COMPARATIVE STUDIES ON AN ALIEN GENOME COMBINANT
STRUCTURAL INVESTIGATION OF STARCH COMPONENTS

by

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INTRODUCTION

Biochemical genetics has relatively recently become an increasingly important field of active research. Because of some obvious technical considerations, rather simple living organisms (bacteria, viruses, molds, yeasts) lend themselves to such studies. The synthesis and characterisation of alien genome combinations (synthetic species) within certain cereal species has opened a new field of investigation in this general area. Understandably the greater the number of chromosomes involved, the greater will be the genetic complexity.

As early as 1888, Rimpau produced a hybrid of Secale cereals (rye) and Triticum species. This hybrid species has been appropriately named Triticale. The earlier "hybrid" genome synthesis were mainly confined to the production of octaploid Triticale by combining the diploid rye genomes with the six genomes of hexaploid wheat eg. Triticum vulgare. Hexaploid Triticale has been produced by combining the two genomes of diploid rye with the four genomes of some of the more primitive tetraploid wheats eg. Triticum durum. Colchicine, an alkaloid, is known to prevent cell differentiation without inhibiting chromosome duplication. A zygote with genome combination of ABR is formed when a tetraploid wheat, Triticum durum (AABB) is crossed with the diploid rye, Secale cereals (RR). In the presence of colchicine, this

zygote undergoes mitotic division without cell differentiation, so that the hexaploid synthetic species Triticale (AABBRR) is produced. Considerable research towards the development of these species is being carried out at the University of Manitoba, Winnipeg, Manitoba. (1)

The present thesis describes investigations which were involved in attempts to establish, by chemical and enzymic approaches, whether an observable change in the structure of the starch components occurred when the genomes of the two parental species, rye (variety Prolific, RR) and durum wheat (variety Stewart, AABB) were combined to give the hexaploid synthetic species Triticale (AABBRR).

The problem could, for example, be investigated under the following parameters:

(1) Will there be any observable alien genome-induced change in the amylose over amylopectin ratio in the starch isolated from these three species?

(2) Could amylose and amylopectin be fractionated in sufficiently large quantities for detailed analysis?

(3) What is the structure of amylose and amylopectin? ie molecular weight and degree of branching?

(4) Will there be any new linkages resistant to enzymic degradation, and what is the percentage of conversion into maltose?

(5) Will there be any new or different linkages resistant to periodate oxidation? If so, could they be characterized?

(6) In the event that chemically observable changes were introduced in the synthetic species, how could this best be interpreted on the basis of present knowledge of biochemical genetics?

LITERATURE REVIEW

Photosynthesis, the assimilation of carbon dioxide in green plants, is the most important biochemcial and biophysical process on this planet from both a qualitative and quantitative point of view. It is not surprising, therefore that starch, which is the main end product of photosynthesis, has historically been a topic of active research.

The hydrolysis of starch by mild acid and by hydrolytic enzymes yields large amounts of glucose and maltose, respectively. This immediately suggests that the basic structural architecture is concerned with a polymer composed of glucose and this has been supported by elementary analysis giving the formula $C_6H_{10}O_5$.

Meyer and his associates (2) were the first to discover that starch was heterogeneous and in fact was composed of two fractions namely amylose (the linear fraction) and amylopectin (the branched fraction). They demonstrated that these two fractions are chemically, physically and enzymically distinguishable.

As first introduced by Meyer's group, one of the typical methods of separating amylose from amylopectin is based on the fact that amylose is soluble in hot water (70-80 C) whereas amylopectin is relatively less soluble. Later, Schoch (3) discovered that amylose is selectively

precipitated with n-butanol with which it forms a crystalline complex. After removal of the amylose complex by centrifugation, amylopectin could then be recovered from the supernatant solution by freeze-drying. Since the introduction of the above method, many other solvents such as n-amyl alcohol, thymol (4) and cyclohexanol (4, 5) were found to be even better precipitants (complexing agents) than n-butanol. Another method using magnesium sulfate as a selective salting out agent for amylose has been developed by Muetgeert. (6)

Various methods have been developed for estimating the molecular weight and investigating the fine structure of starch and its components. Exhaustive methylation followed by subsequent acid hydrolysis is a procedure of wide usage which was developed some time ago by Haworth and Machemer. (7) In this method, a small amount of 2, 3, 4, 6-tetramethyl-α-D-glucose is produced in addition to a large quantity of 2, 3, 6-trimethyl-α-D- glucose. The tetramethyl glucose corresponds to the non-reducing terminal ends in both amylose and amylopectin and thus constitutes a form of end-group analysis. In this manner, the size of the repeating unit for amylopectin was found to be 18-24 α-D- glucose units. (8) Methylated starch amylose usually has one 2, 3, 4, 6-tetramethyl-α-D-glucose end-unit for 150-1000 2, 3, 6-trimethyl-α-D- glucose units.

Another important method involves periodate oxidation and the titration of formic acid which is liberated during the course of oxidation. This procedure was elaborated by Hirst and his collaborators. (9, 10, 11) During periodate

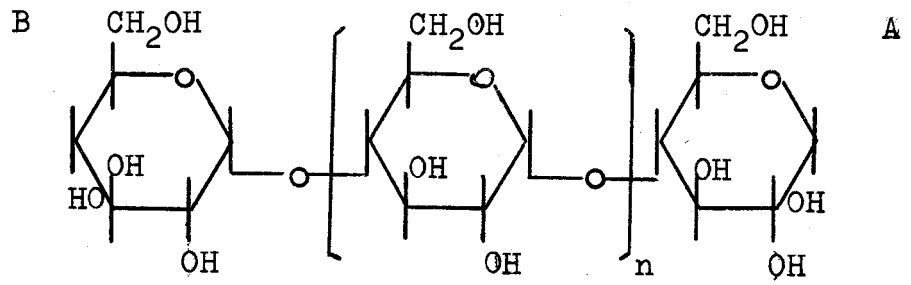
oxidation, both the non-reducing and reducing end-groups ordinarily produce 1 mole each of formic acid, however, 2 moles are formed from the reducing end-group if the intermediate formate ester is hydrolyzed. No formic acid is formed in the central part of the polymer molecule. It follows therefore that, in the case of amylose, formic acid produced may be used as a direct measure of the degree of polymerization (D.P). In amylopectin, the proportion of reducing end-groups is small compared with that of the non-reducing end-groups. Theoretically, only one reducing glucose unit should be present in an undegraded amylopectin molecule, and the formic acid produced from this reducing end group can thus be ignored. However, a quantitative measurement of the formic acid formed should give a measure of the average chain-length of the branches. This was found to vary from 19 - 27. (12)

Smith et al (13, 14, 15) reported that periodate oxidation followed by reduction with either hydrogen and a Raney nickel catalyst or with sodium borohydride (16) in aqueous solution represented a general analytical procedure which can be applied to polysaccharides. The polyalcohol formed after the reduction of the polyaldehyde can then be hydrolyzed partially or completely by using different strengths of acids. By using the above method, Smith (13) found that glycogen and amylopectin contain about 1.0 and 0.5% glucose respectively which is evidently immune to glycol cleavage even after prolonged treatment with sodium periodate. Similarly, amylose contains approximately 0.2 - 0.5 percent

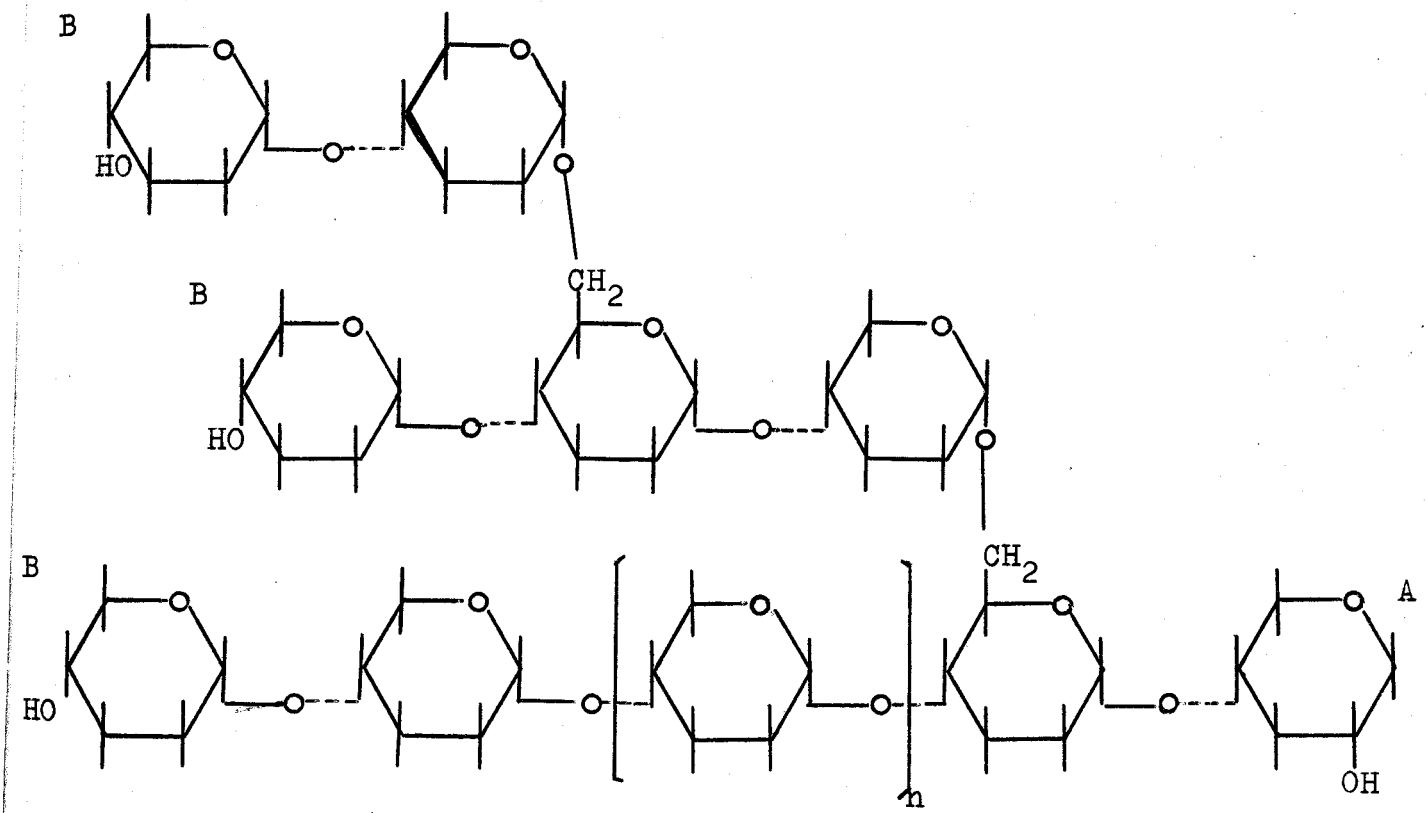
glucose. Glycerol and erythritol formed after acid hydrolysis of the polyalcohol were determined, after paper chromatographic separation, by periodate oxidation and determination of the formaldehyde formed using the chromotropic acid method. (17) The molar ratio of these compounds was calculated and formed the basis for determining the ratio of non-terminating to terminal non-reducing units of the molecule. The above authors obtained a value of 16 for waxy maize starch.

Since the above methods can only derive an average value for the length of the repeating units for a branched polymer, a number of chemical methods have been used in determining the aldehydic end-residues present in polysaccharides. (18, 19, 20, 21) Unrau (22) found that by using sodium borohydride followed by periodate oxidation, two moles of formaldehyde were formed per every reducing end-group of some particular polysaccharides. The formaldehyde determined by the chromotropic acid method could be used to estimate the molecular weight of polysaccharides. (22)

Amylases have contributed significantly in the elucidation of the molecular structure of starch and its components, and other related polysaccharides. In the case of amylose, complete beta-amylolysis may occur as initially reported by Meyer. (21) However, amylolysis has been shown to stop frequently at 70-80% hydrolysis as shown by Peat et al. (23, 24) The existence of abnormal linkages in amylose which act as a barrier to beta-amylolysis has been investigated



Amylose



Amylopectin

Fig.1 Chemical structure of amylose and amylopectin.

A. Reducing end; B. Non-reducing end

by Kjolberg and Manners. (25) For amylopectin, beta-amylolysis is incomplete, yielding residual beta-limit dextrin.

