+14	25.5	+13	+ 6	84	+18	+15
0	After germinating two days	175	+ 6	---	24.6	+ 9	---	75	+ 5	---
2.5		200	+22	+16	27.4	+22	+13	86	+21	+16
1.5		193	+18	+12	26.6	+18	+ 9	94	+32	+27
1.0		189	+16	+10	27.1	+20	+11	90	+27	+22
0.5		193	+18	+12	25.8	+15	+ 6	85	+20	+15
0	After germinating three days	176	+ 8	---	24.0	+10	---	76	+ 7	---
2.5		205	+26	+18	28.3	+25	+15	90	+27	+20
1.5		190	+16	+ 8	27.6	+23	+13	93	+31	+24
1.0		193	+18	+10	25.5	+14	+ 4	95	+34	+27
0.5		184	+13	+ 5	25.1	+11	+ 1	88	+24	+17

the use of GA in barley malting (30, 41, 59, 64).

Effect of Combinations of GA-K and Other Growth Substances on Enzyme Production

The effects of auxins when applied singly and in combination with GA-K are summarized in Table 8. Comparatively low levels (less than 0.0005 per cent) of indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and alpha-naphthalenacetic acid (NAA) had little effect on enzyme production and but slight influence on malt yield. Concentrations in excess of 0.015 per cent often reduced growth materially, thus reducing malting loss, but they also had very adverse effects on enzyme production and but slight influence on malt yield. Concentrations in excess of 0.015 per cent often reduced growth materially, thus reducing malting loss, but they also had very adverse effects on enzyme production. These effects appeared to be comparable to those obtained by Dickson and co-workers (16), Bawden, et al. (4), and Stoller (66). The results obtained by use of high concentrations of the growth substances referred to above are not presented herein as they did not seem to be germane to the current study, the purpose of which has been the development of means of increasing enzyme production. GA-K was again found to increase growth and enzyme activities when present in the steep liquid at levels ranging from 0.00005 to 0.05 per cent. The results obtained from use of the extremely high and low levels were not included since these data was presented elsewhere in the thesis. Treatments involving the simultaneous use of GA-K in the steep water at 0.0005 to 0.001 per cent and 0.0001 to 0.0005 per cent IAA, 2,4-D or NAA were found, in most instances, to increase enzyme activities, while simultaneously curtailing malting losses, induced through the use of GA alone. The action of these combinations on enzyme activity appears to be of a synergistic nature as the

use of the above levels of auxin alone has no consistent, significant effect on enzyme production. Brian and Hemming (13) and Kato (27) have shown by means of tissue culture techniques that combinations of auxins and GA may induce synergistic responses. Chlor, et al. (14) found that certain combinations of 2,4-D and GA were synergistic when the growth of shoots of cotton seedlings was used as the criteria. The growth of roots and shoots induced by combinations of GA with higher levels of auxins obtained in this study were similar to those reported by Linko and Enari (36) when barley was malted, that is, malting yield was curtailed while permitting moderate increases in enzyme activities and modification. Since the conclusion of this study, information recently obtained concerning the work of Stadler and co-workers (64) has indicated they also found that the use of combinations of GA and IAA at low levels induced beneficial results.

Table 8. Effect of growth substances used singly and in combination with potassium gibberellate (GA-K) on the production of alpha-amylase, protease and yield during the malting of wheat.

Treatment		α-Amylase		Protease		
GA-K Conc. %	Growth Subst. %	Activity SKB Units/g.	Change %	Activity HU/g.	Change %	Malt Yield %
0.0	0.0	150	---	68	---	91.0
0.0001	0.0	167	+11	75	+12	90.6
0.0005	0.0	190	+27	86	+26	89.8
0.001	0.0	220	+47	89	+31	89.3
		Indole-3-acetic acid				
0.0	0.0001	152	---	71	+ 4	91.3
0.0	0.0003	150	---	71	+ 4	91.5
0.0	0.0005	142	- 5	68	---	92.0
0.0001	0.0001	162	+ 8	79	+15	91.0
0.0001	0.0003	173	+15	83	+22	91.5
0.0001	0.0005	169	+13	77	+13	91.3
0.0005	0.0001	203	+35	88	+30	90.5
0.0005	0.0003	208	+39	93	+37	90.5
0.0005	0.0005	193	+29	90	+32	91.5

Table 8. (Continued).

