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Linear and cyclic amylooligosaccharides and their relationship to the amylolytic enzymes

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LINEAR AND CYCLIC AMYLOOLIGOSACCHARIDES AND THEIR
RELATIONSHIP TO THE AMYLOLYTIC ENZYMES

by

Melvin Lewis Levine

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Plant Chemistry

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TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
II. REVIEW OF THE LITERATURE	5
A. Starch Oligosaccharides	5
B. The Schardinger Dextrins	13
C. <u>Macerans</u> Amylase	17
D. Mode of Action of β -Amylase	21
E. Mode of Action of α -Amylases	24
III. EXPERIMENTAL	31
A. Preparation of Materials	31
Schardinger β -dextrin	31
Schardinger α -dextrin	32
<u>Macerans</u> amylase	33
β -Amylase	33
Salivary amylase	33
Amylodextrin	33
B. Precipitation of Schardinger β -Dextrin with Organic Precipitants	34
C. Rate Constant for the Acid Hydrolysis of β -Dextrin	34
From increase in reducing power	34
From recovery of unhydrolyzed β -dextrin	35
D. Rate Constant For the Acid Hydrolysis of Amyloheptaose	36

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	Page
E. Production of Amyloheptaose	36
F. Derivatives of Amyloheptaose	38
Potassium salt of amyloheptaonic acid	38
Phenylhydrazine derivative	38
G. Analytical Methods	39
Colorimetric estimation of carbohydrates	39
Chromatographic analysis of mixtures of glucose, maltose and amylotriose	39
Detection of long chain glucose polymers in enzymolysis solution	40
H. Action of <u>Macerans</u> Amylase	41
On amyloheptaose	41
On potassium salt of amyloheptaonic acid	42
On β -dextrin alone and in the presence of co-substrates	42
On α -dextrin alone and in the presence of co-substrates	43
I. Action of β -Amylase	44
On amyloheptaose	44
On the potassium salt of amyloheptaonic acid.	45
On amyloextrin	45
J. Action of Salivary Amylase	46
On amyloheptaose	46
On amyloextrin	46
IV. DISCUSSION OF RESULTS.	47
A. Preparation and Properties of Amyloheptaose	47

	Page
Purification and precipitation of β -dextrin . . .	47
Rate constant for the hydrolysis of β -dextrin . .	52
Characteristics of amyloheptaose	55
B. Action of <u>Macerans</u> Amylase	62
C. Action of β -Amylase	85
D. Action of Salivary Amylase	92
V. CONCLUSIONS	99
VI. LITERATURE CITED	102
VII. ACKNOWLEDGEMENTS	108
VIII. VITA	109
APPENDIX 1	110
APPENDIX 2	114

I. INTRODUCTION

Knowledge of the structure and behavior of starch has been considerably clarified in recent years as a result of the recognition of the heterogeneity of the native and commercial product. Satisfactory methods have recently been developed for separating the two major components, amylose, the linear fraction, and amylopectin, the branched fraction. As a result, most of the results and interpretations presented in the older literature have required re-evaluating and many of the experiments have been repeated on each of the starch fractions.

However, these advances have not completely solved all of the many problems involved. Starch, its fractions, and its partially degraded products are high polymers and as such have many chemical and physical properties peculiar to that class of compounds. The physical properties of starch and its fractions, such as solubility behavior, iodine color and the adsorption of many polar organic compounds, are entirely different from those exhibited by its structural unit, maltose. On the other hand, many of the chemical reactions useful in characterizing starch, such as oxidation, hydrolysis by acids or enzymes, methylation followed by hydrolysis, acetylation and others, may be as successfully carried out in an analogous manner with maltose.

Since there is a great gap in molecular weight between maltose and starch, to reason about the properties of one of the starch components from similar or dis-similar properties of maltose may be quite misleading, if not impossible, in many cases. If a series of intermediate molecular weight amylose or amylopectin homologues were available in a high state of purity, the errors of such interpolation would be reduced since a path over which to follow any property under investigation would be available. Thus it would be relatively simple to determine the degree of polymerization above which any particular property could no longer be deduced from the behavior of lower members of the series. And likewise, one could determine the lowest molecular weight at which properties peculiar to starch or one of its fractions are no longer evident.

The study of the intermediate molecular weight amylose or amylopectin homologues has received only a limited amount of attention and much of the information recorded is of doubtful value because of the lack of knowledge and confusion concerning the two components of starch. Furthermore, the most commonly used method of obtaining intermediate fractions has been by subjecting starch hydrolysates to a series of alcohol precipitations until successive fractions show very little or no change in specific rotation and/or reducing power. The purity of such fractions is questionable. Where

no initial separation has been made the presence of dextrans of different molecular weights from both the amylose and amylopectin fractions is probable. Furthermore, few of these intermediate compounds have been obtained in crystalline form. Although the lack of crystallinity does not necessarily indicate lack of purity, where the starting material is itself known to be heterogeneous, crystallinity would seem desirable.

It was the purpose of these investigations to prepare a low molecular weight member of the amylose series in a high state of purity and to study some of its reactions, particularly those resulting from the action of some of the amylolytic enzymes. The particular oligosaccharide chosen is composed of seven glucose units linked α -1,4- as in maltose. This heptasaccharide will hereinafter be termed amyloheptaose denoting that it is a member of the straight chained amylose series and contains seven glucose units.

Amyloheptaose was chosen for study because it seemed most feasible to obtain in a high state of purity from a crystalline compound of known constitution. The parent compound, Schardinger's β -dextrin, can be readily obtained in very pure form. The conversion of β -dextrin to amyloheptaose involves the hydrolysis of one glucosidic bond in the ring and can be accomplished by comparatively mild treatment.

In connection with the amylase studies it was found desirable to extend the investigations somewhat to include a

limited study of Nägeli's amyloextrin, the micro crystalline hydrolytic product obtained through the extended action of cold 15% sulfuric acid on starch. This material consists largely of relatively short amylose chains.

II. REVIEW OF THE LITERATURE

A. Starch Oligosaccharides

A starch oligosaccharide may be defined as a homogeneous glucose polymer of definite molecular dimensions which contains only those types of linkages normally found in starch. Such compounds may be derived either from amylose or amylopectin. They may contain either α -1,4- or α -1,6-glucosidic linkages or both. They include any single species of the disaccharides or higher glucose polymers.

The preparation of any starch oligosaccharide of proved purity is very rare, but many low molecular weight starch degradation products have frequently been regarded as being homogeneous. They have been obtained almost exclusively from the hydrolytic products resulting from the action of the various amylases on whole starch. The homogeneity of these preparations, which have been isolated primarily by alcohol fractionation procedures, is questionable.

Brown and Millar (1) were among the first to report a homogeneous oligosaccharide. They termed their material "maltodextrin". It was isolated by a series of alcohol precipitations from a malt diastase hydrolysis of starch at 65° C. until the specific rotation had fallen to 168-175°. Their maltodextrin had a specific rotation of 181-183° and a

reducing power equivalent to 42-43 per cent of that of maltose. It was noncrystallizable, was not fermented by brewer's yeast and did not yield a crystallizable phenylhydrazine derivative. It was completely hydrolyzed to maltose by malt extract at 50° C. Ling and Nanji (2) reported some studies on "alpha and beta maltodextrin". They reported that α -maltodextrin had the formula $C_{36}H_{62}O_{31}$, a specific rotation of 180° and a reducing power equivalent to 32.8 per cent that of maltose. For the β -maltodextrin they assigned the formula $C_{24}H_{42}O_{31}$, a specific rotation of 171.6° and reducing power of 43 per cent that of maltose. The specific rotations reported for these two compounds are much too high for straight chain polymers with the degree of polymerization indicated. The specific rotations calculated from the Freudenberg equation (3) are 176 and 164 respectively. For branched molecules they would be even lower. Since Ling and Nanji claimed that isomaltose (which is now considered to contain the α -1,6-glucosidic linkage) was obtained from enzymic hydrolysis products of their maltodextrin, the homogeneity of their preparations is doubtful.

The first separation of what were probably quite pure oligosaccharide derivatives was obtained by Freudenberg and Friedrich (4). After the methylation of acetylated starch they were able to obtain a methylated trisaccharide, $[\alpha]_{578}^{18} - 133^{\circ}$ in water, molecular weight 613(calc. 658), and a

tetrasaccharide, $[\alpha]_{578}^{18} + 140^{\circ}$ in water, molecular weight 812 (calc. 862), by distillation at reduced pressure. These preparations were probably quite pure but since no satisfactory method of demethylation is known their usefulness would be limited. Samec and Klemen (5) identified a trisaccharide in the hydrolysate of a pancreatic amylase digestion of erythroamylose.

Myrback (6) treated corn starch with takadiastase and brewer's yeast and after repeated fractionations with alcohol obtained a subfraction which gave constants indicative of a trisaccharide. He reports a specific rotation of 132° which is less than that of maltose and nowhere near the calculated value of 153° for a linear trisaccharide of the amylose series. Further studies of amylase digests by Myrback and Ahlberg (7) resulted in the establishment of the presence of a trisaccharide containing both the maltose and isomaltose linkages. This trisaccharide was not acted on by brewer's yeast in contrast to the normal trisaccharide which is readily fermented (8). From a malt amylase digest of corn starch, Ortenblad and Myrback (9) isolated fractions, most of which consisted of hexa-, tetra- and trisaccharides. Molecular weights calculated from both reducing values and diffusion measurements were in good agreement. In the case of the trisaccharide Myrback based the uniformity of the material on the fact that several consecutive fractions possessed practically

the same molecular weight of about 500. The fact that the reducing power was somewhat lower than that calculated for a trisaccharide was attributed to impurities from the enzyme solution. Myrbäck made a correction for about 5 per cent non-carbohydrate material to get the proper reducing power for a trisaccharide. It would seem from his method of preparation that the purity of his trisaccharide is questionable. Molecular weights determined from reducing power, rotation and diffusion methods indicate average values only and do not necessarily indicate purity.

The frequency of mention of tetrasaccharides from starch is limited and its isolation in anything like a pure state has only been reported once. Akiya (10) claimed to have isolated a tetrasaccharide from the digestion product of potato starch with the amylase from Bacillus mesentericus vulgatus. He refluxed the hydrolysate with 75 per cent alcohol and then evaporated the alcohol. The material would not reduce Fehling's solution. Acetylation yielded a product with only 12 hydroxyl groups covered instead of the 14 which should be available. By methylating with dimethyl sulfate and sodium hydroxide in acetone he obtained only 38 per cent methoxyl content instead of the calculated 45.5 per cent. His hydrolysis of the methylated product yielded trimethylmethylglucoside, dimethylmethylglucoside, and methylmethylglucoside. These results are probably due to incomplete methylation. According to the

specific rotation he obtained, $[\alpha]_D^{18^\circ} + 168^\circ$, the tetrasaccharide should be linear and contain only α -1,4-glucosidic linkages. Judging from his method of isolation and subsequent studies the identity and purity of his preparation does not seem proven. Myrbäck (8) states that tetrasaccharides are not fermented by yeast. His evidence is based on the attempted fermentation of dextrin fractions with average molecular weights of more than 660.

No reference has been found to indicate that anyone has as yet succeeded in isolating or identifying a pentasaccharide from starch hydrolysates. Waldschmidt-Leitz and Reichel (11) however, reported a "crystalline" amylohexaose by pancreatic amylose digestion of erythroamylose. Their technique involved removal of the dextrin by precipitation with ethyl alcohol and then adding methyl acetate to the concentrated aqueous solution followed by washing with ether. They reported $[\alpha]_D - 183^\circ$. The calculated specific rotation of amylohexaose is 176° . The melting point of their hexaose was $258-263^\circ$ with decomposition and it gave no color with iodine. The material was hydrolyzed completely to maltose by either α - or β -amylase. Their work has not been verified.

Other mention is made of the oligosaccharides in connection with the activation of phosphorylase with low molecular weight glucose polymers but usually no attempt has been made to isolate any particular member. For example, Weibull and

Tiselius (12) performed an adsorption analysis on a low molecular weight dextrin prepared by Ortenblad and Myrbäck (9) which contained chiefly maltose bonds. They obtained fractions which contained only maltose, only maltose and amylotriase, only maltose, amylotriase and amylotetraose, etc., up to and including amylohexaose. Thus they had convincing evidence of the presence of all the amylooligosaccharides up to and including amylohexaose but they did not attempt to obtain any of them pure. They showed that all of the oligosaccharides of the amylose series above maltose had approximately equal efficiency as potato phosphorylase activators.

Cori, Swanson and Cori (13) investigated the activation of potato phosphorylase with amylooligosaccharides obtained from acid hydrolysis of amylose and the Schardinger dextrans. They found that the peak activation is obtained when the average chain length is about five glucose units and intimated that the amylooligosaccharides with chain lengths of four and three glucose units are perhaps ineffective. They also found experimentally by synthesis of longer chains from short ones, that no iodine color is given by preparations having an average chain length of 5.8 glucose units or less. Those having chain lengths of 6.9 to 9.8 glucose units give a red coloration with iodine, 10 to 10.7 give red-purple, 11.3 to 11.8 give purple, and 12.6 to 15.4 give blue-purple iodine

colors. These calculations are based on the reducing power of Schardinger dextrin hydrolysates used as the starting material and the amount of glucose used in the enzymic synthesis. The conclusions in this case would not appear to be well founded.

Hidy and Day (14) studied the effect of acid hydrolysis of synthetic polysaccharides and amylose from corn starch on the yield of activators for potato phosphorylase. They found that dextrans which give no color with iodine, presumably seven to eight glucose units, are effective activators. Proehl and Day (15) obtained amyloheptaose and lower members of the amylose series in mixtures by acid hydrolysis of β -dextrin. They found that the activity of hydrolyzed β -dextrin was approximately 30 per cent greater than α -dextrin hydrolyzed to a comparable degree, as determined by ferricyanide reduction. They concluded that linear dextrans containing six or less glucose residues may not be as capable of activating potato phosphorylase as those containing seven or more glucose units.

Levine, Foster and Hixon (16) reported a number of intermediate molecular weight dextrans isolated by alcohol fractionation of corn syrup dextrans. These fractions varied in molecular weights from 310 to 1902 as calculated from iodine oxidation. The evidence collected seemed to indicate that these dextrans were all members of the amylose series. The individual fractions were all reported to be mixtures.

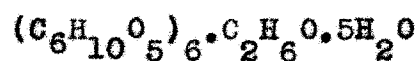
Brown and Morris (17) also studied the product obtained from the extended action of cold mineral acid on starch. They claimed the amyloextrin which they isolated was homogeneous and composed of 14 glucose residues. There is little doubt that their amyloextrin was also a mixture. The chain length average of various amyloextrins may vary considerably with variations in the type and pretreatment of the starch substrate.

Klason and Sjöberg (18) reported an "amyloseoctadextrin", an amyloextrin containing 16 glucose residues, prepared by extended hydrolysis of starch with cold 12 per cent hydrochloric acid. The material, obtained as spherocrystals, had a specific rotation of 187.5° and a reducing power toward Fehling's solution equivalent to 9.08 per cent that of maltose.

Köhler-Hollander (19) obtained an erythroextrin from a digestion of amyloamylose with salivary amylase to a red-violet color. He claimed the product occurred as hexagonal plates, gave a deep reddish violet iodine color, reduced Fehling's solution, had a specific rotation at 23° C. with sodium light of 170° and was tasteless. It was insoluble in alkali and cold water. It was easily hydrolyzed to glucose by pancreatic, malt or salivary amylase. He obtained three fractions from his crystalline material, one consisting primarily of molecules containing 12 glucose units, the second of 18 glucose units and the third of 24 glucose units.

B. The Schardinger Dextrins

These crystalline dextrins were first obtained by Villiers (20) while studying the fermentation of starch with a crude culture of Clostridium butyricum. After precipitating the starch degradation products with alcohol and filtering them off, he put the filtrate aside. He observed that after a few weeks standing a crystalline material had separated from the alcoholic mother liquors. He examined this material and recorded the following properties: The precipitate was white, crystalline and had a faint sweet taste. It was only slightly soluble in cold water but was 15 to 16 per cent soluble in water at 70° C. The specific rotation on the anhydrous basis was 159.42°. It was non-fermentable and non-reducing toward copper. Boiling with dilute mineral acids yielded only glucose although the material was more resistant to hydrolysis than starch or dextrins. It did not react with phenylhydrazine. He assigned the formula:



to the crystalline material obtained from alcoholic solution. It is apparent that he had obtained cycloamyloheptaose, or what is more generally known as Schardinger β -dextrin. He also observed that more than one crystalline dextrin was formed but he did not characterize the others.

Schardinger (21, 22, 23) was the first to make a detailed

study of these crystalline dextrans. He obtained them from Aerobacillus macerans digests of starch. He describes their crystalline iodine complexes, solubility, decomposition temperatures and other chemical and physical properties. He differentiated them into what he termed the α - and β -dextrans. He obtained a specific rotation of 144° for the α -dextrin and 158° for the β -dextrin, both on the anhydrous basis. Following Schardinger little advance was made in elucidating the structure of these dextrans until Freudenberg became interested in the problem.

Freudenberg and Jacobi (24) undertook a careful study of the non-reducing dextrans produced by the action of Aerobacillus macerans on starch paste. By fractionation of the reaction mixture, primarily through the acetylated products, they obtained five non-reducing dextrans differentiated by the specific rotations, solubility and crystalline appearance of the dextrans and their acetates. They record a specific rotation of 148° for the α -dextrin and 158° for the β -dextrin. The existence of the three new dextrans has not been corroborated by other workers.

Freudenberg, Blomquist, Ewald and Soff (25) studied the hydrolysis and acetolysis of the Schardinger dextrans in an effort to obtain more information as to the structure. They observed that there was initially an increase in rotation which

was followed by a steady decrease. The curve obtained by plotting the rotation against time finally coincides with that for starch when α -dextrin is hydrolyzed while when β -dextrin is hydrolyzed it does not. Both dextrans finally give the rotation of glucose. From their analysis of the hydrolysis curves and rotation values they suggest the possibility that both the α - and β -dextrin are ring structures.

