ABSTRACT

During the study of two near-isogenic lines of wheat (Triticum aestivum, var. "Triple Dirk") it was found that endosperm α-amylase levels increased significantly more quickly in the spring line, TD(D) (Triple Dirk "D") than in the winter line, TD(C) (Triple Dirk "C"), during imbibition of the seeds at 25° C. However, after imbibition of the seeds for six weeks at 2° C., no significant difference could be detected between the rates of α-amylase production by the two lines at 25° C. Agarose gel electrophoresis in phosphate buffer (pH 7.3, μ 0.02) resulted in the separation of seven bandes of α-amylase activity. No differences could be detected between the α-amylase species found in the endosperm portions of the spring line and those found in the portions of the winter line.

Gibberellin-glycosides were extracted in methanol from the dry seeds of a wheat (T. aestivum, var. "Marquis") and dissolved in phosphate buffer (pH 8). The aqueous phase was washed with petroleum ether, acidified (pH 3) and any free gibberellins were removed with ethyl acetate. The glycosides were partitioned into 1-butanol and resolved by thin layer chromatography into five spots. ^{14}C-labelled gibbere-

llin-glycosides were prepared by incubating embryo and endosperm preparations of TD(D) for 24 hours with exogenous gibberellic acid (GA_3) and UDP-glucose-(U-^{14}C), and extracting each preparation as above. These glycosyl-conjugates were shown to be detoxified forms of the hormone, since acid hydrolysis was found to increase the levels of gibberellin-like activity in the endosperm-half bio-assay. However, endosperm-halves of TD(C) and TD(D), showed no changes in α-amylase production in response to 10^{-6}M. GA_3 when exogenous UDP-glucose was included in the bio-assay medium, even to a concentration of 10^{-2}M. UDP-glucose. Thus, the differences in α-amylase production in the spring and winter lines of Triple Dirk during their imbibition at 25° C. can be explained neither by differences in their response to the hormone, nor by a higher rate of detoxification of the hormone by glucosyltransferases in the winter line, TD(C), at 25° C.

PRÉCIS

Pendant l'étude de deux variétés "presque-isogéniques" de blé (Triticum aestivum, cult. "Triple Dirk"), nous avons trouvés que les niveaux d'α-amylase en l'endosperme augmentaient plus rapidement chez la variété printanière, Triple Dirk "D" (TD(D)), que chez la variété hivernale, Triple Dirk "C"(TD(C)), durant l'imbibition des graines à 25° C. Mais après l'imbibition des graines pour six semaines à 2° C., aucune différence a été remarqué en production d'α-amylase à 25° C. entre les deux. L'électrophorèse en gel d'agarose en tampon phosphatique (pH 7.3, μ 0.02) a résolu sept bandes d'activité d'α-amylase. Les deux variétés montrirent le même type de bandes.

Des glucosides de gibbérellines ont été extraits en méthanol des graines seches d'un blé (T. aestivum, cult. "Marquis") et dissolus en tampon phosphatique (pH 8). La phase aqueux a été lavée avec de l'éther de pétrole, et acidifiée a pH 3. Des gibbérellines libres a été enlevées en acetate éthylique et les glucosides a été déplacés en 1-butanol et résolus en cinq taches par la chromatographie en couche-mince. Des glucosides radio-actifs (^{14}C) de gibbérellines ont été faits par l'incubation des préparations de

germes et d'endospermes de TD(D) avec de l'acide gibbérellique (GA_3) et de l'UDP-glucose-($U-^{14}C$) pendant 24 heures. Les glucosides a été extraits comme ci-dessus.

Ces glucosides étaient des formes détoxifiées des hormones, à cause de l'augmentation de leur activités gibbérelliques après l'hydrolyse acidique. Mais, les demi-endospermes de TD(C) et de TD(D), couvés avec 10^{-6}M. GA_3, se montraient aucune diminution en production d'α-amylase avec l'UDP-glucose ajouté, ni a un niveau de 10^{-2}M. UDP-glucose. Donc, on ne peut pas expliquer les différences en production d'α-amylase chez les deux variétés durant l'imbibition à 25° C. ni à cause d'un différence innée en leur réaction aux hormones, ni à cause d'un détoxification des hormones unique à la variété hivernale.

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This thesis is dedicated to Jill, my wife, without whose moral support and tireless hours over the typewriter, this manuscript would not have been possible.

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1. Introduction

1.1 Vernalization

Vernalization (jarovisation) is a term coined by T.D. Lysenko in 1931 to describe the acceleration of the flowering of winter cereals with a period of low temperature treatment (0°C. to 10°C.) of the germinating seed or of the vegetative plant (Picard, 1968). Spring cereals have no cold requirement and, under long-day conditions (16-18 hr. of illumination per day), will flower at approximately the 7-leaf stage of development. Winter cereals have varying cold requirements depending upon the variety used and the growth stage at which vernalization is begun. It is the usual practice to apply the treatment to the germinating seed. Fully vernalized winter cereals develop as do the spring varieties, floral initiation occurring at the 7-leaf stage under long day conditions. Under short-day conditions (10 hr. or less of illumination per day), initiation in spring and in vernalized winter forms of "Petkus" rye did not occur until the 12-leaf stage. In the same study, unvernalized "Petkus" winter rye developed to the 24-leaf stage before floral initiation under either short-day or long-day conditions (Gott, Gregory and Purvis, 1955). Purvis and others have studied the effects of various temperatures on the vernalization process. They have found that vernalization

occurs between 0°C. and 10°C., with an optimum range of 0°C. to 7°C., and that devernalization occurs at temperatures above 15°C. The neutral range between 10°C. and 15°C. is without effect, however exposure to these temperatures serves to stabilise the plant against devernalization by subsequent exposure to higher temperatures. The plant also becomes less susceptible to devernalization as vernalization proceeds (Friend and Purvis, 1963; Trione and Metzger, 1970).

The low-temperature physiology of cereals has been widely studied. It has been well established that vernalization is strictly an aerobic process (Purvis, 1961; Picard, 1968; Chailakhyan, 1968). Cold hardening (the increase in frost tolerance) of cereals exposed to low temperatures results in an increase in total carbohydrates with a decrease in reducing sugar. In general, frost tolerance seems to be correlated to levels of oligosaccharides, in particular sucrose, which seems to have a cryoprotective function in the plant (Kneen and Blish, 1941; Rybakova, 1963; Levitt, 1966; Paulsen, 1968; Green, 1972).

Purvis has shown that vernalization of excised winter rye embryos could not take place unless they were given an exogenous source of carbohydrates and that better response and growth occurred in the presence of

sucrose than in the presence of any of the other sugars tested (Purvis, 1961). Using spring varieties as a low-temperature control, it has been established that the increases in total carbohydrates are significantly higher in vernalization than in cold-hardening (Devay, 1962; Trione, 1966). Several workers have studied levels of free amino acids during cold-hardening and during vernalization, and have established that glutamate, aspartate and their amides as well as serine and proline are particularily affected by vernalization (Trione, Young and Yamamoto, 1967; Ste. Marie and Weinberger, 1970; Jones and Weinberger, 1970; Srivastava and Fowden, 1972).

In 1953, Purvis and Gregory succeeded in hastening the flowering of unvernalized winter rye embryos using a chloroform extract of vernalized embryos (Purvis and Gregory, 1953). This stimulated much work concerning the hormonal interrelationships during vernalization, particularily gibberellins, since these have been shown to be able to induce flowering in a number of systems (Paleg and West, 1972). This, however, is not the case for cereals (Purvis, 1960). Nevertheless, several workers have shown that levels of endogenous gibberellins are increased in cereals after vernalization (Suge and Osada, 1966; Chailkhyan, 1968) and that the two are very closely

linked in function (Suge and Osada, 1966; Bolduc, Cherry and Blair, 1970). Recently, Tomita has succeeded in isolating an active principle from aqueous extracts of vernalized winter wheat or winter radish, which is not found in non-vernalized plants and which is capable of hastening the flowering of unvernalized winter wheat plants (Tomita, 1963; Tomita, 1964). This principle appears to be identical with 3'-uridylic acid (Tomita, 1968; Tomita, 1973). Much basic work remains to be done concerning the vernalization process, and the closest we may be to a molecular explanation may be in Tomita's studies. However, Trione and Metzger, using mixed isomers of uridylic acid, have been unable to reproduce Tomita's work (Trione and Metzger, 1971).

1.2 Gibberellins

Since the description by Sawada in 1912 of the stimulation of the growth of Oryza which had "Bakanae" disease resulting from its infection with the fungus, Fusarium moniliforme (Sheldon) Gibberella fugikuroi (Saw.) Wr. , studies on gibberellins have been carried out in many laboratories. The work of Kurosawa, Yabuta and Hayashi culminated in the isolation from the fungus of a relatively pure, non-crystaline substance by Yabuta, in 1935, which he called "Gibberellin". This was resolved into two crystalline components by Yabuta and Sumiki in 1938, gibberellins A and B. The first studies of gibberellins outside of Japan began in 1950 in the United States and in Britain, and, by 1960, the structures of eight gibberellins were known, five of which had been shown to have biological activity (Stowe et al, 1959; Phinney and West, 1960). In 1965, Paleg described 13 naturally occuring gibberellins and stated that gibberellin-like substances have been found in cereal grains and in some higher plants (Paleg, 1965), and Paleg and West have described 24 (Paleg and West, 1972). MacMillan describes gibberellins 1 through 29 as of April 1970, and states that gibberellins and gibberellin-like substances have been found in over 100 dicots, over 30 monocots, and in

several conifers (MacMillan, 1971). In 1971, MacMillan described gibberellins 30 through 36 (MacMillan, 1972).

It is easily seen that the field of gibberellin chemistry is advancing very rapidly at present, however the study of the physiological roles of gibberellins is a slow process. Gibberellin action has been implicated in various aspects of reproductive growth, in the delay of leaf scenescence, in the breaking of seed and bud dormancy, in the reinforcement of apical dominance, in the promotion of stem elongation through both increased cell division and cell elongation, and in the promotion of root growth. Roots have also been implicated as a major site of gibberellin synthesis in the mature plant (Paleg and West, 1972). However, the application of exogenous gibberellins to many plants results in increases in endogenous auxin levels of up to two orders of magnitude (Paleg, 1965) and many of the above effects might be explained on this basis.

Gibberellic acid (GA_3) has been found to replace the light requirement in the germination of Grand Rapids lettuce, and, while the synthesis of RNA is necessary to this response, DNA synthesis is not (Khan, 1967). Johri and Varner found that there was an enhancement of RNA synthesis, assayed in vitro, in nuclei which had been isolated in the presence of GA_3 from the shoot of

a dwarf variety of pea, and that the RNA thus synthesized had a different nearest-neighbour frequency than that from nuclei isolated without GA_3. They found, however, that RNA synthesis was not enhanced if GA_3 were added only after the isolation of the nuclei, which seems to implicate a cytoplasmic component, possibly a gibberellin receptor, necessary to this system (Johri and Varner, 1968). The application of GA_3 to isolated barley aleurone layers has been shown to result in increases in levels of endogenous 3',5'-cyclic-AMP (Pollard, 1970), and it has been demonstrated that GA_3 can enhance, and abscisic acid can inhibit, the translation of pre-existing mRNA in the early germination of wheat embryos, before RNA synthesis occurs (Chen and Osborne, 1970). In the cereal aleurone, which is the best studied of the gibberellin-dependent systems, the application of GA_3 induces the secretion of several hydrolytic enzymes, including α-amylases (Varner, 1964), acid phosphatases (Jones, 1969), proteases, endo-β-glucanases, pentosanases, and ribonucleases (Yormo and Iinuma, 1964). De novo synthesis has been demonstrated in the case of α-amylase and enzyme release has been correlated with polyribosome formation in barley aleurone layers. Abscisic acid, which inhibits this enzyme

release, inhibits polyribosome formation (Varner and Johri, 1968; Evins and Varner, 1972).

Radley has shown that barley scutella secrete GA_1 during the first 48 hours of germination, and that this secretion, since it is not stimulated by exogenous mevalonate, is of a stored form. After three days, GA_3 synthesized in the embryonic axis becomes the predominant form. Both secretions were inhibited by CCC (β-chloroethyltrimethylammonium chloride) (Radley, 1967) which inhibits the final cyclization of copalyl pyrophosphate to ent-kaurene (Shechter and West, 1969). Thus, the initial secretion of GA_1 is probably due to its synthesis from a stored precursor, such as copalyl pyrophosphate. Cohen and Paleg have suggested that the initial secretion of GA_3 (?) by germinating barley embryos is due to the release of "bound" gibberellins, however their results support those of Radley (Cohen and Paleg, 1967).

1.3 Triple Dirk

In the past, research into the vernalization phenomenon, as well as responses of plants to other environmental influences such as water stress, photoperiod, etc, has been oriented either toward basic physiological studies, or toward applied genetic studies. The physiologist has accepted the "spring-winter" classification of cereals with little or no understanding of the genetic backgrounds of his study materials, and the plant breeder has been hampered through a lack of understanding of the physiological reasons for a plants response to low temperature. Dr. Pugsley envisages a clarification of this problem through a co-ordinated genetic and physiological research program (Pugsley, 1968), and has developed the Triple Dirk cultivars to this end. He classifies these strains as "near-isogenic" meaning that they are rigidly defined as to "Vrn" genotype and that every precaution has been taken to maintain a minimum variability in their genetic background.

Dirk wheat, developed by E.J. Breakwell from the cross Ford X Dundee in the 1930's, was selected in the early 1940's as a recurrent parent in an extensive backcrossing program, due to its outstanding yield and quality

characteristics, in order to add resistance to several major wheat diseases. The result of this program was the development, in 1958-59, of a crossbred with the three Kenya stem rust resistance genes Sr_6, Sr_{9b} and Sr_{11}, designated "Triple Dirk" (Pugsley, 1970).

Triple dirk is a daylength insensitive wheat and thereby better suited to Australian conditions than most northern varieties, which are very sensitive to short days. However, it is of full spring habit and extremely sensitive to stem frosting. Thus, in 1959, a backcrossing program was begun with the cross Triple Dirk X Winter Minflor to isolate strains with resistance to stem frosting as well as some flexibility as to sowing time. The donor Triple Dirk was found to have two dominant genes inhibiting the expression of winter habit (Vrn_1, Vrn_2), and three lines were segregated. Triple Dirk (C) possessed the recessive alleles of both genes (vrn_1, vrn_2) and was of full winter habit. Triple Dirk (B) was responsive to vernalization and Triple Dirk (D) was not, each possessing a single dominant allele (Pugsley, 1968, 1970, 1971). A third locus (Vrn_3) has been identified and placed in a Triple Dirk background (Pugsley, 1972). Studies carried on in our own laboratory by N. de Silva have

confirmed that Triple Dirk (C) has a full vernalization requirement (6 weeks at 2°C.) while Triple Dirk (D) is not responsive to vernalization (de Silva, 1974).

Cultivar	Genotype	Response to vernalization
Triple Dirk	Vrn_1, Vrn_2, vrn_3	nil
Triple Dirk (B)	vrn_1, Vrn_2, vrn_3	positive
Triple Dirk (C)	vrn_1, vrn_2, vrn_3	positive
Triple Dirk (D)	Vrn_1, vrn_2, vrn_3	nil
Triple Dirk (E)	vrn_1, vrn_2, Vrn_3	positive

The objective of the work presented herein was to study the aleurone response of Triple Dirk (C) and Triple Dirk (D) in an examination of work previously done on gibberellin levels in winter wheat (Suge and Osada, 1966; Chailakhyan, 1968) and to extend these studies in light of what is now known concerning aleurone physiology and gibberellin biochemistry (cf. sec. 1.2).

2. Materials and Methods

2.1 Grain

Two near-isogenic lines of wheat (Triticum aestivum, var. "Triple Dirk") were used in the present study: a spring line (TD(D)) and a winter line (TD(C)). These were graciously supplied by Dr. A.T. Pugsley of the New South Wales Department of Agriculture.

2.2 Chemicals

All chemicals were used as supplied.

CHEMICAL	SUPPLIER
Bovine serum albumin	Sigma
Amylose	Sigma
Maltose hydrate	Sigma
UDP-glucose	Sigma
Neocuproine HCl	Sigma
Sodium borohydride	Sigma
Chloramphenicol	Empire Laboratories
3,5-dinitrosalicylic acid	Eastman
Gibberellic acid (GA_3)	Eastman
Indubiose A-45	I.B. France
2 N. Folin-Ciocalteau phenol reagent	Fisher Scientific Co.
UDP-glucose-(U-^{14}C)	New England Nuclear

2.3 Protein Determinations

2.3.1 Method of Lowry et al:

2.3.1.1 Reagent A: 2% sodium carbonate in 0.1 N. sodium hydroxide.

Reagent B: 0.5% cupric sulfate pentahydrate in 1% sodium potassium tartarate.