Treatment		α-Amylase		Protease		
GA-K Conc. %	Growth Subst. %	Activity SKB Units/g	Change %	Activity HU/g.	Change %	Malt Yield %
0.001	0.0001	231	+54	92	+36	89.5
0.001	0.0003	231	+54	95	+40	90.5
0.001	0.0005	226	+50	90	+32	90.8
		2,4-dichlorophenoxyacetic acid				
0.0	0.0001	147	- 2	69	---	90.0
0.0	0.0003	147	- 2	69	---	92.0
0.0	0.0005	141	- 6	65	- 4	92.0
0.0001	0.0001	174	+16	77	+12	91.4
0.0001	0.0003	175	+17	79	+12	91.8
0.0001	0.0005	163	+ 9	79	+13	91.8
0.0005	0.0001	206	+37	90	+32	90.9
0.0005	0.0003	206	+37	93	+37	90.5
0.0005	0.0005	196	+31	88	+30	92.0
0.001	0.0001	233	+55	93	+37	90.7
0.001	0.0003	229	+53	95	+40	91.4
0.001	0.0005	216	+44	87	+29	91.7
		Alpha-naphthaleneacetic acid				
0.0	0.0001	151	---	67	---	90.0
0.0	0.0003	147	- 2	69	---	90.0
0.0	0.0005	143	- 5	65	- 4	90.4
0.0001	0.0001	170	+13	76	+12	90.0
0.0001	0.0003	168	+12	76	+12	90.0
0.0001	0.0005	165	+10	77	+13	90.4
0.0005	0.0001	193	+28	88	+30	89.9
0.0005	0.0003	205	+36	93	+37	90.6
0.0005	0.0005	196	+31	93	+37	91.0
0.001	0.0001	215	+43	89	+31	89.6
0.001	0.0003	226	+50	93	+37	90.2
0.001	0.0005	232	+54	91	+34	91.1

The effects on enzyme production induced by maleic hydrazide (MH), 2,3,5 triiodobenzoic acid (TIBA) and coumarin singly and in combination with GA-K are reported in Table 9. These substances were unable to stimulate growth or enzyme production at any of the concentrations employed. This confirms the results obtained by other investigators (4, 16, 26). When used in combination

with GA-K the two components appeared to counteract each other to varying degrees. Brian and Hemming (8) had found that MH blocks the action of GA. The results obtained as a result of the use combinations of coumarin and GA are in agreement with the observations of Mayer (42) who found that while GA could reverse the germinating inhibiting properties of coumarin, it could not reverse the effect on growth. The lack of synergistic responses to treatments involving the use of these substances with GA-K apparently reflects the fact that they are often referred to as anti-auxins or auxin antagonists.

Studies involving other varieties of wheat gave similar results but responses varied in degree, as was found to be the case when GA-K alone was applied to different wheat varieties (Table 4, Fig. 2). The variation in response to treatments of this type serves to emphasize the complexity of the problem and reflects the findings of Brian and Hemming (10) who found that responses were apparently dependent upon the level of endogenous auxin in a given tissue. The present study and others, however, indicate that procedures involving the use of these substance appears to have promise for the malting industry.

The effects of kinetin and kinetin and GA-K acting in concert on the production of enzymes are given in Table 10. Kinetin alone had no effect on seed germination rate or on the production of enzymes. Pollock (52, 53) found that kinetin, and gibberellic acid each would stimulate the germination of dormant barley seeds under certain conditions. Similar effects on dormant lettuce seeds with kinetin were reported by Skinner and co-workers (62). Combinations of kinetin and gibberellic acid were found to increase measurably the rate of germination of wheat seed and the rate of production of alpha-amylase and protease during germination. Interactions between kinetin and gibberellic acid have been reported by workers using tissue culture methods (63) but the report of Skinner, et al. (62) and the present study are the only indications to date

Table 9. Effect of growth substances used singly and in combination with potassium gibberellate on the production of alpha-amylase, protease and malt yield.

Treatment		∝-Amylase		Protease		
GA-K Conc. %	Growth Subst. %	Activity SKB Units/g:	Change %	Activity HU/g.	Change %	Malt Yield %
0.0	0.0	154	---	70	---	90.4
0.0001	0.0	169	+10	76	+ 9	90.5
0.0005	0.0	193	+27	88	+26	89.6
0.001	0.0	220	+43	91	+30	89.0
		Maleic hydrazide				
0.0	0.0001	154	---	74	+ 6	90.0
0.0	0.0003	147	- 4	74	+ 6	90.5
0.0	0.0005	143	- 7	72	+ 3	91.0
0.0001	0.0001	174	+13	76	+ 9	90.0
0.0001	0.0003	170	+10	73	+ 4	90.4
0.0001	0.0005	163	+ 6	75	+ 7	91.1
0.0005	0.0001	191	+24	91	+30	90.3
0.0005	0.0003	191	+24	84	+20	90.5
0.0005	0.0005	185	+20	87	+24	91.0
0.001	0.0001	220	+43	88	+26	90.3
0.001	0.0003	214	+41	90	+30	91.0
0.001	0.0005	208	+35	84	+20	91.4
		Coumarin				
0.0	0.0025	146	- 4	68	- 2	89.7
0.0	0.005	141	- 8	69	- 1	90.0
0.0	0.0075	128	-17	59	-16	91.3
0.0005	0.0025	157	+ 2	71	- 1	90.3
0.0005	0.005	150	- 2	65	- 7	91.3
0.0005	0.0075	139	-10	61	-11	91.3
0.001	0.0025	173	+12	74	+ 6	90.5
0.001	0.005	157	+ 2	71	- 1	91.1
0.001	0.0075	145	- 3	61	- 6	91.5
		2,3,5 Triiodobenzoic acid				
0.0	0.0001	154	---	70	---	90.6
0.0	0.0003	140	- 9	63	-10	91.4
0.0	0.0005	122	-20	56	-20	91.9
0.0001	0.0001	161	+ 5	70	---	90.5
0.0001	0.0003	158	+ 2	65	- 7	91.0
0.0001	0.0005	141	- 9	60	-14	92.0
0.0005	0.0001	185	+20	81	+16	91.3
0.0005	0.0003	175	+14	77	+10	92.0
0.0005	0.0005	157	+ 2	71	+ 1	92.0
0.001	0.0001	217	+41	90	+30	90.1
0.001	0.0003	205	+33	85	+21	91.4
0.001	0.0005	195	+27	77	+10	92.0