Treatment of the Schardinger α -dextrin with sodium or potassium in liquid ammonia and methylation of the resulting alcoholate by Freudenberg and Rapp (26) produced the first completely methylated α -dextrin. Freudenberg and Meyer-Delius (27) prepared the first completely methylated β -dextrin in a similar manner. Hydrolysis of the methylated α - and β -dextrans yielded no other methyl sugar derivative than 2,3,6-trimethylglucose. From the optical behavior of the methyl derivatives and of the dextrans themselves as well as from methylation studies they concluded that the unions of the glucose units with each other are all of the maltose type, that is α -1,4-glycosidic linkages. From previous information they concluded that the α -dextrin is a five membered ring and the β -dextrin is a six membered ring. They explain the initial increase in rotation during hydrolysis of the two dextrans as follows: Since the cyclic dextrans and straight chain dextrans of the same degree of polymerization have different rotations, it follows that ring formation produces changes in rotation. If ring formation,

as with these dextrans, causes a decrease in rotation, hydrolysis, or ring opening, will result in an initial increase in rotation. As hydrolysis continues the opened chains will naturally be hydrolyzed so there will be a steady decrease in rotation after the initial increase.

Freudenberg and Meyer-Delius further confirmed that their dextrans were non-reducing toward Fehling's solution. They observed, however, that iodine, in the iodine oxidation methods for determining free aldehyde groups, does react with them. The amount of iodine used is not constant but seems to be dependent somewhat on the amount of iodine added. They found that β -dextrin finally consumes an amount equivalent to 20-30 per cent of that which would be consumed by an equal weight of free glucose. The α -dextrin shows the same effect but to a lesser degree consuming only up to 10 per cent of the amount of iodine which would be consumed by an equal weight of free glucose. No explanation of this phenomenon was offered.

Freudenberg, Schaaf, Dumpert and Floetz (28) built spacial models of the two dextrans. The model which they built for α -dextrin had the glucose units perpendicular to the plane of the ring. They found that no fewer than five glucose units are required to produce such a ring without tension. The ring of this dextrin has the five oxygen atoms

of the 1,4-bonds arranged in a regular pentagon, carbon atoms one and four lie in one plane and the other carbon atoms lie in a second plane. In β -dextrin, with six glucose units, the corner oxygen atoms are in two planes.

X-ray studies by Kratky and Schneidmesser (29) purportedly corroborated the observation of Fraudenberg that the α -dextrin was composed of a ring of five glucose residues. French (30) and French and Rundle (31) take issue with the interpretation of the data made by Kratky and Schneidmesser. From X-ray diffraction measurements of their own they show that the α -dextrin is a six membered ring and the β -dextrin a seven membered ring. They propose the names cyclohexaamylose for the α -dextrin and cycloheptaamylose for the β -dextrin. They report a specific rotation of 151.4° for α -dextrin and 161.9° for β -dextrin.

Tilden and Hudson (32) and McClenahan, Tilden and Hudson (33) studied the production of the Schardinger dextrans. They report specific rotations of 150.5° for the α -dextrin and 162.5° for the β -dextrin.

C. Macerans Amylase

Until the work of Tilden and Hudson in 1939 (32) the method of production of the Schardinger dextrans involved active growth of Aerobacillus macerans on the starch substrate.

These workers showed that sterile filtrates from Aerobacillus macerans cultures contain an enzyme which converts gelatinized starch to a mixture of the non-reducing α - and β -dextrins of Schardinger. They reported that there was no production of maltose, glucose or other reducing sugars. The enzyme was purified and concentrated by precipitation with acetone and re-solution in 0.1 the original volume of water. The activity of the enzyme was unimpaired by this purification. They further showed that the optimum temperature for enzyme action is 40° C. At 30° C. the rate of reaction is half as fast as at 40° C. and at 50° C. less than twice as fast indicating some inactivation at the higher temperature. The enzyme is active over a pH range of 5.6-6.4. They defined one unit of enzyme as that quantity in 0.5 ml. which will convert 30 mg. of starch in 15 ml. of solution in 30 minutes at 40° C. They standardized the iodine test for α -dextrin using 1 drop of 0.1 N iodine solution and 3 drops of enzymolysis mixture. They observed no significant change in reducing power during the course of the enzymolysis.

Further work by Tilden and Hudson (34) and McClenshan, Tilden and Hudson (33) was done on the preparation, purification and properties of the macerans enzyme. They found that the enzyme action on starch substrates proceeds with a rapid decrease in viscosity and a gradual decrease in specific rotation. They again found that there was no significant

increase in reducing power. They obtained a maximum yield of Schardinger dextrans of 55 per cent. They observed that the β -dextrin is stable toward the enzyme at 20° C. The α -dextrin, however, was converted at least in part, to a higher rotating material with a slight reducing power. The specific rotation rose from 150.5° to 169° during the course of the reaction. They could detect no β -dextrin in this solution. They also found that the only iodine test that they could get on the converted material was identical with that for the α -dextrin. They purified the enzyme by dialysis as well as adsorption on aluminum hydroxide to obtain one preparation concentrated 140 times over the culture filtrate.

Myrbäck and Gjorling (35) studied the action of Aerobacillus macerans culture filtrates, apparently rather weak in enzyme, on potato starch. They obtained large amounts of α -dextrin, detected by the iodine test, up to 216 hours after which the α -dextrin slowly decreased until there was none at all after 864 hours. They first obtained maltose after 48 hours and after 864 hours the amount of maltose had increased to 61 per cent. It, too, gradually decreased in amount after this time. After 2060 hours they first obtained glucose. They also plotted the reducing power against the viscosity during the course of such a reaction and obtained a curve identical with that obtained when α -amylase from malt was used to convert starch, although with α -maltamylase the elapsed time for

the reaction was very much less. They explained these transformations by assuming that the macerans amylase or mixture of amylases, attacks starch first of all through normal hydrolysis as shown by α -maltamylase and then forms the Schardinger dextrans from the relatively small fragments. They further postulated that the macerans enzyme system then hydrolyses the Schardinger dextrans with the production of maltose and glucose. Just what happened between 864 hours, when maltose started to disappear, and 2060 hours, when glucose first appeared, was not clearly explained. However, if no fermentation was taking place, it would seem that glucose should be detectable immediately with the decrease in the amount of maltose. In any case, after such an excessively long time the possibility of bacterial and mold growth or traces of hydrolytic amylases, such as α -malt amylase, affecting the course of the reaction cannot be discounted. Nevertheless, the above workers claim that the enzyme, or enzyme system, obtained from Aerobacillus macerans does have hydrolytic action resulting in the production of end groups and a consequent increase in reducing power. This is opposed to the results previously reported by Hudson and co-workers using much more concentrated enzyme systems and a shorter time.

Kneen and Beckord (36) included the macerans amylase in a study of the conversion of starch to fermentable sugars by bacterial amylases. Their technique involved simultaneous

fermentation and enzymolysis, resulting in the removal of the fermentable sugars as fast as they were formed. The amount of fermentable sugars was expressed as carbon dioxide, measured manometrically. They found that fermentable sugars were produced by macerans enzyme although much more slowly than by most of the other bacterial amylases they studied. They conclude that the initial reaction was a conversion of starch to the non-reducing, non-fermentable Schardinger dextrans followed by a progressive production of fermentable sugars.

D. Mode of Action of β -Amylase

The present concept of the method of attack of starch by β -amylase is based in large part on elucidation of the structure of starch achieved through chemical methods. The concept of the starch molecule as consisting of α -glucopyranose units in linear chains of 25-30 glucose units was developed from methylation studies by Haworth and co-workers (37, 38). Subsequent work (39, 40, 41) has demonstrated that the Haworth concept was too simple and therefore it has been modified. The demonstration that starch consists of two components, one linear and one branched, was achieved by Hess and Krajc (42), and Meyer (43). With the help of the basic concept of starch structure as elucidated by Haworth, Hanes (44, 45) developed

the general picture of β -amylase action. His concept may be summarized briefly as follows. β -Amylase hydrolyzes only α -1,4 glucosidic linkages beginning with the non-reducing end of a chain and clipping off maltose units until the chain is completely hydrolyzed or until an anomalous linkage stops the course of the hydrolysis.

Myrbäck and Mycander (46) showed that starch and dextrans that have been oxidized are saccharified in the same manner with β -amylase as their unoxidized counterparts. This had previously been demonstrated by Brown and Millar (1) who hydrolyzed an oxidized low molecular weight straight chained dextrin to obtain 40 per cent maltose. Their enzyme, however, was undoubtedly impure since they obtained it from malt diastase. Baker (47) showed that only maltose was formed by the action of barley diastase on barley starch and on soluble starch. Ohlsson (48, 49) concluded that the saccharogenic amylase of malt detaches maltose molecules successively from the starch chains. His conclusions were based mainly on the permanence of the iodine color at various stages of the saccharification. Freeman and Hopkins (50) found that β -malt amylase and the amylase of ungerminated barley produced maltose in the early stages of starch hydrolysis in an amount virtually equivalent to the reducing power. The material remaining resembled amylose or starch. They thus

confirmed the view of previous workers that β -amylase removes successive molecules of maltose from starch. Meyer, Bernfeld and Press (51) obtained further confirmation of the hypothesis by observing the action of β -amylase on oxidized corn starch. They obtained virtually the same amount of maltose from the oxidized corn starch as from the unoxidized corn starch tested at the same time. They also showed that the rate at which degradation takes place proceeds regularly as long as the concentration of the non-reducing terminal groups remains constant in relation to the concentration of enzyme.

Meyer, Wetheim and Bernfeld (52) examined the residual dextrin from the degradation of corn starch by β -amylase. By means of methylation procedures they found that all of the terminal groups present in the original corn starch were also present in the limit dextrin. They concluded that since all of the original end groups were still present the action of the β -amylase was stopped at the points of branching in the original starch molecule. Meyer (53, 54) found that amylose, which is presumably not branched, is hydrolyzed almost quantitatively to maltose. Haworth, Hirst, Kitchen and Peat (55) showed that branchings do not disappear during the hydrolysis by β -amylase. Similar observations have been made by Haworth, Heath and Peat (56), Hanes (57, 58) and Samec and Waldschmidt-Leitz (59).

Kuhn (60) demonstrated that the maltose formed by the action of β -amylase is in the beta form regardless of the polysaccharide substrate. Freeman and Hopkins (61) confirmed this observation and concluded that this is due solely to a characteristic of the enzyme and not the presence of the beta linkage in the original substrate.

E. Mode of Action of α -Amylases

The initial action of α -amylases, or the group of amylases which are of the α -type, is characterized by liquefaction of the starch substrate resulting in a rapid decrease in viscosity apparently without the production of appreciable amounts of glucose or maltose (48, 49, 50). Ohlsson (48, 49) showed that the osmotic pressure increased to almost 10 times its original value during the early stages of malt α -amylase action. Myrbäck (62) showed that the molecular weights of the products formed during the early phases of α -amylase activity were in the neighborhood of 1000 to 3000. Freeman and Hopkins (50) showed by alcohol fractionation methods that these products were dextrans rather than mixtures of maltose and essentially unchanged starch.

Following the dextrinization of the starch substrate and coincident with it some extent, saccharification occurs. The principal product of the saccharification is maltose.

However, there are abundant reports that glucose, too, is formed. Somogyi (63, 64) followed the reaction of animal amylases and determined, quantitatively, the appearance of dextrans, maltose and glucose by differential fermentation methods. He found that glucose was formed in the early stages of the reaction, apparently simultaneously with maltose. The production of glucose by various dextrinogenic amylases has also been reported by Ling and Davis (65), Baker (47) and Hanes (66) and others. Hopkins (67) from a review of the previous work maintains that the glucose is derived from the amylopectin or branched portion of the starch after it has been partially degraded. He takes issue with Myrbäck (68, 69) who concluded that glucose was formed immediately during the hydrolysis and directly from starch. Myrbäck also obtained glucose from the action of malt α -amylase on amylose, which had been shown to be hydrolyzed to completion by β -amylase and therefore presumably contains no branch linkages. Hopkins (67) ascribes the production of glucose in Myrbäck's experiments as due to branching in the amylose molecule itself, contamination of the amylose with amylopectin, or the complete breakdown of chain molecules containing odd numbers of glucose residues, thus leaving only the glucose residue. Haworth, Kitchen and Peat (70) also attribute the production of glucose almost entirely to the breakdown of amylopectin.

The limit of hydrolysis of the α -amylases varies somewhat depending on the source. In general, however, it is in the neighborhood of 80-90 per cent of the reducing value of maltose (67). It has been observed, however, that even when appreciable quantities of limit dextrans are left unattacked in the original enzymolysis, if they are freed from the sugars and redispersed the enzyme which produced them can hydrolyze them further (60, 71, 72, 73).

There is no reliable evidence of an α -amylase being separated into components exhibiting different amylolytic characteristics. Purification of pancreatic amylase by alcohol precipitation, dialysis, etc., gave rise to no great variation in the ratio of liquifying to saccharifying power in experiments of Sherman and Schlesinger (74, 75). Much more recent work also supports the view that the starch liquifying and saccharifying activities are due to one and the same enzyme (76, 77).

Various explanations have been offered for the two stages of the reaction of starch with α -amylase but the most credible and refined hypotheses are those presented by Myrbäck and by Meyer and co-workers. Myrbäck (78) explains the hydrolysis by α -amylase, first through a phase in which rapid splitting of the long chains to hexaoses and heptaoses occurs, followed by a second slower phase of hydrolysis of these short chains. Thus about 16 per cent of the linkages are

split more easily than the remaining 84 per cent. Only small quantities of fermentable sugars are formed during the first part of the hydrolysis.

Meyer and Bernfeld (79) studied the hydrolysis of amylose by α -malt amylase. They analyzed the products of hydrolysis by the differential fermentation methods introduced by Somogyi. They found that the velocity constant constantly decreased up to a degradation of 90 per cent calculated as theoretical maltose. The final velocity constant was only a very small fraction of the initial constant. They substantiated Myrback's["] belief that the glucosidic bonds of the lower degradation products such as the hexaoses are degraded more slowly than those of the high molecular weight portions. They explain this partially on the basis of a higher dissociation coefficient for the enzyme-substrate combination for the small molecules than for the big ones. At the start of the reaction maltose cannot be found. At 87 per cent degradation, they found 30 per cent trisaccharide as well as higher oligosaccharides in addition to glucose and maltose. The final slow reaction they attributed to a cleavage of the trisaccharide and other oligosaccharides. They further calculated that, assuming that all bonds in the chain reacted in the same manner and the rate of degradation of maltose and maltotriose was zero, one would expect 50 per cent trisaccharide, 33.3 per cent

maltose and 16.7 per cent glucose in the final mixture. If the triose was then hydrolyzed slowly to glucose and maltose the final yield would be 66.7 per cent maltose and 33.3 per cent glucose. Experimental values indicated that more maltose and less glucose was actually formed. In other words, all bonds are not equally reactive. The less reactive bonds appeared to be the terminal linkages. If it is assumed that the terminal linkages are not hydrolyzed at all, they calculated that the final products from the degradation of a long chain would be 60 per cent maltose and 40 per cent trisaccharide. Experimental results seemed to indicate that this was substantially correct except that amylotriose was slowly hydrolyzed to glucose and maltose but never to the extent of 100 per cent.

Myrbäck and Thorsell (69) investigated the action of dextrinogenic amylases on amylose and obtained somewhat different results than Meyer. They found that when approximately 22 per cent of the glucosidic bonds in amylose are hydrolyzed the rate of splitting suddenly drops to less than 2 per cent of the initial value. Thus there appeared to be a sharply defined phase of dextrinization and saccharification in amylose. A break in the curve at 22 per cent hydrolysis of glucosidic bonds indicates that the hydrolytic products formed at that time have an average chain length of about five glucose units. After removing the fermentable sugars,

glucose, maltose and trisaccharide, they found that at least 65 per cent of all the dextrans formed had a chain length of six to seven glucose units and 32 per cent had a chain length of four to five units. They obtained these results by alcohol fractionation methods. Assuming that all of the four to five glucose unit chains arose by hydrolysis from the six and seven glucose unit chains, they conclude that the dextrans first formed consisted almost entirely of hexaoses and heptaoses. Myrback (78) first explained the preponderance of the production of these five, six and seven membered glucose chains on the structure of starch as a helix similar to that proposed by Hanes (45). He pointed out, however, that the enzyme does not attack the chains from the non-reducing end but can start hydrolysis within the molecule. He also rejected Meyer's hypothesis of random attack of interior linkages at that time.

However, more recently he has put forth a modified version of Meyer's hypothesis (80, 81). In this concept terminal linkages are hydrolyzed very slowly or not at all. The next linkage is hydrolyzed somewhat more rapidly and the next still faster until after about five or six linkages in there is no particular difference in the speed of hydrolysis. On this basis, the fact that very small amounts of fermentable sugars are produced in the early stages of the reaction and

almost 100 per cent hexa- and hepta- saccharides are produced was explainable. Myrback, in contrast to Meyer, regards maltose and maltotriose as non-hydrolyzable, glucose apparently being formed from longer fragments.

Both Myrback and Meyer explain the slowness of hydrolysis of the relatively short fragments not only on the inherent difference in ease of hydrolysis of the bonds near the ends of the chain but also on the basis of the dissociation of the enzyme, substrate complex. The longer fragments give a smaller co-efficient of dissociation than the shorter ones and therefore when the two are in competition the enzyme tends to combine with the longer chains at the expense of the shorter ones. For a mathematical analysis of the α -amylase hydrolysis of amylohexaose see Myrback and Leissner (82).

III. EXPERIMENTAL

A. Preparation of Materials

Schardinger β -dextrin

The crude β -dextrin was obtained from a macerans enzyme digest of potato starch according to the method of Tilden and Hudson (32). A weighed quantity of this crude material was dissolved in four times its weight of boiling distilled water. To the hot solution a small quantity of activated charcoal and Celite 535 was added and the mixture boiled for a few minutes. It was then filtered through a #0 pad in a Hormann pressure filter. The filtrate was allowed to cool to room temperature with occasional stirring, to assure moderately small crystals, and then refrigerated over night. The crystal crop was recovered by filtration with the aid of suction through a medium porosity sintered glass filtering funnel. The crystals were allowed to air dry for 12 to 24 hours. The above procedure was repeated several times increasing the density of the Hormann pad each time up to a #6 pad. On successive crystal crops the moisture, specific conductance and specific rotation were determined. When little or no change in specific conductance and specific rotation occurred on successive recrystallizations the product was considered to be pure, provided it

gave optically clear, water white solutions. The purest reference sample obtained was recrystallized eight times in this manner. Moisture was determined by drying a weighed sample under 2 mm. mercury pressure at 78° C. for 18 hours over P_2O_5 . Specific rotations for wave lengths of 5893 \AA° , sodium D line, and 5460 \AA° , mercury green line, were determined on a 1.2 per cent aqueous solution in a 4 dm. brass polarimeter tube. The amount of ash by ignition with ammonium sulfate was determined on a 0.3 g. sample. Ash as sodium chloride calculated from specific conductance was obtained by measuring the conductance of a 0.3 per cent solution of the dextrin in freshly boiled distilled water.