Reagent C: 1 ml. of reagent A in 50 ml. of reagent B prepared fresh.

Reagent D: 1.0 N. Folin-Ciocalteau phenol reagent.

2.3.1.2 To 1.0 ml. of a protein sample were added 5.0 ml. of reagent C. After 10 min. at room temperature, 0.5 ml. of reagent D was added and the reaction was allowed to proceed for 30 min. at room temperature. A standard curve was prepared over the range between 0 and 200 µg. of bovine serum albumin and treated simultaneously as above. Absorbances were read at 550 nm. (Lowry et al, 1951).

2.3.2 Method of Hartree:

2.3.2.1 Reagent A: 2 g. of sodium potassium tartarate and 100 g. of sodium carbonate were dissolved in 500 ml. of 1 N. sodium hyd-

roxide, then diluted to 1 liter with water.

Reagent B: 2 g. of sodium potassium tartarate and 1 g. of cupric sulfate pentahydrate were dissolved in 90 ml. of water and 10 ml. of 1 N. sodium hydroxide were added.

Reagent C: 1 volume of 2 N. Folin-Ciocalteau phenol reagent was diluted with 15 volumes of water immediately before use.

2.3.2.2 0.9 ml. of reagent A was added to 1.0 ml. of a protein sample. After the tubes were heated for 10 min. at 50°C., then cooled, 0.1 ml. of reagent B was added and the reaction was allowed to proceed at 25°C. for at least 15 min. 3.0 ml. of reagent C were then forcibly added with a syringe and, after 10 min. at 50°C., the mixture was cooled and its optical density determined at 650 nm. A standard curve was prepared using bovine serum albumin over the range between 0 and 100 µg. and identically treated (Hartree, 1972).

2.4 Methods of reducing sugar determination

2.4.1 Modified dinitrosalicylate method:

2.4.1.1 Dinitrosalicylate reagent: 5.0 g. of 3,5-dinitrosalicylic acid were dissolved, with warming, in 100 ml. of 2 N. sodium hydroxide. 150 g. of sodium potassium tartarate were dissolved, with warming, in 250 ml. of water. These two solutions were mixed, made up to 500 ml. with water, and gravity-filtered through Whatman #1 filter paper.

2.4.1.2 2.0 ml. of dinitrosalicylate reagent and 0.1 ml. of 5.0 mM. maltose were added to 2.0 ml. of sample. The same additions were made to a standard curve prepared with maltose over the range of 0 to 2.0 μmoles. The tubes were heated in a water bath at 85°C. for 30 min., then cooled and diluted with 2.0 ml. of water. Absorbances were determined at 540 nm. (Bernfeld, 1955).

2.4.2 Alkaline copper method (Dygert method):

2.4.2.1 Reagent A: 40 g. of anhydrous sodium carbonate (dibasic) and 16 g. of glycine

were dissolved in 600 ml. of water. 450 mg. of cupric sulfate, dissolved in a minimum of water, were added and the solution was made up to 1 liter.

Reagent B: 120 mg. of neocuproine hydrochloride were dissolved in 100 ml. of water and stored in a brown bottle. Solution was discarded if a brown discoloration developed.

2.4.2.2 5.0 ml. of reagent A and 5.0 ml. of reagent B were added to 2.0 ml. of sample. A standard curve over the range between 0 and 500 nmoles of maltose was similarily prepared. The tubes were heated for 15 min. in a boiling water bath, then cooled, and the contents were diluted to 25 ml. with water. Absorbances were determined at 450 nm. (Dygert et al, 1965).

2.5 Methods of enzyme preparation

2.5.1 Initial studies: Single seeds of comparable initial air-dry weight (40 ± 10 mg.) were each placed in a 9 cm. petri dish lined with three layers of Whatman # 1 filter paper, and imbibed at 25°C. with 8.5 ml. of distilled water. After various periods of imbibition (cf. "Results and Discussion", sec. 3.3) fresh weights were determined, and the seeds were de-embryonated. The endosperm portions were each homogenized for 90 sec. in 2.0 ml. of cold (2°C.) 1.0 mM. acetate buffer (pH 4.8) (Varner, 1964) using a motor-driven glass homogenizer. The homogenate was transferred to a centrifuge tube using 3 x 2.0 ml. of additional buffer, and mixed on a vortex mixer for 30 sec. The suspension was centrifuged at 3 x 10^4 g. for 20 min. at 2°C. and the supernatant was quantitatively transferred to a 25.0 ml. volumetric flask and made up to volume using additional buffer.

2.5.2 Studies with intact seeds: 5 seed lots (approximately 180 mg. per lot) were imbibed in the dark at either 2°C. or 25°C. as the experiment required (cf. "Results and Discussion", sec 3.5) –

in 50 ml. screw-top erlenmeyer flasks containing 5.0 ml. of 20mM. succinate buffer (pH 5.3) saturated with calcium sulfate. After their required period of incubation, the seeds were de-embryonated and the endosperms were homogenized in their imbibition medium then centrifuged at 3×10^4 g. for 15 min. at 2°C. The pellets were each resuspended in 5 x 3.5 ml. of 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate, each time centrifuging as above. The combined supernatants were made up to 25.0 ml. with additional buffer.

2.5.3 Studies with endosperm halves: 5-seed lots were weighed, sterilized for 20 min. in 1% "Javex", then imbibed in distilled water. After 6 hours the seeds were split longitudinally and de-embryonated, and the endosperm halves were incubated for 3 days at 25°C. in 10^{-6} M. gibberellic acid (GA_3) in 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate with several concentrations of UDP-glucose. The incubation medium had been previously filter-sterilized through a 0.20 µm. membrane.

Following this incubation, the contents of each flask were homogenized and centrifuged.

The pellets were extracted with 5 x 3 ml. of additional succinate buffer, centrifuging each time. The combined supernatants were made up to 25.0 ml.

2.5.4 Purification of α-amylase:

2.5.4.1 Method of Schwimmer and Balls: 50 g. of dry seeds of the winter wheat, Triticum aestivum (var. "Rideau"), were imbibed with 100 ml. of 20 mM. succinate buffer (pH 5.3), saturated with calcium sulfate, for 4 days at 30°C. on a shaker, then homogenized for 1 min. using a Sorval "Omnimixer" at top speed. The suspension was centrifuged at 5×10^3 g. for 30 min. at 2°C., and the pellet was resuspended in 2 x 100 ml. of additional buffer, each time centrifuging as above. The combined supernatants were heated, with continuous gentle stirring, to 70°C. for 15 min., then cooled and centrifuged (5×10^3 g. for 60 min. at 2°C.).

The supernatant was adjusted to 0.5 saturation with solid ammonium sulfate and to pH 6.0 with 4% ammonium hydroxide.

The suspension was allowed to stand, with occasional agitation, for 3 hours at room temperature then centrifuged at 10^4 g. for 10 min. at 2°C. The pellet was washed with 30 ml. of 15 mM. calcium chloride, 0.33 saturated with ammonium sulfate (pH 6.0) then centrifuged as above. All further operations were performed between 0°C. and 2°C.

The pellet was dissolved in 50 ml. of cold 15 mM. calcium chloride (pH 5.3) and 50 ml. of cold 5% calcium chloride in 80% ethanol were added. After 30 min. the mixture was centrifuged and the supernatant was stirred magnetically with 5 g. of purified potato starch for 90 min., then centrifuged. The pellet was washed with 2 x 10 ml. of 2.5% calcium chloride in 40% ethanol, centrifuging each time. The combined supernatants were stirred for 90 min. with an additional 5 g. of starch and centrifuged. The pellet was washed with 10 ml. of 2.5% calcium chloride in 40% ethanol, and the enzyme was eluted

from the combined starch fractions with several small volumes of 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate (Schwimmer and Balls, 1949).

2.5.4.2 Method of Greenwood and MacGregor:

60 g. of dry seeds of the spring wheat, Triticum aestivum (var. "Marquis"), were imbibed with 100 ml. of 20 mM. succinate buffer (pH 5.3), saturated with calcium sulfate, at 25°C. for 4 days on a shaker, then homogenized for 1 min. using a Sorvall "Omnimixer" at top speed. The suspension was centrifuged at 5 x 10^3 g. for 10 min. at 2°C., and the pellet was resuspended in 2 x 50 ml. of buffer, each time centrifuging as above. The combined supernatants were heated, with ~~continuous~~ gentle stirring, to 70°C. for 15 min., then cooled and centrifuged at 8 x 10^3 g. for 20 min. at 2°C. To the cold supernatant was added freezer-cooled acetone (-20°C.) to 35% (v/v). The suspension was centrifuged at 8 x 10^3 g. for 20 min. at 2°C., and the pellet discarded. Additional freezer-

cooled acetone was added to the supernatant to 40% (v/v) and the suspension was centrifuged at 8 x 10^3 g. for 40 min. at 2°C. The pellet was dissolved in 40 ml. of succinate buffer and centrifuged at 3 x 10^4 g. for 10 min. at 2°C. The supernatant was made up to 50.0 ml. (Greenwood and MacGregor, 1965; Greenwood, Milne and Ross, 1968; Greenwood and Milne, 1968, a and b).

2.5.5 Purification of β-amylase: 60 g. of dry seeds of spring wheat, Triticum aestivum (var. "Marquis"), were homogenized in 75 ml. of water, using a Sorval "Omnimixer" at top speed for 1 min., and transferred to a 1 liter erlenmeyer flask with an additional 225 ml. of water. The mixture was left overnight at 30°C. on a shaker, then centrifuged at 5 x 10^3 g. for 30 min. at 2°C. The supernatant was adjusted to pH 3.7 with 5 N. acetic acid and left between 0°C. and 2°C. for one week. The pH of the mixture was adjusted to 5.3 with 4% ammonium hydroxide, and 2 volumes of saturated ammonium sulfate (pH 5.3) were added. The mixture was centrifuged at 7 x 10^3 g. for

30 min. at 2°C. and the pellet was dispersed in 100 ml. of cold water. After 5 hours between 0°C. and 2°C., the suspension was centrifuged and the supernatant was diluted to 160 ml. and adjusted to pH 5.5 with 4% ammonium hydroxide. 43 ml. of saturated ammonium sulfate (pH 5.5) were added over 20 min., and the suspension was centrifuged at 7×10^3 g. for 10 min. at 2°C. 183 ml. of saturated ammonium sulfate (pH 5.5) were added to the supernatant and the suspension was centrifuged at 8×10^3 g. for 20 min. at 2°C.

The pellet was dispersed in 21 ml. of water and left overnight at 0°C. to 2°C. The solution was then diluted to 45 ml. and adjusted to pH 5.3 with 5 N. acetic acid. 17 ml. of saturated ammonium sulfate (pH 5.3) were added over 20 min. and the suspension was centrifuged at 5×10^3 g. for 20 min. at 2°C. 63 ml. of saturated ammonium sulfate (pH 5.3) were added to the supernatant and the suspension was centrifuged at 8×10^3 g. for 10 min. at 2°C. The pellet was dissolved in a minimum of 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate and transferred to a dialysis sack. Dialysis was carried out,

with continuous gentle stirring, against 3 daily changes of 1 liter of the same buffer at 2°C. The retentate was centrifuged at 2 x 10^4 g. for 30 min. at 2°C. and the supernatant was made up to 50.0 ml. (Piguet and Fischer, 1952; Meyer, Spahr and Fischer, 1953).

2.6 Determination of amylolytic activities

2.6.1 Preparation of Substrate:

2.6.1.1 Preparation of 1.3% amylose: 6.50 g. of amylose were dispersed in 500 ml. of 0.016 M. acetate buffer (pH 4.8) and the mixture was constantly stirred with gentle heating until the amylose had dissolved. The solution was cooled, and dispensed equally among 15-50 ml. screw-top erlenmeyer flasks, and autoclaved at 115°C. for 10 min.

2.6.1.2 Preparation of 1% reduced amylose:

Stock solution: 2.50 g. of amylose were dissolved in 40 ml. of water with gentle heating, then cooled to 2°C. 75 mg. of sodium borohydride in 5 ml. of cold (2°C.) water was added carefully with continuous stirring, then incubated at 25°C. for 1 hour. The mixture was made up to 50.0 ml. and stored for 48 hours at 2°C. before use.

Substrate solution: 10.0 ml. of the stock solution were placed in a 50 ml. volumetric flask, and excess sodium borohydride was destroyed with 0.2 ml. of redistilled

acetone. For the determination of total amylolytic activities, the substrate was made up to volume with 20 mM. succinate buffer saturated with calcium sulfate (pH 5.3). For the determination of α-amylase, 5.0 ml. of 5 x 10^{-5} M. mercuric chloride in buffer were added, and the substrate was made up to volume with additional buffer (Strumeyer, 1967).

2.6.2 Assay of amylolytic activities: One unit of amylolytic activity is that amount of enzyme which will hydrolyze one nanomole of glycosidic bonds in amylose in one minute under standard conditions, the increase in reducing power being determined against maltose.

2.6.2.1 Initial studies: Individual flasks of 1.3% amylose (cf. sec. 2.6.1.1) were equilibrated at 25°C. 1.0 ml. aliquots of an enzyme preparation were equilibrated for 5 min. in a water bath at the same temperature, and the reactions were started with the addition of 1.0 ml. of starch substrate. After 5.0 min., the reactions were stopped with the addition of 2.0 ml. of dinitrosalicylate reagent, followed by 20 sec. of vigourous

mixing on a vortex mixer. Enzyme blanks were prepared by adding the dinitrosalicylate reagent before the starch substrate. Reducing sugar was determined by the modified dinitrosalicylate method (sec. 2.3.1.2).

2.6.2.2 Standard method: Reactions were started with the addition of 25 µl. of enzyme preparation to 2.0 ml. of the appropriate 1% reduced amylose substrate, previously equilibrated at 25°C., (cf. sec. 2.6.1.2) and were stopped at the required time (20 min. for α-amylase; 3 or 5 min otherwise) with the addition of Dygert "A"-reagent. Enzyme blanks were prepared with the addition of "A"-reagent prior to the addition of enzyme. All assays and blanks were determined in duplicate. Reducing sugar was determined by the alkaline copper method (sec. 2.4.2.2).

2.6.2.3 Amyloclastic method: This method was used to check the pattern of the hydrolysis of amylose by the purified α- and β-amylases (cf. sec. 2.5.4 and 2.5.2). Enzyme was

added to tubes containing 20 ml. of 1% reduced amylose at 25°C. (sec. 2.6.1.2) with the mercuric chloride omitted. Reducing sugar was determined upon 2.0 ml. aliquots, which were removed at 0,5, 10,20, and 30 min., by the method of Dygert et al (sections 2.4.2.2 and 2.6.2.2). 1.0 ml. aliquots withdrawn at the same times were added to a 500 ml. flask containing 400 ml. of water, 5 ml. of 1 N. sulfuric acid and 1 ml. of 0.3% iodine in 3% potassium iodide. The mixtures were made up to volume, and absorbances were determined at 620 nm. against distilled water (Smith and Roe, 1949).

2.7 Agrose gel electrophoresis of amylase isozymes

2.7.1 Buffers: Phosphate buffer was prepared as a concentrate by dissolving 4.14 g. of NaH_2PO_4 H_2O and 12.78 g. of K_2HPO_4 $3H_2O$ in distilled water to a final volume of 100 ml. A 1:100 dilution of this concentrate gives an ionic strength of $\mu = 0.02$ and a pH of 7.3.

Succinate buffer for the substrate slides is the standard 20 mM. succinate (pH 5.3) saturated with calcium sulfate used in previous work.

2.7.2 Preparation of slides: Electrophoresis slides were prepared by dissolving 0.5 g. of immunoelectrophoresis grade agarose (I.B. France, Indubiose A-45) in 100 ml. of phosphate buffer (pH 7.3, μ 0.02) at 100°C.. Standard 75 mm. x 25 mm. glass microscope slides were spread with 2 ml. of this solution, forming a 1 mm. thick agarose layer, and were allowed to cool. The slides were stored in a closed box at 2°C. for 24 hours before use. Substrate slides were prepared in a similar manner using 1% microbiological grade agar with 0.5% soluble starch in succinate buffer, and were stored at 2°C. until required. An electrophoresis chamber capable of holding 10 slides and

with a buffer capacity of one liter was used (figure 1).