that this combination is effective with intact plants. Pollock (53), working with dormant barley seeds was unable to demonstrate synergistic effects when mixtures of gibberellic acid and kinetin were used.

Table 10. Effect of gibberellin (GA-K) and kinetin on enzyme production during malting.

Treatment		∝-amylase		Protease	
Kinetin %	GA-K %	Activity :SKB units/g.	Change %	Activity HU/g.	Change %
0.0	0.0	149		67	
0.0001	0.0	151	+ 2	67	---
0.0005	0.0	147	---	69	+ 2
0.0	0.0005	189	+26	85	+27
0.0	0.001	217	+50	90	+33
0.0001	0.0005	197	+32	90	+33
0.0001	0.001	233	+56	95	+40
0.0005	0.0005	200	+34	92	+35
0.0005	0.001	234	+57	95	+40

Effect of Combinations of GA-K and Thiol Compounds on Enzyme Production

The effects of thiol compounds and combinations with potassium gibberellate on wheat seed germination are reported in Table 11. Significant differences in the percentage of seed which germinated following these treatments were not observed but differences in the rate and amount of acrospire and rootlet growth were obtained. All of the thiol compounds as well as GA-K increased the growth of acrospires. Root growth was not affected by GA-K but was increased slightly following treatment with the thiol compounds. The combinations of thiol substances and GA-K appeared to be beneficial in most instances but the combined

effect seemed to be additive, rather than synergistic. Pollock (52, 53) found that combinations of gibberellic acid and hydrogen sulfide added synergistically in breaking dormancy of some lots of barley seeds. The wheat used in this study was fully viable, hence the results obtained cannot be compared with those found with dormant barley.

Table 11. Effect on sulfhydryl compounds and potassium gibberellate on germination, root and acrospire growth.

Treatment					
GA-K Conc. %	SH Compd. Conc. M.	SH Compound	Germination %	Acrospire Index (1)	Root Index (2)
0.0	0.0	Control	98	.75	1.9
0.0	0.005	Glutathione	97	1.3	2.4
0.0	0.005	Cysteine	100	1.0	2.1
0.0	0.005	2,3-dimercaptiopropanol	97	1.3	2.3
0.0	0.005	Thiourea	100	1.1	2.6
0.0	0.005	2-mercaptoethylamine HCL	100	1.1	2.3
0.0005		Gibberellin (GA-K)	100	1.2	2.0
0.0005	0.005	Glutathione	96	1.1	2.3
0.0005	0.005	Cysteine	100	1.6	3.0
0.0005	0.005	2,3-dimercaptopropanol	100	1.4	2.3
0.0005	0.005	Thiourea	100	2.0	3.0
0.0005	0.005	2-mercaptoethylamine HCL	100	2.0	2.7

(1), (2). Lengths of acrospires and roots, respectively, compared to the length of seed as unity.

The effect of thiol compounds alone and in combination with GA-K on the production of alpha-amylase and protease during malting are summarized in Table 12. The addition of 5×10^{-4} M. glutathione (GSH), cysteine, and 2,3-dimercaptopropanol (BAL) to the steep water was found to induce slight increases in growth and enzyme activities. Protease activity appeared to be increased slightly more than that of alpha-amylase. The combination of GA-K and thiol

compounds appeared to induce greater increases than those obtained through the use of GA-K alone. The molar concentrations of thiourea required to achieve a given response were higher than those required of the other compounds in this group. Thiourea, like glutathione, may exist in two tautomeric forms (C-SH and C=S). The GSH used in this study was known to be in the reduced form whereas the thiourea used was probably a mixture of both forms and, hence, lacked the reducing power of GSH. Marre and Arrigoni (40) have suggested that a relationship exists between auxin activity and the ratio between reduced and oxidized glutathione (GSH/GSSG) and that induced changes in this ratio have an effect on growth. While the interaction between compounds of this type and gibberellin is of interest, it is doubtful if any of the substances tested thus far could be used commercially. Cysteine and GSH are probably too expensive for this purpose, while BAL and 2-mercaptoethylamine would be objectionable for esthetic reasons because of their aromas. Thiourea, long thought to be relatively innocuous, has recently been found to be carcinogenic and, thus, must be removed from consideration by the food industry.