Alpha-amylase attacks both amylose and amylopectin in a random manner and differences in enzymic properties of individual alpha-amylase have been found. (26, 27) Of all the starch-splitting enzymes, alpha-amylase is preeminently the one most widely used in industrial processes. In pure research, it gives way to beta-amylase in usefulness as an agent for achieving quantitative conversion of starch.

It is now well established that amylose is an essentially linear molecule consisting of D-glucose units joined by α-1, 4-glucopyranoside linkages and possesses a molecular weight ranging from 10,000 to 100,000. On the other hand, amylopectin is a highly branched molecule of varying chain length and is linked by α-1, 6-bonds to the main α-1, 4-linked chain with a molecular weight ranging from 50,000 to 1,000,000. The basic structure of each is shown in figure 1.

The biosynthesis of starch (28) deserves a few comments. Phosphorylase was long thought to be involved in the synthesis of polysaccharides. However, in the light of present knowledge, phosphorylase is now believed to be a degradative enzyme only. On the other hand, nucleotide sugars are found to be the actual glycosyl donors. Adenosine diphosphate glucose (ADPG) was found by Recondo and Leloir (29) to be the most effective in the biosynthesis of starch.

Amylopectin is subsequently synthesized through the action of the Q-enzyme which was first described by Haworth and Peat. (30). This enzyme acts as a transglycosylase transfering a segment of a chain to position 6 of a glucose unit belonging to a different chain. This transfer is most likely an irreversible and non-phosphorylytic enzymic reaction. A similar enzyme, the branching enzyme is responsible for the synthesis of the branched chain structure of glycogen.

EXPERIMENTAL METHODS AND RESULTS

A. Extraction of Starch from Flour Using the Alkaline Digestion Method

A sample (20 grams) of flour, which was prewetted with absolute alcohol, was added to 560 ml 20% sodium hydroxide solution and subsequently 400 mg of sodium borohydride added as well. The mixture was stirred in a Waring Blendor for 30 minutes at a low speed, after which the mixture was boiled for 4 hours with constant stirring. Antifoam was added to prevent foaming. After cooling in an ice bath, the solution was neutralized with glacial acetic acid and the starch precipitated by addition of ethanol (1:10 in volume) with vigorous stirring. The starch was collected after standing overnight, washed three times with ethanol, four times with acetone and dried in a desiccator. The yield was 13.35 grams, 11.5 grams and 11.6 grams for durum, rye and Triticale, respectively.

B. Determination of the Nitrogen Content of Starches

The nitrogen content of the starch prepared as described above was determined using Nessler's reagent. (31) Ammonium sulfate was used as a standard and the standard curve is shown in Figure I.

C. Fractionation of Amylose and Amylopectin

Montgomery (32) found that pretreatment of starch with certain organic solvents such as glycerol, cellosolve, dioxane or n-butanol tends to facilitate starch fractionation. These solvents have the common properties that they extract fat from starch in the above pretreatment, but more importantly, amylose binding or hydrogen bonding is significantly reduced.

Starch (8grams) was suspended at a 5% concentration in 70% glycerol (90 ml of glycerol plus 40 ml of water) and stirred slowly while heated in a flask placed in a temperature controlled water bath. During pretreatment and extraction, the solution was thoroughly purged with nitrogen gas to minimize possible oxidative degradation. The bath temperature was regulated to permit raising the temperature of the solvent-starch mixture from 30°C to 89°C in 1 to 1½ hours and was kept at that temperature an additional hour. After cooling to 25°C, the suspended starch was precipitated in 10 volumes of ethanol, the starch separated by filtration and washed with ethanol until essentially free of glycerol. The wet starch was slurried in 100 ml of water and added to 400 ml of water at 98°C, then maintained at that temperature for 11-15 minutes with constant stirring. The pH of the solution was held at 6.0 - 6.3 with phosphate buffer. The solution was cooled to room temperature by immersion in an ice bath at 0-2°C. The residue, which consisted mainly of undissolved starch granules and some amylopectin, was removed

Table 1

Some Properties of Isolated Starches

Sample	Nitrogen %	Amylose %*	Blue Value	Beta-limit Dextrin %
Durum	0.167	25.8	0.288	31.0
Rye	0.25	25.4	0.288	27.7
Triricale	0.214	26.8	0.291	27.2

*Average of triplicate values.

by centrifugation. The supernatant was heated to boiling, during which time nitrogen gas bubbled through the solution, and held at boiling temperature for 30 minutes. Cyclohexanol (5 ml) (5) was added, and boiling continued for an additional 30 minutes after which time the solution was allowed to cool to room temperature and let stand overnight. The precipitated amylose-cyclohexanol complex was removed by centrifugation. The supernatant solution left contained mainly amylopectin. Amylose was resuspended in water and purified by a repetition of the above whole process 4 times. The final amylose-cyclohexanol complex was suspended and stirred in methanol for 30 minutes and the amylose collected by suction filtration followed by several washings with acetone. The product was dried in a desiccator; the yield for durum, rye and Triticale was 1.25 grams, 0.97 grams and 1.60 grams respectively.

The same procedure was repeated for the supernatant solution in order to remove traces of amylose. The amylopectin was then further purified by passing the solution through a paper column as described by Winkler. (33)

D. Purification of Amylopectin

A tightly rolled paper column (length 10 cm, diameter 7 mm) was placed in a glass column and a stirring rod forced down the center. The supernatant solution containing amylopectin (see previous section) was diluted with an equal volume of dioxane, making the total volume about 800 ml. The

solution was then passed through the column at a flow rate adjusted to about 5-10 ml per hour. Amylose, if present, would be absorbed selectively by the paper column. Amylopectin, which was not absorbed, would pass through the column and would be free of amylose. The column was finally washed with 50% dioxane solution. The eluate was concentrated under reduced pressure in a rotary evaporator and the polysaccharide precipitated in acetone. After several changes with acetone, the product was dried in a desiccator. The yield for durum, rye and Triticale amylopectin was 2.3 grams, 2.75 grams and 2.2 grams respectively.

E. Determination of the Purity of Amyloses

The purity of the amylose prepared was compared to a reference sample (A potato amylose, product of Nutritional Biochemical Corp.) by the iodine absorption method described by MacCready and Hassid. (34) The absorbance of the standard reference was measured over a series of wavelengths in a spectrophotometer and the optimum wavelength determined (645 mμ). Absorption measurements for the standard curve were consequently made at this wavelength. The absorption curve of the reference amylose and the standard curve are shown in Figure II and Figure III respectively.

About 100 mg of prepared amylose was prewetted with 2 ml of absolute ethanol, then 2 ml of 10% sodium hydroxide was added followed by 30 ml of water. The amylose suspension was stirred in ice bath with nitrogen gas passing through the

suspension until the polysaccharide was dissolved (about 2-3 hours). The volume of the solution was adjusted to 100 ml, 1 ml aliquots were withdrawn, followed by neutralization with hydrochloric acid 1 ml of KI-I_2 solution (I_2 2 mg/ml, KI 20 mg/ml) was added and the solution diluted to 100 ml. The absorbance was measured at 645 mμ. Purity of the amylose was found to be 90%, 91.5% and 92% for durum, rye and Triticale respectively. Since it was suspected that reference sample differed from the amyloses isolated, the absorption curve of these amyloses was compared using higher concentration. The result is shown in Figure IV.

F. Determination of the Purity of Amylopectins

The purity of the prepared amylopectins was determined as described by Wintler (33) without any modification. It was found that the amylopectins prepared were pure and free of amylose.

G. Determination of the Amylose Content in the Isolated Starches

The amylose content of the isolated starches was determined colorimetrically, using the MacCready and Hassid Method. (34) The procedure was essentially the same as in the determination of the purity of amylose. The results obtained are shown in Table 1.

H. Determination of Blue Value

The Blue Value of the isolated starches was determined following the procedure described by Gilbert and Spragg. (35) The values measured for the isolated starch and amyloses are recorded in Table 1 and Table 4 respectively.

I. Periodate Oxidation Studies on the Amyloses and Amylopectins

(i) Periodate Consumption Studies

Small amount of the prepared amyloses and amylopectins were subjected to periodate oxidation according to the method of Abdel-Akher and Smith. (36)

The polysaccharides (about 100 mg) were dissolved in dilute alkali. The solutions were cooled, after neutralization with acetic acid, to 5°C 0.5 M sodium periodate solution (5 ml) was added and the solution then adjusted to a desired volume. The oxidation was allowed to proceed at 5°C in the dark. A blank was treated in exactly the same way. The consumption of periodate (37) was determined as followed: Aliquots of the oxidation solution were transferred to a flask containing 1.5 grams of sodium bicarbonate and an excess of standard sodium arsenite, followed by addition of 1 ml 20% potassium iodide solution. After standing for 15 minutes, the excess sodium arsenite was titrated with standard 0.1N iodine solution using freshly prepared starch as the indicator. The blank was treated in a similar manner and the volume of standard iodine solution used for the

sample was determined by difference from the two titration values. The amount of periodate consumed by the polysaccharides was calculated in moles per glucose unit. The results are shown in Table 2 and Table 3 respectively. A one to one molar ratio was achieved in moles of periodate uptake per mole of glucose for both amyloses and amylopectins. These values are expected for normal amylose and amylopectin.

(ii) Formic Acid Production in the Periodate Oxidation of Amyloses and Amylopectins

The formic acid produced during the periodate oxidation was determined as described by Shasha and Whistler. (38)

The amount of formic acid liberated was used to calculate the number of non-reducing end units in the amylopectins as well as the molecular weight of the amyloses. The results are shown in Table 4 and Table 5 for amylose and amylopectin respectively.

(iii) Formaldehyde Determination Upon Periodate Oxidation of Borohydride-Reduced Amyloses and Amylopectins

The formaldehyde liberated during the periodate oxidation of reduced amylose and amylopectin was determined using the chromotropic acid method. (17) Briefly the procedure (39) was as follows:

Polysaccharide (about 400 mg for amylopectin, 100 mg for amylose) was wetted with ethanol and dissolved in dilute

Table 2

Periodate Oxidation Studies of

Amyloses

Time in Hour	Moles of Periodate Consumed per Mole of Glucose			
	Standard	Durum	Rye	Triticale
24	0.96	0.85	0.92	0.84
48	0.97	0.89	0.90	0.86
72	0.99	0.93	0.96	0.91
96	0.99	0.96	0.99	0.95
120	1.00	0.98	1.02	0.94
144	1.01	1.00	1.03	1.00

Table 3

Periodate Oxidation Studies of Amylo-pectins

Time in Hour	Moles of Periodate Consumed per Mole of Glucose		
	Durum	Rye	Triticale
24	0.80	0.80	0.86
56	0.94	0.94	0.94
102	0.94	0.99	0.99
161	0.99	1.04	1.09
190	1.00	1.08	1.09

alkali. Sodium borohydride (1 ml, conc. 0.5 g/ml) was added. The reaction was allowed to proceed for 24 hours after which the excess sodium borohydride was decomposed by adding 8 ml of 10N acetic acid. The pH of the solution was found to be about 3.78. The solution was cooled to 5°C and 10 ml of 0.5 M sodium periodate was added and the volume adjusted to 100 ml. The oxidation was carried in the dark at 5°C, and periodically a 2 ml aliquot of the oxidation solution was transferred to a test tube and the periodate ions precipitated with saturated lead acetate (3 ml). A length of dialysis tubing containing 5 ml of distilled water was introduced into the tubes and the systems were allowed to equilibrate overnight with shaking. One ml aliquots of the dialysate were transferred to centrifuge tubes and 10 ml of the chromotropic acid added. Lead sulfate that formed was removed by centrifugation and the resulting clear solution was transferred to test tubes and the contents heated for 30 minutes in the absence of light. The absorbance was measured at 570 mμ in a spectrophotometer. The amount of formaldehyde present was determined by reference to a standard curve from erythritol which is shown in Figure V. At the end of the oxidation, periodate consumption was determined as described previously.

J. Enzymic Degradation Studies

(i) Alpha-Amylase Degradation

(a) Determination of Alpha-Amylase Activity. The alpha-amylase used was a commercially available bacterial

enzyme.* The method used was that of Fischer and Stein (40 as described by Whelan. (41) It basically consists of incubation of a starch and enzyme mixture followed by measurement of reducing sugars with alkaline sodium 3, 5-dinitrosalicylate reagent.