Schardinger α -dextrin

The crude α -dextrin was acetylated by the acetic anhydride, sodium acetate method (83). The crude acetate was dissolved in ten times its weight of toluene and the solution filtered hot. The α -dextrin acetate crystals which separated on cooling, were recovered and again crystallized from toluene. This product was dissolved in methanol filtered hot through a bacterial filter pad with the aid of pressure and allowed to crystallize. The purified material was then de-acetylated in boiling methanol to which a small amount of KOH had been added. The precipitate was filtered off and recrystallized from water by adding methanol to the hot solution and allowing it to cool.

Macerans amylase

Macerans amylase was prepared according to the procedure of Hudson and co-workers (32, 33). This involved growing Aerobacillus macerans in a large volume of potato starch medium. The culture was then passed through the supercentrifuge to remove the major quantity of suspended matter. The centrifugate was then filtered through a Hormann pad, precipitated with acetone, centrifuged to remove the supernatant liquors and finally dissolved in about one tenth the original volume. The material was preserved under toluene in the refrigerator.

β -Amylase

The β -amylase used was prepared from soy beans by Newton (84). The amylase was used as obtained, no further purification being attempted.

Salivary amylase

Ten to 15 ml. of saliva was filtered through an alundum filtering crucible to remove suspended matter. This procedure resulted in an optically clear preparation much more fluid than the native, semi-viscous saliva. No further purification was attempted.

Amylodextrin

Nägeli's amylo-dextrin (85) was prepared by the extended

action of cold 15 per cent sulfuric acid on commercial potato starch. The resulting material was recrystallized several times from a hot aqueous solution to which an equal volume of methanol had been added.

B. Precipitation of Schardinger β -Dextrin with Organic Precipitants

A 0.3 to 0.5 per cent solution of β -dextrin was prepared by dissolving the appropriate amount in distilled water and diluting to one liter. 35 to 50 ml. of this solution were transferred to a 125 ml. iodine flask and about 5 ml. of the organic precipitant added. The flask was then stoppered and the resulting mixture shaken mechanically for 20 hours. At the end of this time the precipitate was filtered off and the rotation determined on the filtrate. The concentration of β -dextrin in the filtrate was then calculated using the specific rotation of pure β -dextrin as 163.0° . The effect of refrigerating the sample for 24 hours at 3° C. after shaking was also determined. The results are presented in Table 1.

C. Rate Constant for the Acid Hydrolysis of β -Dextrin

From increase in reducing power

One part of dry β -dextrin was mixed with four parts of

exactly 0.001 N HCl in a test tube. The test tube was sealed with a rubber stopper and placed in a boiling water bath. After five minutes the tube was vented, to assure that excess pressure would not blow out the stoppers, and swirled to complete solution of the dextrin. After a definite period of time the tube was removed, the contents transferred to a 300 ml. Erlenmeyer flask and the reducing power determined by the alkaline ferricyanide method of Farley and Hixon (86). The results of the experiment are given in Table 2.

From recovery of unhydrolyzed β -dextrin

A typical determination was conducted as follows. Exactly 10 grams of dry β -dextrin were placed in a large glass stoppered test tube and 40 ml. of 0.001 N HCl were added from a burette. The tube was heated carefully with a bunsen burner to put the dextrin in solution, and sealed by wiring a glass stopper in place. It was then hung over gently boiling water in a large flask fitted with a reflux condenser. The temperature inside the flask was 99.2°C. The tube was removed several times during the first few hours of hydrolysis and inverted to insure complete solution and mixing. After 19 days, 21 hours and 45 minutes, the tube was removed from the water bath, opened and the acid neutralized with the calculated amount of sodium hydroxide. After cooling to room temperature the solution was

refrigerated for 4 hours. The precipitate was collected quantitatively in a weighed alundum filtering crucible. The filtrate was transferred to an iodine flask, toluene added, the stoppered flask shaken for 24 hours and then refrigerated for 24 hours. Again the precipitate was collected in a weighed alundum filtering crucible. The two precipitates were dried in the vacuum oven at 70° C. to constant weight.

D. Rate Constant For the Acid Hydrolysis of Amyloheptaose

This constant was determined by calculation from the rate constant for the hydrolysis of β -dextrin and the reducing power of the hydrolysate according to the method of French (30).

E. Production of Amyloheptaose

100 grams of dry β -dextrin were dissolved in exactly 400 ml. of 0.001 N HCl in a liter glass stoppered flask. A reflux condenser was fitted to the flask and the flask about half submerged in a boiling water bath. The flask was occasionally swirled to complete solution and assure mixing. After 7 hours in the boiling water bath the flask was removed, the acid immediately neutralized with the calculated amount of lithium carbonate and the unhydrolyzed β -dextrin allowed to crystallize by cooling to room temperature and then refrigerating for 24

hours. The precipitated dextrin was removed by filtration through a medium porosity sintered glass filtering funnel. The remaining β -dextrin was removed by saturating the filtrate with p-xylene, shaking the resulting solution for 24 hours followed by refrigerating for 24 hours and filtering. This filtrate was then concentrated to one fifth its original volume in vacuo. Six additional hydrolyses were carried to this stage in 100 grams β -dextrin lots and the concentrated filtrates combined. The resulting solution was again covered with p-xylene, shaken for 24 hours, the precipitate filtered off, and the resulting filtrate concentrated to a thin syrup in vacuo. The amyloheptaose was precipitated from the resulting syrup by adding 4 volumes of absolute ethanol. The syrupy precipitate was redissolved in a small amount of water and reprecipitated by adding 4 volumes of absolute ethanol. The amyloheptaose was reprecipitated in this manner five successive times. The final precipitate was dehydrated with absolute ethanol to which a small amount of butanol had been added. The purpose of the butanol was to prevent condensation of water and the consequent turning of the amyloheptaose to a syrup as the ethanol evaporated during the filtration. After drying in vacuo the specific rotation, alkaline ferricyanide reducing power, and molecular weight by the Kline and Acree iodine oxidation (87) were determined.

F. Derivatives of Amyloheptaose

Potassium salt of amyloheptaonic acid

Approximately 0.5 gram of amyloheptaose were dissolved in 5 ml. of distilled water and 25 per cent excess of approximately 0.1 N iodine and 0.1 N KOH were added in two steps, the iodine being added first each time. The reaction mixture was allowed to stand for about 10 minutes and then precipitated by adding 200 ml. of absolute ethanol. This precipitate was dried, ground in an agate mortar to a fine powder and then extracted for 48 hours with acetone to remove any potassium iodide remaining from the reaction. The amount of potassium present in the compound was determined by ashing with ammonium sulfate.

Phenylhydrazine derivative

The phenylhydrazine derivative was prepared by a modification of the method of Bergmann and Machemer (88) described in a previous publication from this laboratory (16). One gram of amyloheptaose was dissolved in 5 ml. of liquid phenylhydrazine by heating in an oil bath at 130° C. After two hours the reaction mixture was poured into 50 ml. of benzene. The precipitate was filtered off, thoroughly washed with ethyl ether, and dried in vacuo at 50° C. for 18 hours. The dried material

was ground to a fine powder in an agate mortar and then extracted for 48 hours in a soxhlet with ether. Nitrogen was determined by the micro Dumas method described by Johns (89).

G. Analytical Methods

Colorimetric estimation of carbohydrates

A detailed description of the method used is given in Appendix 1. Four ml. of diphenylamine reagent was added to 1 ml. of 95 per cent ethanolic sugar solution and the mixture heated in a boiling water bath for 30 minutes. The solution was then cooled under running water and made up to 25 ml. with methanol. The per cent transmission of light with a wave length of 640 millimicrons was determined with a Coleman Spectrophotometer. The concentration of carbohydrate was then determined by reading from the curve in Appendix 1.

Chromatographic analysis of mixtures of glucose, maltose and amylotriase

Detailed procedure for the chromatographic analysis of sugar mixtures is given in Appendix 2. Only a brief outline of the method will be given here. The column consisted of 12 grams of adsorbent made by mixing 8 grams of Florex XXX and 4 grams of Celite 535 and packing into the column in a thick slurry of ethanol. The adsorption column was 16 mm. in diameter

and 19 cm. in length. Five ml. of 95 per cent ethanolic solution containing up to 0.1 g. of the carbohydrate mixture to be analyzed was introduced into the column. The column was developed by forcing the requisite amount of 90 per cent ethanol through the column under 65 to 70 cm. of mercury pressure. Samples of the eluate were collected periodically as the developer came through the column. The amount of sugar in each portion of eluate collected was estimated by the diphenylamine color test. The identity of the sugar was established by the volume of developer which carried it through the column. The concentration in the original solution was determined from the greatest concentration present in the eluate.

Detection of long chain glucose polymers in enzymolysis solution

Solutions containing approximately 1 per cent α -dextrin and 0.17 per cent glucose were subjected to the action of macerans enzyme for varying periods of time. Control solutions of distilled water, one per cent α -dextrin, one per cent amyloheptaose and 0.1 per cent amylo-dextrin were prepared.

5 ml. of each solution was mixed in a test tube with 10 ml. of 0.01 N I_2 solution, 0.5 M in KI. Three or four milliliters of the resulting solution were introduced into a colorimeter cuvette. Then a square, optical glass insert was dropped into the cell in order to decrease the thickness of the liquid

film sufficiently to allow the adsorption spectra to be determined on the dark iodine solution. The absorption spectra of the iodine solutions were determined using 0.0001 N iodine solution as the blank. The per cent transmission was plotted against the wave length (Fig. 2).

H. Action of Macerans Amylase

On amyloheptaose

An approximately two per cent solution of amyloheptaose in phosphate buffer, 0.1 N in NaH_2PO_4 and 0.01 N in Na_2HPO_4 , adjusted to pH 5.8-6.0, was mixed with 1 ml. of filtered macerans amylase containing 10 to 20 units per ml. of enzyme. The reaction was allowed to proceed in different determinations for different lengths of time at room temperature. In order to prevent the growth of microorganisms, particularly mold, the reaction mixture was covered with toluene or p-xylene. The initial course of the reaction was followed polarimetrically in 2 dm. tubes. The end products of the reaction, or the products present at any time during the reaction, were determined by one or more of the following methods: chromatographic-colorimetric method for glucose, maltose and amylotriose; osazone formation for glucose and maltose; iodine test for the qualitative presence of α -dextrin; the potentiometric iodine

titration of Dube (90) for the quantitative estimation of α -dextrin. The change in rotation and the change in reducing power were also determined. Results are given in Table 4.

On potassium salt of amyloheptaonic acid

The procedure followed was similar to that used above. The only determinations made on the enzymolysis mixture were qualitative determinations of the production of α -dextrin and the change in reducing power toward alkaline ferricyanide.

On β -dextrin alone and in the presence of co-substrates

A typical experiment to determine the action of the macerans amylase on β -dextrin alone and in the presence of co-substrates is given below. The following stock solutions were first prepared:

0.8300 grams dry β -dextrin in 50 ml. phosphate buffer
1.1510 grams maltose hydrate in 50 ml. phosphate buffer
1.000 gram sucrose in 50 ml. phosphate buffer

All of the solutions were heated to boiling before making up to the volume given above. The following amounts of the above solutions were pipetted into 25 ml. volumetric flasks in order to prepare solutions for the testing of the action of macerans amylase:

- Solution 1. 15 ml. β -dextrin solution
5 ml. maltose solution
2. 15 ml. β -dextrin solution
5 ml. sucrose solution
3. 15 ml. β -dextrin solution
4. 5 ml. maltose solution
5. 5 ml. sucrose solution

One ml. of filtered macerans amylase containing 20 units per ml. enzyme activity was added to each of the above solutions. The solutions were then made up to volume with phosphate buffer. The final solutions were 0.1 M in NaH_2PO_4 and 0.01 M in Na_2HPO_4 and were adjusted to approximately pH 6. The course of the reactions were followed polarimetrically in 2 dm. polarimeter tubes. The production of α -dextrin was followed by the iodine test. The reducing power was determined on the solutions containing maltose after 18 hours. In addition to maltose and sucrose, the effect of adding glucose and gluconic acid was also observed. The results are given in Table 5.

On α -dextrin alone and in the presence of co-substrates

The method used in carrying out these tests was essentially the same as given for similar tests with β -dextrin. The following modifications were made. Detection of the β -dextrin by the iodine test in the presence of much α -dextrin is very difficult. Therefore the solutions remaining in the volumetric flasks,

after filling the polarimeter tubes, were covered with p-xylene. In this case the p-xylene acts as a precipitant for the β -dextrin while it leaves the α -dextrin in solution. Thus the presence of small amounts of β -dextrin can be detected. The action of macerans amylase on the α -dextrin alone and on the α -dextrin in the presence of several co-substrates was studied.

I. Action of β -Amylase

On amyloheptaose

Enough amyloheptaose was weighed out to make up 25 ml. of an approximately 1 per cent solution. The weighed material was dissolved in a small amount of water and then enough NaH_2PO_4 and Na_2HPO_4 added to make the final solution 0.1 M in the former and 0.01 M in the latter. One ml. of β -amylase containing 0.01 g. of enzyme per 10 ml. of solution was then added to the amyloheptaose solution. The saccharogenic power of the amylase was 1072 (84). The enzymolysis solution was made up to volume with distilled water. The reaction was followed polarimetrically in a 2 dm. tube at room temperature. Reducing power by the alkaline ferricyanide method was determined on the final enzymolysis mixture. The end products were also subjected to a yeast fermentation.

Ultimate analysis of the end products of the action of β -amylase on amyloheptaose was obtained by means of the chromatographic-colorimetric technique.

On the potassium salt of amyloheptaonic acid

0.1820 grams of the potassium salt of amyloheptaonic acid were dissolved in 25 ml. of distilled water, the pH being adjusted to 6 with acetic acid. 2 ml. of this solution were taken for a reducing power determination by the alkaline ferricyanide method. 0.005 grams of soy bean β -amylase (saccharifying power 1072) were dissolved in 10 ml. of the remaining solution. After 18 hours the reducing power was again determined by the alkaline ferricyanide method.

On amyloextrin

A 1 per cent solution of amyloextrin, specific rotation 188.9° and reducing power equivalent to 3.41 ml. of 1.0 N potassium ferricyanide solution, was treated as given above for the potassium salt of amyloheptaonic acid. The change in rotation during the course of the reaction was observed and the reducing power at the end of the reaction was determined.

J. Action of Salivary Amylase

On amyloheptaose

An approximately 1 per cent solution of amyloheptaose buffered with phosphate to pH 6 as given previously was treated with 1 ml. of filtered saliva. The course of the reaction was followed polarimetrically in a 2 dm. tube. When the rotation had become constant, usually the reaction was allowed to run over night or about 18 hours, the resulting end products were determined by one or more of the following methods: specific rotation, reducing power, or the chromatographic-colorimetric method. Blanks on maltose were run to determine whether or not the saliva were free of maltase.

On amyloextrin

The procedure followed was essentially the same as that followed with the amyloheptaose.

IV. DISCUSSION OF RESULTS

A. Preparation and Properties of Amyloheptaose

Purification and precipitation of β -dextrin

The conversion of Schardinger's β -dextrin, or cycloamyloheptaose, into amyloheptaose is shown in the following reaction:



(1) Cycloamyloheptaose + water \longrightarrow amyloheptaose

The amyloheptaose formed will then be hydrolysed until, under appropriate conditions, it has all been converted to glucose. Therefore it is necessary to know the rate constant for the formation of amyloheptaose from cycloamyloheptaose as well as that for the destruction of amyloheptaose. Two methods presented themselves for determining the rate constant for the hydrolysis of cycloamyloheptaose. The first method is based on the determination of the amount of straight chain material formed after a definite time of hydrolysis. Since amyloheptaose is subject to further hydrolysis the reaction must be allowed to proceed only a relatively short period of time. The amount of amyloheptaose formed under such conditions is small. In order to facilitate subsequent removal of the acid catalyst to concentrations of acid, 0.001 NHCl, were used.

The most plausible means of measuring the amyloheptaose formed is by one of the reducing methods. The alkaline ferricyanide method of Farley and Hixon (86) was chosen since it is rapid, relatively simple to carry out and relatively sensitive. The second method of determining the rate constant for the hydrolysis of β -dextrin is based on the quantitative recovery of the β -dextrin free from hydrolysis products. The most satisfactory method to achieve this appeared to be by precipitating the last traces of the dextrin with an organic precipitant. Trichloroethylene and toluene have been used extensively as precipitants but no quantitative data are recorded concerning their efficiency. Therefore, it was deemed desirable to investigate these and other possible precipitants quantitatively so that the most satisfactory one could be employed. The method for determining the efficiency involved measuring residual, unprecipitated dextrin by means of rotation.

It is apparent from the foregoing discussion that both methods for determining the rate constant for the hydrolysis of β -dextrin require a pure starting material. The presence of reducing substances would introduce an error in the first method and the presence of optically active substances other than the β -dextrin itself would introduce an error in the second method. Furthermore the presence of starchy impurities in the β -dextrin would lead to impurities of unpredictable nature in the amyloheptaose prepared from it.

The purification of β -dextrin is relatively simple since the material has a very favorable ratio of solubility in cold and hot water and it crystallizes very nicely from solution. Thus the removal of all starchy impurities can be readily accomplished. A reference sample of extreme purity was prepared by eight successive recrystallizations from water. The specific rotations for the reference sample are:

$$\begin{array}{ll} [\alpha]_D & 163.5 \pm 0.5^\circ \\ [\alpha]_{Hg} & 189.5^\circ \pm 0.4^\circ \end{array}$$

The specific rotations for sodium light given by French (31) and Hudson (33, 34) are slightly lower than this value being 161.9° and 162.5° respectively.

The purity of the reference sample is further confirmed by the fact that it gives optically clear, water white aqueous solutions, contains no ash by ignition with ammonium sulfate and less than 0.005% ash calculated as sodium chloride from specific conductance measurements (91).

The removal of β -dextrin from solution can be accomplished with a large number of organic precipitants although their efficiency varies widely. The ideal precipitant would be specific for the β -dextrin, completely miscible with water, easily removed from the precipitated complex, non-acid, rapid, readily available and inexpensive. Furthermore, it would precipitate the β -dextrin in a form which was easy to filter. The most important of these considerations are specificity and completeness of precipitation. Most of the other desirable

characteristics have proven to be unattainable although if the immiscible precipitants are allowed to act slowly a precipitate with satisfactory filtering qualities usually results.