Effect of Combination of GA-K and Krebs Cycle Acids on Enzyme Production

The effects of steeping wheats in solutions of certain Krebs cycle acids on enzyme production are reported in Table 13. Earlier studies have indicated that concentrations of fumaric and succinic acids at concentrations in excess of 0.005 M. were inhibitory, while levels below 0.0001 M. had little or no effect, hence, they were not used in later trials. The 0.0001 to 0.0005 M. concentrations appeared to be optimal for the stimulation of the production of both enzymes. Additional trials of a limited nature indicated that alpha-keto glutaric acid and malic acids, when used at approximately the same concentrations,

Table 12. Effect of thiol compounds and gibberellic acid (GA) used singly and in combination on the production of alpha-amylase and protease during malting.

Treatment (Conc.)		α-amylase		Protease	
Thiol Compound M.	GA %	Activity SKB units/g.	Increase due to treatment %	Activity HU/g.	Increase due to treatment %
0.0	0.0	153		69	
0.0	0.001	223	45	88	28
GSH					
0.0005	0.0	157	3	72	4
0.001	0.0	157	3	72	4
0.0005	0.001	230	50	93	35
0.001	0.001	235	53	95	37
Cysteine					
0.0005	0.0	151		72	4
0.001	0.0	156	2	72	4
0.0005	0.001	230	50	93	35
0.001	0.001	235	53	95	37
BAL					
0.0005	0.0	155	2	74	7
0.001	0.0	161	5	73	5
0.0005	0.001	233	52	93	35
0.001	0.001	240	56	95	37
2-mercaptoethylamine HCL					
0.0005	0.0	157	3	69	
0.001	0.0	155	2	71	3
0.0005	0.001	229	50	91	32
0.001	0.001	234	53	91	32
Thiourea					
0.005	0.0	154	1	72	4
0.01	0.0	154	1	71	3
0.02	0.0	159	4	70	1
0.04	0.0	155	2	72	4
0.005	0.001	230	50	91	32
0.01	0.001	235	53	93	35
0.02	0.001	238	56	95	37
0.04	0.001	235	53	95	35

were beneficial but that pyruvic and citric acids did not induce increases in enzyme production. Ruge (57) has stated that the degeneration of seeds upon aging was due to the loss of readily oxidizable substances, thus, reducing respiration. This theory would seem to explain the stimulatory effects found during the course of the current work. Responses to combinations of fumaric or succinic acid and GA-K are also given in Table 13. The combinations induced increased activity but the effect of mixtures appeared to be additive rather than synergistic. The beneficial effects obtained by the use of these acids alone confirm the results of Blagoveshcheskii and Petrochenko (6). The use of these substances in dilute concentrations, particularly in combination with gibberellins, may be of practical importance in malting. The additional response obtained through these combinations could be a manifestation of the condition mentioned previously, namely that GA treated seeds respire faster (48) and grow more rapidly, hence, require greater quantities of readily utilizable nutrients (70).

Present studies (Table 7) and those of other workers (41, 60) have indicated that significant increases in enzyme activities may be induced when the grain is sprayed with GA during the germination period. Preliminary experiments had indicated that maximum response was obtained when this type of treatment was applied at the start of the germination period, hence, the data reported (Table 14) are concerned only with those obtained by that procedure. Optimum levels of GA-K when applied in this fashion appeared to be approximately 1 mg. per 50 g. of wheat. Mastovsky and co-workers (41) found that greater stimulation was possible by the admixture of glucose with GA. The writer was unable to induce similar results. The study with barley (41) indicated that "glucose syrup" was used, hence it is possible that the major benefits derived from their treatment were caused by something other than glucose per se, in the syrup. The failure

to obtain a complementary action with glucose could also be due to a lack of need for supplemental carbohydrates by the seed used in this study. Urea, however, when used singly and in combination with GA-K, caused increases in growth and as a consequence enzyme activities were increased. It is assumed that urea hastens growth in this instance as it does when used as a commercial fertilizer.

Table 13. Effect of fumaric and succinic acids and gibberellic acid on enzyme production during malting.

Treatment[a]		α-amylase Activity		Protease Activity	
Acid Molarity	GA p.p.m.	SKB units/g.	% change	HU/g.	% change
0	0	150		67	
0	10	229	+52	90	+34
Fumaric					
0.0001	0	165	+10	75	+12
0.0001	10	240	+60	95	+43
0.0005	0	161	+ 8	71	+ 6
0.0005	10	240	+60	93	+40
Succinic					
0.0001	0	159	+ 7	71	+ 6
0.0001	10	240	+60	94	+42
0.0005	0	163	+ 9	74	+10
0.0005	10	244	+63	96	+45

a. Fumaric and succinic acids were added to the steep water 12 hours before conclusion of steep period, while GA was added but one hour before end of steep time.