The enzyme was incubated in 0.002M glycerol phosphate buffer (pH 5.9) so that 1 ml of solution contained 0.5 - 1.5 units of enzyme. Starch solution (1 ml) at 25°C was added with mixing, to 1 ml of enzyme solution. After exactly 3 minutes the reaction was terminated by addition of 2 ml of the salicylate reagent. The mixture was heated in a boiling water bath for 15 min., then cooled in a cold water bath, and diluted with 20 ml of water. The absorbance was measured at 540 mμ in a spectrophotometer with reference to a blank. A calibration curve (Figure IV was established with maltose hydrate (0 - 2 mg, no starch or buffer was added to the maltose). The activity of the enzyme was found to be 8.76 units/mg. protein.

(b) Hydrolysis of Starch, Amylose and Amylopectin. Polysaccharides (100 - 150 mg) was dissolved in dilute alkaline solution, neutralized with hydrochloric acid using phenolphthalein as an indicator. The solution was diluted to 100 ml using 0.015M sodium glycerol phosphate buffer (pH 5.7) after which 0.2 ml of alpha-amylase solution was added (100.0 mg in 10 ml). The surface was covered with a

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* A product of Nutritional Biochemical Corporation.

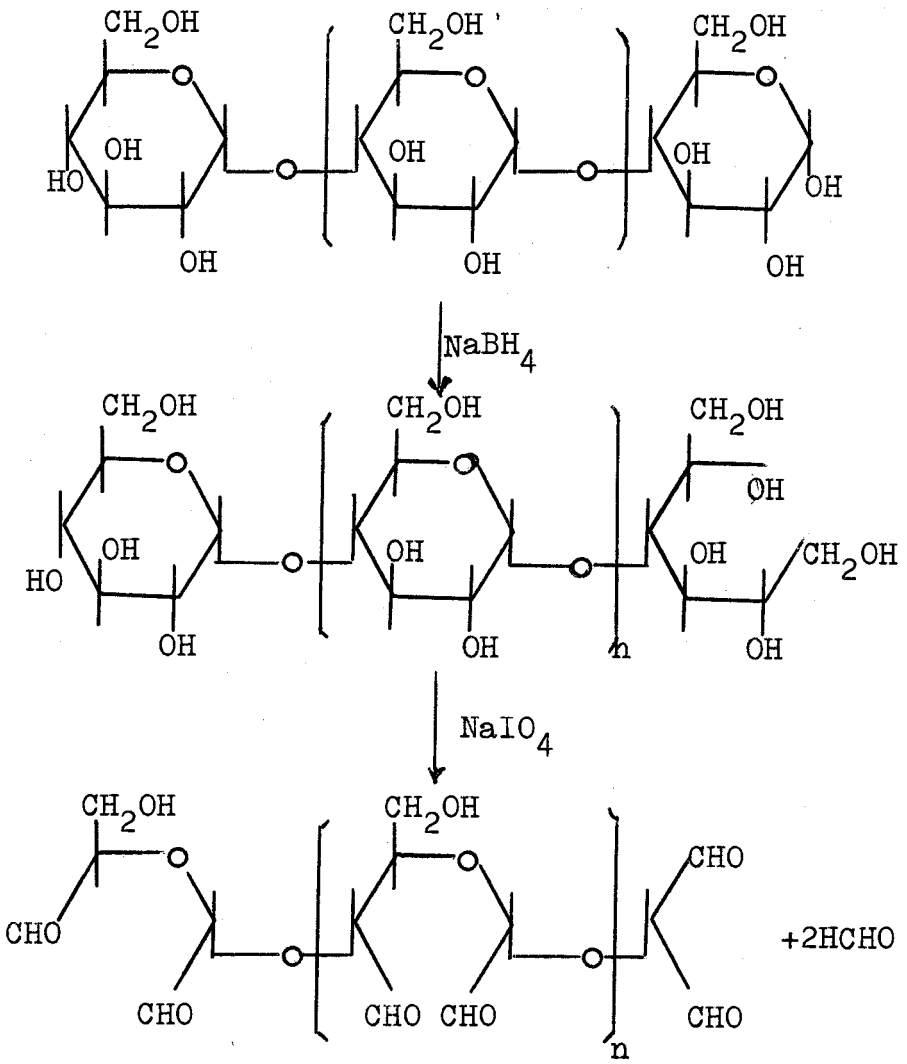

Figure 2 Production of Formaldehyde during Periodate Oxidation

Table 4

Some Properties of the Isolated Amyloses

Sample	Purity	Blue Value	Molecular Weight a.	Molecular Weight b.	Degree of Polymerisation a.	Degree of Polymerisation b.
Durum	90%	1.03	2.54×10^4	3.46×10^4	157	192
Rye	91.5%	--	2.42×10^4	3.20×10^4	150	178
Triticale	92%	1.07	2.44×10^4	3.97×10^4	151	220
Standard	100%	1.16	--	--	-	-

a. Results of formic acid titration

b. Results of formaldehyde determination

Table 5

Some Properties of the Isolated

Amylopectins

Sample	No. of non-reducing end group	Molecular Weight	Beta-limit dextrin%
Durum	21.3	1.35×10^5	27.0
Rye	19.0	1.15×10^5	26.2
Triticale	19.0	1.20×10^5	28.1
Standard	--	--	30.7

Table 6

The Determination of Formaldehyde Liberated from Reduced Amyloses During Periodate Oxidation

Sample	Time in Hour	Formaldehyde liberated	M.wt. of amyloses	Degree of Poly-merisa-tion	Periodate uptake
Durum		550ug	3.46×10^4	192	--
Rye	24	600ug	3.15×10^4	176	--
Triticale		450ug	4.20×10^4	232	--
Durum		552ug	3.42×10^4	190	--
Rye	72	598ug	3.20×10^4	178	--
Triticale		475ug	3.97×10^4	220	--
Durum		550ug	3.46×10^4	192	--
Rye	120	598ug	3.20×10^4	178	--
Triticale		475ug	3.97×10^4	220	--
Durum		550ug	3.46×10^4	192	--
Rye	144	598ug	3.20×10^4	178	--
Triticale		475ug	3.97×10^4	220	--
Durum		--	--	-	1.33
Rye	168	--	--	-	1.27
Triticale		--	--	-	1.29

Table 7

The Determination of Formaldehyde Liberated from Reduced Amylopectins During Periodate Oxidation

Sample	Time in Hour	Amount of Formaldehyde liberated	M.wt. of Amylopectin	Periodate uptake
Durum		164ug	1.64×10^5	--
Rye	50	234ug	1.15×10^5	--
Triticale		180ug	1.25×10^5	--
Durum		164ug	1.64×10^5	--
Rye	100	234ug	1.15×10^5	--
Triticale		225ug	1.20×10^5	--
Durum		198ug	1.35×10^5	--
Rye	150	234ug	1.15×10^5	--
Triticale		225ug	1.20×10^5	--
Durum		198ug	1.35×10^5	1.21
Rye	200	364ug	0.74×10^5	1.27
Triticale		225ug	1.20×10^5	1.19

layer of toluene. Two control mixtures were incubated, one containing substrate with inactivated enzyme (boiled in water bath for 15 minutes) the other containing maltose instead of the test substrate. Aliquots were periodically removed and the reducing values measured as stated in section (a).

The polysaccharide concentration was determined using the phenol-sulfuric acid method (42) using glucose as the standard (Figure VII.) and the results intepreted as total glucose content. At the end of the first incubation period after which there was no further observable increase in reducing value, more enzyme (1 ml, 0.25 gm in 25 ml) was added and the volume adjusted to 100 ml using the same buffer, and the system incubated again. When a constant reducing value was finally obtained, it was found that the enzyme was still active since a sample of this solution when added to the control solution (substrate with deactivated enzyme) hydrolyzed the starch within 2 hours as observed by a change in the iodine absorption color. The incubated solutions were heated in a boiling water bath for 15 minutes to deactivate the enzyme and then passed through cation resin (Amberlite IR 120(H^+)),[a] anion resin (Duolite A4(OH^-))[b] and again cation resin in that order. The eluate was evaporated

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a. Product of Rohm and Haas Company, Philadelphia, U.S.A.

b. Product of Chemical Process Company, California, U.S.A.

and analyzed by descending paper chromatography using Whatman No. 1 paper, ethyl acetate: acetic acid: water (8:2:2) as the solvent and 36 hours irrigation time. The spots were located by using the p-anisidine trichloroacetic acid spray agent. (43) Glucose, maltose, maltotriose, maltohexose and oligosaccharides were found to be present and their relative concentrations were determined by the phenol-sulfuric acid method after elution of these from the paper with water. (42) The results of the paper chromatographic analysis of the alpha-amylolysis products are shown in Table 11.

(ii) Beta-Amylase Degradation

(a) Determination of Beta-Amylase Activity. The beta-amylase used was a barley enzyme which was obtained from Nutritional Biochemical Corp. The method used for the determination of its specific activity was that of Whelan's. (44)

To a solution which contained 25 ml of soluble starch was added sodium acetate-acetic acid buffer (0.2M, pH 4.8) (3 ml) and the mixture brought to 35°C. An aliquot (2 ml) of preheated enzyme solution (35°C) was added and the digest incubated exactly 30 min. A 2 ml portion was removed and immediately added to 2 ml of alkaline 3, 5-dinitro salycylate regent and the mixture heated for 5 min. The reducing value was measured as described in the determination of alpha-amylase activity. One unit of enzyme was defined as the amount of protein liberating 1 mg of maltose in 30 min. The activity of the enzyme was found to be 27 units/mg. protein.

Table 8

Alpha-Amylolysis of Amyloses

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Standard
½	61.8	63.1	62.4	65.7
1	71.6	63.2	74.9	75.9
3	71.6	65.8	80.0	73.1
6	73.6	76.4	77.9	73.1
24	82.6	81.6	87.5	82.5
50	85.6	82.6	88.5	81.1
72	91.9	91.0	93.5	96.0
96	89.5	90.8	93.5	91.3
120	89.5	93.5	94.6	96.1
2nd Incubation				
1	100.0	98.5	100.0	100.0
24	102.0	103.0	100.0	102.0

Table 9

Alpha-Amylolysis of Amylopectins

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Standard
3	58.5	59.8	59.8	66.8
6	62.1	62.1	62.1	71.4
30	69.4	73.3	71.7	76.7
42	74.5	74.4	75.5	84.2
96	80.3	80.5	80.0	87.8
120	74.3	74.0	74.3	79.5
2nd Incubation				
1	67.5	71.5	69.5	65.5
22	76.2	74.0	75.5	84.5
44	74.0	75.0	75.5	84.5
66	75.0	75.8	75.5	84.5

Table 10

Alpha-Amylolysis of Starches

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Soluble Starch
½	49.4	47.9	48.2	33.2
1	50.0	52.2	50.0	36.2
3	57.4	59.5	59.3	40.6
24	65.3	63.5	64.0	44.1
48	70.0	69.4	71.5	53.9
72	74.6	74.6	73.5	54.3
96	79.3	77.6	78.2	57.0
144	80.0	77.6	79.0	59.0
2nd Incubation				
1	73.0	73.0	73.0	55.8
24	78.0	74.8	74.1	55.8
48	79.0	78.0	77.7	59.0

Table 11

Paper Chromatographic Analysis of

Alpha-Amylolysis Products

Sample	Oligo Saccharides	G_6 R_G = 0.125	G_3 R_G = 0.266	G_2 R_G = 0.51	G_1 R_G = 1.00	Total Percentage	Recovery
A. Amyloses							
Durum	6.27	9.17	42.1	33.3	9.16	100.0	94%
Rye	6.85	12.20	46.1	26.8	8.14	100.7	100%
Triticale	6.07	11.60	44.6	28.8	9.13	100.4	103%
Standard	3.28	10.90	48.5	26.48	11.0	100.2	77%
B. Amylopectins							
Durum	27.7	5.85	29.5	27.3	9.96	100.1	101%
Rye	27.6	5.97	30.9	25.7	9.90	100.0	86%
Triticale	28.8	5.94	30.0	24.7	10.60	100.0	87%
Standard	19.0	6.63	37.0	27.0	10.40	100.0	82%
C. Starches*							
Durum	29.6		33.8	27.5	9.10	100.0	67%
Rye	26.2		28.8	32.6	12.4	100.0	68%
Triticale	27.8		31.5	29.4	11.5	100.0	-
Soluable	40.7		27.4	23.5	8.50	100.0	-