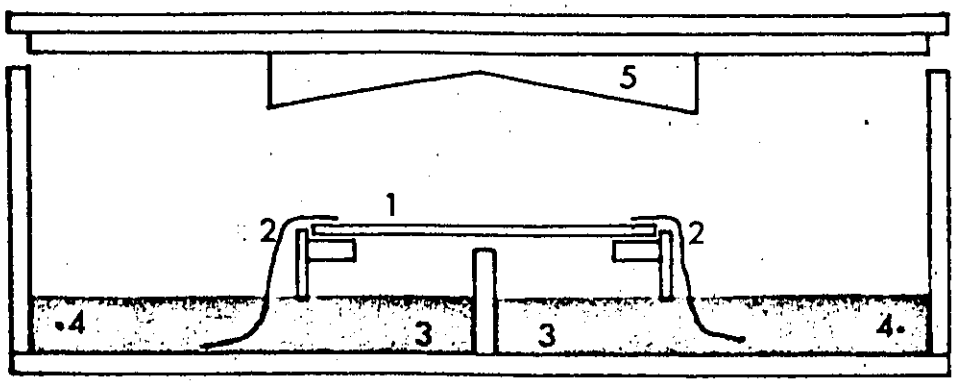
2.7.3 Electrophoresis: Immediately before the expt., a 1 cm. square of Whatman #1 filter paper was inserted in the electrophoresis gel at the line of origin and was allowed to soak up buffer until completely moist. This removes approximately 10 µl. of buffer from the slide, which is replaced with a similar amount of sample injected into the well thus formed using a 10 µl. syringe. Slides thus prepared were placed in the electrophoresis chamber, with their origins towards the cathode, and connections with the electrode compartments were achieved using double thicknesses of buffer-soaked Whatman #1 filter paper. Fresh phosphate buffer was used in the electrode compartments for each run. A current of 3 mA. per slide was obtained from a Heath model IP-17 regulated high-voltage power supply. Electrophoresis was continued for 2 hours at $4^{o}C$.

After the completion of the electrophoresis, the sample slides were placed face-down on substrate slides, and were thus incubated

for 1 hour at 37°C. Both the sample slides and the substrate slides were dipped in I_2:KI and rinsed with distilled water. A record was maintained by photographing the slides in red light, using a Wratten R2 filter, on Kodak Panatomic X film, as the original clarity of the bands is rapidly lost upon drying.

Figure 1 : Electrophoresis chamber used for the separation of bands of amylolytic activity on agarose gel.

1. Standard 25 mm. X 75 mm. glass slide coated with agarose gel.
2. Filter paper wick.
3. Buffer.
4. Platinum electrode.
5. Condensation panel in top.


5
1
2
2
4
3
3
4

2.8 Gibberellin-glycosides

2.8.1 Preparation:

2.8.1.1 Preparation of un-labelled gibberellin-glycosides:

140 g. of dry grains of the spring wheat, Triticum aestivum (var. "Marquis"), were homogenized in 150 ml. of petroleum ether using a Sorvall "Omnimixer" at top speed for 3 min., and the solvent was removed by vacuum filtration. The powder was extracted with 2 X 150 ml. of methanol, each extraction taking 24 hours at 2°C. The methanol was collected by vacuum filtration, and the combined extracts were concentrated to dryness in vacuo. The residue was dispersed in 100 ml. of 0.04 M. potassium phosphate buffer (pH 8.6) and washed with 2 X 50 ml. of petroleum ether, which was discarded. The aqueous phase was acidified to pH 3 with 1 N. hydrochloric acid and washed with 60 ml. of ethyl acetate (which was discarded), then extracted with 5 X 10 ml. of 1-butanol. The combined butanolic extracts were concentrated to dry-

ness in vacuo, taken up in a minimum of methanol, and dried under nitrogen in a previously tared tube, yielding 79 mg. of a yellow oil. This sample was dissolved in 2.0 ml. of methanol and stored at -20°C.

Separation of components was achieved by thin layer chromatography (T.L.C.) on 250 µm. layers of silica gel H, activated at 110°C. for 60 min. and developed with chloroform: methanol: acetic acid: water (40:15:3:2). The spots were initially visualized under ultraviolet light after spraying the plates with sulfuric acid: water (7:3), and heating to 100°C. for 5 min. Glycosides were subsequently detected by spraying with 0.05% α-naphthol in methanol: water (1:1) and heating to 100°C. to develop the blue colour (Tamura et al, 1968; Yokota et al, 1969; Harada and Yokota, 1970).

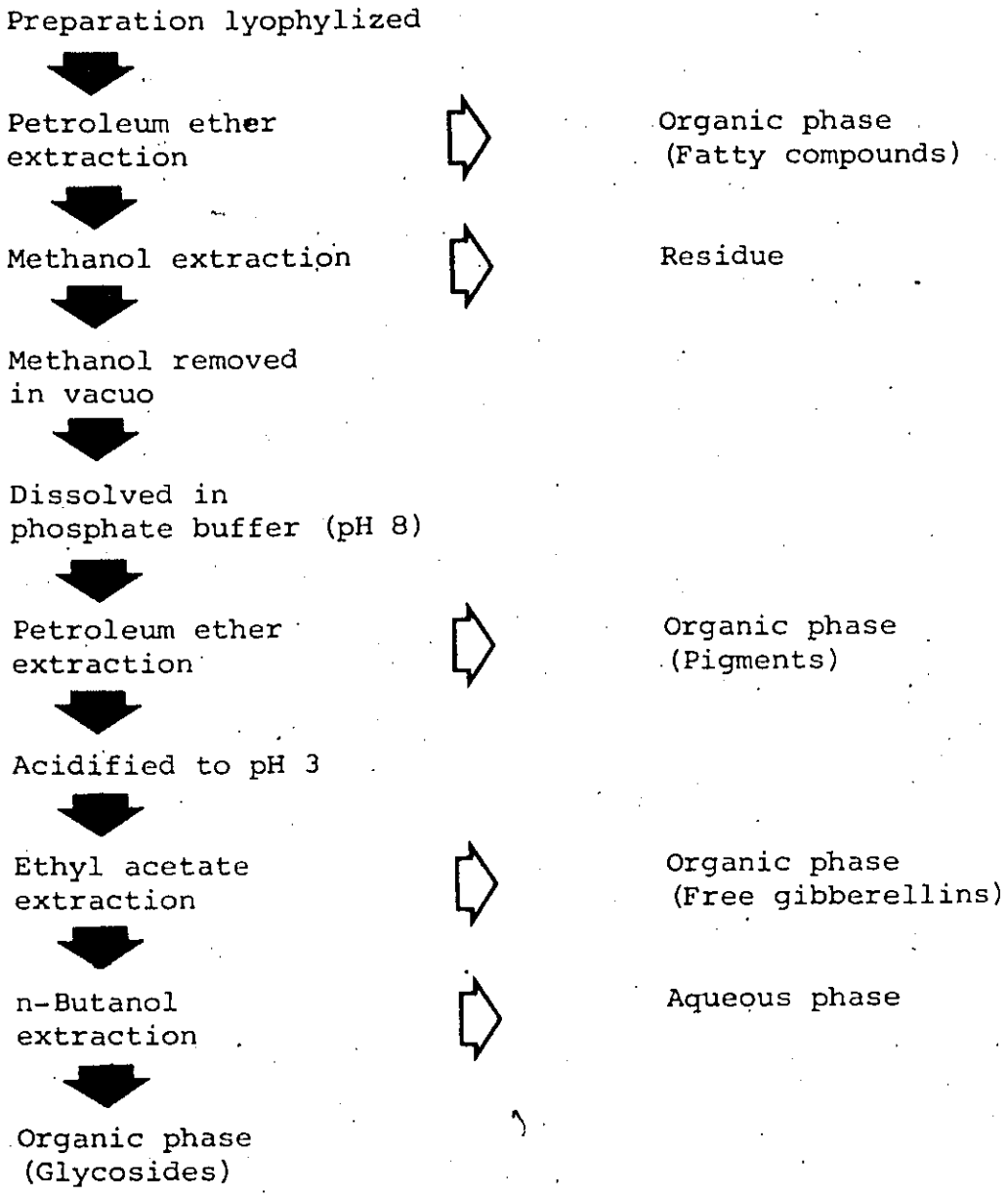
2.8.1.2 Preparation of gibberellin-glycosides labelled with glucose-U-^{14}C: 50 seeds

(1.445 g.) of seeds of TD(D) were disinfected with 1% "Javex" for 15 min. and imbibed with excess distilled water on a shaker at 25°C. for 2 days. The seeds were dissected and germs and endosperms were placed in 2-125 ml. screw-top flasks containing 90 mg. (250 μmoles) of gibberellic acid (GA_3) each in 20 ml. and 30 ml., respectively, of 20 mM. succinate buffer saturated with calcium sulfate and containing 2 mg.% of chloramphenicol and 0.20 μc./ml. of uridine-5'-diphosphoglucose-(U-^{14}C). The flasks were incubated on a shaker (80 cpm) at 25°C. After 24 hours the preparations were lyophylized, then homogenized in 10 ml. of petroleum ether, and centrifuged. The pellets were extracted with 3 X 15 ml. of methanol, each extraction requiring 24 hours at 2°C. The combined methanolic extracts were concentrated to dryness in vacuo, resuspended in a minimum of 0.04 M. potassium phosphate buffer (pH 8.6) and washed with 3 X 2 ml. of petroleum ether. The aqueous

phase was acidified to pH 3 with 5 N. sulfuric acid and washed with 3 X 2 ml. of ethyl acetate, then extracted with 6 X 2 ml. of 1-butanol. The combined butanolic extracts were concentrated to dryness in vacuo, dissolved in 5.0 ml. of methanol and stored at -20°C. (Eichenberger and Newman, 1968).

Radio-activity was determined by liquid scintillation counting in 10 ml. of 0.3% PPO and 0.01% POPOP in toluene: Triton X-100 (7:3) using a two-channel Beckman LS-233 (Turner, Amersham-Searle bulletin). Standard preset discriminator "windows" for ^{3}H and $^{3}H+^{14}C$ were used and quenching was corrected for by the channels ratio method (Bush, 1963; Rogers and Moran, 1966; Blanchard, Wagner and Takahashi, 1968). Samples of each preparation were spotted on a 5 cm. X 20 cm. plate, and separated in chloroform: methanol: acetic acid: water (40:15:3:2). Glycosides were detected using α-naphthol spray, scraped from the plates and counted.

Figure 2 : Flow-chart for the isolation of gibberellin-glycosides.


Preparation lyophylized
Petroleum ether extraction
Organic phase (Fatty compounds)
Methanol extraction
Residue
Methanol removed in vacuo
Dissolved in phosphate buffer (pH 8)
Petroleum ether extraction
Organic phase (Pigments)
Acidified to pH 3
Ethyl acetate extraction
Organic phase (Free gibberellins)
n-Butanol extraction
Aqueous phase
Organic phase (Glycosides)

2.8.2 Bioassay:

2.8.2.1 Bioassay of un-labelled gibberellin-glycosides: Approximately 10 µl. of sample was applied across 3 cm. of the origin of a 5 cm. x 20 cm. plate and developed in chloroform: methanol: acetic acid: water (40:15:3:2). The plates were then divided into 3 zones (fig. 11) and half of each zone was subjected to 16 hours of hydrolysis in 5 ml. of 1 N. sulfuric acid at 100°C., then extracted once with 5 ml. of ethyl acetate. The solvents were removed from the samples in 25 ml. screw-top flasks and the samples were dispersed in 5.0 ml. of 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate.

Single seeds (approximately 26 mg.) of TD(D) were weighed, then disinfected with 1% "Javex" for 15 min. and imbibed in distilled water for 6 hours. The seeds were split longitudinally, de-embryonated and the endosperm halves were placed in their respective pre-

pared flasks and incubated for 3 days at 25°C. After incubation, the contents of each flask were homogenized, and centrifuged. The supernatants were assayed for α-amylase content according to the standard method. (cf. sec. 2.6.2.2)

2.8.2.2 Bioassay of resolved ^{14}C-labelled gibberellin-glycosides: 1.0 ml. of the embryo preparation and 0.4 ml. of the endosperm preparation were applied across the origins of 2-20 cm. x 20 cm. plates coated with 500 μ of Silica gel H and activated for 1 hour at 110°C. Components were resolved in chloroform: methanol: acetic acid: water (40:15:3:2), and the glycosides were eluted from the gel with methanol using 75 mm. funnels plugged with glass wool; likewise, the remainder of the surface of the plates was eluted. The methanol was removed from each sample under nitrogen and replaced with 5.0 ml. of additional methanol.

2.0 ml. of each sample was placed in a screw-top test tube and, after the removal of the solvent, each was hydrolyzed in

2.0 ml. of 1 N. sulfuric acid at 100°C. for 18 hours. The hydrolysates were extracted with 3 x 1.5 ml. of ethyl acetate. The organic phases were evaporated to dryness and each residue was taken up in 2.0 ml. of methanol. The solvent was removed from 0.5 ml. aliquots of each of the hydrolyzed and un-hydrolyzed preparations in screw-top test tubes and the residues were dissolved in 1.0 ml. portions of 20 mM. succinate buffer (pH 5.3) saturated with calcium sulfate. Individual seeds of TD(D) were weighed and sterilized in 1% "Javex" for 30 min. After 6 hours of imbibition in distilled water, the seeds were split longitudinally, de-embryonated, and the endosperm halves were incubated at 25°C. in their respective bioassay tubes. After 3 days, the contents of each tube were homogenized with an additional 4.0 ml. of succinate buffer, then centrifuged. The supernatants were assayed for α-amylase by the standard method (cf. sec. 2.6.2.2).

3. Results and Discussion

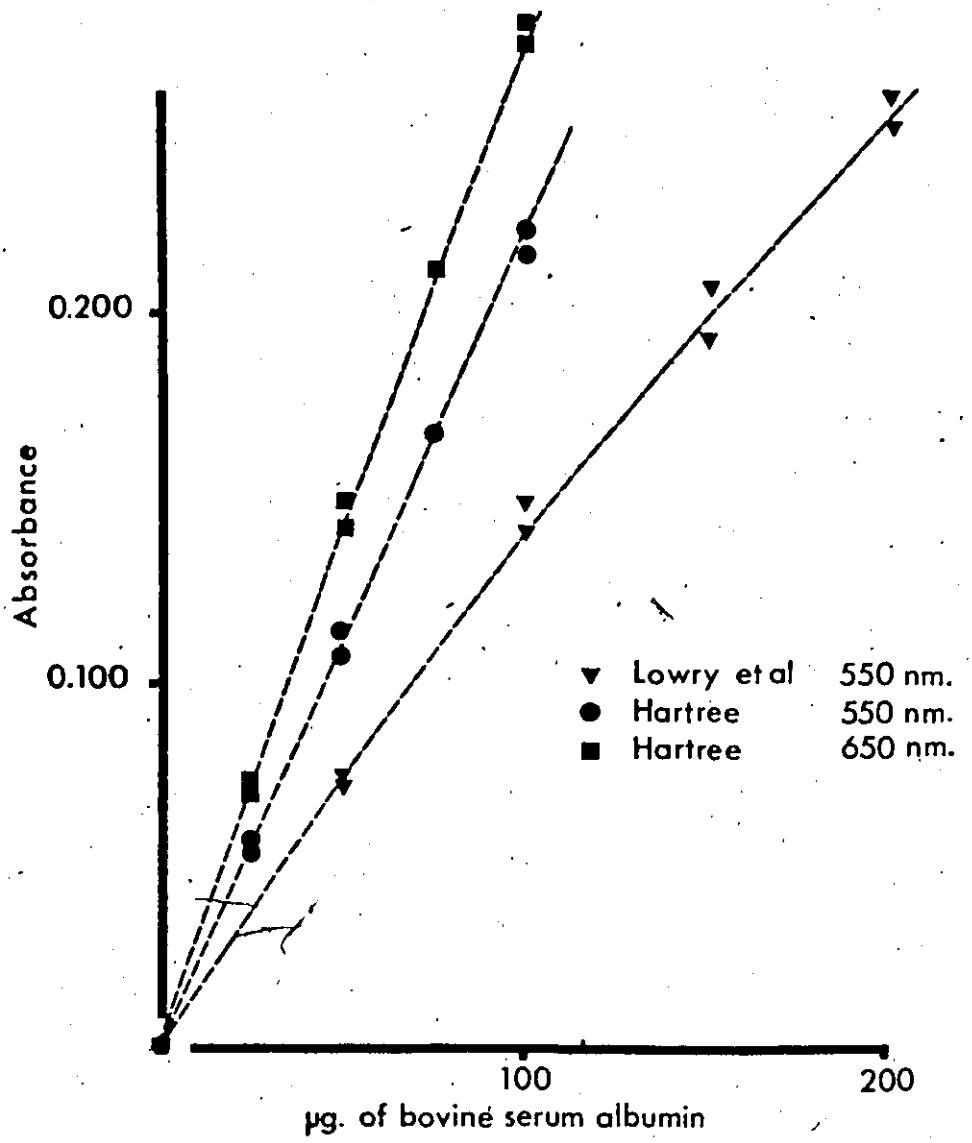
3.1 Comparision of methods of protein determination

Two methods of protein determination, that of Lowry et al (1951) (sec. 2.3.1) and that of Hartree (1972) (sec. 2.3.2) were compared. For comparison, both were read at 550 nm., the Hartree determination being read at 650 nm. as well. These data are shown in table 1 and graphically in figure 3. Colour development was found to be more intense with the method of Hartree than with that of Lowry et al. For this reason, the method of Hartree was found to be a more precise and reliable for use with low levels of protein (20 to 50 µg.) and was used throughout these studies (sec. 2.3.2).