Effect of Some Gibberellic Acid Derivatives on Enzyme Production

The data concerning the relative effectiveness of several gibberellates on the production of alpha-amylase and protease appear in Table 15. All of the gibberellic acid salts stimulated growth and enzyme production to essentially

Table 14. Increasing enzyme activities of malt by spray treatment of the grain during germination.

Treatment	α-amylase Activity SKB units/g	% Change	Protease Activity HU/g	% Change	Malt Yield %
Control (no spray)	148		67		90.5
Control (sprayed with water)	151	+ 2	69	+ 3	90.0
0.5 mg. GA-K	187	+25	80	+20	89.5
0.75 mg. GA-K	196	+32	83	+23	88.7
1.0 mg. GA-K	191	+29	80	+20	89.3
5 mg. glucose	148		69		91.2
1 mg. glucose	150	+ 1	69		90.5
0.5 mg. glucose	153	+ 3	67		90.7
5 mg. urea	153	+ 3	71	+ 6	90.7
1 mg. urea	157	+ 6	73	+ 8	90.5
0.5 mg. urea	157	+ 6	73	+ 8	90.5
0.75 mg. GA-K + 5 mg. glucose	193	+30	83	+23	90.0
0.75 mg. GA-K + 1 mg. glucose	196	+32	81	+21	89.5
0.75 mg. GA-K + .5 mg. glucose	196	+32	83	+23	89.5
0.75 mg. GA-K + 5 mg. urea	200	+35	84	+25	89.9
0.75 mg. GA-K + 1 mg. urea	205	+38	86	+28	90.2
0.75 mg. GA-K + .5 mg. urea	200	+35	86	+28	90.3

the same degree or greater than the original GA or the commercial GA-K. The alkaline earth salts induced approximately similar results but the cobalt gibberellate was found to be slightly less effective than the other compounds tested. The silver, copper and iron gibberellates were found to be slightly more effective than the other substances. The increased effectiveness of the copper, iron, and manganese salts cannot be explained at this time but the results obtained corroborate the data of Moffatt and Radley (44). It is possible that the metal part of the gibberellate may increase the efficiency of the gibberellate by facilitating penetration into the seed or its movement within and/or utilization by the seed. The response to the silver gibberellate was surprising as silver has not been reported to be necessary for plant nutrition. Furthermore, silver is known to be an enzyme "poison".

Combinations of gibberellic acid and the metal carbonates or hydroxides were used in the steep liquor in order to check the possibility that the metallic ions could increase the efficiency of the GA without being bound to it. These data are given in Table 16. These combinations were found to induce essentially the same responses obtained when gibberellic acid alone was used, hence it appears that the metallic ions must be combined with GA in order to cause the effects described above.

SUMMARY AND CONCLUSIONS

1. Treatment of wheat seeds with potassium gibberellate (GA-K) during steeping or during germination materially increases the rate of germination and production of alpha- and beta-amylases and protease during malting.
2. The ability of GA-K to induce the formation of highly active malts

Table 15. Comparison of the effectiveness of gibberellic acid and some functional derivatives with respect to the stimulation of the production of alpha-amylase and protease during malting.

Compound[a]	α-amylase Activity		Protease Activity		Melting Points	
	SKB Units/g	% control	HU/g	% control	Observed °C	Reported by Moffatt & Radley (21) °C
Control	156	100	70	100		
GA	203	130	85	121		
GA-K (Commercial)	203	130	88	126	227-230 d.	225-229 d.
GA-K (Experimental)	199	128	82	117	226-228 d.	225-229 d.
GA-Na	195	125	82	117	232-236 d.	232-238 d.
GA-Ca	208	133	89	129	261-265	260
GA-NH_4	200	128	83	119	165-168 d.	167-170 d.
GA-Ag	218	140	94	134	203-205 d.	198-199 d.
GA-Cu	223	143	94	134	248-251 d.	245-248 d.
GA-Co	189	121	88	126	203-205 d.	204-206 d.
GA-Fe	227	147	94	134		
GA-Mn	215	138	90	129	258-264 d.	261-266 d.
GA-Mg	205	131	85	121	174-176	

a. The gibberellins were present in the steep water at .0005 per cent for the final hour only of the steep hour.

Table 16. Effects of combinations of gibberellic acid with certain metallic hydroxides and carbonates which were used in the preparation of the gibberellates.