* This section includes oligosaccharide and G_6

R_G = 0. -0.173

(b) Hydrolysis of Starches, Amyloses and Amylopectins with Beta-Amylase. (44) Polysaccharide (100 mg - 200 mg) in a 100 ml volumetric flask was prewetted with 2 ml ethanol, 10 ml of distilled water was added, followed by addition of 1 ml of 6N sodium hydroxide. After thorough mixing, the suspension was heated in a water bath under nitrogen. At 1 min. intervals, the flask was removed and agitated vigorously. Heating and shaking were continued until a homogeneous solution was obtained. After cooling to room temperature, the solution was neutralized with 3N sulfuric acid. Sodium acetate-acetic acid buffer (0.2M pH 4.7) (10 ml) was added, followed by addition of 1% serum albumin (5 ml), 0.01M reduced glutathione (5 ml) and aliquots of beta-amylase. The digest was diluted to 100 ml with water, the surface covered with a layer of toluene and the mixture incubated at 35°C. Two control mixtures were incubated, one containing substrate and enzyme inactivated by 5 min. heating in a boiling water bath, the other containing maltose instead of the test substrate. The reducing power of the controls were measured at the same time as the test mixtures. The first control allowed for the reducing power of the non-substrate components, especially glutathione, the second allowed for the detection of maltose enzyme activity. At the end of the first incubation, more enzyme was added to effect further hydrolysis. When no further observable increase in reducing values was evident, it was found that the enzyme was still active. This was shown to be the case in that a significant increase in reducing value was observed

after 24 hours when the enzyme (2 ml) was added to a standard amylose solution. The incubated solutions were heated in a boiling water bath for 5 minutes after which the denatured enzyme and serum albumin proteins were removed by centrifugation. Aliquots of the supernatant solution were transferred to dialysis tubing and dialyzed for 72 hours against distilled water. Beta-limit dextrin, because of its relatively high molecular weight remained inside the dialysis tubing. The amount of beta-limit dextrin present was determined using the phenol-sulfuric acid method, and the results shown in Table 1 and Table 5 for starches and amylopectins respectively.

K. Smith Degradation Studies

Chromatographic separation was carried out on Whatman No. 1 and N. 3MM paper for analytical and preparative purposes respectively using the descending technique and the following solvents:

A. Ethyl acetate:acetic acid:water (8:2:2)

B. Ethyl acetate:pyridine:water (8:3.5:2)

Compounds were detected with p-anisidine trichloroacetic acid,(43) ammoniacal silver nitrate (45) and periodate-permanganate (46) reagents. Evaporation was carried out under reduced pressure with a bath temperature about 40°. Melting points reported are uncorrected.

Table 12

Beta-Amylolysis of Amyloses

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Standard
½	72.0	80.0	65.5	40.7
3	80.6	88.2	85.0	78.4
6	83.0	91.9	90.4	90.4
24	94.5	98.5	97.5	100.0
48	98.1	98.1	100.0	102.8
72	98.4	103.5	103.5	101.8
96	101.8	103.5	103.5	110.0

Table 13

Beta-Amylolysis of Amylopectins

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Standard
48	40.0	56.0	56.0	58.2
100	61.2	60.0	59.0	58.5
116	61.3	60.0	58.0	57.5
2nd Incubation				
21	59.2	57.5	60.5	62.0
72	59.2	58.0	60.5	62.0
94	61.7	60.8	60.5	62.0

Table 14

Beta-Amylolysis of Starches

Time in Hour	% Conversion to Maltose			
	Durum	Rye	Triticale	Soluble Starch
48	59.0	58.0	59.0	20.2
96	59.0	60.5	60.5	25.2
120	57.0	59.0	59.0	25.2
2nd Incubation				
27	64.0	73.5	68.5	26.8
72	64.0	66.6	68.0	31.2
94	64.6	66.6	68.0	31.2

(i) Periodate Oxidation

Amylose or amylopectin (200-500 mg) was dissolved in dilute alkali, sodium borohydride was added (40 mg) and the solution was stored overnight. The solution was subsequently acidified with 10 N acetic acid (8 ml), the pH of the first solution was 3.8-4.1. After cooling to 5°C, 10 ml of 0.5 M sodium periodate was added, the volume of the solution adjusted 100 ml, and the oxidation was allowed to proceed at 5°C in the dark. The periodate uptake at the end of the oxidation was determined using the arsenite method. (37) The periodate and iodate ions were precipitated in the form of the barium salt using barium carbonate. After removal of the precipitate, the polyaldehyde was reduced with sodium borohydride and stored for 24 hours. Excess borohydride was then destroyed by addition of acetic acid and the resulting solution evaporated. Borate was removed by repeated evaporation with methanol containing 1% acetic acid.

(ii) Complete Acid Hydrolysis

A portion (half the amount for amylose and about ¼ for amylopectin) of the residue was boiled with 1N sulfuric acid for 8 hours after which the solution was neutralized with barium carbonate and filtered. The filtrate was passed through cation resin (Amberlite IR 120(H^+)) and anion resin (Duolite A 4(OH^-)) beds in that order and the eluate evaporated. The residue was chromatographed in solvent A and B. Glycerol, erythritol and traces of glucose were found to be present. The molar ratio of the components were determined

as described in section (iv).

(iii) Partial Acid Hydrolysis

The remaining portions of the residues were deionized as above and the eluates evaporated. The residue was subsequently dissolved in 25 ml 0.2N hydrochloric acid and stored for 11 hours at room temperature. The acidic solution was neutralized with lead carbonate and the precipitate removed by filtration. The filtrate was deionized, evaporated to dryness and chromatographed using solvents A and B. The molar ratio of the components was determined as described in section (iv).

(iv) Determination of Erythritol, Glycerol and Glucose Present in the Residue (22)

Aliquots of the residue were chromatographed in solvent A for 14 hours. The section corresponding to erythritol was excised and eluated with 20 ml of distilled water. After shaking for half an hour, the mixture was filtered through glass wool. Aliquots of the filtrate were transferred to centrifuge tubes, 1 ml of 0.5 M sodium periodate was added and the oxidation was allowed to proceed for 15 minutes at room temperature after which the periodate and iodate ions were removed by adding 2 ml of saturated lead acetate. The resulting precipitate was removed by centrifugation for 20 minutes, the clear supernatant was transferred to ordinary tubes and aliquots suequently pipetted into another series of centrifuge tubes. The volume was adjusted

to 1 ml with distilled water and 10 ml of chromotropic acid added. After the removal of lead sulfate the formaldehyde present was assayed as described previously (see section I).

The section containing glycerol was eluated with 10 ml of distilled water and the amount of formaldehyde liberated after oxidation of the glycerol was determined in the same way. Since glucose occurred only in trace amounts, standard glucose was applied as the marker and the corresponding location was eluated with 10 ml of water. The amount of glucose present was determined by the phenol-sulfuric acid method.

(v) Identification of the Components

Whatman No. 3MM paper was used to resolve larger quantities of the residue in solvent A.

Glycerol: To the dry sirup was added a 10% molar excess of p-nitrobenzoyl chloride and pyridine (3 ml) and the mixture was heated for one hour. Excess aroyl halide was decomposed by the addition of a few drops of water followed by addition of an excess of saturated sodium bicarbonate. After storing overnight, the solid was collected by filtration, washed with water and recrystallized from acetone. The tri-p-nitrobenzoate of glycerol had m.p. and mixed m.p. 187-190°C.

Erythritol: This compound was converted to the tetra-p-nitrobenzoate as described for glycerol, melting point and mixed m.p. 248-250°C.

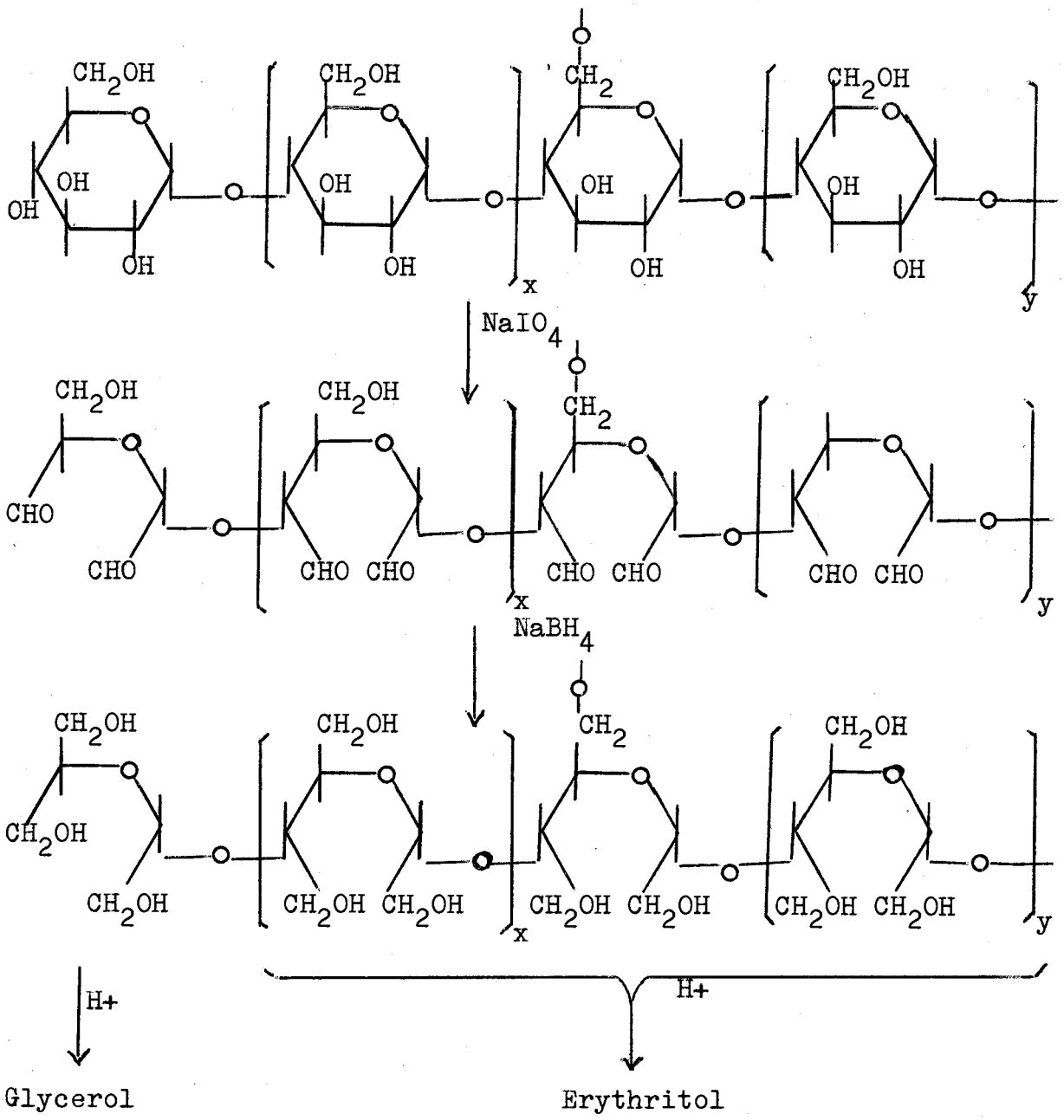


Figure 3 Formation of Glycerol and Erythritol in Smith Degradation

Table 15

Paper Chromatographic Analysis of Amylose Hydrolysates from Smith Degradation

Sample	Erythritol			Glycerol			Glucose		
	weight mg	m. moles $X10^{-3}$	molar* ratio	weight	m. moles $X10^{-3}$	molar* ratio	weight	m. moles $X10^{-3}$	molar* ratio
(A) Partial Acid Hydrolysis									
D.A	36.4	300	125	0.226	2.40	1.00	0.250	1.38	0.580
R.A	18.8	154	145	0.098	1.06	1.00	0.150	0.83	0.780
T.A	38.0	312	127	0.228	2.46	1.00	0.274	1.52	0.620
(B) Complete Acid Hydrolysis									
D.A	32.6	267	160	0.160	1.78	1.00	0.155	0.86	0.501
R.A	40.6	335	131	0.256	2.66	1.00	0.140	0.77	0.294
T.A	--	--	--	--	--	--	--	--	--

* These values were obtained by taking the m. moles of glycerol as unity.