Table 1 : Comparison of the methods of Lowry et al (1951) and of Hartree (1972) for the determination of protein, using bovine serum albumin (B.S.A.)

Micrograms of B.S.A.	Lowry		Hartree			
	$O.D._{550}$	$O.D._{550}$	$O.D._{550}$	$O.D._{550}$	$O.D._{650}$	$O.D._{650}$
0	0.001	0.000	0.000	0.000	0.000	0.000
25	-	-	0.057	0.055	0.073	0.071
50	0.074	0.072	0.113	0.107	0.149	0.140
75	-	-	0.167	0.167	0.212	0.212
100	0.148	0.141	0.216	0.222	0.272	0.278
150	0.193	0.208	-	-	-	-
200	0.260	0.252	-	-	-	-

Figure 3: Comparison of the methods of Lowry et al (1951) and of Hartree (1972) for the determination of protein (Data from table 1).


0.200
0.100
Absorbance
100
200
µg. of bovine serum albumin
Lowry et al 550 nm.
Hartree 550 nm.
Hartree 650 nm.

3.2 Development of the modified dinitrosalicylate method

(sec. 2.4.1) Due to the low amounts of reducing power being detected in the assays of amylolytic activities, Bernfeld's (1955) method was first modified by a reduction of the volume of water added in the final dilution from 16 ml. to 2 ml., such that a higher change of absorbance would be obtained per micromole reducing sugar. Also, due to the large number of tubes in each assay series, a large water bath with a maximum stable temperature of 85°C. had to be used for the heating step, a sufficiently large boiling water bath being unavailable.

Under these conditions, the technique seemed to be unable to detect a residual level of reducing sugar of between 0.2 and 0.4 micromoles as shown in curve A of figure 4. For this reason, the method was further modified to include the addition of 0.10 ml. of 5.0 mM. maltose to all tubes prior to the heating step, which addition seems to eliminate the blanking problem, as in curve B in figure 4.

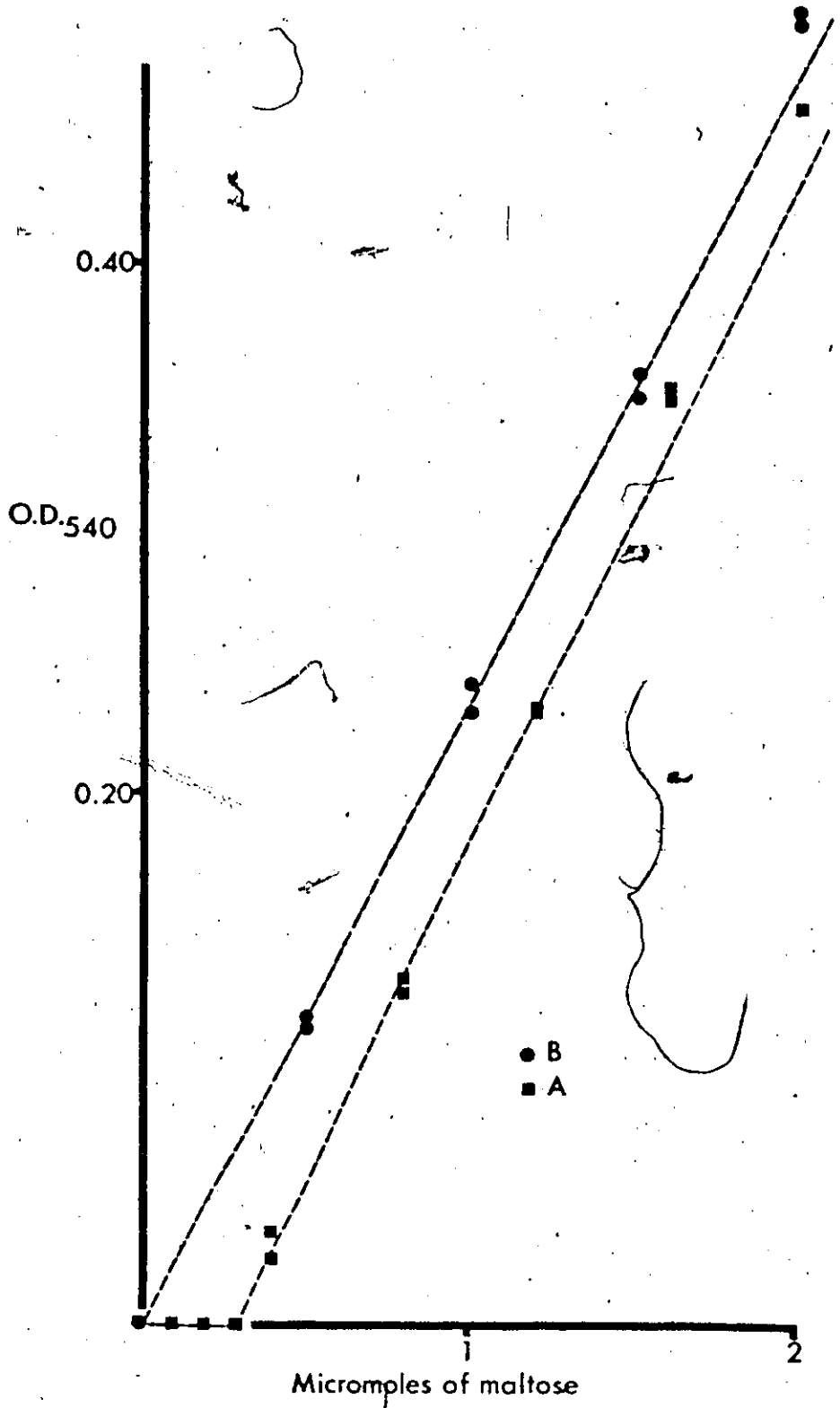
Table 2 : Development of the modified dinitrosalicylate method for the determination of reducing sugar. Two standard curves prepared (A), without the addition of 0.5 µmoles of maltose before heating, and (B), with the additional maltose.

Micromoles of maltose	A		B	
	1	2	1	2
0.0	0.000	0.000	0.000	0.002
0.1	0.000	0.000	-	-
0.2	0.000	0.000	-	-
0.3	0.001	0.002	-	-
0.4	0.025	0.035	-	-
0.5	-	-	0.112	0.118
0.8	0.126	0.130	-	-
1.0	-	-	0.231	0.242
1.2	0.230	0.232	-	-
1.5	-	-	0.350	0.359
1.6	0.350	0.354	-	-
2.0	0.460	0.461	0.492	0.496

Figure 4 : Development of the modified dinitrosalicylate method for the determination of reducing sugar (Data from table 2).

A. Without addition of maltose.

B. With maltose addition.


0.40
0.20
O.D.540
B
A
1
2
Micromples of maltose

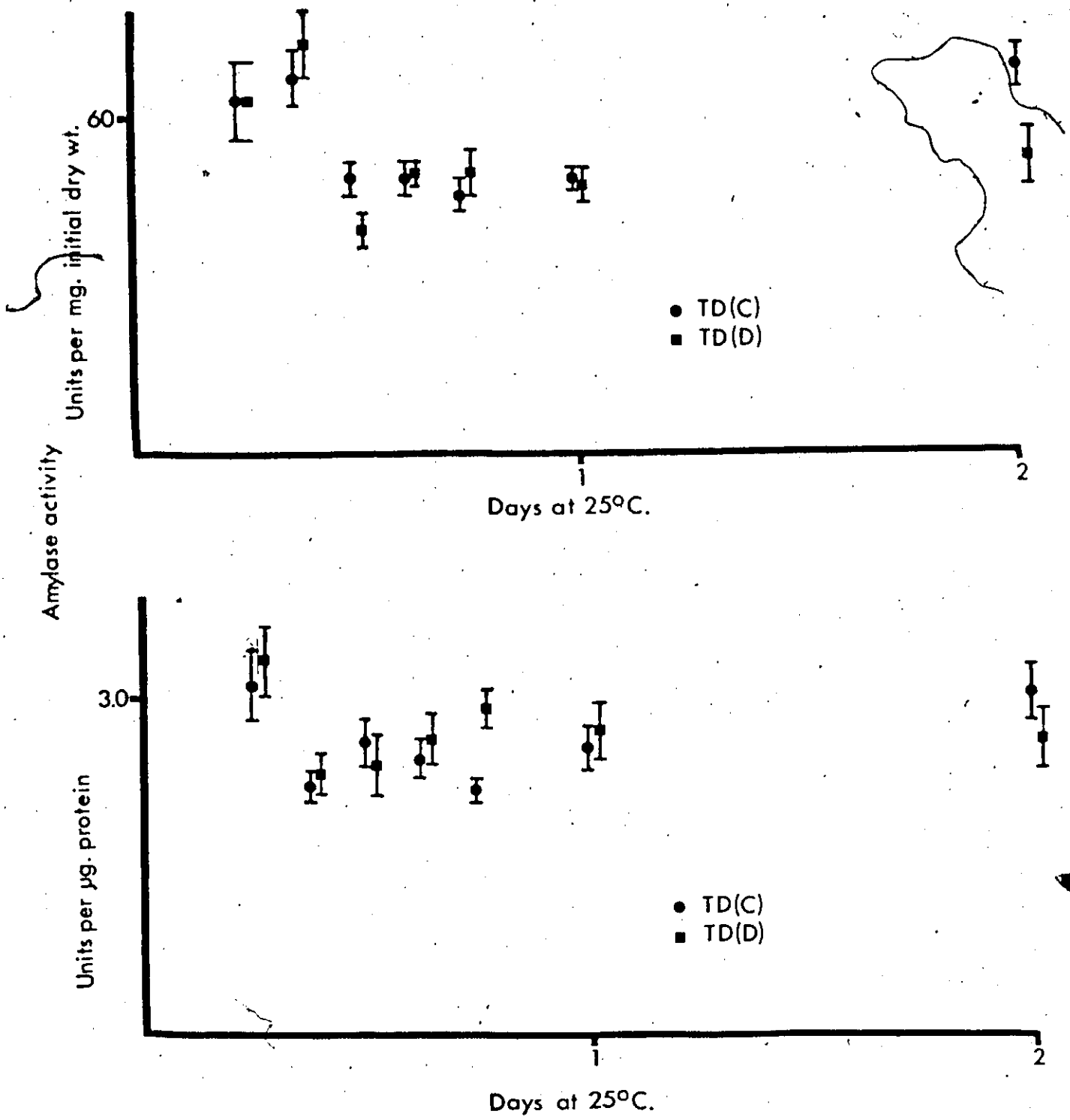
3.3 Initial Studies

Method	Sec.
1. Preparation of enzyme	2.5.1
2. Method of assay	2.6.2.1

The amylolytic activities of the endosperms of seeds of TD(C) and TD(D) during the first twenty-four hours of imbibition were studied in an attempt to localize the initial stages of α-amylase synthesis. However, as shown in figure 5, the activities did not appear to change significantly during this period.

As the activities were assayed after as little as six hours of imbibition, it became apparent that β-amylase was responsible, and that any increases in α-amylase would probably be masked by the high background levels of β-activity. Thus an improved method of assay was sought (sec. 3.4).

Figure 5 : Amylolytic activities found in the endosperms of seeds of TD(C) and TD(D) during the early stages of imbibition (sec. 3.3) Intervals shown are the standard error of the mean.


Amylase activity
Units per mg. initial dry wt.
60
TD(C)
TD(D)
1
2
Days at 25°C.
Units per µg. protein
3.0
TD(C)
TD(D)
1
2
Days at 25°C.

3.4 Development of the standard method for the assay of α-amylase

Method	Section
1. Purification of α-amylase	2.5.4
2. Purification of β-amylase	2.5.5
3. Methods of assay	2.6.2.2
	2.6.2.3

3.4.1 Preparation:

Although the method of Schwimmer and Balls is used by a number of laboratories for the routine preparation of α-amylase from barley, the method (sec. 2.5.4.1) does not appear to be suitable for the isolation of the wheat enzyme. Its use resulted in a continuous reduction in specific activity, as shown in table 3. The destruction of α-amylase by ethanol has been observed by others (Fischer and Haselbach, 1951; Ayrapaa and Nihlén, 1954), thus another method, that of Greenwood and MacGregor, was finally used (sec 2.5.4.2). This method gave good yield, with a 13-fold purification of α-amylase on the basis of specific activity, with a single fractionation (table 4). The β-amylase isolation (sec. 2.5.5) resulted in a doubling of the specific activity

(table 5). The patterns of hydrolysis of amylose substrates, as determined with the amyloclastic method (sec. 2.6.2.3), were used to verify the identity of the enzymes. As shown in table 6 and figure 6, a decrease in the absorbance of the starch-iodine complex was observed in the case of α-amylase, whereas no such change was observed for the β-preparation, even though a much higher level of β-activity was used in the determinations. As the intensity of the absorbance of the starch-iodine complex at 620 nm. depends directly upon the length of the amylose chain, these results are consistent with those expected for α- and β-amylase, respectively.

3.4.3 Inhibition studies: Since our studies are mainly concerned with the aleurone response of wheat, it would be desirable to be able to measure α-amylase activity without interference from β-amylase. Roy and Underkofler showed that β-amylase was inhibited by 0.1% cyanide (Roy and Underkofler, 1950). They noted that some residual β-activity appeared to remain if α-amylase were present, however it has been demonstrated that α-amylase will show definite saccharogenic activity with

Table 3 : Purification of α-amylase by the method of Schwimmer and Balls

Fraction	Total activity (units)	Recovery (%)	Specific activity (units/mg. protein)
1. Supernatant from heating to 70°C.	1.3×10^6	100	620
2. Supernatant from ethanol precipitation	1.3×10^4	1.0	170
3. Eluant from starch	3.7×10^3	0.3	41

Table 4 : Purification of α-amylase by the method of Greenwood and MacGregor.

Fraction	Total activity (units)	Recovery (%)	Specific activity (units/mg. protein)	Purification factor
1. Original extract.	(8.5×10^5)		(266)	
2. Supernatant from heating to 70°C.	1.9×10^5	100	41	1
3. Fraction precipitated between 35% and 40% (v/v) acetone.	7.2×10^3	9	517	13

Table 5 : Purification of β-amylase.