Treatment[1]	α- amylase activity SKB units/g.	% of control
Control	147	
Gibberellic acid (GA)	222	152
Potassium hydroxide	150	102
Potassium hydroxide + GA	217	147
Sodium hydroxide	145	99
Sodium hydroxide + GA	219	149
Ammonium hydroxide	143	98
Ammonium hydroxide + GA	221	150
Calcium carbonate	152	103
Calcium carbonate + GA	226	154
Magnesium carbonate	152	103
Magnesium carbonate + GA	226	154
Lithium carbonate	145	99
Lithium carbonate + GA	215	146
Copper carbonate	150	102
Copper carbonate + GA	225	153
Cobalt carbonate	152	103
Cobalt carbonate + GA	217	147
Silver carbonate	150	102
Silver carbonate + GA	225	153
Manganese carbonate	147	100
Manganese carbonate + GA	219	149

1. Components were added at levels to give a GA concentration of 0.001 per cent and metallic ion concentration comparable to that which would be present in the form of the gibberellate.

in a short time would seem to make its use attractive to commercial maltsters as the production of malt per processing unit in a given period should be materially increased.

3. The response to GA-K is greatest during the early portion of the germination period, thus suggesting a need for renewal of treatment with GA-K or the depletion of some essential ingredient in the seed which is necessary for continued rapid growth.
4. The enhancement of the effect caused by GA-K when Krebs cycle acids, -SH compounds, or urea are included in treatment indicates that their effect may occur because of the possible depletion of naturally occurring substances of these types during the initial period of rapid growth induced by GA treatments.
5. The data obtained from studies involving combinations of GA-K and growth regulating substances confirm those of previous workers who demonstrated that substances having auxin activity may react synergistically with GA while auxin antagonists block the action of GA.
6. Much more work of both a practical and fundamental nature is required in order to fully understand and exploit the effects possible through the use of gibberellic acid in malting.

SUGGESTIONS FOR FUTURE WORK

The primary purpose of this investigation was to devise methodology for the application of gibberellic acid to wheat malting in order to increase the enzyme activity of the finished malt. The results obtained have suggested several additional areas for future investigations.

Further refinements in the mode of application appear to be necessary in order that lesser amounts of GA might be expended to achieve satisfactory results. Present data indicate that savings may be possible through the use of GA-K in combination with other substances and by treatments performed during the germination period rather than during the steeping period. More intensive studies of the variables involved would be required in order to determine the commercial feasibility of such treatments.

Gibberellin treated malts were found to be darker colored following kilning than control malts and often had a "sharper" flavor. A study to determine the reasons for these differences would seem to be desirable. Treatments of this sort could prove to be of practical value as a means of altering the flavor of the malt.

The growth of fungi during the malting of GA-K treated wheats was often more extensive than that present on control wheats. Numerous studies have shown that GA has no direct effect on microorganisms, hence, it is apparent that the treatments have altered the composition of the host tissue (seeds) making it more amenable to fungi. A study designed to elucidate this relationship is desirable.

Paleg (49, 50) has suggested that gibberellic acid is capable of releasing amylolytic enzymes from barley endosperm without germination. Further information concerning the mechanism(s) which indice this effect and, indeed, most of the other responses to GA treatment is necessary in order to understand the effect of GA on seed germination.

Lazer, et al. (34) have indicated that exogenously applied gibberellin is not utilized by the grain but apparently stimulates the release of endogenous gibberellic acid or GA-like substances which are responsible for the increased growth rate. Further work in this area obviously is required in order to understand the effect of GA on the malting process.

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APPENDIX

Measurement of Alpha-amylase Activity

The procedure for determining alpha-amylase activity was a modification of the Wohlgemuth method as modified by Sandstedt, et al. (61) and Redfern (56). It utilized as a substrate, soluble starch, which had been subjected to the action of beta-amylase for 24 to 72 hours, hence, it was a beta limit dextrin, the degradation, of which, cannot be continued without the presence of alpha-amylase. The limit dextrin was a sufficiently large molecule that it formed a blue complex with iodine. Upon being acted upon by alpha-amylase it was further reduced in size until it lost its ability to give a blue color with iodine. The time required to degrade the limit dextrin to the extent that when combined with iodine, a standard copper-red hue was produced was used as the basis for this determination.

Stock Iodine Solution. 5.5 g. iodine, 11 g. Potassium iodide, dilute to 250 ml.

Dilute Iodine Solution. -2 ml. of the above, 20 g. Potassium iodide, dilute to 500 ml. Made fresh daily.

Buffer Solution. 120 ml. glacial acetic acid, 164 g. anhydrous sodium acetate, dilute to 1 liter.

Calcium Chloride Solution. 0.2 per cent.

Buffered Beta-limit Dextrin. Dissolve 10 g. of soluble starch in boiling water by slowly pouring suspension of starch[1] in a small amount of water into 250 ml. of boiling water. Continue to boil, with stirring for 2 minutes. Cool. Add 24 ml. of buffer, 250 mg. of beta-amylase[2] and sufficient water to

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[1]Soluble (Lintner) starch, specially prepared for amylase determinations. Merck & Co., Rahway, N. J.