An examination of Table 1 shows that the most effective precipitant found was p-xylene. There is one disadvantage connected with its use, however. It tends to form a fairly stable emulsion which makes filtration very difficult, much more so than in the case of toluene, for instance.

The use of p-xylene and toluene for removing the β -dextrin from solution has received application in these researches in a number of cases. Aside from its value in providing a method for obtaining the rate constant for the hydrolysis of the β -dextrin, it has been used to obtain complete removal of that dextrin from the amyloheptaose preparation. In the studies of the action of enzymes, particularly the macerans enzyme, it has been used for a dual purpose. In many cases the primary purpose has been to prevent the development of microorganisms. A secondary purpose has been to cause the precipitation of β -dextrin in the presence of α -dextrin. Most of the organic precipitants examined are relatively ineffective as precipitants for the α -dextrin. p-Xylene and toluene, for instance, can be added to a 1% solution of α -dextrin and will not cause even a trace of precipitation at room temperature. Furthermore these precipitants will not

Table 1

Concentration of β -Dextrin Remaining in Solution
after Precipitation with Organic Precipitants

Precipitant	β -dextrin remaining	β -dextrin remaining
	after 20 hours shaking at room temperature	after 20 hours shaking at room temperature and 24 hours refrigerating at 3° C.
	grams/100 ml.	grams/100 ml.
1. p-xylene	-----	0.0040
2. m-xylene	-----	0.0077
3. bromobenzene	0.021	0.0077
4. α -bromonaphthalene	0.021	0.0077
5. xylene	0.021	0.0092
6. trichloroethylene	0.025	0.0077
7. nitrobenzene	0.029	-----
8. naphthalene	0.031	-----
9. nujol	0.034	-----
10. o-xylene	-----	0.0100
11. toluene	0.049	0.018
12. methyl iodide	0.055	-----
13. phenylsalicylate	0.060	-----
14. cyclohexane	0.061	0.021
15. chloroform	0.072	0.021
16. benzene	0.074	0.015
17. carbon tetrachloride	0.095	-----
18. skelly B	0.112	-----
19. ethylene dibromide	-----	0.040
20. ethylene chloride	0.117	-----
21. skelly A	0.133	-----
22. p-dichlorobenzene	0.176	-----
23. azobenzene	0.201	-----
24. ethyl ether	no ppt.	0.057
25. nitromethane	"	no ppt.
26. dioxane	"	"
27. ethyl acetate	"	"
28. acetone	"	"
29. ethyl alcohol	"	"
30. anthracene	"	"
31. 2,4 dinitrophenylhydrazine	"	"
32. ethyl benzoate	"	"
33. triphenyl chloromethane	"	"
34. palmitic acid	"	"
35. n-butyl alcohol	"	"
36. benzyl alcohol	"	"

precipitate low molecular weight dextrans to any appreciable extent.

Rate constant for the hydrolysis of β -dextrin

Schardinger's β -dextrin is completely non-reducing as determined by copper and alkaline ferrocyanide methods. Therefore all reducing power present after a hydrolysis is due to the production of reducing groups by opening up the ring and further hydrolyzing the open chain amyloheptaose. Two distinct reactions should follow each other successively. The first one results in the opening up of the β -dextrin ring to give a straight chain reducing dextrin of seven glucose residues, amyloheptaose. The second reaction is the hydrolysis of amyloheptaose to give additional reducing groups in the form of shorter chains and finally glucose.

- (1) Cycloamyloheptaose + water \rightarrow amyloheptaose
- (2) Amyloheptaose + water \rightarrow amylohexaose + glucose
or amylopentaose + maltose
or amylotetraose + amylotriose
or finally 7 glucose.

In determining the rate constant for the destruction of β -dextrin to give amyloheptaose we are primarily interested in the first reaction. If the hydrolysis is allowed to proceed for comparatively short periods of time we can obtain a rate constant from the increase in reducing power. If we assume that all the reducing power arises from the amyloheptaose. From this reducing power we may calculate the weight of amyloheptaose

present since 1 gram of amyloheptaose will reduce 10.48 ml. of 1.0 N $K_4Fe(CN)_6$ (16). The amount of β -dextrin destroyed will be equal to:

$$\frac{\text{Molecular Weight of } \beta\text{-dextrin} \times \text{wt of amyloheptaose}}{\text{Molecular Weight of Amyloheptaose}}$$

The rate constant can be calculated from the first order reaction equation:

$$C = C_0 e^{-kt}$$

Table 2 gives the rate constants calculated in this manner for 3 different times of hydrolysis. The values obtained are admittedly somewhat high. However, they should give good approximations of the correct value. It is seen that the velocity constants obtained are in fair agreement with each other although they tend to rise with time. It is also apparent the true velocity constant for the destruction of the β -dextrin must be somewhat smaller than the smallest value obtained.

Table 2

Rate Constant for the Hydrolysis of β -Dextrin
Determined From Reducing Power

Wt. of dry β -Dextrin (grams)	Time of Hydrolysis (min.)	Ml. of $Ce(SO_4)_2$ (0.1018N)	Amyloheptaose Produced (grams)	β -Dextrin Remaining (grams)	k ($\times 10^4$)
0.4395	180	0.45	0.0044	0.4352	0.469
0.4371	360	0.85	0.0082	0.4287	0.648
0.4350	540	1.55	0.0150	0.4203	0.687

The second method for determining the velocity constant for the destruction of β -dextrin is based on the quantitative recovery of the unchanged dextrin from the hydrolysis mixture. p-Xylene, the most effective of the precipitants investigated, was used to precipitate the last traces of β -dextrin. Table 3 shows the results of three independent determinations of the velocity constant.

Table 3

Rate Constant for the Hydrolysis of β -Dextrin
Calculated from the Recovery of Unhydrolyzed
 β -Dextrin

	Determination Number		
	1	2	3
Original weight β -dextrin, g.	8.7036	10.0000	10.0000
Weight unhydrolyzed β -dextrin, g.	7.3828	2.6548	6.8473
Time of Hydrolysis, min.	4,280	28,560	7,130
Velocity Constant, $k \times 10^4$	0.40	0.46	0.50

The hydrolyses for determinations 1 and 2 were made in a ground glass stoppered test tube while that for determination 3 was carried out in a ground glass jointed erlenmeyer flask under reflux. The values are in fair agreement for the three determinations. Determination 2, which was hydrolyzed for the longest period of time, is felt to be most accurate since slight errors in recovery or weighing will give relatively small percentage errors in the calculation of

the hydrolysis constant.

Examination of the data verifies the fact that the hydrolysis is a first order reaction since k , the rate constant, calculated from the first order reaction equation is found to be constant for times varying from 4,280 minutes to 28,560 minutes. Furthermore, the values obtained for the velocity constant as given in Table 3 check very well with those obtained by the measurement of the increase in reducing power as given in Table 2. The values obtained by the recovery of the unchanged β -dextrin are, in general, somewhat lower than those obtained by measuring the reducing power. This is what was expected since some of the amyloheptaose formed was bound to be hydrolyzed, thus producing more reducing groups and consequently an increase in the apparent amount of amyloheptaose.

In conclusion, the best value obtained for the hydrolysis constant of the β -dextrin, namely 0.46×10^{-4} , while perhaps not absolutely accurate, is felt to be very satisfactory for the purpose in hand, i.e., as a working basis for the preparation of amyloheptaose.

Characteristics of amyloheptaose

The first property of amyloheptaose which must be deduced in order to prepare the material is the rate constant for its hydrolysis. The determination of this rate constant

offered considerably more difficulty than that for the β -dextrin since no amyloheptaose was available. The method used for determining this constant, however, has been outlined by French (31), as follows, with slight modification. The rate of formation of amyloheptaose, KC_0e^{-kt} , is equal to the rate of destruction of the β -dextrin. The rate of destruction of the amyloheptaose, k_2A , is dependent on the amount present, A , as well as the velocity constant for its hydrolysis, k_2 . The rate of increase of amyloheptaose, then, will be equal to its rate of formation minus its rate of destruction or:

$$\frac{dA}{dt} = k_1C_0e^{-k_1t} - k_2A$$

French calculated the amount of amyloheptaose that would be formed and its purity for various times of hydrolysis of β -dextrin assuming that the velocity constant for the hydrolysis of the glucosidic bond in amyloheptaose is the same as that for the glucosidic bond in β -dextrin. From measurements of the reducing power of β -dextrin hydrolysates, however, it now seems that the glucosidic bond in amyloheptaose is hydrolyzed about five times as fast as in β -dextrin.

Using the corrected value of k_2 , it was calculated that if the hydrolysis of β -dextrin was carried to the extent of 4 per cent, the amyloheptaose in the hydrolysate should be over 90 per cent pure. In preparing the amyloheptaose the β -dextrin was hydrolyzed only to the extent of 2 per cent. The amyloheptaose

recovered from such a hydrolysate should be 95 per cent pure, or better.

A batch of amyloheptaose amounting to 13.3 grams, dry weight, was prepared by hydrolyzing the β -dextrin to the extent of 2 per cent. This preparation had the following constants:

$[\alpha]_D$	175.5°
Reducing power, ml. 1.0 N $K_4Fe(CN)_6$	10.15
Molecular weight calculated from reducing power	1190
Molecular weight calculated from iodine oxidation	1206
Theoretical molecular weight	1152

The Freudenberg equation (3) relates the molecular rotation of an intermediate member of an homologous carbohydrate series with that of the appropriate disaccharide and the infinitely large member of the series as follows:

$$[\alpha]_m = [\alpha]_2 - (n-2) \frac{[\alpha]_\infty}{\infty}$$

where M is the molecular rotation and n is the number of glucose residues in the particular carbohydrate under consideration. This equation can be modified for the particular case in hand, i.e., for the estimation of the molecular and specific rotation of the straight chain amyloheptaose as follows:

$$[\alpha]_{\text{amyloheptaose}} = [\alpha]_{\text{maltose}} - \frac{(7-2) [\alpha]_\infty}{\infty}$$

If the limiting value for the specific rotation of starch is assumed to be 200°, the molecular rotation of amyloheptaose is

equal to 206,500 and the specific rotation calculated is 179.6° . The actual value obtained is about 2.2 per cent too low. This observation, coupled with the fact that the molecular weights determined by both iodine titration and alkaline ferricyanide reduction were too high, would seem to indicate that there was some inert material present. This material apparently affected neither the rotation nor the reducing power. The exact nature of this substance was not determined but it would appear to be moisture. The presence of inorganic impurities is doubtful because of the purity of the starting material and the fact that the hydrochloric acid used for the hydrolysis was neutralized with lithium carbonate specifically because of the solubility of lithium chloride in alcohol. After the several reprecipitations from alcohol solution it is doubtful if any lithium chloride would still remain. The presence of alcohol, too, is doubtful. If any were present it would tend to make the molecular weight as determined by the iodine oxidation method too low rather than too high, as was found, because iodine will oxidize alcohol in alkaline solution.

Although the purity of the amyloheptaose based on the calculated extent of hydrolysis, its rotation and reducing power would seem to be satisfactory, additional evidence in regard to homogeneity was obtained by the preparation of two derivatives and a chromatographic analysis.

The potassium salt of amyloheptaonic acid was first prepared. It was obtained as a pure white, amorphous powder by alcohol precipitation from the reaction mixture. No attempt was made to crystallize it. Analysis of this salt by ashing with ammonium sulfate gave 3.34 per cent ash calculated as potassium. Assuming that all of the ash is potassium sulfate and that the potassium was all present originally as the salt of the amyloheptaonic acid, the molecular weight of this derivative was 1167. The theoretical molecular weight calculated for this salt is 1208. The agreement between these two values is relatively good. The preparation reduced alkaline ferricyanide solution to some extent. This was unexpected since potassium gluconate is completely non-reducing toward this reagent. The cause of the reducing power was not determined.

The phenylhydrazine derivative of amyloheptaose was next prepared. The reaction between phenylhydrazine and the short chain dextrans has been shown to be useful in estimating molecular weight in the case of the corn syrup dextrans up to a chain length of about 6 to 7 glucose units (16). Above this point it seemed that in addition to the normal formation of the hydrazone or hydrazide there was an appreciable adsorption of the phenylhydrazine. The adsorbed reagent was not removed by exhaustively extracting with ether. In the case of the amyloheptaose, it was not known whether there would be appreciable adsorption or not, but since it had been previously

demonstrated that the molecular weights determined from nitrogen analysis are in fair agreement with those determined by oxidation methods and rotation, it appeared that the method might offer additional proof of the molecular size. Since the method of preparation involves heating at a high temperature and subsequent handling in non-aqueous media it also seemed likely that any volatile solvent, presumably water or alcohol, held by the amyloheptaose would be removed. Furthermore, since neither water nor alcohol were used at any stage in the preparation of the phenylhydrazine derivative it seemed that there was no possibility of fractionation to occur.

The phenylhydrazine derivative of amyloheptaose was a light yellow, amorphous powder. The amount of nitrogen found was 2.23 per cent. Assuming the compound to be a phenylhydrazone the calculated molecular weight was 1252. The theoretical molecular weight for the phenylhydrazone of amyloheptaose is 1240. The agreement between these two values is seen to be very good.

Further evidence of purity of the preparation was obtained by chromatography. 20 milligrams of amyloheptaose was chromatographed by the method given in the Appendix 2 on a 19 cm. long column of Florex XXX and Celite 535 in 3 to 1 ratio. The eluate was tested periodically for carbohydrate. All these tests were negative. A total of 85 ml. of 90 per

cent ethanol was used for developing. The column was then pushed from the tube and cut in 2 cm. sections. Each section was eluted with 90 per cent alcohol and 1 ml. of the eluate was tested for carbohydrate by means of the diphenylamine reaction. The top 2 cm. portion of the column had no carbohydrate. The next two 2 cm. portions of the column did have carbohydrate. All the rest of the column had no trace of carbohydrate. The fact that no carbohydrate appeared in the eluate while the column was being developed is very strong evidence that no glucose, maltose or amylotriose is present. These sugars can be detected in as small a concentration as 0.01 mg. per ml.

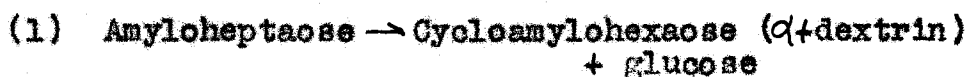
Since amylotriose is washed completely through the column under the conditions employed, it would be reasonable to expect that amylotetraose would be moved well down the column and that amylopentaose also would be moved far enough down the column to give a separation from amylohexaose and amyloheptaose. Therefore, if the tetra-, penta- and hexa-saccharides were present in appreciable amounts, one would expect the carbohydrate to be spread over a large part of the column. The fact that all the carbohydrate in the column was concentrated in a small, relatively well defined area, therefore, testifies to its essential homogeneity.

B. Action of Macerans Amylase

The formation of the Schardinger dextrans by the action of macerans amylase on starch apparently does not proceed through a true hydrolysis since no more water is present in the resulting products than was originally present in the starch. However, very little is actually known about the course of the reaction since very little critical study has been made of the enzyme system involved. To date most of the macerans enzyme preparation have been merely filtrates from Aerobacillus macerans cultures, sometimes concentrated by precipitation with acetone and re-solution. Obviously, such crude preparations may contain several enzyme systems elaborated by the growing organism with the enzyme or enzymes responsible for the formation of the Schardinger dextrin being the principal ones. Conclusions arrived at from the results of a reaction occurring in a few hours may be attributed to the predominant enzyme. If the enzymolysis is allowed to proceed for several weeks, however, the significance of the results would be uncertain. In the studies being presented here it is recognized that the enzyme preparation may be contaminated with traces of other amylolytic enzymes besides the enzyme responsible for the formation of the non-reducing, cyclic dextrans. Therefore, any reaction which was not observable within a few hours has been regarded as

questionable and of unknown significance.

The action of macerans amylase on amyloheptaose should provide some information as to the size of starch fragment which the enzyme is capable of attacking, and, if such an attack occurs, the course of the reaction. Providing the reaction does take place, one would expect it to be as follows:



As a result of such a reaction there should be several easily observable changes. The apparent reducing power toward alkaline ferricyanide should decrease since the reducing power of glucose is only 50% greater than that of maltose while the reducing powers of the higher dextrans are approximately proportional to their degree of polymerization based on the reducing power of maltose. Furthermore, one would observe a drop in specific rotation of the enzymolysis mixture. The specific rotation of the amyloheptaose is 179.6° ; that of α -dextrin is 151.4° (31) and that of glucose is 53° . An equimolar mixture of glucose and α -dextrin would have an apparent specific rotation of 139.8° . If the above reaction went to completion one would get a drop of almost 40° .

Other methods of determining whether or not macerans enzyme will attack amyloheptaose include the detection of the end products, α -dextrin by the iodine test and glucose by preparation of the osazone or by chromatography.

The results of several experiments on the action of macerans amylase on amyloheptaose at room temperature are presented in Table 4. Figure 1 shows the actual change in specific rotation which occurs in the early stages of experiment #5, Table 4.

There was an actually observed drop of 15.5° in the specific rotation of the reaction mixture instead of the calculated drop of 40° . This indicated that the reaction did not go to completion according to equation (1). The reducing power for experiments #3 and #5 was determined on the final enzymolysis mixture and found to be lower than the reducing power of amyloheptaose. This observation as well as the leveling off of the specific rotation at about 160° indicates that no hydrolytic action is involved. The presence of α -dextrin in these enzymolyses could be detected after 10 minutes by the iodine test.

An attempt was made to demonstrate the presence of glucose in the end products of experiment #5 by preparing the osazone from the filtrate of the enzymolysis mixture from which the Schardinger dextrans had been precipitated by trichloroethylene. An osazone insoluble in hot water and with the correct melting point for glucosazone was obtained. Mixed melting points with known samples of glucosazone and maltosazone also indicated that the product was glucosazone. However, its crystalline appearance was atypical.

An interesting and disturbing result was obtained when the trichloroethylene precipitate was recovered. 60 per cent of the weight of the original amyloheptaose was accounted for in this precipitate. As a check on its identity the specific rotation was determined in the usual way. The specific rotation was 162° , in good agreement with the specific rotation of β -dextrin. An iodine test on the material yielded only the β -dextrin iodine complex, not the slightest trace of the α -dextrin being detectable. However, when an iodine test was made on the filtrate from the trichloroethylene precipitate a weak test for α -dextrin was obtained. This indicated that most of the α -dextrin originally formed had been converted into the β -dextrin.