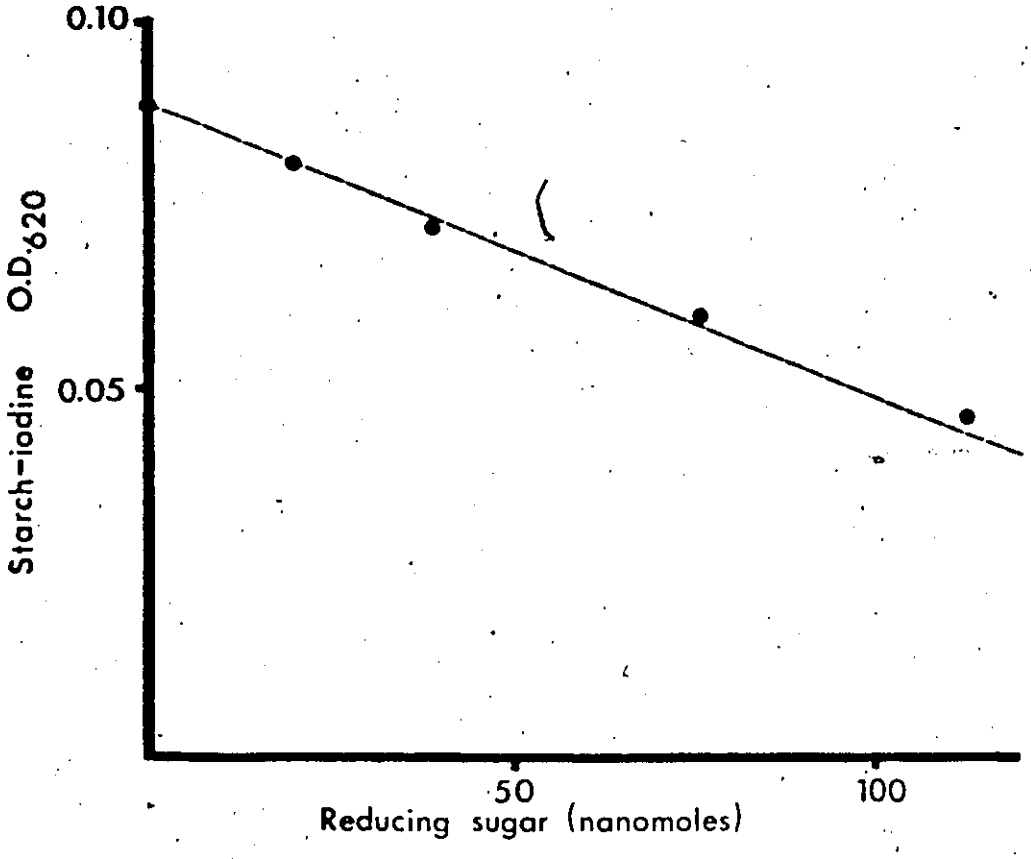
Fraction	Total activity (units)	Recovery (%)	Specific activity (units/mg. protein)	Purification factor
1. Original extract.	(3.4×10^6)		(1.8×10^3)	
2. Supernatant after 6 days at pH 3.7.	1.0×10^6	100	5.2×10^2	1.0
3. First ammonium sulfate fraction.	4.6×10^5	46	1.0×10^3	1.9
4. Second ammonium sulfate fraction.	1.9×10^5	19	1.1×10^3	2.2

Table 6 : An illustration of the patterns of amylose degradation by purified α- and β-amylases.

Time (min.)	α-Amylase		β-Amylase	
	Reducing sugar (nanomoles)	Starch-I_2 ($O.D._{620}$)	Reducing sugar (nanomoles)	Starch-I_2 ($O.D._{620}$)
0	0.0	0.088	0	0.087
5	19.6	0.080	745	0.083
10	38.8	0.072	841	0.086
20	75.1	0.060	904	0.088
30	112.0	0.047	947	0.088

Figure 6 : Degradation of amylose by α-amylase

(sec. 3.4.1)


0.10
0.05
Starch–iodine O.D.620
50
100
Reducing sugar (nanomoles)

the assay method used (Kneen and Sandstedt, 1941; Reed and Thorn, 1971). The difficulties of this study are (1) that no analysis of the effects of cyanide on α-amylase was undertaken, and (2) that the cyanide was in a high enough concentration to render the reaction mixture extremely alkaline. However, this led us to look for a method to selectively inactivate β-amylase through a chemical modification of the molecule. The only other reasonable course would be the use of a specific substrate, such as β-limit dextrin. However, α-amylolytic degradation of such a substrate yields β-substrate, which could introduce substantial error into the determination (Marshall and Whelan, 1971; Bilderback 1973).

Thiol groups have been implicated in the endogenous control of β-amylase activity (Thoma et al, 1965; Spradlin and Thoma, 1970; Thoma, Spradlin and Dygert, 1971). The chemical modification of these sulfhydryl groups leads to a loss of β-activity. β-Amylases have been shown to be sensitive to heavy metals (Cu, Hg), and the usual sulfhydryl reagents (eg. PCMB, NEM, iodoacetamide) (ibid; French, 1960; Riordan and Valee,

1972). Although the activity of α-amylase is not regulated through free sulfhydryl groups, it has been reported to be sensitive not only to heavy metals, such as mercury and copper, but also to PCMB (Fischer and Stein, 1960; Greenwood and MacGregor, 1965; Greenwood and Milne, 1968; Niku-Paavola and Nummi, 1971; Thoma, Spradlin and Dygert, 1971).

In our own studies, pre-incubation of α- and β-amylases with various concentrations of cupric or mercuric ions resulted in the inhibition of both enzymes (table 7). However, it became apparent that in all inhibition studies seen, the enzyme had been incubated with the inhibitor prior to being assayed. Since many enzymes are stabilized by the presence of substrate, it was decided to include the inhibitor, mercuric chloride, in the substrate during its preparation (sec. 2.6.1.2). It was found that β-amylase activity was virtually eliminated with 5×10^{-6}M. mercuric chloride, which concentration had no effect upon α-amylase, even after a total exposure of 30 min. at 25°C. (tables 8 and 9). Thus this concentration of mercuric chloride was routinely used in the substrate for the determination of α-amylase levels.

Table 7 : Inhibition of α- and β-amylases by Cu^{++} and Hg^{++} ; with inhibitors added prior to substrate.

Inhibitor.	Concentration (M.)	α-Amylase		β-Amylase	
		Units	% inhibition	Units	% inhibition
None	—	16	0	23	0
Cu^{++}	10^{-4}	15	6	21	9
Cu^{++}	10^{-3}	16	0	3.3	76
Hg^{++}	10^{-5}	2	87	0	100
Hg^{++}	10^{-4}	2	87	0	100
Hg^{++}	10^{-3}	0	100	0	100

Table 8 : Inhibition of β-amylase by $HgCl_2$, included in substrate.

Concentration of $HgCl_2$	Percentage inhibition	
	α-Amylase	β-Amylase
1×10^{-8} M.		2
1×10^{-7} M.		7
1×10^{-6} M.	0	83
2×10^{-6} M.	0	94
5×10^{-6} M.	0	99
1×10^{-5} M.	some	100

Table 9 : Assay of α-amylase in the presence of 5 X 10^{-6}M. $HgCl_2$.

Units of enzyme added	Enzyme units determined after:			
	5 min.	10 min.	20 min.	30 min.
0.78	0.72	0.89	0.71	0.75
3.9	4.5	4.9	4.6	4.4
7.8	7.6	7.3	6.8	6.2

3.5 Development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) during imbibition at 2°C. and at 25°C.

Method	Section
1. Preparation of enzyme	2.5.2
2. Method of assay	2.6.2.2

The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) was studied at 25°C., at 2°C. and at 25°C. after various exposures to 2°C.

It was found that α-amylase levels in TD(D) increased more rapidly ($P > 0.975$) than those in TD(C) at 25°C. (tables 10 and 11; figure 7; cf. tables 12 and 13). At 2°C., there appeared to be no significant difference between the development of α-amylase levels in TD(C) and that in TD(D) in any period up to 6 weeks. The levels after 6 weeks at 2°C. were roughly equivalent to those found after 3 days at 25°C. Thus, for further study, 2 weeks of exposure to 2°C. was considered to be the equivalent of 1 day of exposure to 25°C. (table 14, figure 8; cf. tables 15 and 16).

After full vernalization (ie. 6 weeks of exposure to 2°C.) there appeared to be no significant difference between the development of α-amylase levels in TD(C) and that in TD(D) at 25°C. Indeed, if anything, the levels

in TD(C) develop much more quickly than those in TD(D) which would be a complete reversal of the situation in non-vernalized seeds (table 17, figures 8 and 9). A comparison of the development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) with and without 6 weeks of exposure to 2°C. reveals that cold exposure accelerates the increase of α-amylase levels in TD(C), but has no significant effect upon those in TD(D), assuming that 2 weeks at 2°C. is the equivalent of 1 day at 25°C. (figure 9).

A temperature shift study was undertaken to illustrate the shift in α-amylase development in TD(C) with respect to TD(D). After 0, 2, 4, and 6 weeks of exposure to 2°C., the seeds were incubated for 5 (nominal), 4, 3, and 2 (nominal) days at 25°C., respectively. α-Amylase levels in TD(C) and in TD(D) and their differences, TD(D)-TD(C), were determined (tables 18 and 19, figure 10; cf. tables 20 and 21). The study showed that the differences between TD(C) and TD(D) were significantly decreased ($P > 0.995$) due to the exposure of the seeds to low temperature.

Since gibberellins induce α-amylase synthesis by cereal aleurone layers (Varner, 1964) and since this response appears to be specific for this type of hormone

(Carr and Goodwin, 1970), it follows that, at 25°C., the levels of endogenous gibberellin-like activity are lower in the winter variety than in the spring variety, and that this difference in hormone levels is eliminated after vernalization. The cold exposure seems to increase the levels in the winter variety only, having little or no effect upon the levels in the spring variety.

At first glance, this would seem to support the views of other workers (Suge and Osada, 1966; Chailakhyan, 1968), however, the weakness in these studies is that gibberellins are determined by bioassay techniques, and detoxified forms of the hormones could not be satisfactorily detected. Considering the present view that gibberellins act through a mechanism similar to that of steroid hormones in animals (Carlson, 1972), and the observation of gibberellin conjugates in a number of plant systems (MacMillan, 1970), this possibility must be investigated.

Table 10 : The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) imbibed at 25°C.

Days of imbibition		α-Amylase units per mg. of initial dry weight	
		TD(C)	TD(D)
2		12.6	13.9
		11.8	10.1
		10.4	13.8
	Means	11.6	12.6
3		15.5	32.6
		44.8	49.0
		41.7	27.2
	Means	34.0	36.2
4		60.8	127.2
		60.7	96.7
		25.4	71.5
	Means	48.9	98.5
6		66.9	126.8
		169.1	202.9
	Means	118.0	164.9

(Continued...)

Table 10 : (Continued.)

	8	157.9	137.9
		153.6	137.8
	Means	155.8	137.9
	11	45.4	52.4
		57.6	55.1
	Means	51.5	53.8

Figure 7 : The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) imbibed at 25°C. (Data from table 10).

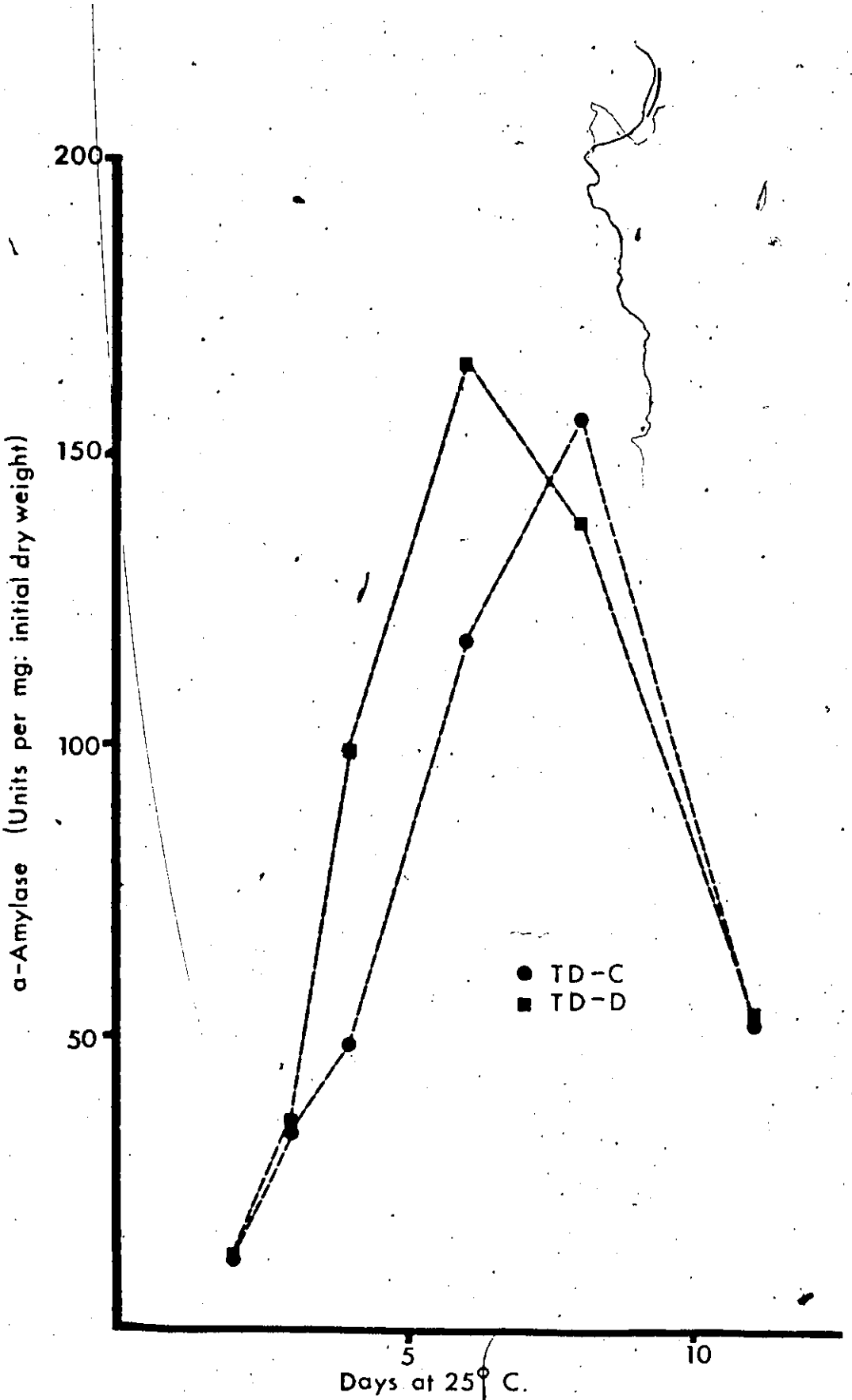

200
150
100
50
α-Amylase (Units per mg: initial dry weight)
TD-C
TD-D
5
10
Days at 25° C.

Table 11 : Analysis of covariance of regressions of α-amylase levels on days of imbibition (data from table 10) between TD(C) (2-8 days) and TD(D) (2-6 days).

	DF	SS	MS	F	
TD(C)					
Total	12	40869.4			
Group	4	34286.4	8571.6	10.42	P > 0.995
Regression	1	33788.3	33788.3	203.50	P > 0.999
Residual	3	498.1	166.0	0.20	n.s.
Error	8	6583.1	822.9		
		Slope = 24.9			
		Intercept = -41.6			
TD(D)					
Total	10	38440.6			
Group	3	33721.9	11240.6	16.68	P > 0.995
Regression	1	32898.7	32898.7	79.93	P > 0.975
Residual	2	823.2	411.6	0.61	n.s.
Error	7	4718.7	674.1		
		Slope = 39.8			
		Intercept = -71.1			
Between slopes	1	3329.1	3329.1	12.60	P > 0.975
Average residual	5	1321.3	264.3		

Table 12 : A survey of specific activities of α-amylase in the endosperms of seeds of TD(C) and TD(D) imbibed at 25°C.

Days of imbibition	α-Amylase units per ug. of protein	
	TD(C)	TD(D)
2	0.30	0.31
	0.27	0.19
	0.23	0.32
3	0.30	1.06
	0.86	0.68
	0.91	0.44
4	1.06	2.48
	1.39	2.07
	0.55	1.62
6	1.19	3.18
	3.89	9.67
8	2.32	3.64
	3.35	6.76
11	1.72	1.02
	3.07	2.29

Table 13 : A survey of protein levels in the endosperms of seeds of TD(C) and TD(D) imbibed at 25°C.

Days of imbibition	Mg. of protein per g. of initial dry weight	
	TD(C)	TD(D)
2	41.4	44.5
	42.8	53.9
	44.6	43.1
3	52.2	46.4
	52.3	48.0
	46.0	61.2
4	57.4	51.3
	43.5	46.7
	46.3	44.0
6	56.1	39.9
	43.5	21.0
8	68.2	37.9
	45.9	20.4
11	26.5	8.7
	18.8	24.1

Table 14 : The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) imbibed at 2°C.

Weeks of imbibition	α-Amylase units per mg. of initial dry weight	
	TD(C)	TD(D)
2	7.4	4.8
	14.5	2.4
Means	11.0	3.6
4	11.5	15.9
	11.3	4.4
Means	11.4	10.2
5	—	11.5
	15.7	11.2
Means	15.7	11.4
6	48.9	21.5
	25.8	8.9
Means	37.4	15.2

Table 16 : A survey of protein levels in the endosperms of seeds of TD(C) and TD(D) imbibed at 2°C.

Weeks of imbibition	Mg. of protein per g. of initial dry weight	
	TD(C)	TD(D)
3	41.5	29.4
	38.6	30.6
4	49.3	45.3
	47.5	45.3
5	57.7	40.2
	59.2	44.0
6	42.1	36.8
	49.8	48.5

Table 17 : The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) at 25°C. after 6 weeks of imbibition at 2°C.