[2]Beta-amylase - Special standard prepared by Wallerstein Laboratories, New York, N. Y.

bring volume to 500 ml. and saturate with toluene. Store for at least 24 hours and not more than 72 hours at 30° C. before using.

Apparatus. Water bath, adjusted to 30° C. Hellige Color Comparator[3] and glass color standard.

Preparation of Malt Extract. Five grams of ground malt was extracted for an hour with 100 ml. of 0.2 per cent calcium chloride solution at 30° C. After centrifugation and filtration, 10 ml. of extract was diluted further with 0.2 per cent calcium chloride. The standard procedure specifies a 1-10 dilution at this stage but due to the variability in activities of malts the actual dilution which was carried out was variable.

Determination of Activity. To a 20 ml. portion of the limit dextrin, 10 ml. of diluted malt extract was added, noting the time of addition. At intervals, 1 ml. aliquots of the digestion mixture was rapidly pipetted into 5 ml. of dilute iodine solution and the color of the mixture was compared with that of the glass color standard of the comparator. The progress of the reaction was followed until the color of iodine-reaction mixture combination matches that of the standard, at which time the reaction was complete and the time noted to the nearest 1/4 minute. Best results were obtained if the total reaction time was 10-30 minutes. Thus, it was a necessity to vary the dilution of the extract. The transfer of enzyme to the reaction flask and of aliquots of reaction mixture to iodine solution must be rapid and accurately timed.

Calculation of Activity. The equation below is a sample calculation. Stetl The product of the amount of starch employed (in this instance 0.4 g.) and 60 (minutes) by the malt equivalent in grams times the dextrinization time (minutes). In this instance the malt contained 30 alpha-amylase (SKB) units per g.

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[3]Hellege, Inc., Garden City, L. I., N. Y.

$$\frac{0.4 \times 60}{0.05 \times 15} = 30 \text{ units/g.}$$

Determination of Proteolytic Activity

Buffer Stock Solution. 120 ml. of glacial acetic acid and 164 g. of anhydrous sodium acetate were dissolved in sufficient water to give a final volume of one liter. Buffer for use in determinations was prepared by diluting the stock solution 1-20.

Bacto-hemoglobin. "Difco" brand hemoglobin as supplied by Difco Labs, Detroit, Mich. The pH of a slurry of 2.5 g. of hemoglobin, 5 g. of pumice and 50 ml. of dilute buffer must be 4.70 + .05. When the pH varied, the stock buffer was adjusted accordingly.

Digestion Procedure. Five grams of finely ground malt, 2.5 g. (dry weight) of Bacto-hemoglobin and ca. 5 g. (1 teaspoon) of fine pumice were mixed by rotation to obtain an intimate mixture. Two such mixtures were prepared for each malt in 125 ml. Erlenmeyer flasks. Fifty ml. of diluted buffer solution (previously adjusted to 40° C.) were added to each flask with thorough agitation. The flasks were placed in a water bath at 40° C. equipped with a shaker to provide for continual movement of flasks. The time required for the solubization of unprecipitatable nitrogenous material was arbitrarily considered to be 15 minutes. Consequently the "digestion" of the blank was halted after 15 minutes by the addition of 10 ml. of trichloroacetic acid solution. The other sample of each pair was allowed to digest for 5 1/4 hours before the addition of trichloroacetic acid. Flasks were returned to the water bath for 30 minutes after the admixture of trichloroacetic acid. Suspensions were then filtered through No. 5 Whatman paper.

Nitrogen Determination. The soluble nitrogen content of aliquots

(normally 10 ml.) of the filtrate was determined by the standard Kjeldahl-Gunning procedure. Care was taken to add the water and alkali to flasks prior to the distillation step in such a manner than the trichloroacetic acid which condensed in the neck of the vessel was washed down. If this was not done the trichloroacetic acid was distilled into the standard acid in the receiving bottles consequently influencing the results. The un-neutralized standard acid was back-titrated with 0.0714 N NaOH.

Calculation of Activity. Protease activity was based on the amount of non-protein nitrogen which was released from hemoglobin by enzymic action of malt during a net digestion period of five hours. The net back-titration value was obtained by subtracting the value obtained from the 5 1/4 hour sample from that obtained for the 15 minute (blank) sample. The value used in the subsequent calculations was based on the analysis of 10 ml. aliquots, hence, when smaller aliquots were used the values must be adjusted prior to performing calculations. The "net" titration value (as ml. of 0.0714 N NaOH) was then raised to the 3/2 power. This value was then multiplied by 6 (the total volume of the digest + 10 ml. aliquot) and by 1000/mg. of enzyme source. The figure thus obtained was protease activity expressed as hemoglobin units (HU) per gram of the enzyme preparation.