Similar observations have been previously noted by McClennahan, Tilden and Hudson (33) and by French (31). Hudson and co-workers found that in the early stages of the enzymolysis the amount of α -dextrin increases rapidly and then, during the later stages, it decreases while the amount of β -dextrin increases slowly at first and continues a steady increase throughout the enzymolysis. French also noted that in the presence of toluene the end products from macerans enzyme digestions were practically all β -dextrin.

The amounts of the reducing carbohydrates reported in experiments 1, 2, 3 and 4 were obtained chromatographically. The amount of α -dextrin was determined by the iodine titration method developed by Dube (90). Experiments 2 and 3 were

Table 4

Reaction Products from the Action of Macerans
Amylase on Amyloheptaose

Experiment No.	1	2	3	4	5
Time	30 min.	2 hours	10 days	3 mos.	2 days
Wt. amyloheptaose, g.	0.5000	0.5008	0.5008		0.5091
Vol. enzyme, ml.	1	1	1		1
Activity of enzyme (Units/ml.)	20	20	20		10
Final Volume, ml.	25	25	25		25
Conc. glucose, mg./g. sample	8.05	22.10	49.0	20.6	
Conc. maltose, mg./g. sample	5.85	21.80	31.20	22.2	
Conc. Amylotriose, mg./g. sample	5.29	20.30	22.80	19.70	
Conc. alpha dextrin, mg./g. sample	195	243	68	172	
Wt./5 ml. chromato- graphed, g.	4800	0.1431	0.2003		
Ml. 1.0 N $K_4Fe(CN)_6$, reduced, amylo- heptaose	10.15	10.15	10.15		10.15
Ml. 1.0 N $K_4Fe(CN)_6$, reduced, enzy- molysis mixture			9.13		9.73
% end groups accounted for as:					
glucose	5.58	15.40	34.3		
maltose	2.14	7.63	10.9		
amylotriose	1.23	4.70	5.3		
Total	8.95	27.73	50.5		

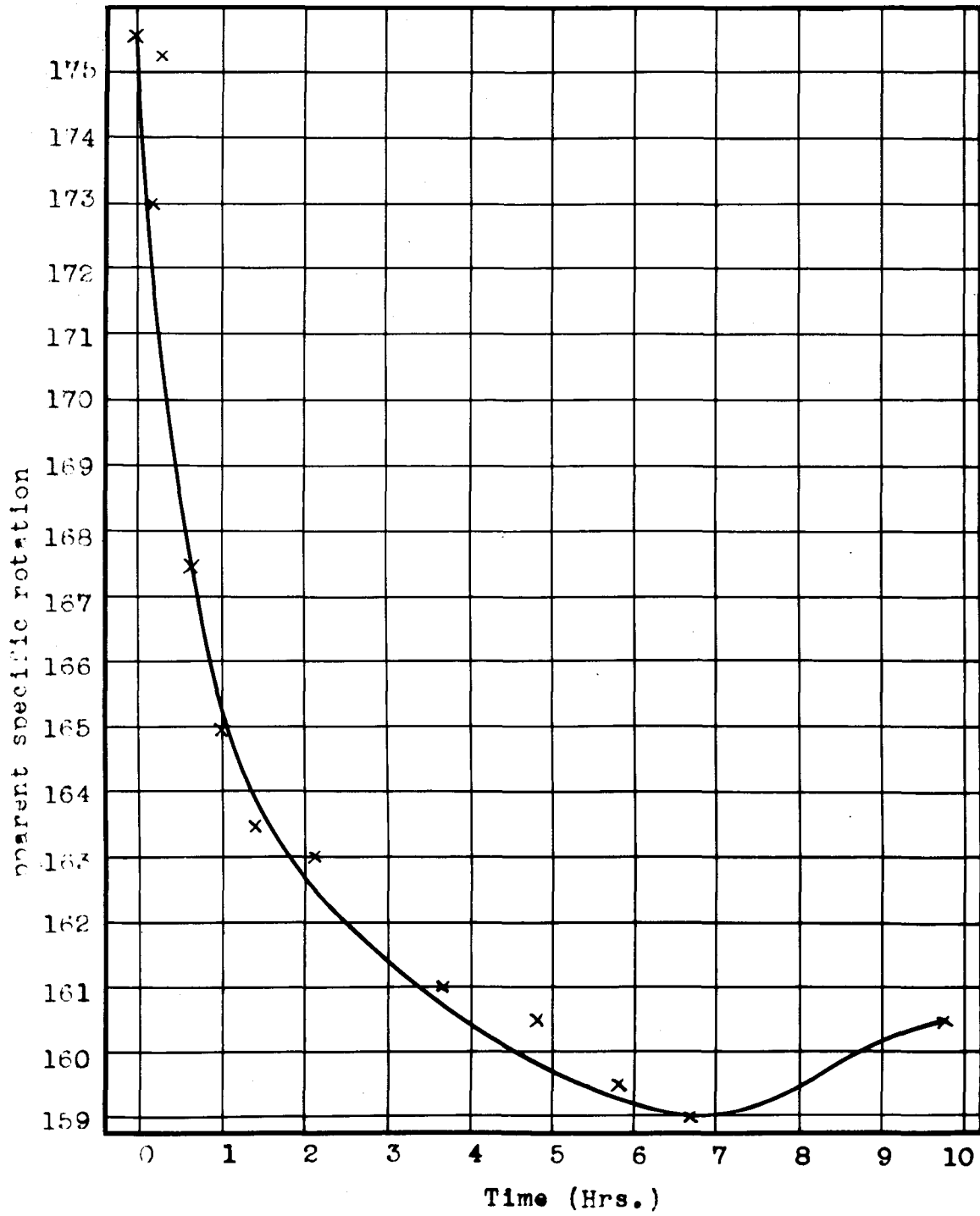


Fig. 1. Rotatory change of amyloheptaose
in presence of macerans amylase

conducted on the same solution. The significance of the analysis presented for experiment 4 is questionable since at the time the solution was prepared it was not contemplated doing any additional work on it and therefore its history is uncertain. The enzymolysis solutions in experiments 3, 4 and 5 were covered with toluene within a few hours of preparation. The heavy precipitate of β -dextrin which had formed at the time of analysis was filtered off, and the clear filtrate was analyzed.

Examination of the table reveals the following pertinent information of a qualitative nature. (1) All of the lower sugars, glucose, maltose and amylotriase, occur relatively early in the reaction. (2) Glucose is produced more rapidly than either maltose or the amylotriase. (3) The amount of glucose compared to the total amount of end groups found remains relatively constant. (4) The amount of maltose increases rapidly at first and then levels off more quickly than glucose. (5) The amount of amylotriase increases very little after 2 hours. (6) The amount of α -dextrin increases very rapidly at first, levels off and then decreases markedly under the influence of toluene. (7) The amount of α -dextrin present throughout the reaction is more than can be accounted for on the basis of the reaction of the amyloheptaose splitting solely to glucose and α -dextrin. (8) The total amount of end group accounted for as glucose, maltose and amylotriase

increases with time.

The presence of glucose can most simply be explained on the basis of the reaction (1) given on page 63. The production of maltose and amylotriase in the early stages of the reaction is somewhat more baffling. If, as Freudenberg's work indicates (24), there are more than two Schardinger dextrans then it may well be that a five membered ring dextrin exists and maltose could then be accounted for by the reaction:



The possibility of the existence of such a non-reducing dextrin can not be eliminated but so far the work of Freudenberg has not been confirmed by others.

Although such a reaction might account for the early production of maltose, the production of amylotriase can not be accounted for by a similar mechanism since the formation of a cycloamylose with four glucose units would seem to be improbable on the basis of the Freudenberg models. It also seems unlikely that any of these products are produced in the early stages of the reaction as a result of enzymic hydrolysis since such a change would necessarily give rise to additional end groups and consequently a marked increase in reducing power. In addition the splitting of amyloheptaose into smaller molecules would give rise to a continual and steady decrease in rotation, until the rotation of maltose or glucose would be approached. Neither of these phenomena occur to any extent.

Hydrolysis then, does not seem to be the explanation for the production of the mono-, di and trisaccharides in the course of the reaction.

The only other reasonable explanation would involve the resynthesis of oligosaccharides longer than the original amyloheptaose through the reaction of the cyclic dextrans formed with glucose formed by the reaction 1 or amyloheptaose originally present.

In order to determine whether the enzyme was capable of opening up the Schardinger cyclic dextrans in the presence of short molecules, particularly glucose and maltose, some investigations were made along this line. The results of the action of macerans enzyme on the Schardinger α - and β -dextrans alone and in the presence of various co-substrates are shown in Table 5. The evidence for reaction was based on two factors determined periodically. The first factor was a change in rotation while the second was the production of α -dextrin, when β -dextrin was used for the substrate, and the production of β -dextrin, when α -dextrin was used as the substrate.

The initial observable reaction where β -dextrin was the substrate would be an increase in the rotation of the mixture due to the opening up of the cyclic molecule according to the following reactions; (3) if no co-substrate is necessary and (4) if a co-substrate is necessary.



The change in specific rotation for reaction (3) would be from 163° to 179.6° if the reaction went to completion. For reaction (4) it would be from 157.3° to 184.3° if the starting materials were present in equimolar amounts and the reaction went to completion. Either of these changes should be easily detectable even if the reaction proceeds to only a few per cent.

Following the formation of straight chains, reaction (1), page 63, or reaction (5) should occur to give rise to α -dextrin and a short chain amylose:



The occurrence of either reaction (1) or (5) should be easily detectable by means of the iodine test for cycloamylohexaose. The first traces of α -dextrin may be difficult or impossible to detect by this means since there is an extremely large excess of β -dextrin present. However, the presence of the α -dextrin could be detected as soon as there was an increase in rotation.

Table 5 shows the actual changes in rotation observed and the elapsed time before an iodine test for α -dextrin was obtained with various co-substrates. It will be observed that in the case of β -dextrin alone or with glucose,

Table 5

Action of Macerans Amylase on the Schardinger
Dextrins in the Presence and Absence of
Co-substrates

Experiment No.	1		
Wt. of β -dextrin, grams	0.249	0.249	0
Wt. of maltose, grams	0.100	0.0	0.100
Ml. of enzyme, 10 u/ml.	1	1	1
Final volume, ml.	25	25	25
Angular rotation after, min.			
5	4.42	3.33	1.17
33	4.42	3.34	1.18
81	4.47	3.35	1.18
272	4.56	3.35	1.18
1069	4.66	3.33	1.18
Length of tube, dm.	2	2	2
Positive iodine test for α -dextrin after, min.	31 (If β -dextrin-iodine complex is first filtered off) 81 (In presence of β -dextrin)		

Experiment No.	2				
	a	b	c	d	e
Wt. of β -dextrin, g.	0.249	0.249	0.249	0.000	0.000
Wt. maltose, g.	0.115	0.000	0.000	0.115	0.000
Wt. sucrose, g.	0.000	0.100	0.000	0.000	0.000
Ml. Enzyme, 20 u/ml.	1	1	1	1	1
Final volume, ml.	25	25	25	25	25
Length of tube, dm.	2	2	2	2	2
Angular rotation after, min.					
28	4.51	4.04	3.30	1.23	0.82
64	4.54	4.03	3.28	1.24	0.82
112	4.56	4.05	3.30	1.23	0.82
1156	4.76	4.04	3.28	1.23	0.82
1362	4.79	4.05	3.28	1.23	0.81
First test for α - dextrin, after, min.	63	--	--	--	--
Ml. 1.0 N $K_4Fe(CN)_6$ reduced after 24 hrs. *					
On basis of total carbohydrate	10.02			29.6	
On basis of reduc- ing sugar added	31.80			29.6	

*One gram of maltose should reduce 29.9 ml. of 1.0 N $K_4Fe(CN)_6$

Table 5, Continued

Experiment No.	3				
	a	b	c	d	e
Wt. β -dextrin, g.	0.249	0.249	0.249	0	0
Wt. glucose, g.	0.048	0.0	0.0	0.0529	0
Wt. gluconic acid, g.	0.0	0.047	0.0	0.0	0.052
Ml. Enzyme, 20 u/ml.	1	1	1	1	1
Final vol., ml.	25	25	25	25	25
Length of tube, dm.	2	2	2	2	2
Angular rotation after,					
min. 14	3.44	3.26	3.23	0.37	0.06
79	3.44	3.27	3.23	0.37	0.05
249	3.43	3.26	3.23	0.36	0.05
397	3.43	3.26	3.22	0.36	0.05
1440	3.48	3.26	3.23	0.35	0.05
First test for α -dextrin, hrs.	29	--	--	--	--

Experiment No.	4		
	a	b	c
Wt. β -dextrin, g.	0.249	0.249	0.249
Wt. glucose, g.	0.040	0	0
Wt. maltose hydrate, g.	0	0.079	0
Ml. enzyme, 20 u/ml.	1	1	1
Final vol., ml.	25	25	25
Length of tube, dm.	2	2	2
Angular rotation after, min.			
7	3.41	4.07	3.21
39	3.40	4.10	3.21
168	3.41	4.14	3.22
273	3.41	4.16	3.21
375	3.41	4.19	3.20
1410	3.46	4.28	3.23
Ml. 1.0 N $K_2Fe(CN)_6$ re- duced per 4 grams carbo- hydrate after 1410 min., on basis of reducing sugar added	45.60	29.5	0

Table 5, Continued

Experiment No.	5				
	a	b	c	d	e
Wt. α -dextrin, g.	0.213	0.213	0.213	0	0
Wt. glucose, g.	0.053	0	0	0.053	0
Wt. maltose, g.	0.0	0.110	0	0	0.110
Ml. Enzyme, 20 u/ml.	1	1	1	1	1
Final vol., ml.	25	25	25	25	25
Length of tube, dm.	2	2	2	2	2
Angular rotation after,					
min. 14	2.56	3.48	2.28	0.24	1.14
144	2.88	3.72	2.29	0.24	1.14
233	2.94	3.82	2.28	0.24	1.14
317	2.97	3.83	2.29	0.25	1.15
1317	3.04	3.83	2.40	0.24	1.15
3162	----	3.78	2.44	0.24	1.15
Ml. 1.0 N $K_4Fe(CN)_6$					
reduced per grams					
carbohydrate after					
24 hours,					
On basis of total					
carbohydrate	10.52	8.44	3.32	43.80	30.05
On basis of reduc-					
ing sugar added	48.40	30.20	3.32	43.8	30.05

Experiment No.	6	
Wt. α -dextrin, grams	0.249	0.262
Wt. glucose, grams	0.046	0
Wt. potassium gluconate, g.	0	0.045
Ml. Enzyme, 20 u/ml.	1	1
Final vol., ml.	25	25
Length of tube, dm.	2	2
Angular rotation after, min.		
4	2.87	2.82
15	2.98	
25	3.06	2.84
53	3.18	2.85
88		2.85
249	3.41	
358	3.44	
1220		2.98
1403	3.50	

Table 5, Continued

Experiment No.	7						
	a	b	c	d	e	f	g
Wt. α -dextrin, g.	0.280	0.280	0.280	0.280	0.280	0.280	0.00
Wt. cellobiose, g.	0.086	0	0	0	0	0	0.086
Wt. sucrose, g.	0	0.086	0	0	0	0	0
Wt. calcium maltobionate, g.	0	0	0.087	0	0	0	0
Wt. glucose, g.	0	0	0	0.042	0	0	0
Wt. α -methyl glucoside, g.	0	0	0	0	0.042	0	0
Ml. enzyme, 20 u/ml.	1	1	1	1	1	1	1
Final vol., ml.	25	25	25	25	25	25	25
Length of tube, dm.	2	2	2	2	2	2	2
Angular rotation after,							
min. 12	3.45	3.53	3.78	3.27	3.71	3.03	0.33
25	3.45	3.56	3.82	3.36	3.79	3.05	----
34	3.45	3.60	3.87	3.40	3.84	3.06	0.35
58	3.61	3.63	3.93	3.50	3.92	3.05	----
105	3.82	3.79	4.08	3.63	4.03	3.06	----
195	3.90	3.87	4.13	3.77	4.04	3.09	----
284	3.90	3.92	4.17	3.79	4.08	3.10	----
1322	3.90	4.08	4.24	3.97	4.10	3.24	0.34

gluconic acid and sucrose there is no change in rotation within six hours while when maltose is used as a co-substrate in the reaction, there is a definite observable change in rotation within this time which is far outside the experimental error of such determinations. A definite test for α -dextrin was also obtained within about two hours when maltose was used as the co-substrate. With the other co-substrates no such test was obtained within this time. Whether or not glucose actually functions as a co-substrate in the reaction is open to question. After 20 hours or more a slight change in rotation was observed and a test for α -dextrin could be obtained.

Similar tests were made on α -dextrin using a wider variety of co-substrates. The rate of action of macerans enzyme with α -dextrin was much greater than that with β -dextrin in the presence of the same co-substrates. Furthermore, several sugars were at least as reactive in this case as maltose, and perhaps more so. When α -dextrin was the starting material, it was not possible to detect small quantities of β -dextrin by means of the iodine test. Therefore the solutions were covered over with p-xylene in order to precipitate small quantities of β -dextrin without precipitating α -dextrin from solutions in the concentration employed. The formation of β -dextrin could thus be detected within about six hours.

There was no apparent change in rotation within five or

six hours when α -dextrin alone was present or when potassium gluconate was added. However, after 21 hours there was a small increase in rotation and small amounts of β -dextrin formed in both cases. Since the magnitude of the increase in rotations for both substrates was about equal it would seem that potassium gluconate has no influence on the reaction. The possibility thus exists that in the case of α -dextrin, macerans amylase may be able to open the ring in the absence of activators. The development of a slight reducing power within 24 hours tends to support this view. McClenahan, Tilden and Hudson (33) had previously made a similar observation although they did not detect the formation of β -dextrin.

Of particular significance is the ability of such varied compounds as cellobiose, sucrose, calcium maltobionate and α -methyl glucoside to act as co-substrates for the reaction of macerans amylase with α -dextrin. The only feature these sugars have in common is a 2, 3, 4, 6 unsubstituted glucopyranose structure. Apparently this is the critical feature necessary for activation. The presence of a reducing end group, of an α -1,4- linkage, or only glucose units in the molecule all seem to be unnecessary since all the sugars tried except maltose have one or more of these features missing. The only sugar tested which does not act as co-substrate for the reaction with α -dextrin, potassium gluconate, is also the only one without the pyranose ring structure.