Table 16

Paper Chromatographic Analysis of Amylopectin Hydrolysates from Smith Degradation

Sample	Erythritol			Glycerol			Glucose		
	weight mg	m. moles $X10^{-3}$	molar* ratio	weight mg	m. moles $X10^{-3}$	molar* ratio	weight mg	m. moles $X10^{-3}$	molar* ratio
(A) Partial Acid Analysis									
D.Ap	58.0	475	13.95	3.13	34.0	1.00	1.00	5.50	0.163
R.Ap	67.2	550	12.70	3.98	43.2	1.00	1.02	5.50	0.127
T.Ap	61.8	506	13.40	3.56	37.6	1.00	0.90	5.00	0.132
(B) Complete Acid Analysis									
D.Ap	17.5	143	13.20	1.01	10.8	1.00	0.40	2.20	0.206
R.Ap	21.3	175	13.45	1.20	13.0	1.00	0.45	2.50	0.193
T.Ap	15.7	129	12.92	0.92	10.0	1.00	0.31	1.72	0.172

* These values were obtained by taking the m. mole of glycerol as unity.

DISCUSSION

A. Fractionation of the Starch Components

Starch usually exists in the form of rather insoluble particles more particularly known as starch granules. The morphology of starch granules of various species is quite characteristic. These granules can be disrupted to form dispersions when heated in water especially at a pH above 7.0. The ease of dispersion depends largely on the botanical source of starches. Cereal starches have been found to disperse less readily and usually require some appropriate type of chemical pretreatment. The principal basis of this treatment is to disrupt or weaken the hydrogen bonding of amylose and consequently facilitate the fractionation of starch into its structural components.

Various methods have been used to fractionate amylose and amylopectin from starch. All these methods are based on the differential solubility of the fractions (amylose and amylopectin) in various media. Those involving organic solvents as complexing agents are most widely used.

Cyclohexanol was preferred in the present studies because it tends to form an amylose-cyclohexanol complex readily and repeated fractionations could be performed within a relatively short period of time to obtain a rather highly purified amylose. As originally noted by Bourne, (4)

it was found that relatively pure amylopectin could be prepared from the supernatant. Precautions have been taken to prevent oxidative degradation of the polymeric material during the fractionation process. To this end, sodium borohydride was added during the extraction of starch from flour and high nitrogen gas atmospheres were used extensively in the pretreatment and fractionation procedure. The solutions were maintained at pH6.0-6.3. During these processes investigators have stressed the need for such mild conditions primarily because these high polymers are readily degraded upon heating in the presence of air particularly under alkaline conditions. This degradation is more serious for amylose than for amylopectin; this is because amylose is believed to have a small number of bonds that are unusually oxygen labile. (47) It is indeed difficult to determine with confidence that no degradation has taken place, and it is even more difficult to visualize the nature of these oxygen-labile bonds. Gilbert (47) has emphasized the hydrolytic role of neutral water (pH6.0) at 100°C. He estimated that approximately 3 glycosidic bonds would be hydrolyzed per molecule. This extremely low degree of degradation could, however be disastrous in the studies of high molecular weight polymers. The possibility therefore still remains that the native polymers will be greater in molecular weight than the materials isolated even when relatively mild fractionation procedures were employed.

B. Purity of Amylose and Amylopectin Isolated

The apparent purity of the isolated amyloses was found to be lower than that of a "standard" amylose. Though as early as 1948, Bourne et al (4) reported that cyclohexanol-amylose complex contained a higher proportion of amylopectin than does the thymol-amylose complex, this is believed to be unlikely in the investigation reported here because of the repeated fractionations that were employed. This contention is further supported by Sarko et al (5) who reported recently that by using the cyclohexanol fractionation method, 95% purity was easily obtained by two fractionations while a third fractionation ensured essentially 100% purity.

It appears that the apparent discrepancies may be a function of the particular botanical sources particularly since the reference standard was a potato amylose which differs in chain length from the amyloses studied. Bailey and Whelan (48) have demonstrated that the "Blue Value" and E_{max} are related to the chain length. In light of this, it is not surprising to find that the isolated amyloses differ slightly in the B.V and E_{max} values from those for potato amylose. The B.V of the potato amylose was slightly greater than that of the amyloses isolated. Furthermore, the absorption spectra of these amyloses revealed that the potato amylose absorbed at slightly higher wavelength than the others. This might imply that the potato amylose has a higher D.P.

It was found in this investigation that the method used for the purification of amylopectin was quite satisfactory in that amylopectin free of amylose could readily be prepared.

C. Determination of the Amylose and Amylopectin Ratios

Amylose and amylopectin are sharply differentiated by their physical reaction with iodine and this forms the basis of all practicable methods for estimating amylose in pure starch. This procedure depends either upon the potentiometric determination of the amount of iodine absorbed under specific conditions or upon the measurement of the color intensity of the amylose-iodine complex. The former appears to be more accurate but the latter is simpler and more convenient. The colorimetric method was used in the present studies since comparative measurements were required.

The apparent percentage of amylose varies from 1% in waxy maize starch to 66% for a starch from wrinkled-seed peas with intermediate values for various other species. The amount of amylose in a starch is not fixed in any particular way, but depends primarily on genetic factors and the maturity of the plants. As the inheritence of waxy grains in cereals is conditioned by a recessive gene, wx, plants homozygous for wx produce amylose-free starch. Recently, mutant genes have been identified which give a high positive correlation between amylose and total starch content. Several genes are apparently associated with amylose content of the starch of maize and other plants.

The present investigation showed that durum starch contains 25.8% amylose, rye starch 25.4% amylose and triticale starch 26.8% amylose. These values are consistent with the reported values for wheat starch. (49)

The constant values observed among the three species is a good indication that these species are very similar in their genetic control mechanisms involved in the biosynthesis of starch. If the overall gene action had been different, various values for amylose content would have been expected.

D. The Molecular Weight of the Isolated Amyloses and Amylopectins

The molecular weight of the prepared amyloses was determined using procedures involving the formic acid titration and formaldehyde determination after periodate oxidation. The values obtained are summarized in Table 4. (See page 23) It is noted the values from both methods are in good agreement and the three species have about the same molecular weight.

Since it has been generally observed that the chemical end-group procedures for the determination of amyloses and amylopectins give lower D.P values than the physical methods, (50, 51) it is therefore not surprising that the D.P. of the isolated amyloses was relatively low. These values were in close proximity to that of corn amylose (D, P 140-300), (22) but differ from that of potato amylose. In fact, of all the various starches, the cereal starches (corn, wheat,

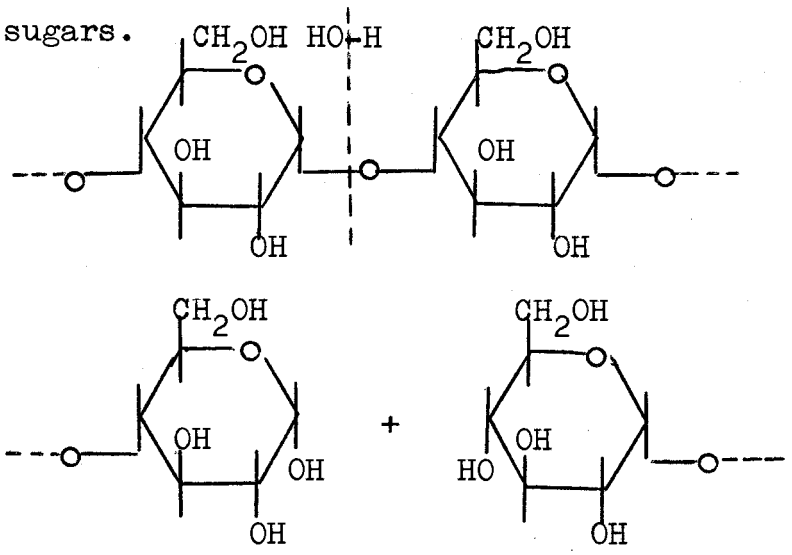
barley) generally give the lowest molecular weight, and the tuber and root starches show the highest molecular weight. (51) Although a D.P. value of 500 of wheat amylose has been reported by Greenwood, (52) this may result from differences in the procedures employed for preparing the starches and consequent differences in the extent of degradation.

In the case of the amylopectins, the degree of branching and molecular weight of the three species are again essentially the same (Table 5, page 24). The degree of branching, which was 19-21 units, was consistent with the value reported by Greenwood (49) for wheat amylopectin and the molecular weights were within the range reported for amylopectins prepared under ordinary conditions.

As stated in the previous section, the amylose content of starch is to a certain extent genetically controlled. There is a definite similarity of the molecular weight of amylose and amylopectin as well as the degree of branching of amylopectin of the parental species. There is no observable alien genome-induced change in the amylose content among the synthetic species. This observation is therefore not unexpected and is supported further by the results of alpha- and beta-amylolysis and the behaviour of these materials toward Smith periodate degradation. It is rather clear that no detectable difference existed in the materials studied. Any subtle differences would be presumably minor and could not be distinguished by the chemical and enzymic methods used.

E. Alpha-Amylolysis of Starch and its Components

The general properties and the action patterns of various alpha-amylases have been discussed in some detail by Fischer and Stein. (53) Alpha-amylase is found in all types of living organisms and it catalyses an essentially random hydrolysis of the α-1,4-glucosidic linkages in starch and glycogen. The reaction is for instance, characterized by a rapid decrease in iodine staining powers and molecular size of the glucan with a concomitant production of reducing sugars.



This hydrolysis takes place by C_1-oxygen scission and not by oxygen-C_4 scission. The reducing sugars set free retain the α-configuration. A mechanism involving double replacement has been proposed to explain the retention of the configuration. (53)

It has been noted that there are wide differences in the action patterns of alpha-amylases from various sources, and the nature of the α-limit dextrins depends largely upon

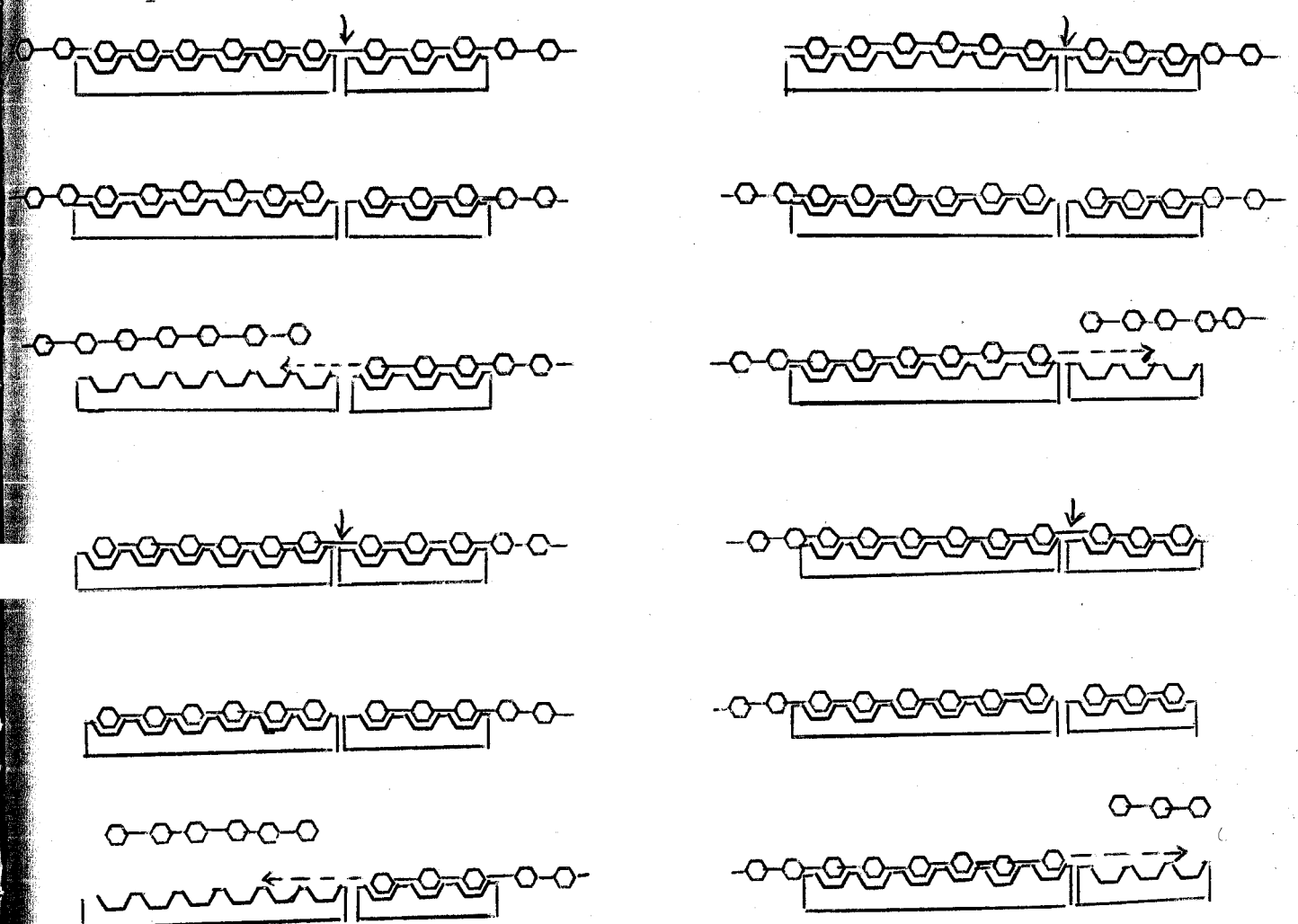
the source of alpha-amylase and the concentration used in hydrolysis.