Determination of Gibberellic Acid in Steep Liquid

The assay of gibberellic acid was based on the formation of a fluorogenic substance resulting from the interaction of gibberellic acid and concentrated sulfuric acid.

Reagents. Sulfuric acid, 85 per cent.

Quinine sulfate, .25 mg./100 ml. in 0.1 N sulfuric acid.

Standard gibberellic acid solutions contained 5, 10, 15, and 20 gamma per ml.

Equipment. Fluorophotometer equipped with a Corning 5113 lamp filter and a 3387 photocell filter.

Procedure. The quinine sulfate solution was used to set the fluorometer potentiometer scale. A standard curve was prepared by measuring the four concentrations of gibberellic acid indicated above and a blank (sulfuric acid). One ml. of the standard solutions were mixed rapidly with 25 ml. of 85 per cent sulfuric acid in a 50 ml. Erlenmeyer flask while immersed in an ice bath. Readings were made after solutions reached room temperature. The standard curve was linear and passed through the origin when values were corrected for the sulfuric acid blank. The unknowns were determined in like manner, diluting when necessary in order to give readings in the range covered by the standard curve.

Preparation of Gibberellic Acid Derivatives

Ammonium gibberellate monohydrate was prepared by evaporating 100 mg. of gibberellic acid in 6.5 ml. of 0.068 N ammonium hydroxide to dryness at 22-25° C. under reduced pressure. The sodium and potassium gibberellate (sesquihydrates) were produced by reacting 100 mg. of gibberellic acid in 6 ml. of methanol with 3 ml. of 0.02 N hydroxide solutions. The liquids were then evaporated at 20° C. under reduced pressure.

The other salts were prepared by shaking 100 mg. of gibberellic acid in 10 ml. of water with an excess of the appropriate metallic carbonates for 10 hours at approximately 30° C. (ambient laboratory temperature). Liquids removed by centrifugation and filtration were dried under reduced pressure at 20-25° C. Final drying was done in a vacuum desiccator over phosphorous pentoxide.

All of the compounds formed were colorless except for the copper, manganese and cobalt gibberellates which were blue-green, pale fawn, and purple, respectively. The silver gibberellate, as expected, turned brown when left in the light.

THE EFFECTS OF GIBBERELLINS AND OTHER PLANT GROWTH REGULATORS ON THE DEVELOPMENT OF ALPHA- AND BETA-AMYLASES AND PROTEASES DURING THE MALTING OF WHEAT

by

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Wheat seed germination rate was greatly increased following steeping in potassium gibberellate (GA-K) solutions. Coleoptile (acrospire) growth alone was stimulated. There was either no or little effect on root growth, depending upon the concentration employed.

The optimum steep liquor concentration for the production of alpha- and beta-amylases was approximately 0.005 per cent while that for protease was about 0.001 per cent. The response to GA-K was somewhat dependent upon variety but did not seem to be correlated with the inherent property of the wheat to produce a malt having high enzymic activity. Treatment of wheat with appropriate levels of GA-K made possible the production of malts having enzyme activities equivalent to those of the controls which had been germinated for 1-3 additional days. Alpha-amylase activity could be more than doubled, beta-amylase increased by as much as 86 per cent over the control, and protease activity increased by 50 per cent or more when proper treatments were employed.

Response to GA-K was most pronounced during the early stages of growth, there being a greater difference between enzyme levels of treated and control malts during the first 1-2 days than during the latter portion of the germination period. Maximum stimulation was achieved following soaking for the final 18-24 hours of the steep period but very significant increases were noted when shorter periods of contact were employed. Exposure to GA-K during the final steep period or its application during the early portion of the germination period appeared to be the most feasible from an economic standpoint. The fact that apparently a relatively small amount of GA-K was absorbed from the steep liquor serves to emphasize the desirability of the spray procedure.

The response to the use of combinations of GA-K and plant growth regulators was dependent upon the types of substances used and the concentrations employed. The use of low levels of indole-3-acetic acid, alpha-naphthaleneacetic

acid, or 2,4-dichlorophenoxyacetic acid and GA-K were found to stimulate growth and enzyme production, whereas higher concentrations of the auxin-like substances overcame to varying degrees the stimulatory effects of GA-K. Triiodobenzoic acid, coumarin, and maleic hydrazide appear to counteract the effect of GA-K at low concentrations.

Treatments with mixtures of thiol compounds and GA-K resulted in increased enzyme activities and, thus, were of theoretical interest but appeared to be of little or no potential commercial importance. A similar conclusion may be drawn when the apparently synergistic relationship between GA-K and kinetin is considered.

Combinations of GA-K and fumaric or succinic acids, when used at the proper levels, were found to be more effectual than any of these substances when used alone. The effect of these combinations appeared to be additive rather than synergistic.

Copper, iron, manganese, and silver gibberellates were more effective in increasing enzyme activities than gibberellic acid or GA salts of the alkaline earth elements.