Despite the variations in the constitutions of the several sugars which are effective as activators, the actual reaction with macerans amylase and α -dextrin may be identical in all cases. The first step in the reaction may be pictured as the formation of a complex of enzyme and α -dextrin which results in the opening of the ring and consequent formation of linear dextrin enzyme complex, the reducing end of the linear dextrin being tied up. The next step in the reaction would result in the formation of a new α -1,4 bond by attaching the reducing end of the linear dextrin to the non-reducing end of the co-substrate. The new linear dextrin formed is then apparently released into the medium where it may act as the substrate for the formation of β -dextrin or as a co-substrate for the reaction with more α -dextrin. The deciding factor then in the ability for any particular sugar to act as a co-substrate appears to be the presence of a 2, 3, 4, 6 unsubstituted glucopyranose structure.

The results of experiments on the macerans amylase indicate that a reversible reaction is involved in the formation of the non-reducing cyclic dextrans and therefore, that the enzyme is synthetic as well as degradative. Hydrolysis is not involved in the formation of the cycloamyloses or of glucose, maltose and amylotriase in the above experiments. The α -dextrin appears to be formed first. The formation of β -dextrin from α -dextrin is initiated under the influence

of short chain co-substrates or results from the relatively short chain dextrans themselves. The equilibrium favors the apparently more stable β -dextrin.

The path through which the reaction of one Schardinger dextrin must go to form the other one and short chain molecules may be illustrated by the following series of reaction using β -dextrin and maltose as the substrate. These reactions may also be used to account for the formation of glucose, maltose and amylotriase in the reactions of macerang amylase on the amyloheptaose described previously.

- (6) Cycloamyloheptaose + maltose \rightleftharpoons amylnonaose
- (7) Amylnonaose \rightleftharpoons cycloamylohexaose + amylotriase
- or Amylnonaose + cycloamyloheptaose \rightleftharpoons amylohexadecaose
- (8) Cycloamyloheptaose + amylotriase \rightleftharpoons amylodecaose
- and Amylohexadecaose \rightleftharpoons 2 cycloamylohexaose + amylotetraose
- and Cycloamylohexaose + maltose \rightleftharpoons amylooctaose
- and Cycloamylohexaose + amylnonaose \rightleftharpoons amylopentadecaose
- (9) Amylooctaose \rightleftharpoons cycloamyloheptaose + glucose
- and Cycloamyloheptaose + amylodecaose \rightleftharpoons amyloheptadecaose
- and Cycloamyloheptaose + amylotetraose \rightleftharpoons amyloundecaose
- and Amylooctaose + cycloamylohexaose \rightleftharpoons amylotetradecaose
- (10) Amyloundecaose \rightleftharpoons cycloamylohexaose + amylopentaose
- and Amylotetradecaose \rightleftharpoons cycloamyloheptaose + amyloheptaose
- (11) Amyloheptaose + cycloamylohexaose \rightleftharpoons amylotridecaose
- Amylopentaose + cycloamylohexaose \rightleftharpoons amyloduodecaose

(12) Amylotridecaose \rightleftharpoons cycloamyloheptaose - amylohexaose

A similar set of reaction would be obtained if amyloheptaose or α -dextrin plus a co-substrate were used as the starting materials. Examination of this set of reactions shows that all the glucose polymers from glucose up through at least amyloheptaose are formed in the reaction and probably much larger straight chain molecules. This mechanism accounts very nicely for the presence of glucose, maltose and amylotriose found in the reaction of macerans amylase on amyloheptaose described earlier. Glucose, maltose and amylotriose were also detected, by the chromatographic-colorimetric technique, as reaction products when β -dextrin and maltose were reacted with macerans amylase.

The demonstration that relatively long chain oligosaccharides are formed during the course of the reaction is necessary since they are intermediates in forming the short chain amyloses. Advantage was taken of the fact that very small quantities of relatively long chains should give a detectable increase in color with iodine as well as a shift in the absorption spectrum so that more light from the red side of the spectrum is absorbed. Figure 2 shows the absorption spectra of various solutions mixed with equal quantities of iodine. It will be seen that 1 per cent α -dextrin solutions and 1 per cent amyloheptaose solution give slight iodine colors compared with the iodine blank. The reaction products of a

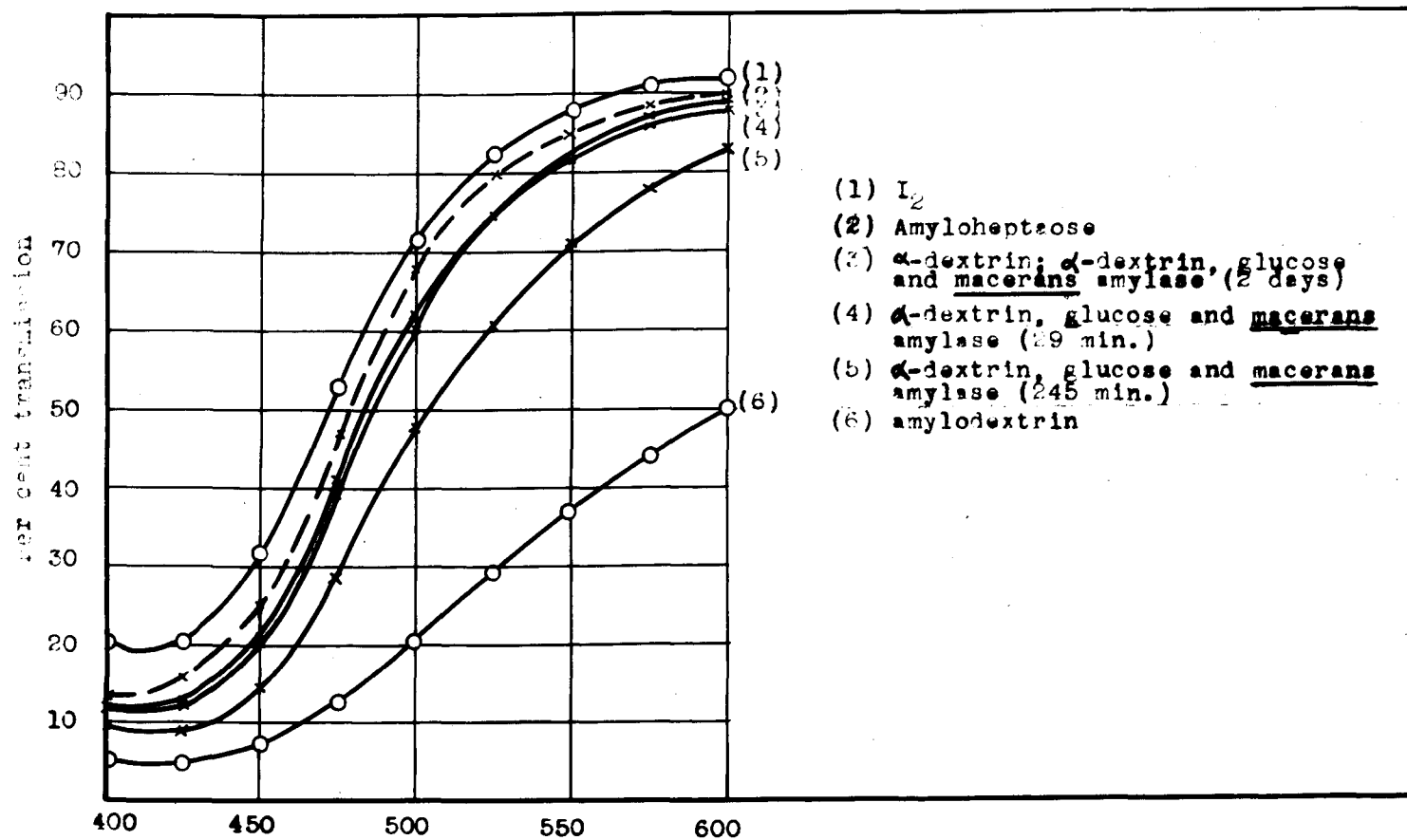


Fig. 2. Absorption spectra of various macerans amylase digests

1 per cent α -dextrin solution with 1/6 of 1 per cent glucose gives no increase in density of iodine coloration after 25 minutes as determined on the Coleman spectrophotometer. At this stage in the reaction the rotation had risen 0.19° . After 249 minutes, however, there is a definite increase in absorption of the reaction mixture mixed with iodine indicating the formation of relatively long chains. The rotation, at this time, had increased 0.54° , almost three times the rise obtained after 25 minutes. Therefore, it would seem that the main reaction which occurred during the first 29 minutes was the opening up of the ring, with the aid of glucose, to form amyloheptaose. This is necessarily followed by the reaction of the amyloheptaose formed with more cycloamylohexaose to form amylotridecaose which would give an iodine color. A 0.1 per cent solution of amylo-dextrin having an average chain length of 18 glucose units was run for comparison.

It was observed that after two days under p-xylene the increased iodine coloration had disappeared. This indicates that most of the long chains have been converted to short chains and the β -dextrin. A heavy crystalline precipitate of β -dextrin was present after this time. It had previously been observed that after 48 hours, on an experiment with amyloheptaose under toluene, that 60 per cent of the weight of the amyloheptaose was converted to β -dextrin. It was also

shown that the per cent of end groups accounted for as glucose, maltose and amylotriase increased steadily with time until, after 10 days, 50 per cent of them were accounted for as these sugars. It would be reasonable to expect, then, that most of the long chains would have disappeared within two days under the influence of precipitants.

Another interesting observation was made on the action of macerans amylase on the oxidized amyloheptaose. There was a rapid reaction according to the change in rotation. Furthermore, a trace of α -dextrin was detected in 10 minutes. The reaction mixture was covered with toluene and there was no apparent formation of β -dextrin from the oxidized amyloheptaose within 48 hours while there was definite formation of β -dextrin in the unoxidized amyloheptaose. Since the potassium salt of amyloheptaonic acid was found to have some reducing power, the possibility exists that the α -dextrin was formed from unoxidized amyloheptaose. It would seem unlikely that amyloheptaonic acid would be acted upon when α -dextrin and gluconic acid are unreactive.

From the foregoing discussion it would seem that the action of macerans enzyme on short linear chains results in the formation of α -dextrin first. This is followed by re-synthesis of relatively long linear chains from the α -dextrin

under the influence of straight chains present in the substrate. These long chains are then degraded to β -dextrin and shorter chains. The reaction is reversible and ultimately an equilibrium is set up among the various components of the system. Hydrolysis is not involved in these reactions.

The greatest need at present is a careful study of the enzyme system involved. Methods must be developed to procure a pure enzyme, known to be free of all traces of the hydrolytic amylases. Furthermore, future studies must be done under aseptic conditions in order to eliminate entirely the necessity of using toluene or other preservatives and to avoid the possible introduction of extracellular enzymes elaborated by microorganisms growing in the reaction mixtures. In many cases the enzymolysis solutions studied became cloudy within 24 to 48 hours indicating microbiological growth. Although such bacterial contamination would not be sufficient to alter the course of reactions observed within a few hours, it would preclude the possibility of studies extending over several days or weeks.

By using the purified enzyme under aseptic conditions not only may the mechanism of enzyme action be more accurately determined but a new tool for the synthesis of oligosaccharides will be available. For example, the action of the enzyme could

be stopped after a relatively short period of time to give a relatively easily separable mixture of oligosaccharides. For instance, the reaction might be stopped after such time that only reaction (6) and (7), page 79, would have taken place to any extent. The resulting mixture would contain only maltose, amylotriase, amylononaose and amylohexadecaose. It should be feasible to separate amylononaose and amylohexadecaose by alcohol fractionation, perhaps, and most certainly by chromatographic techniques. It is even possible that these two carbohydrates may be relatively easily crystallizable since they approach in size the amyloextrins.

Furthermore, the possibility exists that anomalous linkages may be introduced into linear chains by reacting the Schardinger dextrins with sugars such as sucrose or cellobiose. The value of such abnormal polysaccharides in enzyme studies as well as chemical and physical studies is obvious.

C. Action of β -Amylase

The mode of action of β -amylase has been thoroughly investigated and there seems little doubt that its essential features have been explained. The amylase attacks the α -1,4 glucosidic bond in linear molecules starting at the non-reducing end and splitting off maltose until it reaches the end of the chain or until the residue is amylotriase. The

assumption that amylotriase is not hydrolyzed by β -amylase is based on the fact that no glucose has been isolated from such a digestion and on calculations from reducing power and specific rotation measurements. In the case of chains of finite lengths, then, it should be possible to get a fairly accurate estimate of the degree of polymerization by analyzing the products from β -amylase digestions. Table 6 shows the results of β -amylase digestions of some different substrates.

It would be expected that amyloheptaose would be hydrolyzed by β -amylase to give two molecules of maltose and one molecule of amylotriase. The molecular weight determination by the iodine oxidation method of the final enzymolysis mixture was 462. This indicated that the amount of amylotriase formed was apparently more than the calculated amount. The average molecular weight of the theoretical mixture should be 410. However, from the specific rotation measurement the amount of maltose was correspondingly high compared to the theoretical amount. These two results, a high average molecular weight from iodine oxidation and a low average molecular weight from specific rotation, are merely the reflection of the same discrepancy observed in the original amyloheptaose, and are, therefore, not to be given too much consideration.

The actual amount of maltose present was determined by chromatographing 30 mg. of the hydrolysis mixture according

to the method given in Appendices 1 and 2. The actual analysis is plotted in Figure 3. It can be seen from this curve that no glucose is present and that there are two distinct maxima. The first is obviously due to maltose (compare with the curve in Figure 7, Appendix 2) while the second is amylotriase. The evidence indicating that the second maximum in the curve is amylotriase rests not only on its position and the constants determined on the original hydrolysis mixture, but also on a direct determination. From a separate chromatographic analysis, 15 ml. of the eluate, starting with the 56th ml., was evaporated to dryness and a micro-iodine titration run on the dry sample. The amount of carbohydrate present in the eluate collected was determined colorimetrically and this value used to determine the molecular weight from the iodine titration. The molecular weight determined by this method was 566. The calculated molecular weight of amylotriase is 504. There is an error of about 12 per cent. This could well be within the experimental limits of the two methods combined. The colorimetric method is not reliable to more than about 5 per cent of the actual quantity and the iodine titration on micro-quantities of reducing sugars was found by actual trial to be of about the same order of reliability.

Before making the chromatographic analysis of the enzymolysis products an attempt was made on a separate sample

Table 6

Reaction Products from Beta Amylase Action of Amyloheptaose, the Potassium Salt of Amyloheptaonic Acid and Amylodextrin.

	Amylo- heptaose	K Salt of Amylohep- taonic Acid	Amylodextrin (18 glucose res. by reducing power)
Weight of substrate, g.	0.5000	0.0728	0.5000
Vol. enzymolysis soln., ml.	25	10	25
Wt. beta amylase, g.	0.005 g	0.005	0.005
Ml. 0.100 N $K_3Fe(CN)_6$ reduced per gram of original substrate	----	16.10	28.80
Mol. Wt. by I_2 oxidation	462	----	----
sp. rot. on basis of wt. of substrate (uncorrected for water of hydrolysis)	147°	----	147°
% maltose, found chromatographically	57	----	----
% maltose, calc. from reducing power	62.5	57.5	96.5
% maltose, theoret- ical ¹	57.5	53.5	91.7 ²
% amylotriose, found chromatographically	43	----	----
% amylotriose, calc. from reducing power	37.5	----	3.6
% amylotriose, theoret- ical ¹	42.5	----	8.3 ²

1. Assuming that beta amylase starts at one end of the chain and clips off successive maltose residues and will not hydrolyze amylotriose.
2. Assuming that the average chain length is 18 glucose units and half of the molecules contain an even number of glucose residues and half an odd number.

to ferment out the maltose with ordinary bakers yeast in the vain hope that the amylotriase would be left in the solution. After 24 hours at room temperature the solution was clarified by filtration and the specific rotation determined. It was found that the specific rotation had dropped to about 5 per cent of the original value. This indicated that at least 95 per cent of the sugars present were fermentable. Glucose and maltose are both fermentable by ordinary yeast preparations but the fermentation of higher amyloses has not been conclusively demonstrated. Myrbäck (78) states that amylotriase is fermentable by yeast but that amylotetraose is not. This experiment would seem to confirm the fermentability of amylotriase. The question of the fermentability of amylotetraose, however, must be held in abeyance until a relatively pure preparation is available.

The results of the β -amylase digestion of the potassium salt of the amyloheptaonic acid confirms the already well established fact that the enzyme works from the non-reducing end of the chain and that oxidation of the reducing group has no effect whatsoever on the course of the reaction.