The enzyme used in the present studies was from Bacillus subtilis. It is a dimer in that two molecules are joined through a zinc mediated linkage. The enzyme is activated by chloride ions. The action pattern and specificity of this enzyme has been studied extensively by Robyt and French. (54) By analyzing the degradation products using paper chromatography, the above workers were able to show that this enzyme has a dual specificity for the formation of maltotriose and maltohexose. They postulated that the binding sites are completely filled when nine glucose units are bound. The mode of formation of maltotriase and maltohexose units is illustrated. The present studies generally were in agreement with those of the above investigation, however, the dextrins were hydrolysed to a greater extent than the results noted by the above workers. (54)

Though 100 percent conversion to maltose has been achieved for amyloses, paper chromatographic studies revealed the presence of oligosaccharides of greater size than maltose. A similar result has been reported by Whelan using barley-malt amylase. (41)

In the case of amylopectin, different hydrolysis values have been reported using various sources of alpha-amylase. The degree of amylolysis depends on the sources of enzyme, its concentration and incubation time. This is mainly because alpha-amylolysis proceeds in two stages, i.e.

the random scission of glucosidic bonds to oligosaccharides which are then further hydrolysed to maltose and glucose in the second stage. These two stages are not due to two different and se parate reactions but merely reflect a difference in the affinity of the enzyme for large and small molecules. With malt and B. subtilis enzyme, the latter stage of hydrolysis is much slower than for enzymes from other sources and incubations for several months have been reported. (55)



The Formation of G_6 The formation of G_3

Figure 4 - Diagrammatic Representation of the Action of an Amylase from B. Subtilis

Some intriguing features are noted in these alpha-amylolytic studies. The percentage of conversion to maltose of amyloses, amylopectins and starches prepared were identical. The kinetics of hydrolysis as well as the distribution of alpha-limit dextrins were essentially the same. Standard amylose, amylopectin and soluble starch used as reference differed markedly from these species. This implies very strongly that the materials prepared are structurally similar and consequently behaved in an identical way in enzyme degradation studies.

F. Beta-Amylolysis of Starch and its Components

Beta-amylase is a plant enzyme which hydrolyzes α-1-4 D-glucosidic linkages in amylaceous polymers. The enzyme attacks alternate glycosidic bonds in a normal starch chain, starting from the non-reducing end, and continues until the entire chain is converted into maltose or until further action is blocked by a physical or chemical irregularity in the chain. It has consequently been used extensively in investigating the fine structure of polysaccharides.

The general properties and action pattern of beta-amylase has been discussed in detail by French (56) and other workers. (57) The subject represents a fascinating segment of starch enzymology, for which the final chapters are probably yet unwritten.

In the present studies, amylase was degraded completely by beta-amylase, amylopectin 60-61% and starch 64-68%.

A small difference is evident in the values for whole starch, but caution is required concerning this result since it might be merely a reflection on the purity of the samples.

Beta-amylolysis of amylose presents a more complicated picture. Various values ranging from 70% upwards have been reported. (23) It is now known that abnormal linkages that are resistant to beta-amylolytic attack exist. (58) Individual amyloses appear to differ in both D.P. and the beta-amylolysis limit, indicating that variation exists in the relative proportion and distribution of barrier to beta-amylolysis. Greenwood, Manners and Coworkers (59) were able to demonstrate that native amylose was heterogeneous, some molecules consisting of a linear chain of unmodified glucose units, whilst others contain some type of structural anomaly. In addition, this anomaly is associated primarily with the high molecular weight fractions while the low molecular weight fractions are essentially linear.

"Aerobic" isolation of amylose may introduce barriers that are resistant to beta-amylase hydrolysis. This picture is further complicated by the enzymic preparation used. As originally shown by Peat & Whelan (24) the contamination with Z enzyme tends to enhance beta-amylolysis. These workers noted that whereas the Z enzyme could be heat inactivated in the early stages of purification of beta-amylase, it could not be removed by this method when it was present in a more

highly purified beta-amylase preparation. (57) A further source of uncertainty in the beta-amylolysis of amylose is caused by the retrogradation of amylose. This error could be eliminated by determining the amylose that was dissolved and the results interpreted with this value taken into consideration.

Since the amylose isolated in the present studies was of relatively small molecular weight, it was expected that the molecule was found to be essentially linear. If abnormal linkages existed, it could only have occurred in the vicinity of the reducing end of the molecule.

The beta-amylolysis limit of amylopectin was consistent with the values reported by various workers. (23, 60) Greenwood noted that the beta-amylolysis limit for the amylopectins do not appear to vary significantly. The amount of beta-limit dextrins isolated from amylopectin and starch of the three species are in reasonable agreement. It would be of considerable value if the structure of these dextrins could be further characterized.

An interesting feature to be noted is that the beta-amylolysis of starch is only slightly greater than that of amylopectin. If starch is composed only of amylose and amylopectin, the beta-amylolysis would be expected to be much higher. This observation has been attributed to the existence of a third polysaccharide (5-10%) in some starch, notably in potato, rubber seed and wheat (49, 61) having intermediate properties. This seems indeed plausible, since

it is unlikely that nature should synthesize completely linear and completely branched molecules simultaneously without the occurrence of some type of intermediate.

G. Smith Degradation Studies

The results of the partial and complete acid hydrolysis of amyloses and amylopectins are shown in Table 15 and 16 respectively.

The molar ratio of erythritol to glycerol could be used as a measure of the D.P of amylose. Glycerol could be derived only from the non-reducing end-group of the chain while erythritol comes from the remaining internal chain. The sum of the molar ratio of these components will represent the D.P of the molecule. The values obtained (125-160) agrees reasonably well with those obtained from other methods. In the case of amylopectin, this ratio constitutes the basis for the determination of the ratio of non-terminal to terminal non-reducing units of the molecules and a value of 14-15 was achieved as compared to the value of 19-21 obtained from formic acid titration. The former values are generally lower.

It is of considerable importance to note that glucose was produced in both the partial and complete acid hydrolysis of the Smith degraded amyloses and amylopectins. The amount of glucose present was determined by the phenol-sulfuric acid method. This result is rather unexpected. If any unusual linkages resistant to periodate oxidation were present

in the materials studied (as is indicated by the presence of glucose in the complete acid hydrolysis), one would expect the presence of some non-reducing component such as glucosyl erythritol in the partial acid hydrolysate. However, the present investigation revealed that the above stated component was not detected by the spray reagents used. Two possibilities exist, namely the incomplete oxidation of the polysaccharides or the presence of substituted furanoside. Wheat amylose has been found to retrograde much more readily than potato amylose and complete dissolution of amylose is extremely difficult. Furthermore, periodate oxidised amylose tends to aggregate in solution as reported by Erlander et al. (62) This tends to make the partially oxidized molecule unavailable toward further and eventually complete oxidation. In the case of amylopectin, preferential oxidation may occur so that the inner chain is less readily attacked by periodate because of steric hindrance. These unoxidized portions of the chain may then be hydrolysed rather readily because of some unknown reasons. The other possibility is that it is known that a furanoside is more readily hydrolysed than an ordinary pyranoside. The presence of furanoside is rather unlikely since no furanose forms have as yet been found in amylose and amylopectin, furthermore, it is doubtful that glucofuranose residues are formed during the biosynthesis of amylose and amylopectin by ADPG which has a pyranose ring structure and is specific in this regard. The presence of

glucose in both mild and complete acid hydrolysates cannot therefore be easily rationalized.

Considering the results obtained in this investigation, it can be stated that in general the amylose and amylopectin of the starches from the parental and the synthetic (alien genome combinant) species did not have any gross structural differences. This does not preclude the possibility that modification may be present in the fine structure of the amylose and amylopectin of the synthetic species. It cannot be claimed with any great degree of confidence that the methods used would detect difference in the fine or tertiary structure of the polymers.

SUMMARY

(1) Present investigations show that there is no detectable alien genome induced change in the structure of the starch synthetic and two parental species studied. Any difference, if such existed, was minor and could not readily be recognized by ordinary analytical methods.

(2) The amylose content of the three species was identical.

(3) The amylose and amylopectins isolated are similar in their molecular weight and in their degree of branching. Furthermore, they behave identically toward chemical and enzymic degradation.

(4) No conclusive evidence was found for the existence of any abnormal linkages in amylose and amylopectin.

(5) Since chemical methods usually result in some non-specific degradation and modification of the polymeric chain, any fine structural difference is unlikely to be detected by these methods. On the other hand, subfractionation of the materials and subsequent ultracentrifugal studies and viscosity measurement may be promising in revealing the nature of these subfractions. This is particularly true since amylose and amylopectin are heterogeneous with respect to molecular size and possibly molecular shape.