Days at 25°C.	α-Amylase units per mg. of initial dry weight	
	TD(C)	TD(D)
1	153.3	126.0
	112.8	45.1
Means	133.1	85.6
2	160.7	150.8
	118.7	101.9
Means	139.7	126.4
3	171.2	151.4
	123.6	135.7
Means	147.4	143.6

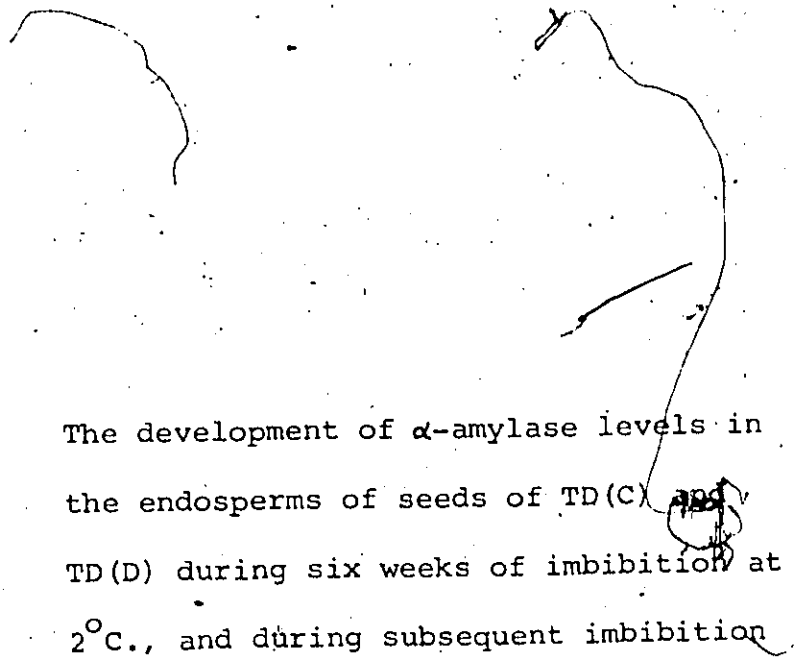


Figure 8 : The development of α-amylase levels in the endosperms of seeds of TD(C) and TD(D) during six weeks of imbibition at 2°C., and during subsequent imbibition at 25°C. (Data from tables 14 and 17).

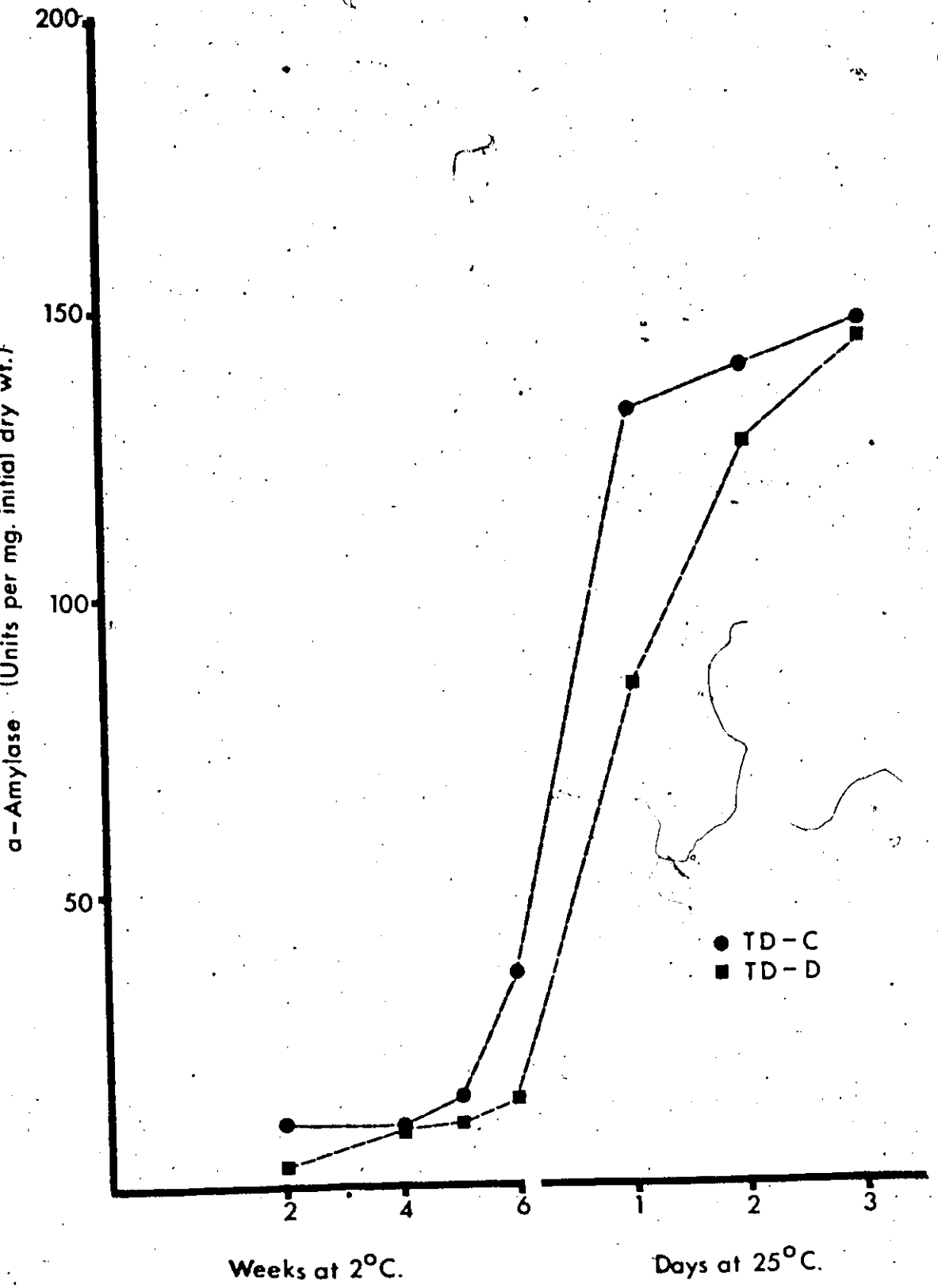

200
150
100
50
α-Amylase (Units per mg. initial dry wt.)
TD-C
TD-D
2
4
6
1
2
3
Weeks at 2°C.
Days at 25°C.

Figure 9 : The superimposition of figures 7 and 8 on the basis of the assumption that 2 weeks at 2°C. is the equivalent of 1 day at 25°C.

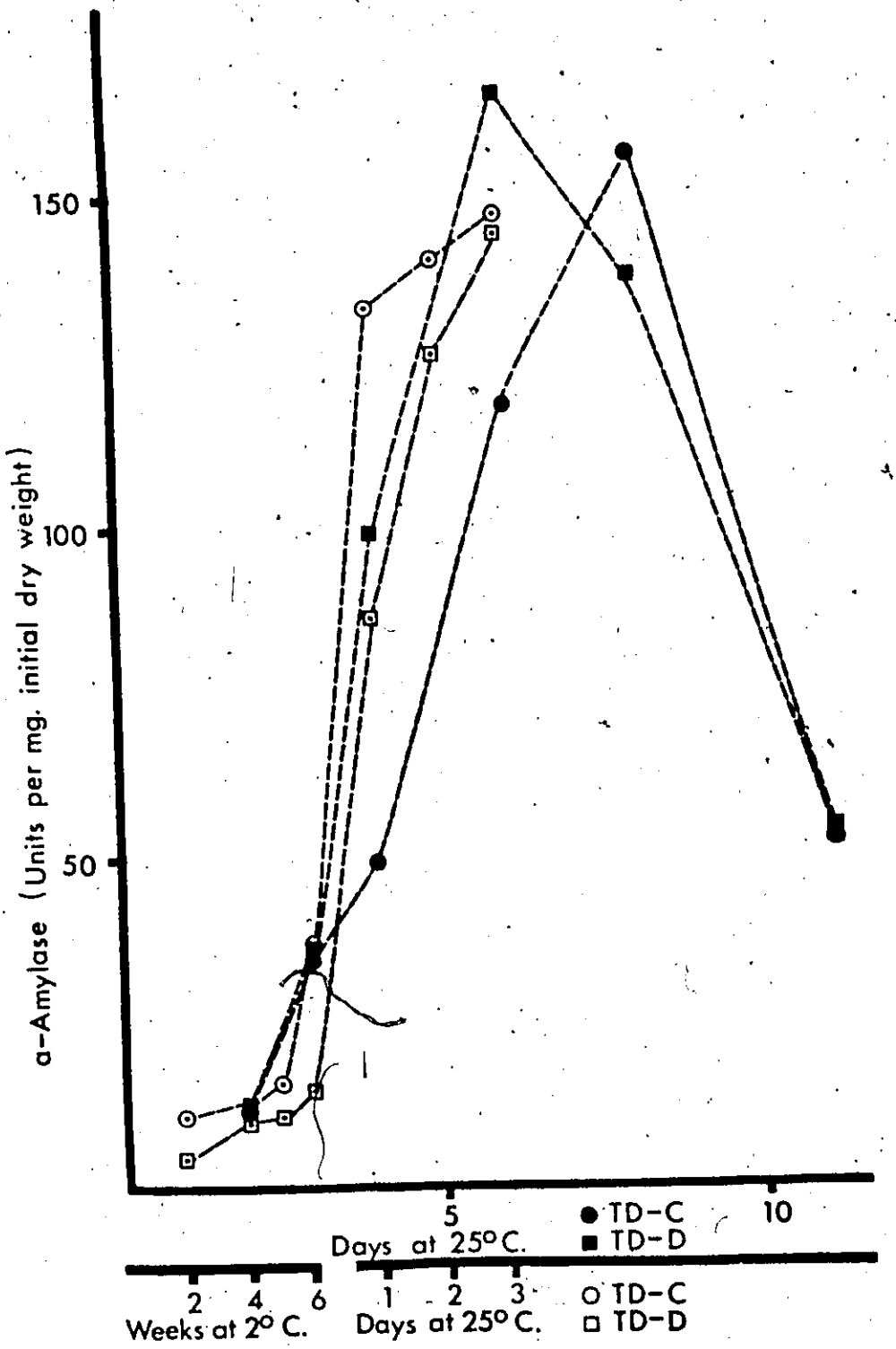

150
100
50
α-Amylase (Units per mg. initial dry weight)
5
10
Days at 25° C.
● TD-C
■ TD-D
2
4
6
Weeks at 2° C.
1
2
3
Days at 25° C.
○ TD-C
□ TD-D

Table 18 : Changes of α-amylase levels in TD(C) with respect to TD(D) at 25°C. after various exposures to 2°C.

Weeks at 2°C.	Days at 25°C.		α-Amylase units per mg. of initial dry weight		
	Nominal	Actual	TD(C)	TD(D)	(D)-(C)
0	5	4	60.8	127.2	66.4
			60.6	96.7	36.1
			25.4	71.5	46.1
		6	66.9	126.8	59.9
			169.1	202.9	33.8
		Means	76.6	125.0	48.4
2	4	4	117.9	184.6	66.7
			88.4	119.1	30.7
		Means	103.2	151.9	48.7
4	3	3	124.3	147.0	22.7
			149.6	168.4	18.8
		Means	137.0	157.7	20.8

(Continued...)

Table 18 : (Continued.)

6	2	1		153.3	126.0	-27.3
				112.8	45.1	-67.7
		2		160.7	150.8	-9.9
				118.7	101.9	-16.8
		3		171.2	151.4	-19.8
				123.6	135.7	12.1
			Means	140.1	118.5	-21.6

Figure 10 : Changes of α-amylase levels in TD(C) with respect to TD(D) at 25°C. after various exposures to 2°C. (Data from table 18).

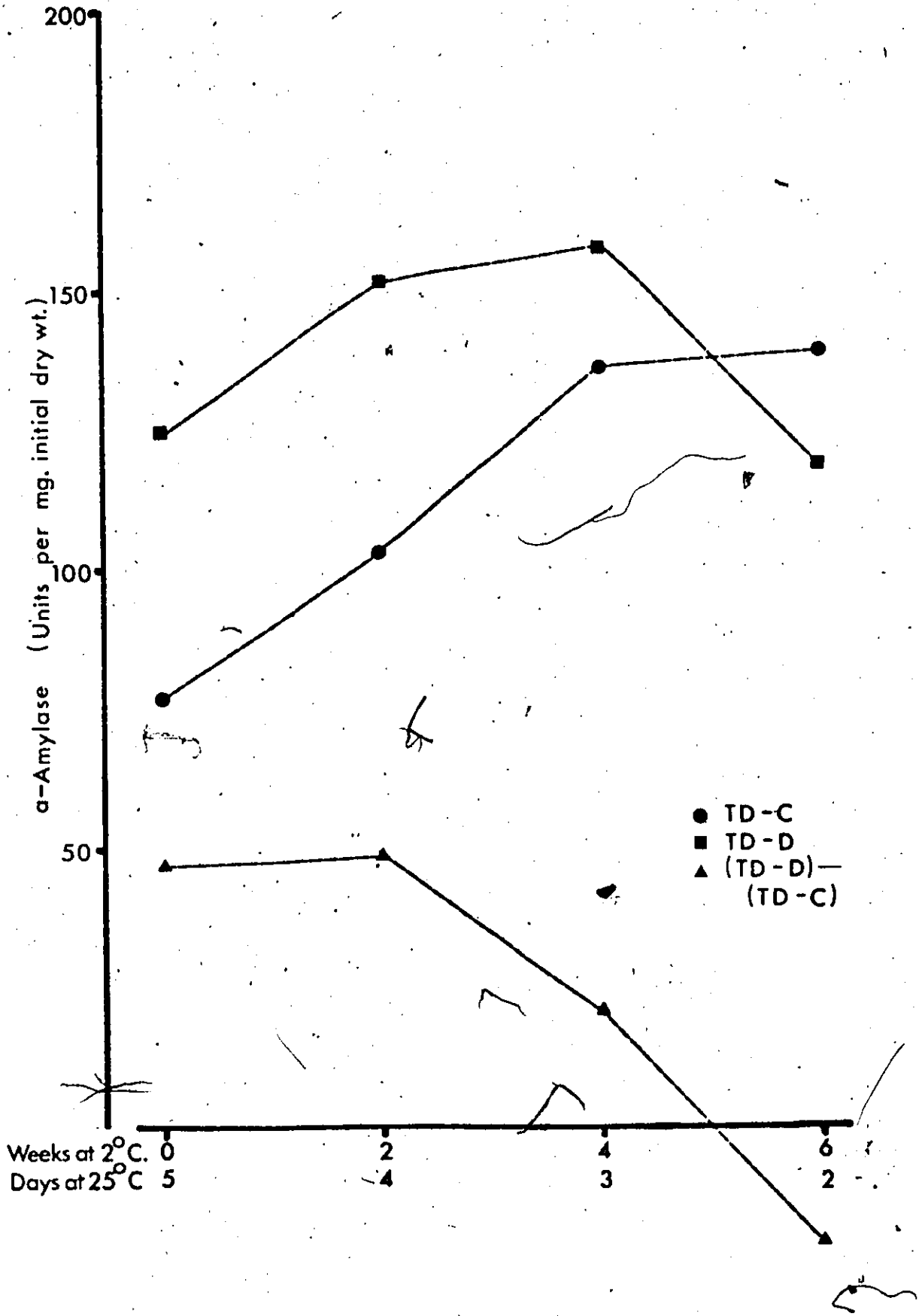

200
150
100
50
α-Amylase (Units per mg. initial dry wt.)
TD-C
TD-D
(TD-D)—
(TD-C)
Weeks at 2°C. 0 2 4 6
Days at 25°C 5 4 3 2

Table 19 : One-way analysis of variance with linear quadratic regression of the difference between TD(C) and TD(D) ((D)-(C); data from table 18) (Y) on the number of weeks of exposure of the seeds to 2^{o}C. (X).

	DF	SS	MS	F	
Total	14	20667.5			
Group	3	14129.8	4709.9	7.92	P > 0.995
Regression	2	14087.9	7043.9	168.11	P > 0.900
Residual	1	41.9	41.9	0.07	n.s.
Error	11	6537.7	594.3		

Correlation $R^2 = 0.682$ $P = 0.990$

$$Y = 42.9 + 7.4 X - 3.0 X^2$$

Table 20 : A survey of specific activities of α-amylase in the endosperms of TD(C) and TD(D) at 25°C. after various exposures to 2°C.