The results of the digestion of amyloextrin, which is a straight chain microcrystalline material having an average degree of polymerization of about 18 glucose units, also confirms previous knowledge that straight chain amyloses are degraded completely to maltose, if the chain has an even

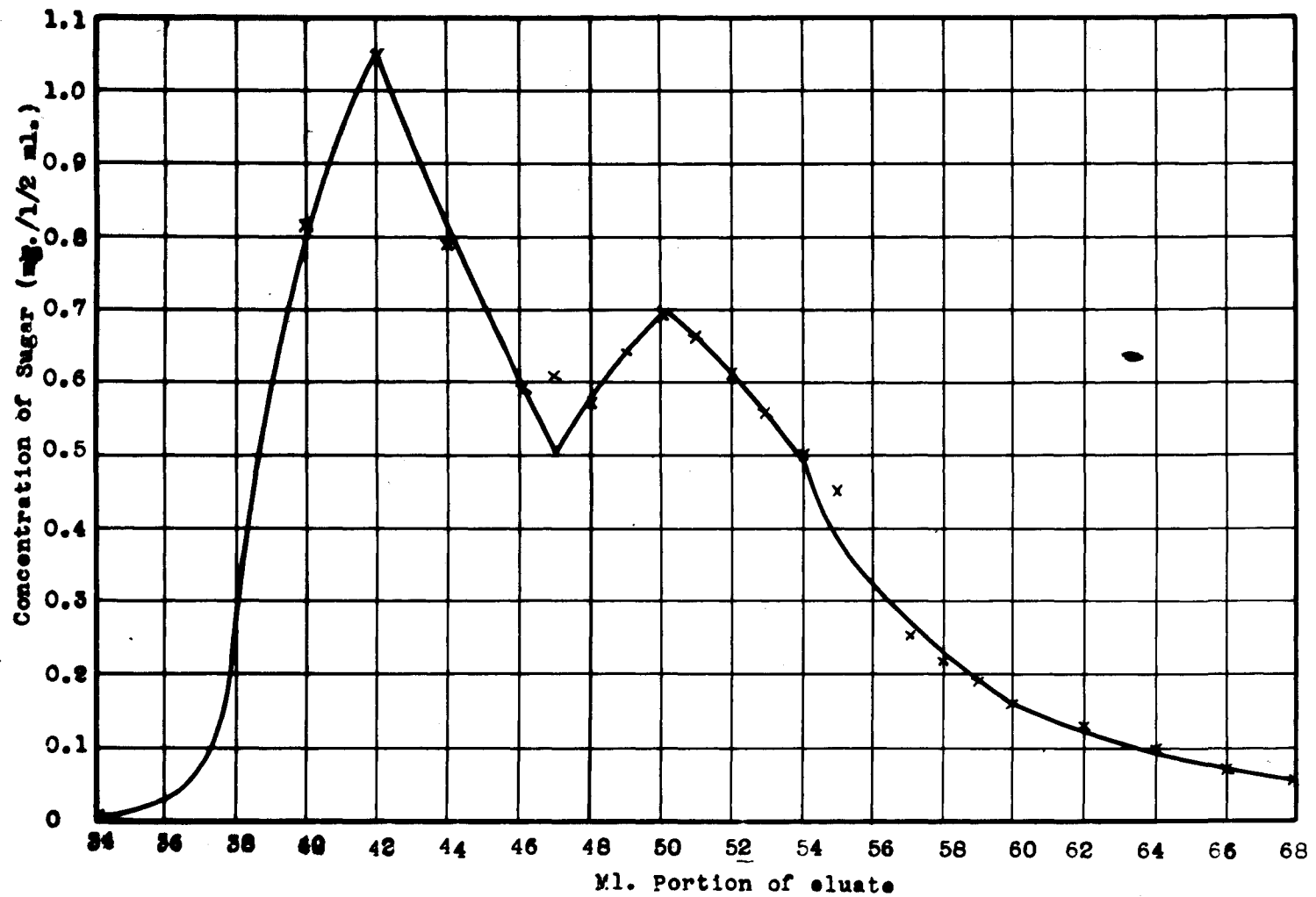


Fig. 3. Chromatographic colorimetric analysis of β -amylase digestion of amyloheptaose

number of glucose units. If the chain has an odd number of glucose residues, amylotriase will also be present in the hydrolysis products. Although amylo-dextrin is a crystalline material it can not be assumed that it is homogeneous in the sense that it has chains of a single degree of polymerisation. It may be assumed that all the chain lengths are in the neighborhood of 18 glucose units and half have an odd number of glucose residues. Then there would be one odd numbered chain, or one molecule of amylotriase formed, for every 36 glucose units, or about 8.3 per cent amylotriase. Assuming that only amylotriase is formed, other than maltose, the amount calculated from reducing power would be equal to 3.7 per cent or one molecule for every 81 glucose units originally present. The agreement between the theoretical amount of amylotriase and that calculated from reducing power of the final enzymolysis solution is only fair.

The question of whether or not amylo-tetraose is attacked by β -amylase has not been answered by these experiments. From the normal reaction of β -amylase with amylo-heptaose no residues with an even number of glucose units is ever involved so there is no chance of a tetrasaccharide being formed. From the action of β -amylase on amylo-dextrin, when even numbered chains are involved, the length of the chains would make the presence of unhydrolyzed amylo-tetraose difficult to detect. The final reducing power and rotation,

however, indicate that no appreciable amount of amyloetraose could be present and therefore it must be hydrolyzed to maltose.

In order to get more conclusive evidence as to the hydrolyzability of amyloetraose it would be desirable to start with amyloetraose itself or with a relatively homogeneous short chained amylose containing only even numbered glucose residues. The best source of such a material would seem to be from the controlled hydrolysis of α -dextrin according to a procedure similar to that used for the β -dextrin. A relatively pure amylohexaose could then be obtained. Then, if the tetrasaccharide was not hydrolyzable it could be easily determined, even without its isolation, by following the change in reducing power and rotation.

D. Action of Salivary Amylase

According to the hypothesis of Meyer and Bernfeld (79), terminal glucosidic linkages are not attacked by α -amylase while all interior linkages are attacked with the same probability in the primary hydrolysis of straight chained members of the amylose series. This primary breakdown is followed by a slow hydrolysis of the amylotriase while maltose is not attacked. These workers further calculated that from a chain of amylose, if the terminal linkages were not attacked and if amylotriase was not hydrolyzed, one

would end up with 60 per cent maltose and 40 per cent amylotriase.

Assuming this hypothesis is correct, if one calculates the amount of maltose and amylotriase which would be produced from the primary hydrolysis of amyloheptaose one gets 57.5 per cent maltose and 42.5 per cent amylotriase. In the primary hydrolysis terminal linkages will not be hydrolyzed, and the maltose and amylotriase produced are not attacked by α -amylase. With this assumption only one set of final products is possible, regardless of how the molecule is first attacked.

(1) Amyloheptaose \rightarrow maltose + amylopentaose
and Amylopentaose \rightarrow maltose + amylotriase

And adding 1, a and b together:

Amyloheptaose \rightarrow 2 maltose + 1 amylotriase

or (2) Amyloheptaose \rightarrow amylotriase + amylotetraose
and Amylotetraose \rightarrow 2 maltose

and adding 2, a and b together:

Amyloheptaose \rightarrow 2 maltose + 1 amylotriase.

If the reaction went no further than that shown, analysis of the products from hydrolysis by means of specific rotation, reducing power or the chromatographic-colorimetric method would agree with the calculated values. The final specific rotation of such a mixture, calculated on the basis of the original amyloheptaose, would be 149° .

The results of a number of salivary amylase digestions

Table 7

Reaction Products of Salivary Amylase Action on Amyloheptaose, Amylodextrin,
and Maltose.

Substrate	Amyloheptaose				Amylodextrin			Maltose	
	1	2	3 ¹	3 ¹	4	5	6	7 ²	8 ³
Sample No.			a	b					
Weight of substrate, g.	0.2500	0.2529	0.2586	0.2586	0.3025	0.2493	0.3080	.2600	.1020
Vol. of salivary amylase, ml.	1 ⁴	1 ⁴	1 ⁵	1 ⁵	1 ⁴	1 ⁴	1 ⁵	1 ⁵	1 ⁵
Final volume, ml.	25	25	25	25	25	25	25	25	25
Specific rotation, substrate	170.7	175.5	175.5	175.5	-	188.9	188.9	131	131
Specific rotation, products, calc. on wt. of substrate	146.0	133.5	154.5	137.0	149.5	133.5	142.0	131	131
Ml. 1.0 N K ₄ Fe(CN) ₆ reduced by products/ 0.1 g. substrate	-	31.8	29.7	32.2	-	29.3	34.9	-	-
Time of digestion, hrs.	17.5	18	0.4	23	17.6	18	26	18	26
% glucose found chromatographically	0	14.9	2.4	11.3	0	7.5	10.2	-	-
% maltose found	63.5	55.2	45.6	55.3	63	64.2	78.7	-	-
% trisaccharide found	36.5	29.9	51.8	33.2	37	28.5	10.8		
% substrate accounted for as glucose, mal- tose and trisaccharide	42.2	86.8	75.5	73.0	38.3	85.0	60.0		
Specific rotation, calc. from analysis, on basis of (162) _n	181.6	136.8	150.2	139.9	151.5	143.2	136.0		

Table 7 (Continued)

Substrate	Amyloheptaose				Amylodextrin			Maltose	
	1	2	3 ¹ a	3 ¹ b	4	5	6	7 ²	8 ³
Ml. 1.0 N $K_4Fe(CN)_6$ reduced, calc. from analysis on basis of (162)n	-	32.2	28.2	31.5	-	30.4	33.9	-	-

1. 3 a and b are same solution with aliquots removed for analysis at 2 different times.
2. Run as blank on same amylase as sample #3.
3. Run as blank on same amylase as sample #6.
4. Salivary amylase collected from Dr. French.
5. Salivary amylase collected from Mr. Levine.

of amyloheptaose, amyloextrin and maltose are given in Table 7. Analysis of the enzymolysis products was made by means of the chromatographic-colorimetric method given in Appendix 1 and 2. All of the starting materials were not accounted for, the most complete recovery being 86.8 per cent. Most of the recoveries were much poorer than that. In view of this fact the analyses can not be used to infer quantitative information concerning the course of the reaction. However, they can be used as the basis for qualitative speculation.

There would seem to be little doubt that glucose was formed in relatively small quantities during the course of the reaction. That its origin was maltose would seem to be pretty well eliminated since in the tests on maltose with salivary amylase, no conversion to glucose took place. That it appeared relatively early in the hydrolysis was apparent since at least 2.4 per cent of the original amyloheptaose was recovered as glucose after 24 minutes. At this point the extent of hydrolysis had not proceeded as far as the 60-40 maltose-amylotriose stage, as judged from the change in rotation. The rotation had dropped at this time about 60 per cent of its total change. The main reaction was a breaking up of the molecule into maltose and amylotriose with a much slower simultaneous side reaction occurring which gave rise to glucose. The source of the glucose is quite definitely those molecules larger than maltose, including amylotriose.

The fact that only relatively small amounts of glucose are formed even after a relatively long time of enzyme action indicates that terminal linkages are hydrolyzed much more slowly than interior linkages. This is in accordance with the concepts of both Meyer (79) and Myrbäck (80, 81).

The results of the digestion of amyloextrin, whose average chain length is about two and a half times that of amyloheptaose, gives about the same qualitative information as was obtained with amyloheptaose. One thing which is noticeable, however, is the relatively larger amounts of maltose and a much smaller amount of amylotriose in the end products than is found with amyloheptaose.

In order to obtain more definite quantitative information concerning the reaction of α -amylase, there is required more fundamental work on the enzyme itself as well as the development of a more satisfactory analytical technique. As far as is known, the purity of all α -amylase preparations is open to much question. It is recognized that the homogeneity of the salivary amylase used in these experiments is questionable since no attempt at purification was made. The relative strength of the enzyme may differ for different times of collection from the same individual and most surely differs for different individuals. Furthermore, the presence of other starch or sugar splitting enzymes may cause side reactions.

The information obtained from the investigation of the products from salivary amylase and β -amylase digestions of amyloheptaose and amyloextrin would seem to provide a relatively good method for preparing amylotriase in a high state of purity. From the β -amylase studies it was found that even numbered linear chains are hydrolyzed completely to maltose. Amylotriase is not hydrolyzed. From the salivary amylase investigations it was found that the major portion of the hydrolysis products are maltose and amylotriase. If α -amylase digestion were stopped when the specific rotation of the substrate has dropped to the theoretical value for a 60-40 mixture of maltose and amylotriase, the amylotriase should constitute at least 30-35 per cent of the carbohydrate present. This mixture will also have glucose, maltose and oligosaccharides larger than amylotriase. A β -amylase digestion at this point should degrade all chains longer than amylotriase to maltose and amylotriase, leaving amylotriase unhydrolyzed. At the end of the β -amylase digestion only glucose, maltose and amylotriase should be present.

Amylotriase should be separable from such a mixture by one of a number of ways. One method would be by a straight alcohol fractionation. The yield of pure amylotriase resulting from such a fractionation would undoubtedly be quite low. Another method of separation would be chromatographically.

The yield of amylotriose by this method should be high but the purification of large quantities of carbohydrate by such a method is tedious. The third method of separation would be by distillation of the propionates according to the method of Hurd and Liggett (92). This method involves special apparatus and is not useful or convenient for very large scale separations. The most satisfactory method would seem to be alcohol fractionation.

V. CONCLUSIONS

1. The specific rotation of Schardinger's β -dextrin is $[\alpha]_D = 163.2^\circ \pm 0.5^\circ$ and $[\alpha]_{5460} = 189.9^\circ \pm 0.4^\circ$.
2. The most efficient precipitant for β -dextrin is p-xylene. Other relatively efficient precipitants include m-xylene, bromobenzene, α -bromonaphthalene, trichloroethylene, nitrobenzene, nujol, o-xylene and toluene.
3. The velocity constant for the hydrolysis of β -dextrin is 0.46×10^{-4} per min. The velocity constant for the hydrolysis of the α -1,4-glucosidic bond in amyloheptaose is approximately five times as great as for similar bonds in β -dextrin.
4. The amyloheptaose preparation is definitely free from glucose, maltose and amylotriose. The low value of the specific rotation $[\alpha]_D = 175.5^\circ$ compared to the calculated value of 179.6° is due to the presence of inert material, probably water.
5. Macerans amylase is capable of attacking amyloheptaose with the production of α -dextrin and glucose, maltose, amylotriose and other oligosaccharides without the production of additional end groups.
6. Macerans enzyme is capable of converting β -dextrin into α -dextrin with the aid of maltose as a co-substrate.
7. Macerans enzyme is able to convert α -dextrin into

β -dextrin with the aid of glucose, maltose, calcium maltobionate, α -methylglucoside, sucrose and cellobiose as co-substrates for the reaction.

8. The production of the Schardinger dextrans from linear chains and the interconversion of these dextrans in the presence of co-substrates is accomplished by the macerans amylase without hydrolysis and the consequent production of new reducing end groups.

9. Macerans enzyme is synthetic in its action as well as degradative, producing linear chains of sufficient length to give detectable iodine colorations. Amyloheptaose and the Schardinger dextrans do not give appreciable iodine coloration in solution.

10. Sucrose, glucose and gluconic acid do not serve as co-substrates for the conversion of β -dextrin to α -dextrin by the action of macerans enzyme.

11. Previously proposed mechanisms for the action of β -amylase on amylose chains have been confirmed. β -amylase clips off maltose units from the non-reducing end of the molecule converting even numbered straight chains entirely to maltose and odd numbered straight chains to maltose and one molecule of amylotriose.

12. The primary products from the reaction of salivary amylase on amyloheptaose and amylo-dextrin are maltose and amylotriose. Some glucose is present apparently as a result

of the slow hydrolysis of terminal linkages in amylotriase and higher oligosaccharides. Maltose is apparently not hydrolyzed by the salivary amylase preparation used.

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VITA

Melvin Lewis Levine was born in Ames, Iowa, December 2, 1916, the fourth child of Adele (Dion) and Max Levine. His early education was obtained in the public schools of Ames, Iowa. He entered Iowa State College in 1934 and received the degree of Bachelor of Science in Chemistry in 1938. He entered the University of Maryland in 1938 and received the degree of Master of Science in Bacteriology in 1940. He re-entered Iowa State College in 1939 and pursued advanced studies in chemistry under Dr. R. M. Hixon until called to the Service in the Fall of 1941. He returned to Iowa State College in the Winter of 1946 to continue his studies under Dr. Dexter French. In 1942 he married Margaret Mary Fox of Ames, Iowa.

APPENDIX 1

COLORIMETRIC ESTIMATION OF CARBOHYDRATES BY
THE DIPHENYLAMINE TEST

The diphenylamine test is based on the production of a blue color when hexoses or hexose polymers are heated with diphenylamine in the presence of concentrated hydrochloric acid and glacial acetic acid. The method was originated by Ihl (93) for use with fructose. Many quantitative methods using the test have been since developed for fructose (94, 95, 96). The blue color produced as well as the diphenylamine reagent are insoluble in water so ordinarily the methods involve the extraction of the color with some organic solvent such as isoamyl alcohol (95) and comparison of the extract with a standard. The method developed for use with glucose and glucose polymers uses the reagent given by Brown and Zerban (97) as originally developed by Dische (98). This reagent consists of 100 ml. of concentrated hydrochloric acid, 80 ml. of glacial acetic acid and 20 ml. of a 10 per cent solution of diphenylamine in ethyl alcohol.

The quantitative estimation of glucose and its polymers is carried out as follows. One ml. of 95 per cent ethanolic sugar solution and 4 ml. of the reagent are heated together in a 25 ml. volumetric flask in a boiling water bath for 30 minutes. The reaction mixture is cooled rapidly in cold

water and made up to 25 ml. with methanol. The transmission at 640 millimicrons is then determined in a Coleman Spectrophotometer against a blank of the reagent prepared in the same manner but using 1 ml. of 95 per cent alcohol free of sugar. 640 millimicrons was found to be the wave length which gave the minimum transmission when a preliminary absorption spectrum was run using glucose as the sugar.

The per cent transmissions were determined for various concentration, from 0.01 to 1.00 mg. per ml., of glucose, maltose and β -dextrin. The results are plotted in Figure 4 on semi-log paper. The three sugars tested give very nearly the same color density for the same weight. The fact that a straight line is obtained on semi-log paper indicates that the reaction follows the Beer-Lambert laws. Slight departures of the individual sugars from the average curve are of no particular significance in the quantitative chromatographic estimation of the sugars. Any inaccuracy in respect to any particular sugar is compensated for by standardizing the quantitative curves from the chromatographic separation of the sugars against the curve shown in Figure 4. It is also apparent that the percentage errors at low concentrations of sugars will be greater than will those at relatively higher concentrations.