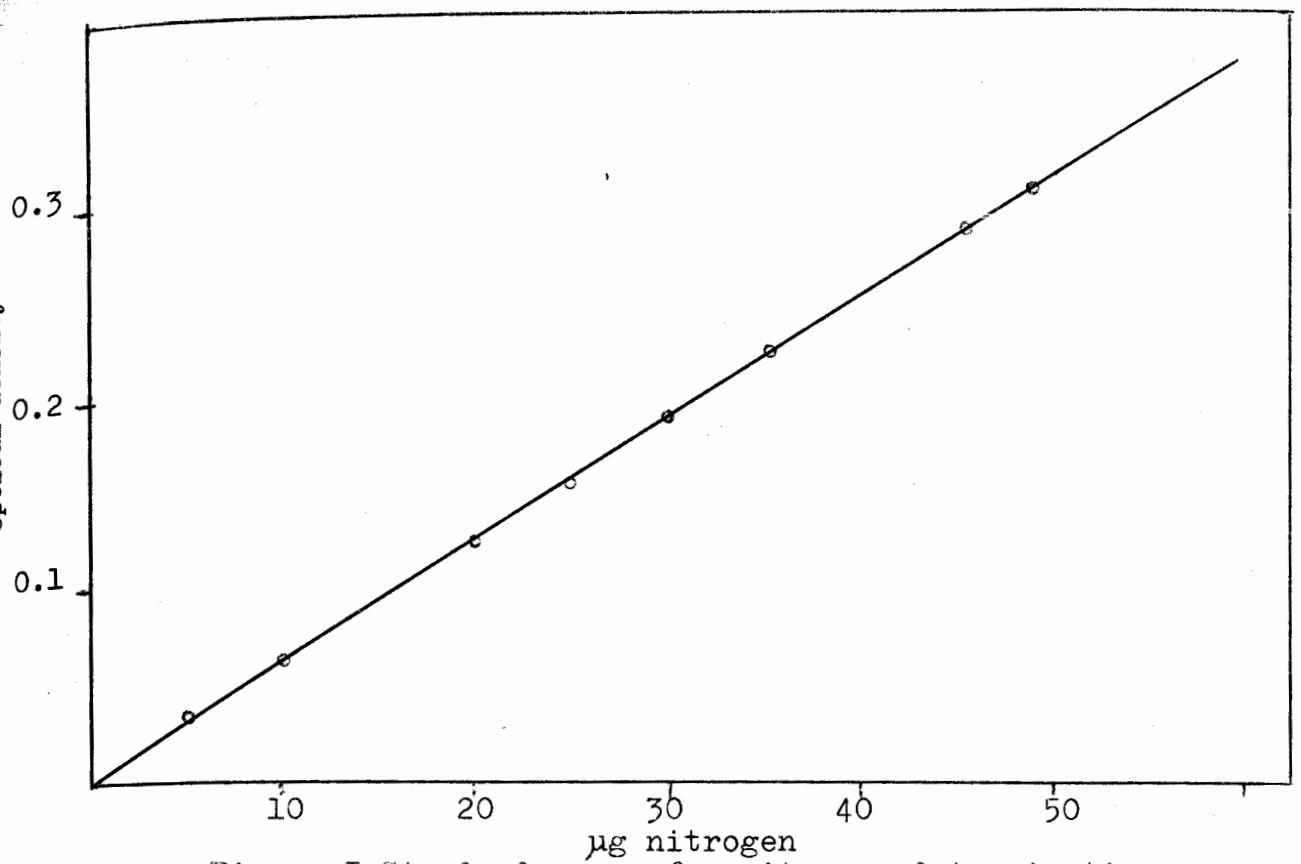


Figure I Standard curve for nitrogen determination

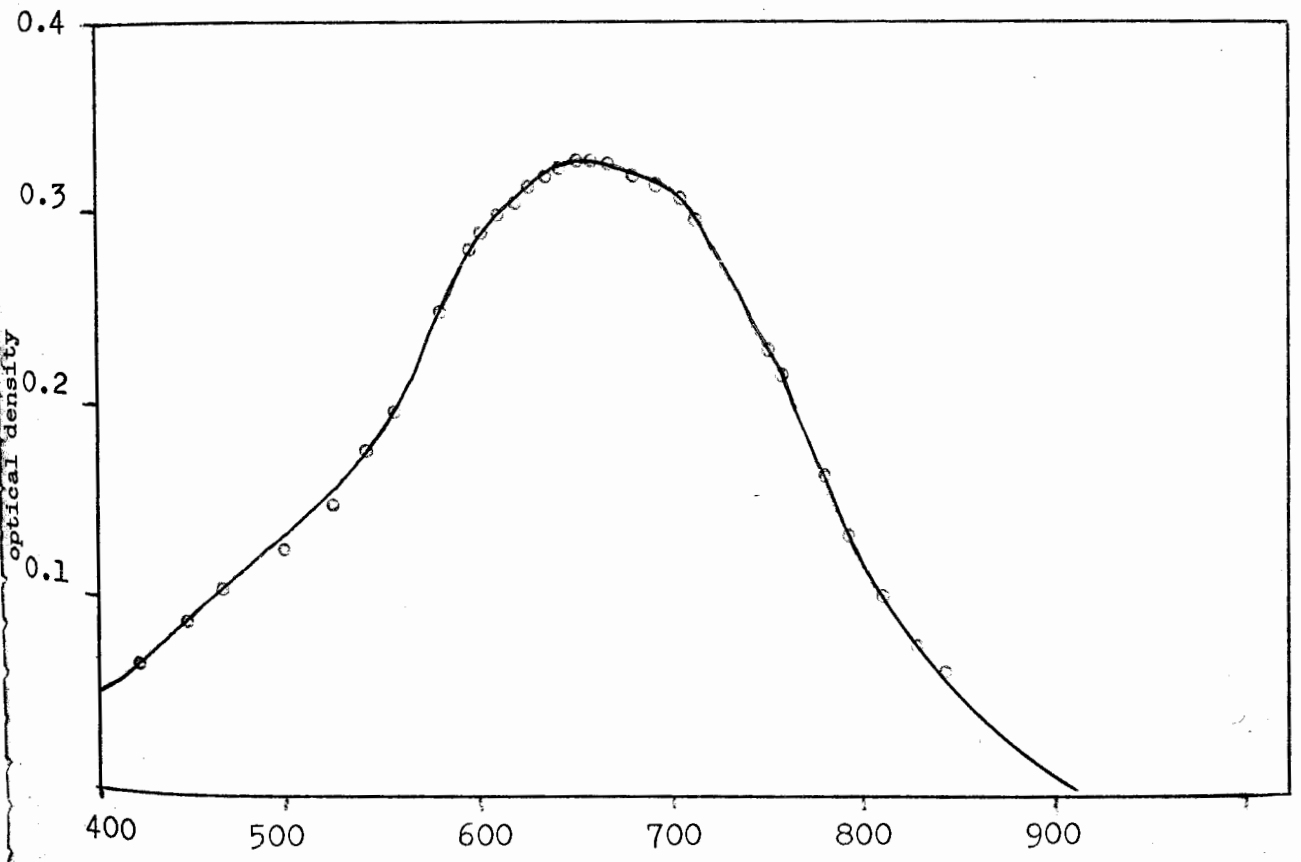


Figure II. The absorbance of standard amylose-iodine complex at different wavelength

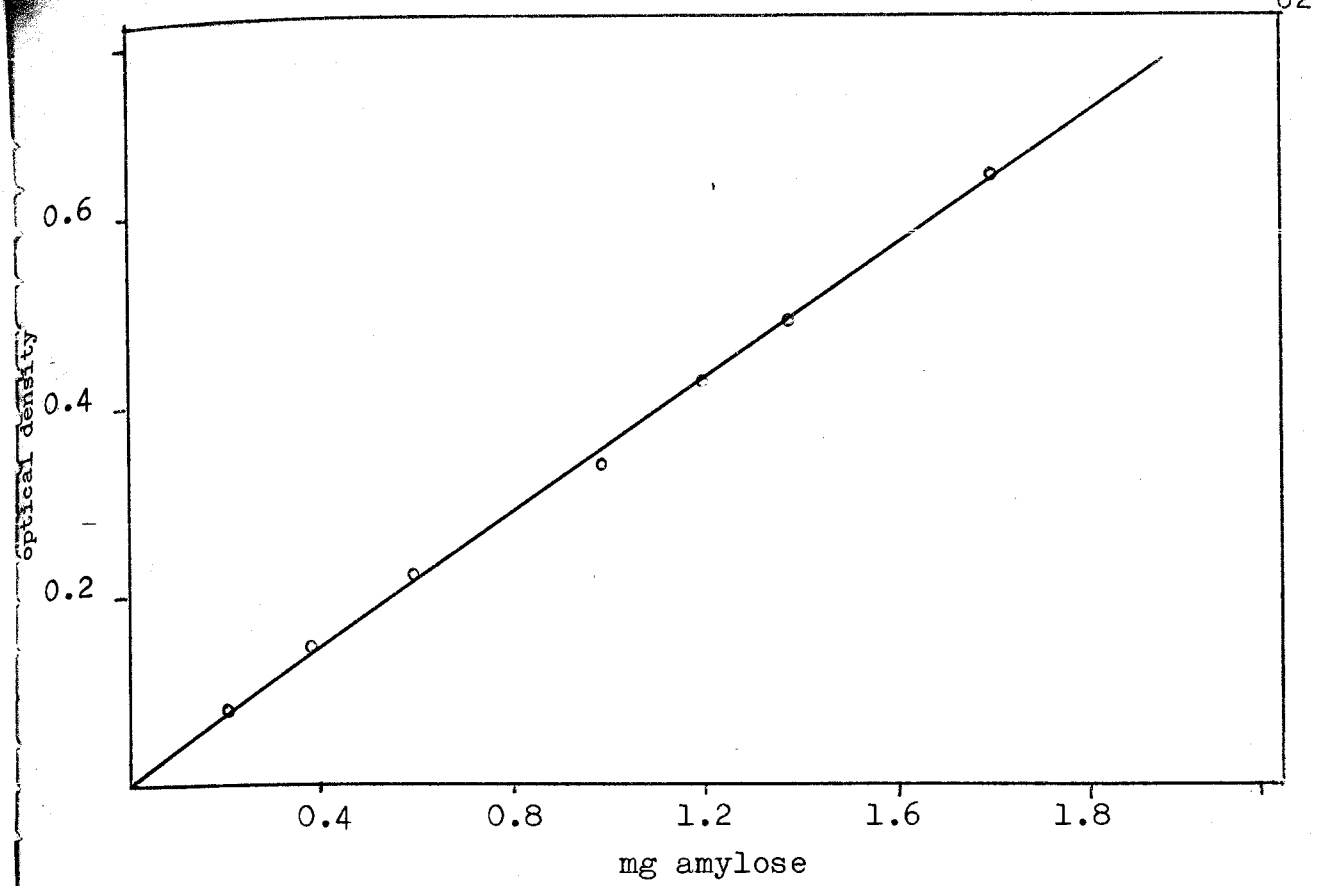


Figure III The standard curve of amylose determination

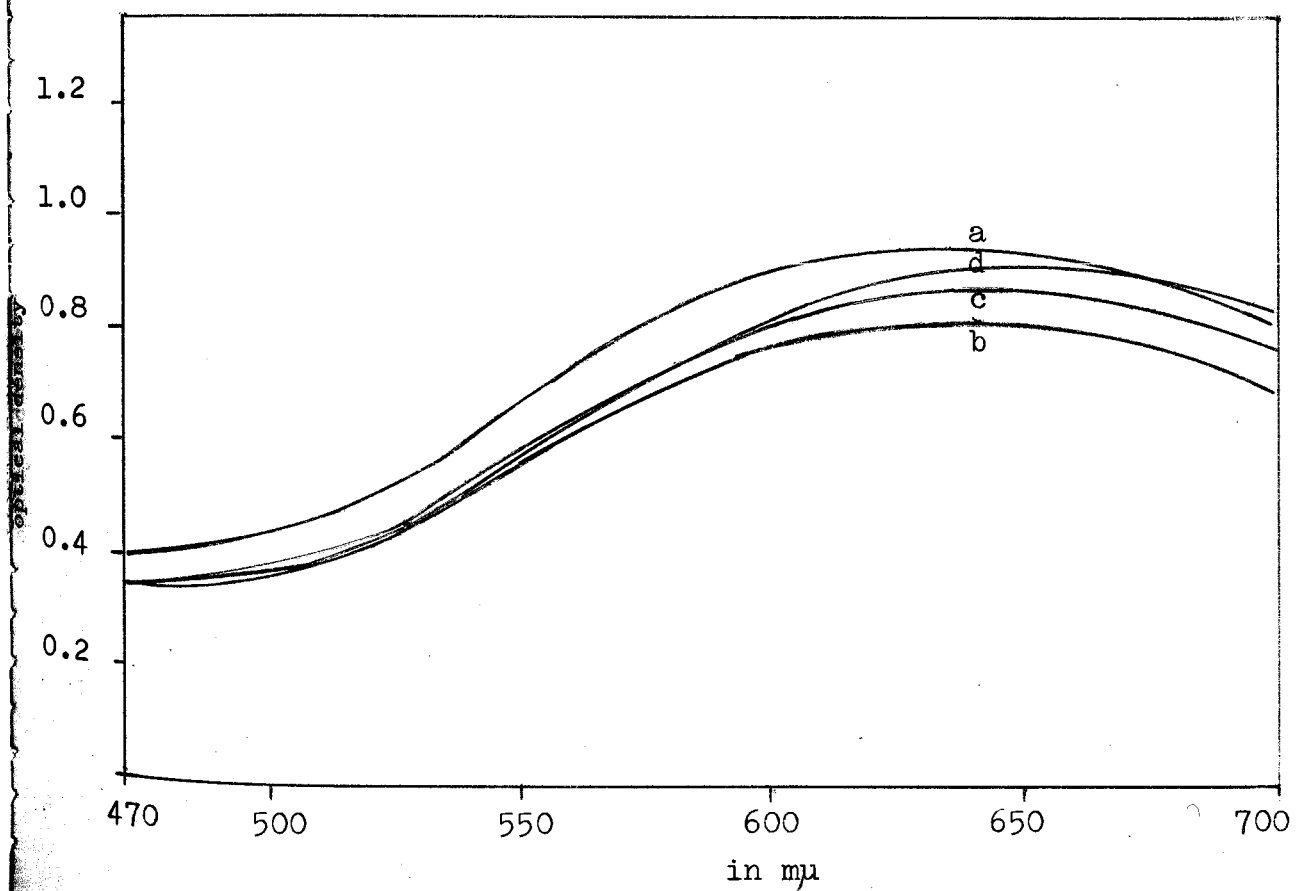


Figure IV The absorption of amylose-iodine complex at different wavelength a. durum b. rye c. triticale d. standard