Weeks at 2°C.	Days at 25°C.	α-Amylase units per ug. of protein	
		TD(C)	TD(D)
2	4	3.23	3.00
		1.90	5.94
4	3	2.17	2.28
		2.10	3.16
6	1	2.16	2.40
		1.71	0.68
6	2	3.79	3.07
		2.29	1.92
6	3	3.91	3.49
		2.39	2.37

Table 21 : A survey of protein levels in the endosperms of seeds of TD(C) and TD(D) at 25°C. after various exposures to 2°C.

Weeks at 2°C.	Days at 25°C.	Mg. of protein per g. of initial dry weight	
		TD(C)	TD(D)
2	4	36.5	39.7
		46.7	31.1
4	3	57.2	64.6
		71.4	53.3
6	1	70.9	52.6
		66.1	66.3
6	2	42.4	49.1
		51.8	53.0
6	3	43.8	43.4
		51.7	57.4

3.6 Agrose gel electrophoresis

The original method (Sick and Nielsen, 1964; Sick, 1965; Frydenberg and Nielsen, 1965) for the separation of barley amylases used 1% purified agar. Although the electrophoresis was performed at pH 7.3, which is on the alkaline side of the isoelectric points of these enzymes, their migration was cathodic due to the high endo-osmotic flow in agar. This was confirmed for the enzymes from Triple Dirk, however electrophoretic mobilities were low and a maximum of three bands were obtained, with poor resolution. It was noted that the agar used had poor mechanical stability, even at a concentration of 1%, and that numerous minute insoluable particles were present. By contrast, the agarose used had a high degree of mechanical stability, even a 0.5% concentration, and was readily soluable. Migration of the wheat amylases in the agarose was anodic and resolution was good (7 bands, figure 11).

Electrophoretic mobilities were even lower in agarose than in agar. An increase in pH to 8.0 resulted in the amylases' migration as a single band, and a further increase to pH 8.7 caused the destruction of any amylolytic activity after 15 to 30 min. of electrophoresis. Increasing the agarose concentration from 0.5% to

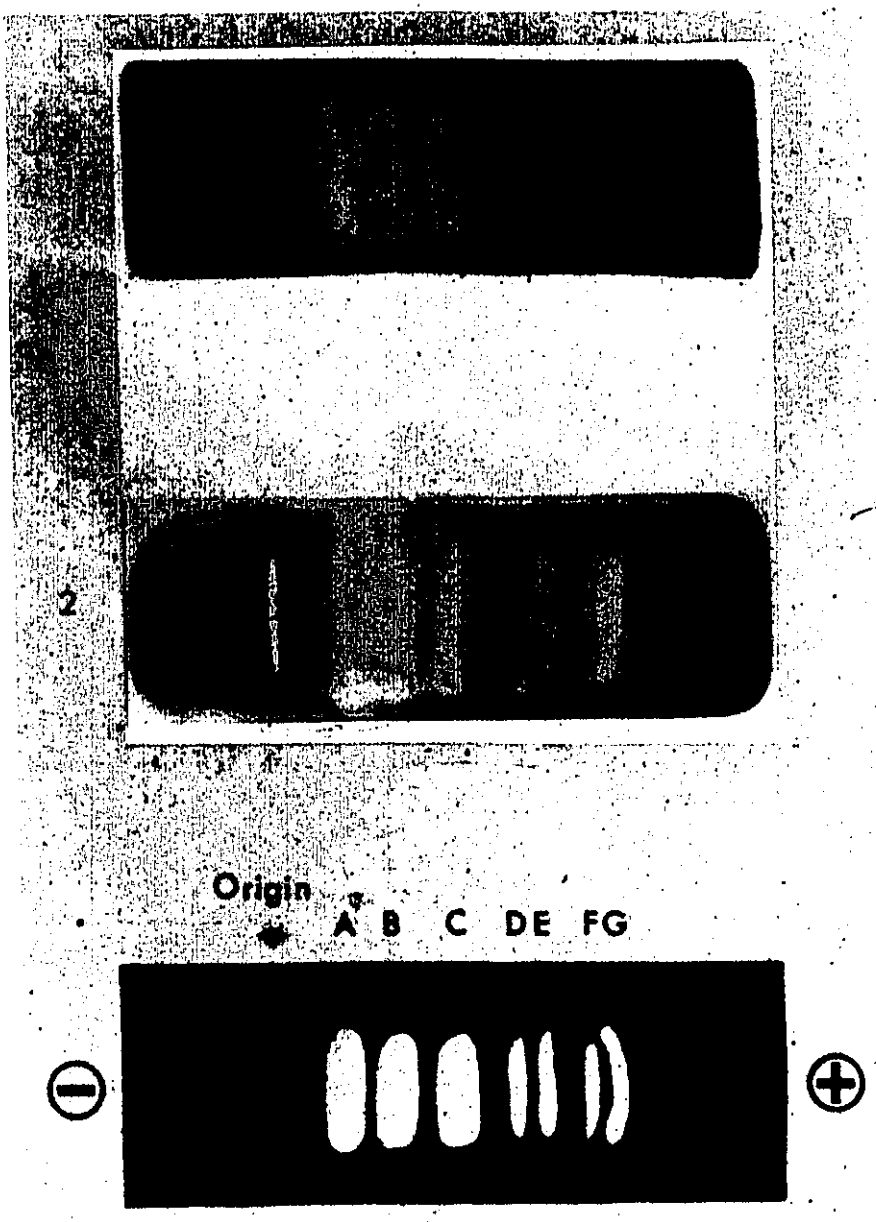
1% resulted in an overall decrease in mobility, but did not affect the relative mobilities of the bands. An increase of current from 3 mA. to 5 mA. per slide gave higher mobilities with a loss of resolution. Endo-osmotic flow was sharply increased with a swelling of the gel layer to a thickness of 1.5 mm. to 2 mm. If this current was maintained for 1 hour (and often much earlier), the gel began to buckle, then tear. Thus the method was standardized to pH 7.3, μ 0.02 in 0.5% agrose with a current of 3 mA. per slide for 2 hours at 4°C. This resulted in consistently clear resolution of seven bands with better than 95% reproducibility.

It was found that the electrode buffer could only be used once. Repeated use resulted in high electrophoretic mobilities and a lack of resolution, even if the electrodes were reversed or if the buffer from the electrode compartments was completely mixed before the second run. This was explained by an overall increase in buffer pH during the run. After a single 2 hour run with 10 slides at 30 mA., the pH of the mixed electrode buffers had gone from 7.3 to 7.6.

All seven bands found appear to be α-amylases. No bands displaying the characteristic red-staining of β-limit dextrin with iodine were found. The staining of

Figure 11 : The separation of zones of amylolytic activity on 0.5% agarose gel (pH 7.3, u 0.02) by electrophoresis at 3 mA. for 2 hours. The seven bands are designated A to G from the origin towards the anode.

1. Substrate slide
2. Electrophoresis slide


1
2
Origin
A B C DE FG

these seven bands varied only in the intesity of blue colour.

Heating of the enzymes to 70°C. for 15 min. before electrophoresis had no effect upon the separation pattern. Similar treatment after electrophoresis destroyed all α-amylolytic activity (Frydenberg and Nielsen, 1965). The incorporation of 5×10^{-6} M. mercuric chloride in the substrate slide had no effect upon the enzyme activities. At 5×10^{-5} M. mercuric chloride, noticable inhibition of all bands occured (sec. 3.4.2).

The appearance of the bands for TD(C) and for TD(D) is related only to the levels of α-amylase activity, and no varietal differences could be found. Imbibition at 2°C. did not appear to alter the pattern (table 22).

Table 22 : A survey of the patterns of electrophoretically-separable bands of α-amylase activity found in endosperms of TD(C) and TD(D) imbibed as described in section 3.5 (band intensity indicated as +, weak to +++, strong).

Weeks at 2°C.	Days at 25°C.	TD(C) A	B	C	D	E	F	G	TD(D) A	B	C	D	E	F	G
0	2	++	+	+	+				++	+	+	+			
	3	+++	++	++	+	+	+	++	+++	++	++	+	+	+	++
	4	+++	+++	++	+	++	+	++	+++	+++	+++	+	++	++	++
	6	+++	+++	++	+	++	++	+++	+++	+++	+++	+	+++	++	+++
	8	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+	++	+++	+++
	11					+		++	+	+	+		+	++	++
2	0	+	+	+											
	4	+++	+++	+++		++	++	+++	+++	+++	+++		++	+	
4	0	++	+	+					+	+					
	3	+++	++	++		+			+++	++	++		+		
5	0	++	++	+					+	+	+				
6	0	+++	+++	++			+	++	+	+	+				
	1	+++	+++	+++	+	++	+	+++	+++	+++	+++	+	++	+	+++
	2	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	++	+++	++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

3.7 Investigations involving gibberellin-glycosides

Method	Section
1. Preparation of glycosides	2.8.1
2. Bioassay of gibberellin-like activity	2.8.2

As stated above (sec. 3.5), acetyl and glucopyranosyl conjugates of several gibberellins have been identified in plants (MacMillan, 1970). Although the conjugation of a plant hormone does not necessarily decrease its biological potency (Fox et al, 1973), preliminary results with gibberellin-glucosides seem to indicate that their potency is drastically decreased (Sembdner et al, 1970; Yamane et al, 1973). There is also evidence to suggest that glucosyl conjugates of phytohormones function in storage, perhaps by protecting the hormone from degradation, and may also serve in hormone translocation (MacMillan, 1972; Fox et al, 1973).

In our own preliminary studies, a modification of the method of Yokota was used (figure 2; sec. 2.8.1.1) (Tamura et al, 1968; Yokota et al, 1969; Harada and Yokota, 1970) to isolate any gibberellin-glycosides present in dry grains of the spring wheat, Triticum aestivum, var. "Marquis". The product of

this procedure gave only five spots on T.L.C. plates, all of which were positive to α-naphthol spray, indicating probable carbohydrate moieties associated with these spots (figure 12). Using the endosperm-half bioassay, gibberellin-like compounds were detected on the plates, and levels of detectable gibberellin-like compounds increased drastically following preliminary acid hydrolysis (table 23). Thus gibberellin-conjugates were present in the dry seed.

No work has been done regarding the donor of the glucosyl moiety to the gibberellin-glucosides, however uridine-5'-diphosphoglucose (UDP-glucose) has been shown to be the donor to steryl-glucosides in plants (Eichenberger and Newman, 1968; Lavintman and Cardini, 1970, 1971 and 1972). Using a method patterned after theirs for the labelling of steryl-glucosides, label was successfully introduced from UDP-glucose-(U-^{14}C) into gibberellin-glycosides during the imbibition stages of TD(D) demonstrating that both the germ and endosperm fractions are capable of synthesizing gibberellin-glycosides during germination (tables 24 and 25; figures 13 and 14). A total of eight glycosides were labelled by the endosperm preparation, only four of which were labelled by the

embryo preparation (table 25). The patterns of labelling shown in the histograms are obviously very different (figure 14). Again, gibberellin-like compounds were demonstrated in the spots by bioassay, and preliminary acid hydrolysis of the samples significantly increased ($P > 0.99$) the levels of detectable gibberellin-like activity (table 26, figure 15).

Figure 12 : The separation of un-labelled gibberellin-glycosides on Silica gel H using chloroform: methanol: acetic acid: water (40:15:3:2). Spots are numbered according to their chromatographic correspondence to those found in the labelled preparations (cf. figure 13).

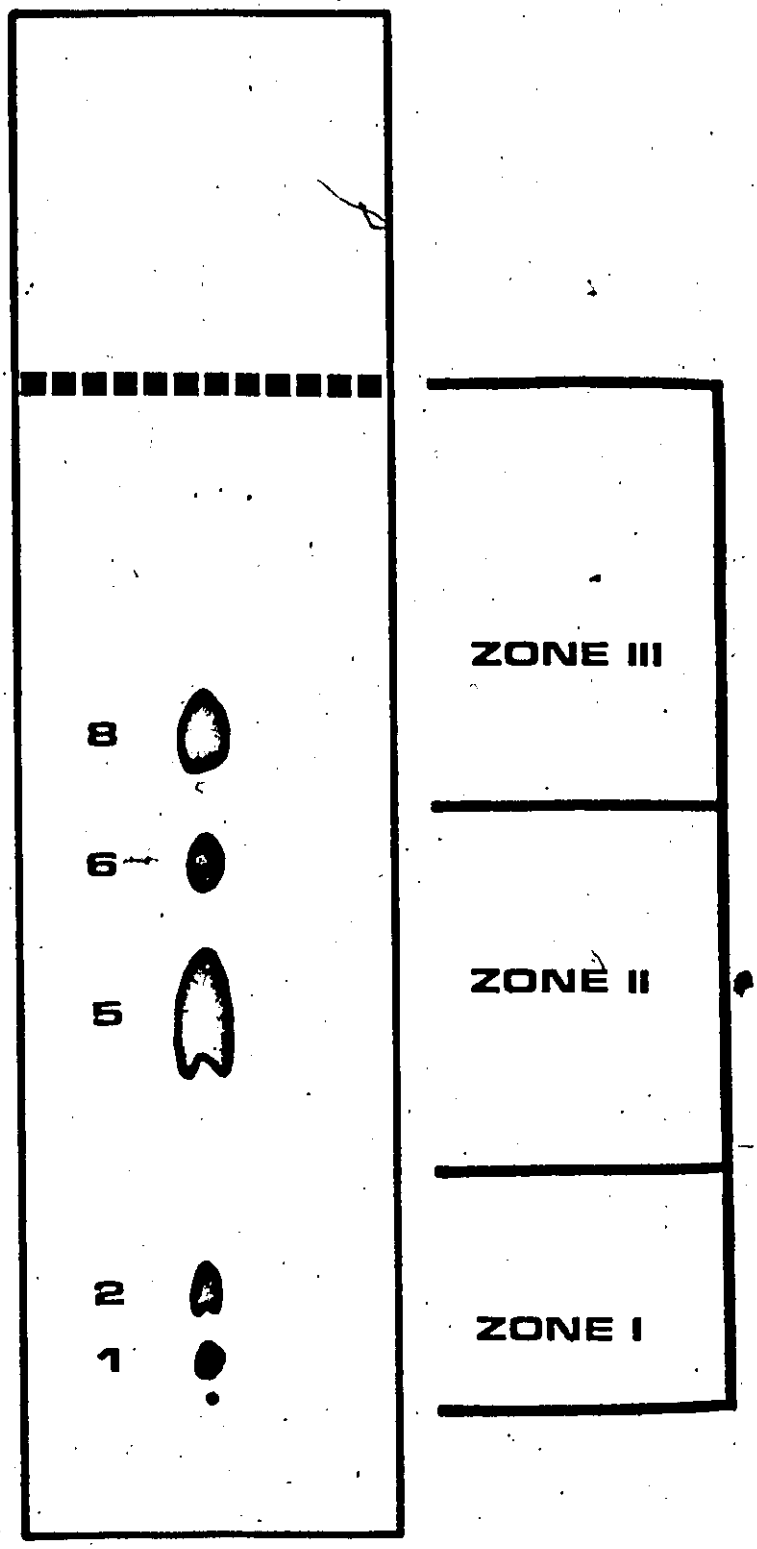

ZONE III
ZONE II
ZONE I
8
6
5
2
1

Table 23 : α-Amylase levels induced in endosperm halves of TD(D) incubated with un-labelled gibberellin-glycosides and with the gibberellins released by acid hydrolysis of those glycosides.

Zone (cf. fig. 12)	Un-hydrolyzed (α-Amylase units)	Hydrolyzed (α-Amylase units)
I	0	213
II	65	389
III	98	115

Table 24 : The distribution of radio-activity during the extraction of the embryo and endosperm preparations of TD(D) after their incubation with UDP-glucose-(U-^{14}C).

Fraction	Embryo preparation		Endosperm preparation	
	Total DPM	Percentage	Total DPM	Percentage
1. First petroleum ether wash.	8.6×10^2	0.1	3.4×10^3	0.1
2. Water-insoluble residue from methanol extract.	1.4×10^3	0.1	1.8×10^4	0.4
3. Second petroleum ether wash.	6.3×10^3	0.4	4.8×10^4	1.0
4. Ethyl acetate wash.	2.5×10^4	1.7	8.2×10^4	1.8
5. Water-soluble impurities.	1.4×10^6	94.0	4.2×10^6	89.9
6. Butanolic extract.	5.4×10^4	3.7	3.2×10^5	6.9
Totals:	1.5×10^6	100.0	4.7×10^6	100.0

Figure 13 : The separation of ^{14}C-labelled gibberellin-glycosides from the embryo and endosperm preparations of TD(D) on Silica gel H using chloroform: methanol: acetic acid: water (40:15:3:2).