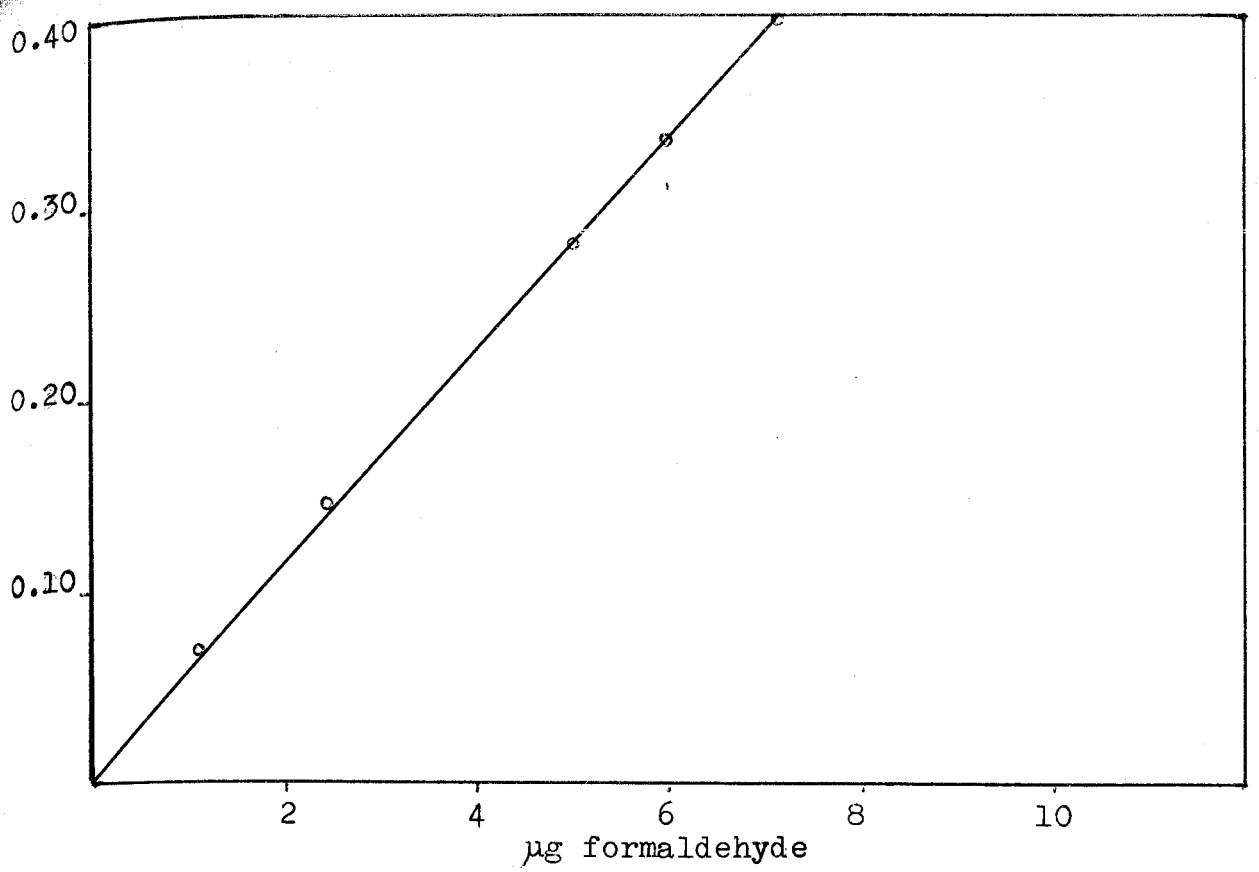


Figure V Standard curve for formaldehyde determination

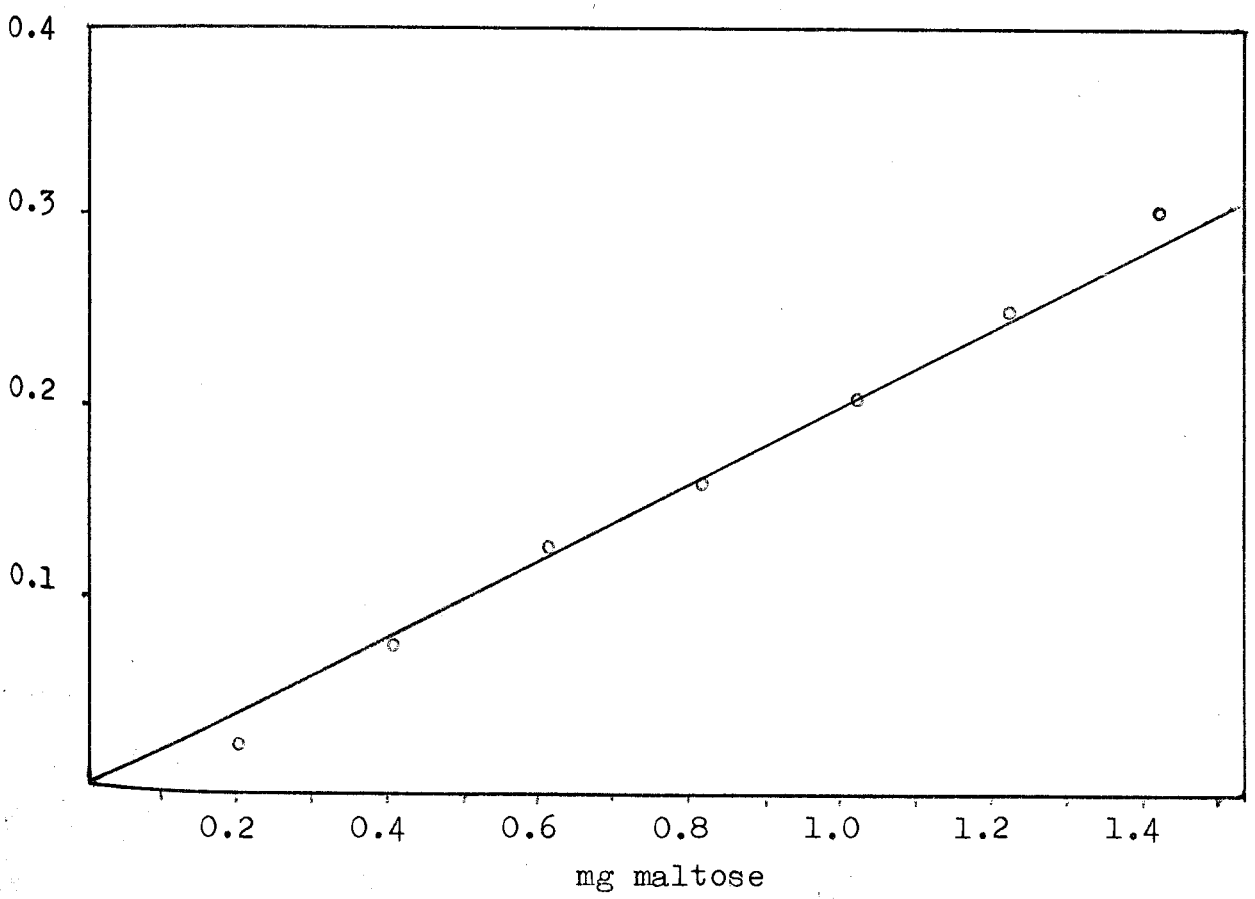


Figure VI Standard curve for maltose determination

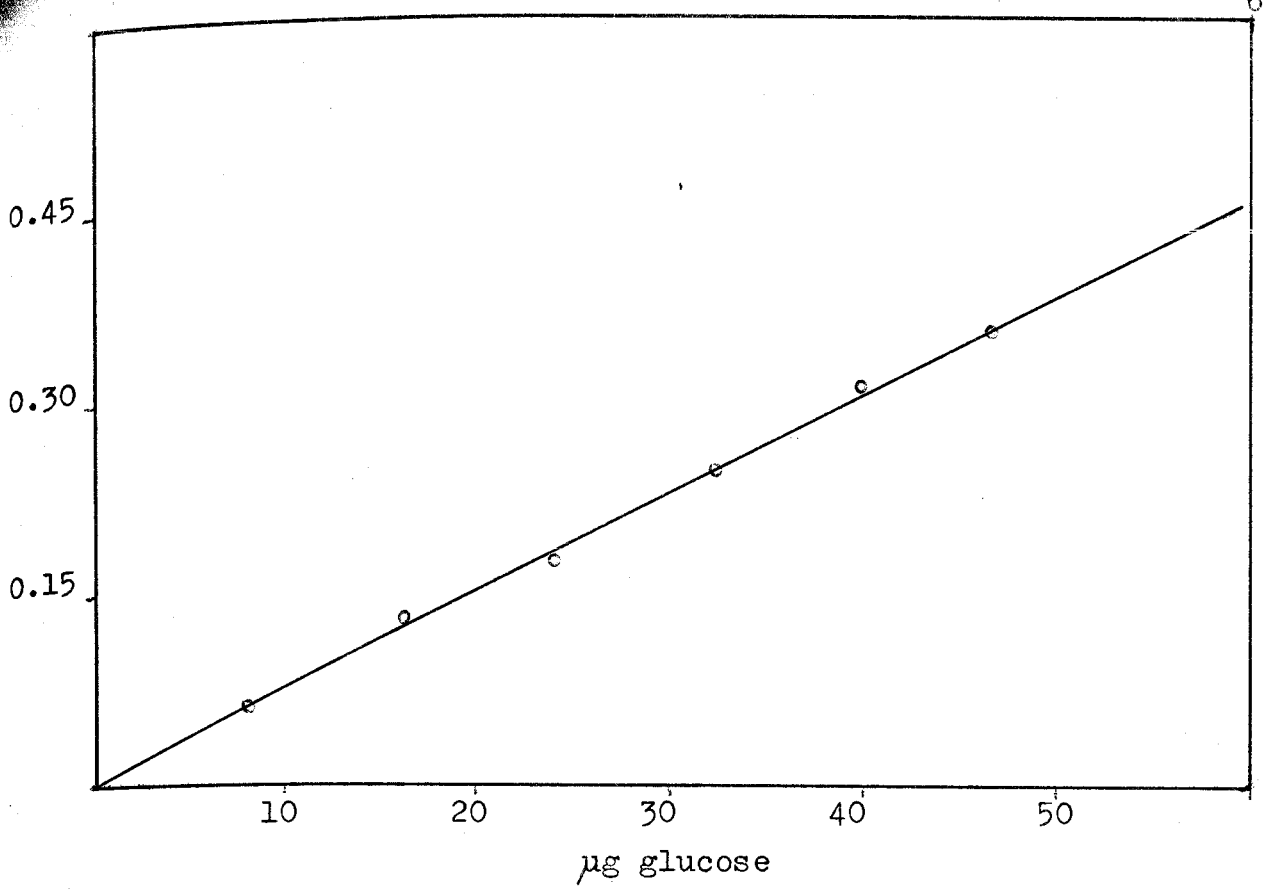


Figure VII Standard curve for glucose determination

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SIMON FRASER UNIVERSITY

DEPARTMENT OF CHEMISTRY

Final report of Master of Science study and research supervisory committee and final oral examination committee.

1) Name: HEW, Choy Leong.

2) Major field of study: Biochemistry

3) Minor field of study: Organic Chemistry

4) Title of M.Sc. thesis: Comparative Studies on an Alien Genome Combinant. Structural Investigation of Starch Components

5) Undergraduate degree: Bachelor of Science

6) Institution: Nanyang University, Singapore.

This is to certify that Mr. Choy Leong Hew, candidate for the M.Sc. degree, has satisfactorily fulfilled the required study and research requirements of the Department of Chemistry of Simon Fraser University. The above graduate student gave a satisfactory oral presentation of his research and rendered an adequate defence of the thesis. The submitted thesis is further certified as being in satisfactory form.

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