A. Embryo preparation

B. Endosperm preparation

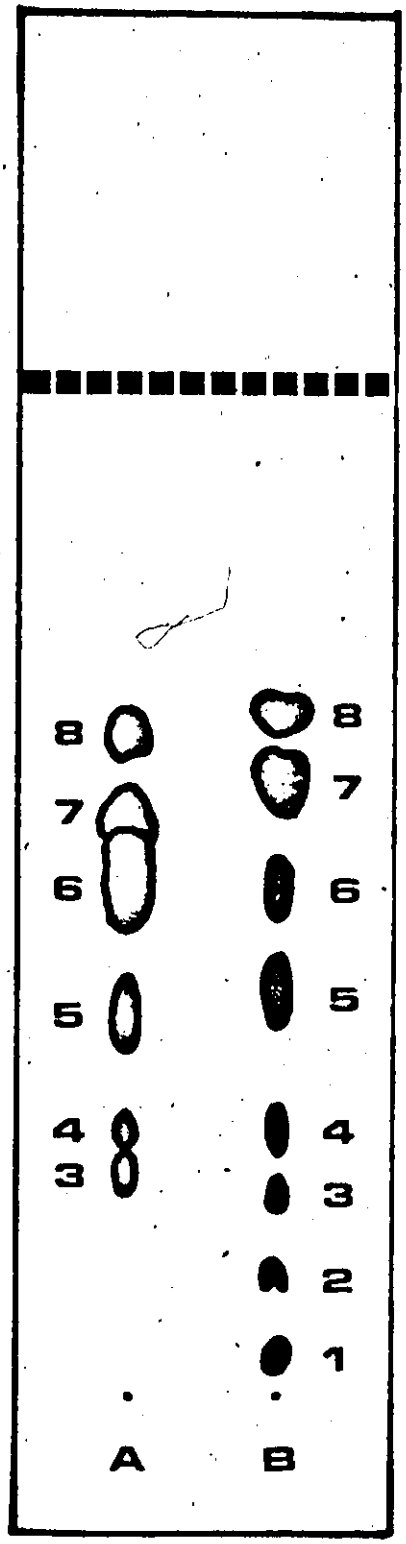
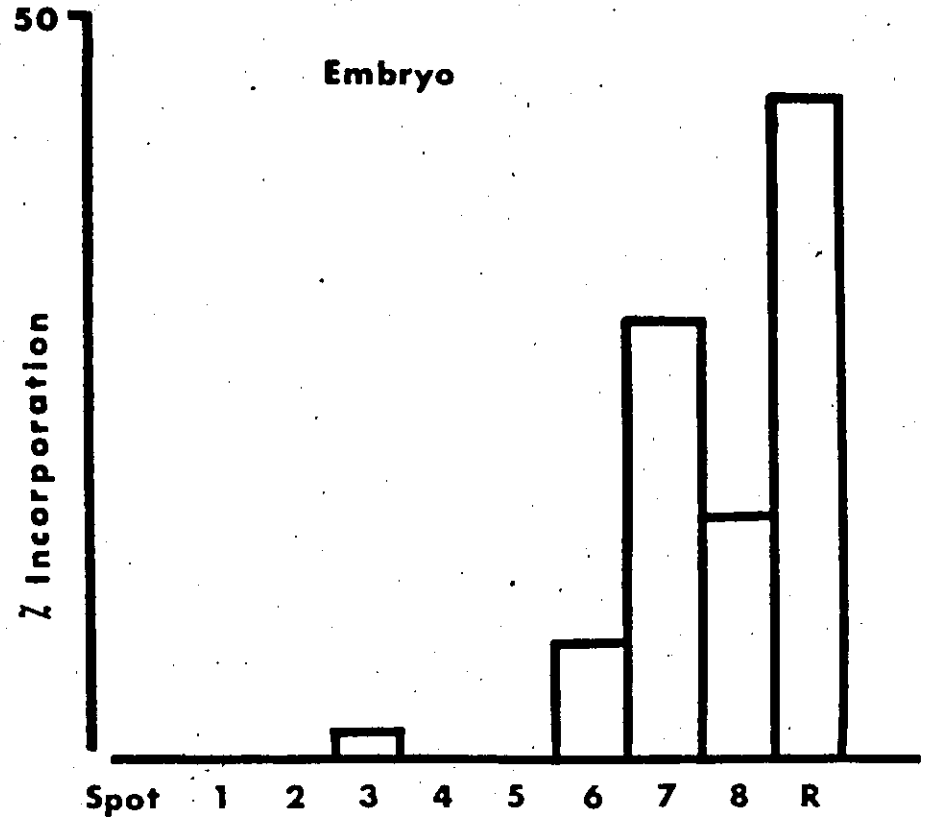

8
7
6
5
4
3
8
7
6
5
4
3
2
1
A
B

Table 25 : The distribution of radio-activity in samples from the embryo and endosperm preparations after their separation on Silica gel H in chloroform: methanol: acetic acid: water (40:15:3:2).

Spot	Embryo preparation		Endosperm preparation	
	Total DPM	Percentage	Total DPM	Percentage
1	0	0	8.8×10^3	44.2
2	0	0	1.0×10^3	5.0
3	1.5×10^2	1.8	1.5×10^3	7.6
4	0	0	4.7×10^2	2.4
5	0	0	9.3×10^2	4.6
6	6.5×10^2	7.8	8.5×10^2	4.2
7	2.5×10^3	29.6	6.0×10^2	3.0
8	1.4×10^3	16.4	2.2×10^2	1.1
Remainder of plate.	3.7×10^3	44.4	5.6×10^3	27.9
Totals:	8.4×10^3	100.0	2.0×10^4	100.0

Figure 14 : Histogram showing the distribution of radio-activity in the gibberellin-glycosides from the embryo and endosperm preparations (Data from table 25).
R = "Remainder of plate"


50
Embryo
% Incorporation
Spot 1 2 3 4 5 6 7 8 R

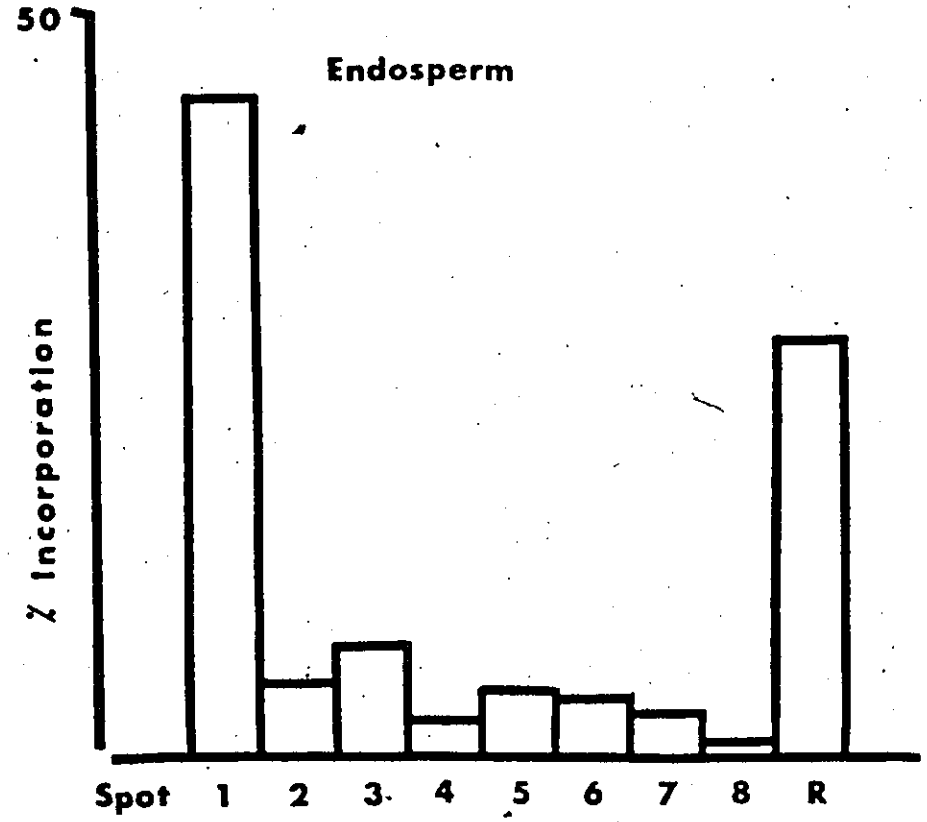
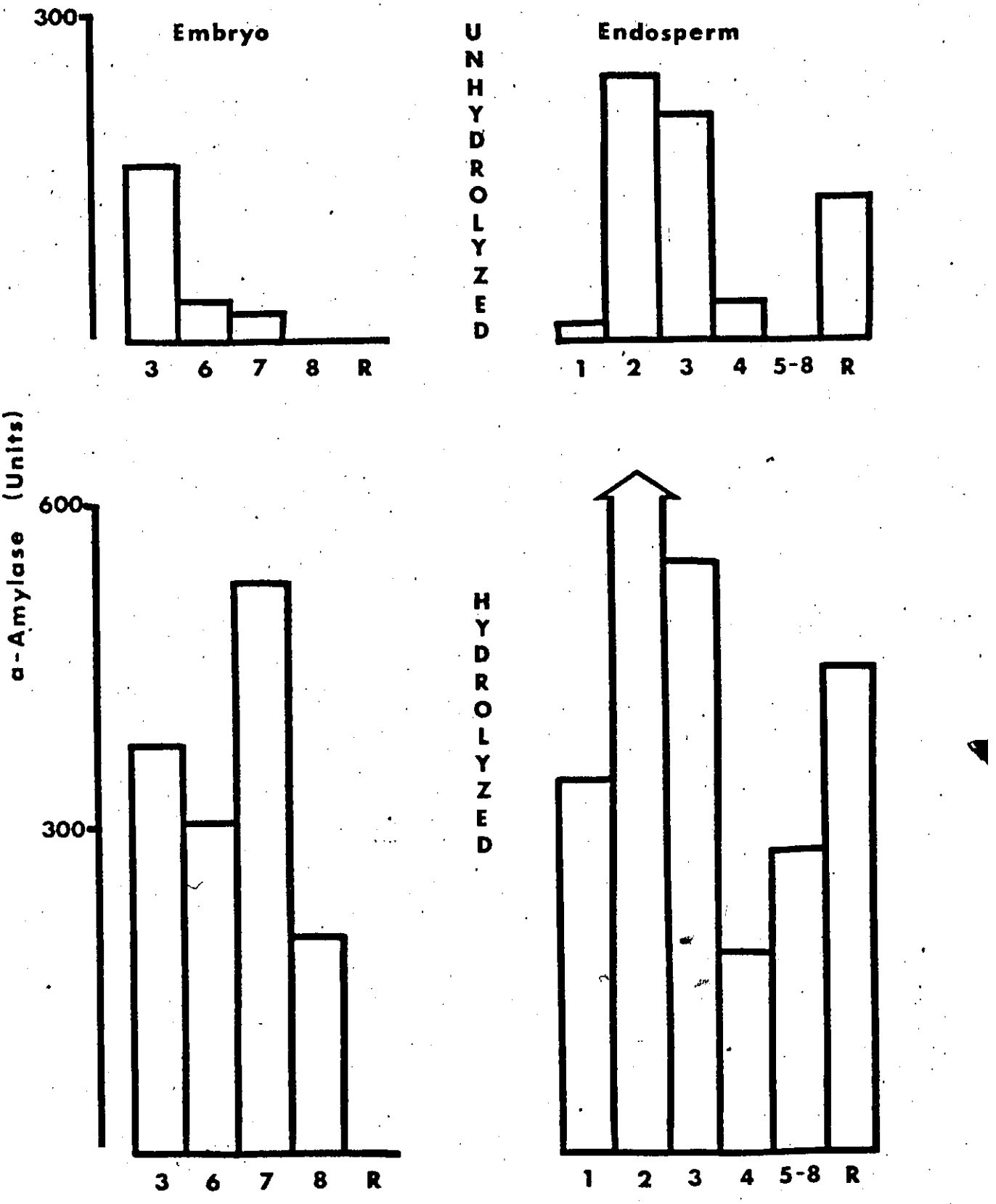

50
Endosperm
% Incorporation
Spot 1 2 3 4 5 6 7 8 R

Table 26 : α-Amylase levels induced in endosperm-halves of TD(D) incubated with ^{14}C-labelled gibberellin-glycosides and with the gibberellins released by acid hydrolysis of those glycosides.

Preparation	Spot	Un-hydrolyzed (α-Amylase units)	Hydrolyzed (α-Amylase units)
Embryo	3	162	376
	6	37	306
	7	27	527
	8	0	200
	Remainder of plate.	0	0
Endosperm	1	16	344
	2	245	1514
	3	210	547
	4	37	183
	5-8	0	278
	Remainder of plate.	134	451

Figure 15 : Histogram showing the distribution of "gibberellin-like" activity (as α-amylase units induced in an endosperm-half bio-assay) in the embryo and endosperm preparations before and after hydrolysis (Data from table 26).

R = "Remainder of plate"


300
Embryo
3 6 7 8 R
UNHYDROLYZED
Endosperm
1 2 3 4 5-8 R
a-Amylase (Units)
600
300
3 6 7 8 R
HYDROLYZED
1 2 3 4 5-8 R

3.8 The effect of UDP-Glucose on gibberellic acid induced α-Amylase production.

Method	Section
1. Preparation of enzyme	2.5.3
2. Determination of enzyme activity	2.6.2.2

As stated above (sec. 3.5), the lower production of α-amylase in TD(C), with respect to TD(D), at 25°C. may be due to the detoxification of endogenous gibberellins, possibly by a specific glucosyltransferase. It has been shown that this wheat is capable of incorporating glucose from UDP-glucose into gibberellin-conjugates and that these conjugates have higher gibberellin-like activity after acid hydrolysis (sec. 3.7).

Therefore, endosperm halves of TD(C) and TD(D) were incubated in 10^{-6} M. gibberellic acid (GA_3) with UDP-glucose from 0 M. to 10^{-2} M. (table 27). However, no difference between the α-amylase levels found in TD(C) and those found in TD(D) could be detected, nor did UDP-glucose appear to have any effect upon those levels.

Table 27 : α-Amylase levels induced in endosperm-halves of TD(C) and TD(D) by 10^{-6}M. GA_3 with various concentrations of UDP-glucose.

Concentration of UDP-glucose. (M.)	α-Amylase units per mg. of initial dry weight.	
	TD(C)	TD(D)
0	28.5	10.4
	27.9	29.0
	24.6	21.7
	20.2	4.5
		43.1
1×10^{-4}	26.3	22.3
	27.9	11.6
		25.4
1×10^{-3}	13.9	10.0
	32.0	23.6
		25.9
2×10^{-3}	16.2	19.9
	17.7	20.1

(Continued...)

Table 27 : (Continued.)

5×10^{-3}	8.0	15.8
	27.4	28.6
1×10^{-2}	9.4	9.1
	19.6	12.8
	23.0	32.8
	18.2	21.3
	Overall mean = 20.8	

4. Summary and Conclusions

4.1 A novel methodology for the determination of α-amylase activity in the presence of β-amylases in wheat has been described (sec. 3.4).

4.2 It was found that α-amylase levels in the endosperm of the winter variety, TD(C), increased significantly more slowly than did the levels in the endosperm of the spring variety, TD(D), during imbibition of the seeds at 25°C. (sec. 3.5, figure 7, tables 10 and 11).

4.3 Exposure to cold (2°C.) for six weeks appears to accelerate the production of α-amylase in TD(C), eliminating the differences in enzyme levels between the spring and winter varieties (tables 14 and 17, figures 8 and 9). This acceleration of α-amylase development in TD(C) by cold exposure occurs towards the end of the full vernalization treatment (tables 18 and 19, figure 10).

4.4 There are no discernable varietal differences in the electrophoretic patterns between the α-amylases from the two varieties.

4.5 Suspected gibberellin-glucosides have been isolated from dry grains (sec. 3.7, table 23, figure 12) and the synthesis of ^{14}C-labelled gibberellin-like conjugates, incorporating label from UDP-glucose-(U-^{14}C), has been observed (tables 24 and 25, figure 13).

4.6 These suspected gibberellin-glucosides are a detoxified

form, as acid hydrolysis significantly increases the gibberellin-like activities, as determined by the endosperm-half bioassay (tables 23 and 26, figure 15).

4.7 No evidence could be found to indicate that the detoxification of gibberellins through the action of a glucosyltransferase was responsible for the differences observed in the development of α-amylase levels in the two varieties at 25°C.

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