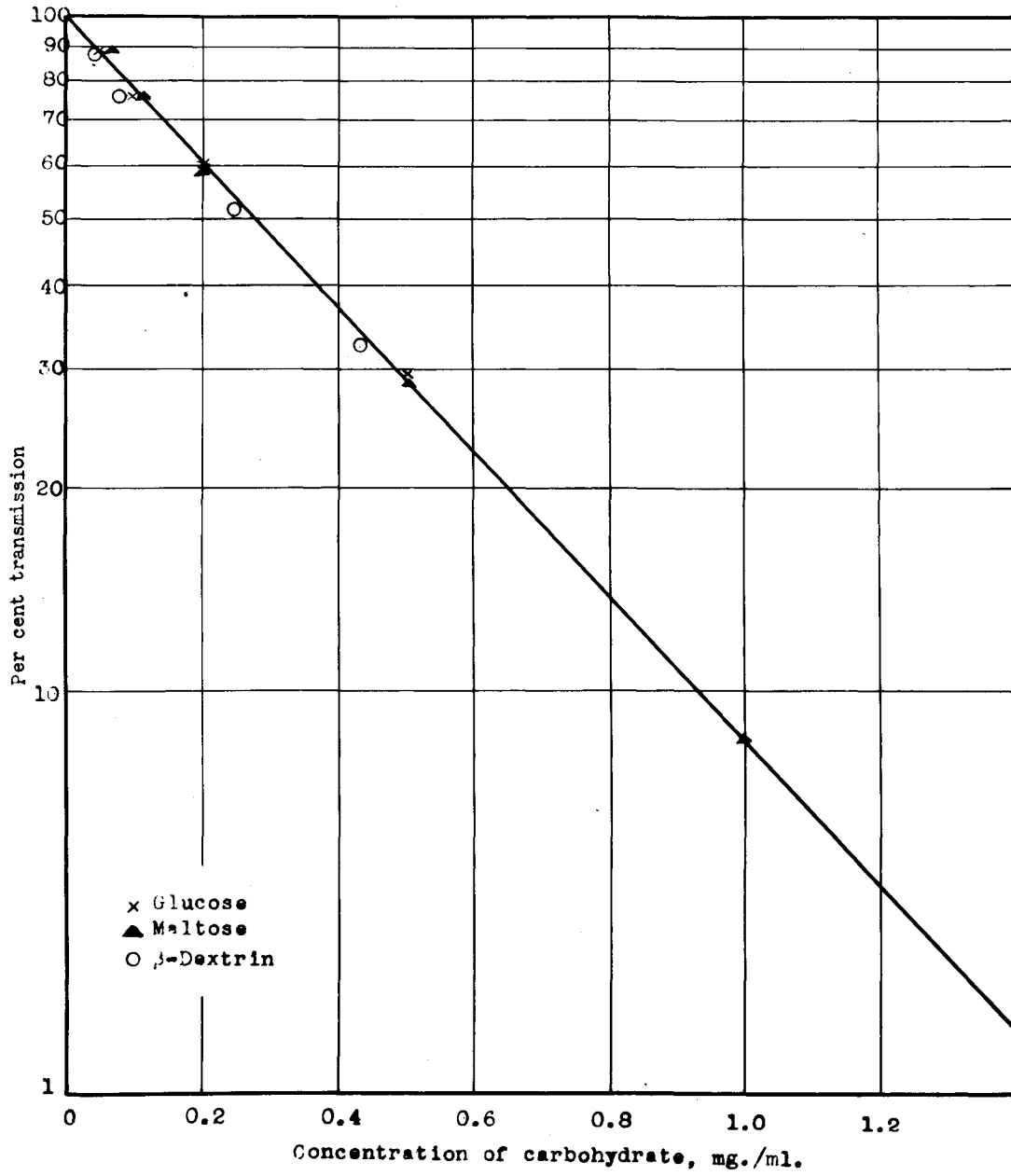


Fig. 4. Percent transmission of various concentrations of glucose, maltose and β -dextrin

Several limitations of the method as developed are apparent. In the first place, since the test is run on sugars dissolved in 95 per cent ethanol, there is a definite limitation of applicability to those carbohydrates somewhat soluble in alcohol. The test gives no differentiation between sugars. This is disadvantageous if there is a possibility of more than one sugar species being present in the carbohydrate. However, for the purposes developed, this is relatively unimportant since all sugars being examined are glucose polymers.

The great advantage of the method is that one may rapidly determine, with an error of about 5 per cent, the carbohydrate content of very small samples. This was the purpose for which the test was developed and the subsequent work with the method has shown it to be extremely useful in this respect.

The usefulness of the test could be extended considerably by first evaporating the sugar solutions to dryness. Thus, the necessity of getting the carbohydrate into 95 per cent alcohol would be avoided and consequently the scope of the test, and perhaps its sensitivity, would be considerably increased.

APPENDIX 2

CHROMATOGRAPHIC ANALYSIS OF MIXTURES FOR
GLUCOSE AND MALTOSE

The chromatographic technique used throughout these researches is a modification of the method developed by Lew, Wolfrom and Goepf (99, 100). These workers investigated the separation of a large number of carbohydrates and arranged them into a "chromatographic adsorption series", listing the compounds in descending order of adsorptive strength. For adsorbent they used a 5 to 1 mixture of Florex XXX and Celite 535 packed in a column 0.9 x 6 cm. They introduced onto the column 1 mg. of the sugars to be separated in 0.5 ml. of developer solution. In the separation of glucose and maltose they used 95 per cent alcohol and achieved separation by developing with 4 ml. of the same strength alcohol. They determined the position of the sugars on the column by streaking the extruded column with a reagent such as alkaline permanganate. After locating the bands, the column was sectioned, the sugar eluted. Where the sugar was present in microquantities estimation of the amount present was made by measuring reducing power by a suitable volumetric method. If sufficiently large quantities of carbohydrate were separated, actual crystallization of the fractions was achieved.

The chief modification of the above method was to develop the column exhaustively with a lower concentration of alcohol than was used to put the carbohydrate on the column. Successive portions of the eluate were then analyzed by the quantitative diphenylamine colorimetric method previously outlined (Appendix 1). The concentration of the sugar was then plotted against the milliliter portion of eluate in which it came through the column.

The following procedure was used in making all analyses. 5 ml. of 95 per cent ethanolic sugar solution or suspension was introduced onto a 12 gram column of a 3 to 1 mixture of Florex XXX and Celite 535 packed wet in a 16 mm. diameter column. Pressure was applied until the surface of the liquid had fallen to 1 to 2 mm. above the surface of the adsorbent column. Sufficient 90 per cent ethanol was then added through a large dropping funnel connected with the top of the adsorbent column to complete the development. Pressure was again applied and the volume of eluate coming through the column measured in a graduated cylinder. Half milliliter portions were collected periodically and used to determine the amount of sugar by the diphenylamine method after making up to 1 ml. with absolute alcohol. The concentration of sugar was then plotted against the milliliter portion of eluate from which it was collected. Figure 5 shows the curve obtained

with several concentrations of glucose. Figure 6 gives the curve for maltose and Figure 7 gives the curve for a known mixture of glucose and maltose with the individual curves for glucose and maltose plotted for reference. Figure 8 shows the maximum amount of sugar in the eluate plotted against the concentration of sugar per 5 ml. introduced onto the column for glucose and maltose. From this curve it is possible to read the actual concentration of sugar introduced onto the column from the elution curve obtained colorimetrically.

The following pertinent observations are significant in using the method for qualitative and semi-quantitative work. (1) The position of the curve for glucose and maltose is relatively constant regardless of the initial concentration of the sugar solution used. (2) The amount of developer passed through the column which gives maximum sugar concentration increases as the concentration of the sugar introduced onto the column decreases. (3) The maximum sugar concentration in the eluate for a given concentration of sugar introduced onto the column is constant and independent of the presence of other sugars. (4) The ratio of the maximum concentration of sugar in the eluate to the original concentration of sugar introduced onto the column is constant for any given sugar.

The main disadvantage of the method as developed is

the limited solubility of most polysaccharides in 95 per cent alcohol. When glucose and maltose are used, the method is satisfactory for chromatographing as much as 100 mg. of sugar. However, with the higher oligosaccharides, much lower concentrations must be used. Furthermore, when much insoluble material is present the chances of retaining the more soluble sugars in considerable amounts by occlusion are great. This, naturally, leads to low results in the analysis. It is felt that more satisfactory quantitative results could be obtained by this method by introducing the carbohydrate onto the column in more dilute alcoholic solutions and developing with the same alcohol concentration.

It is possible that the size and perhaps the composition of the column would have to be altered to meet the changed conditions. However, many advantages would be gained by extending the method to cover all the oligosaccharides up to and including amyloheptaose or even higher polysaccharides.

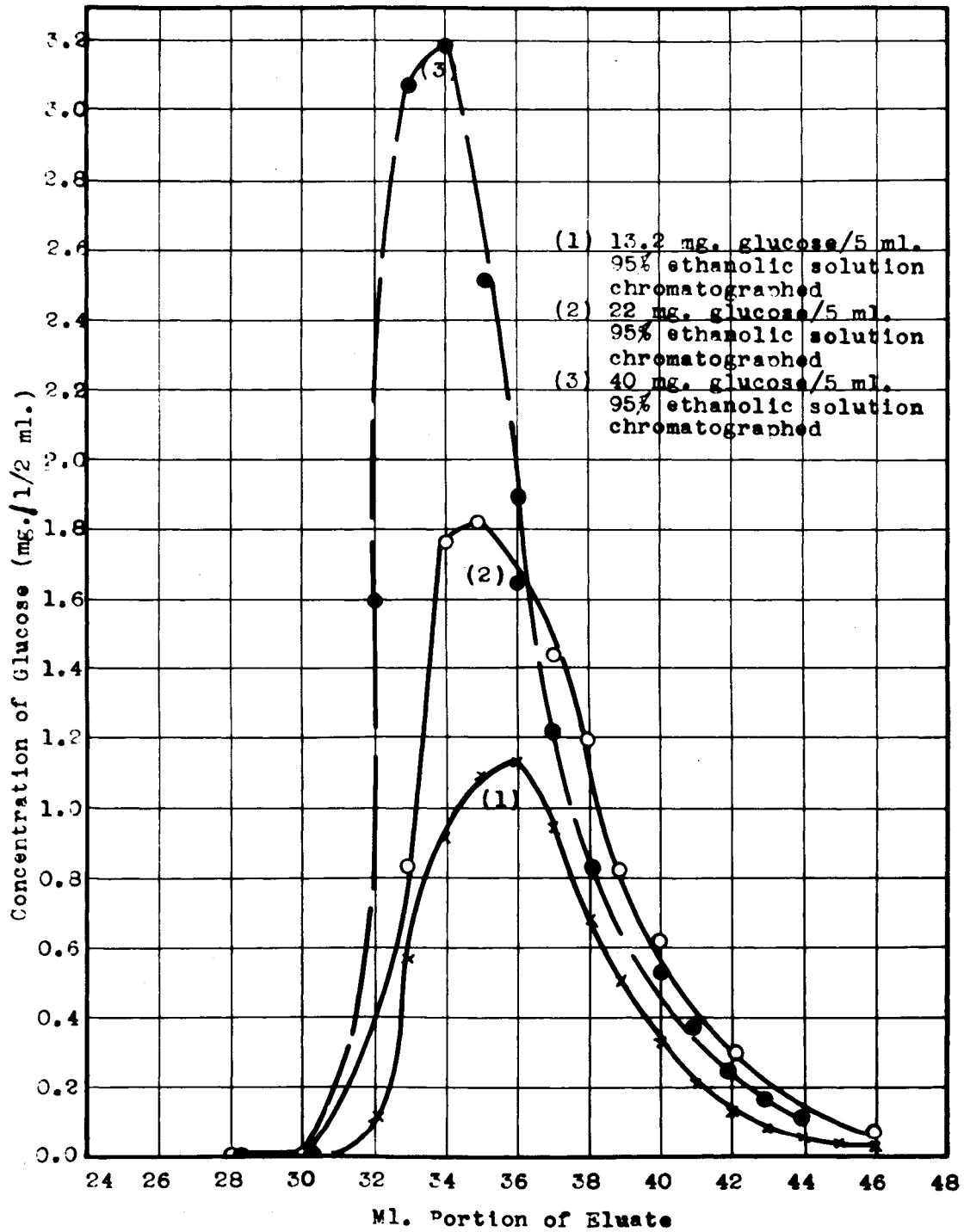


Fig. 5. Concentration of glucose in eluate from chromatographic column

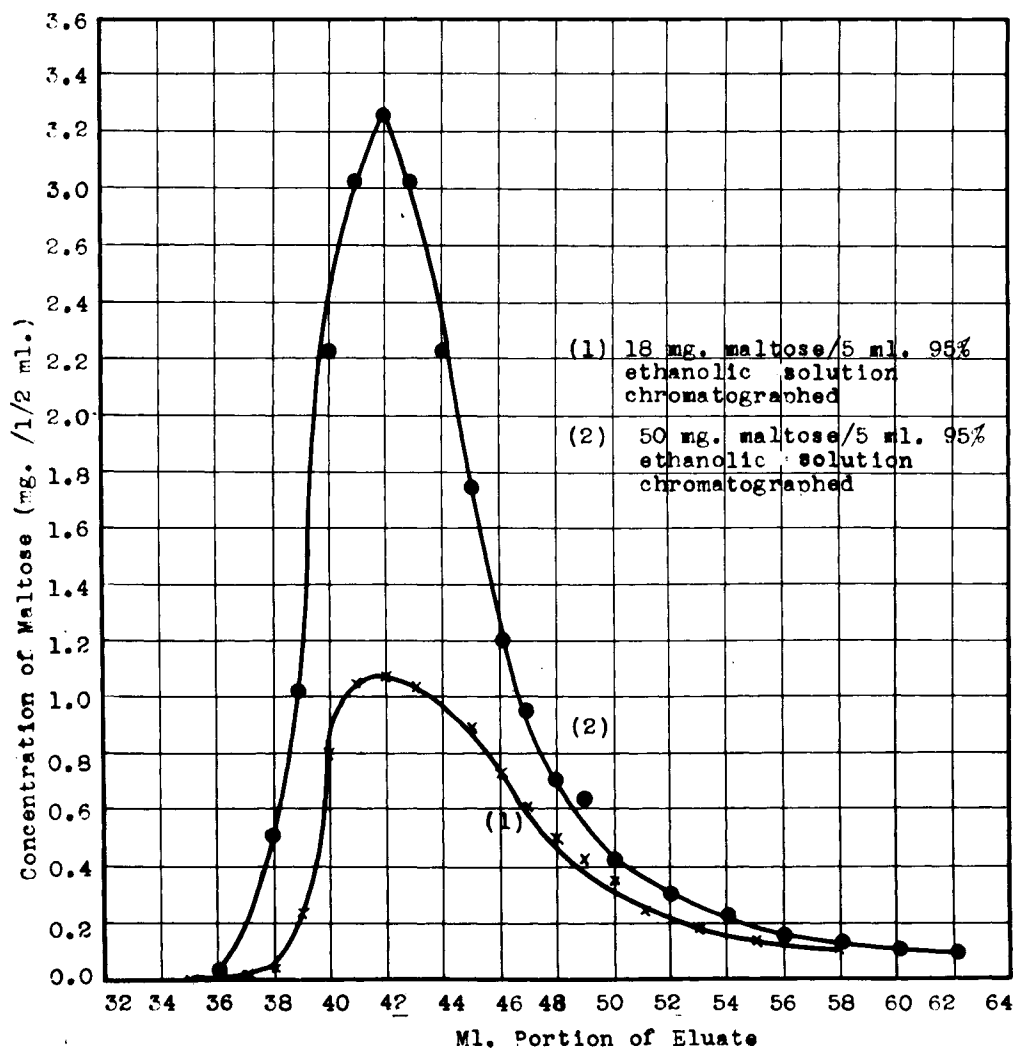


Fig. 6. Concentration of maltose in eluate from chromatographic column

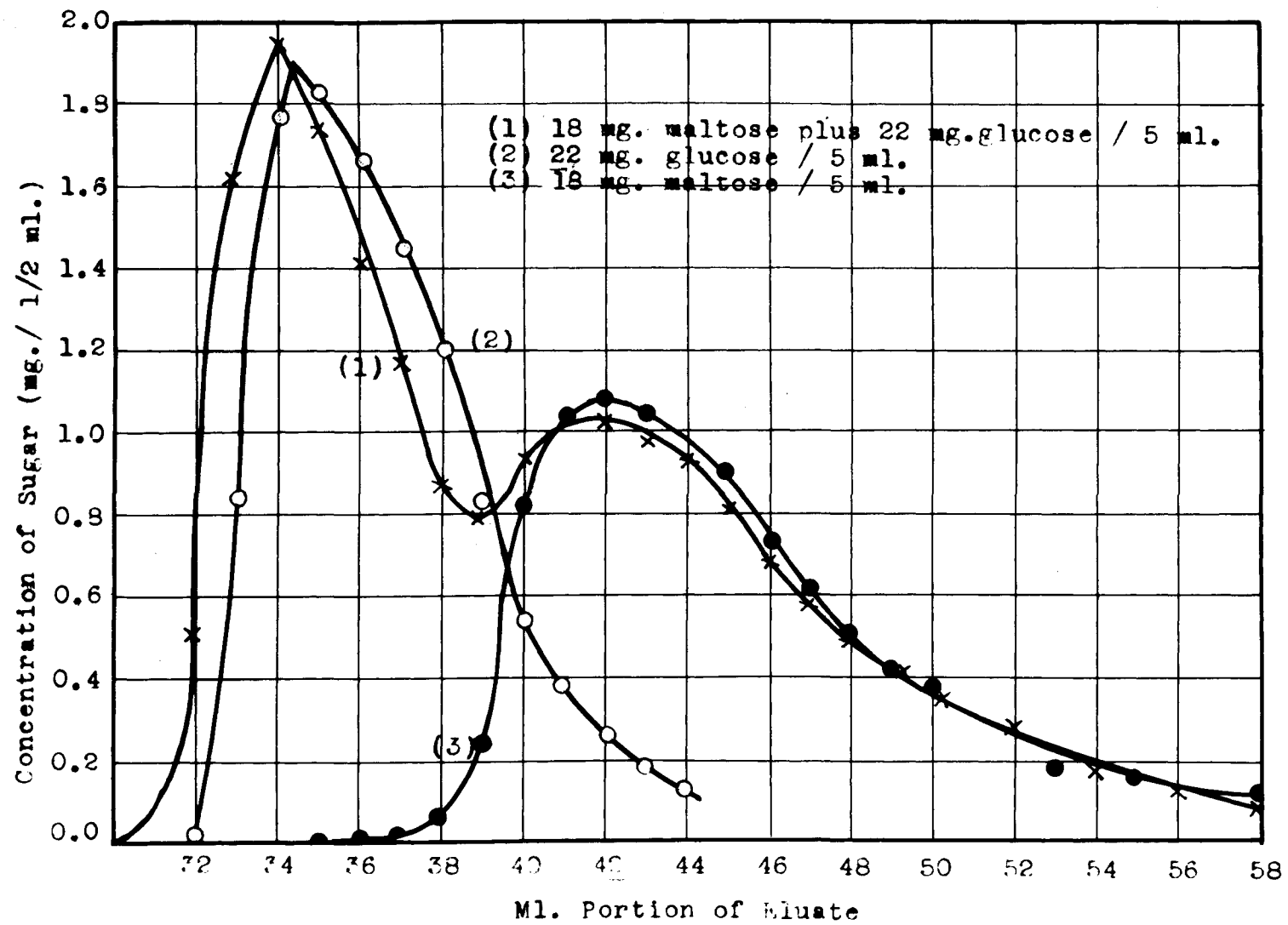


Fig. 7. Chromatography of glucose and maltose

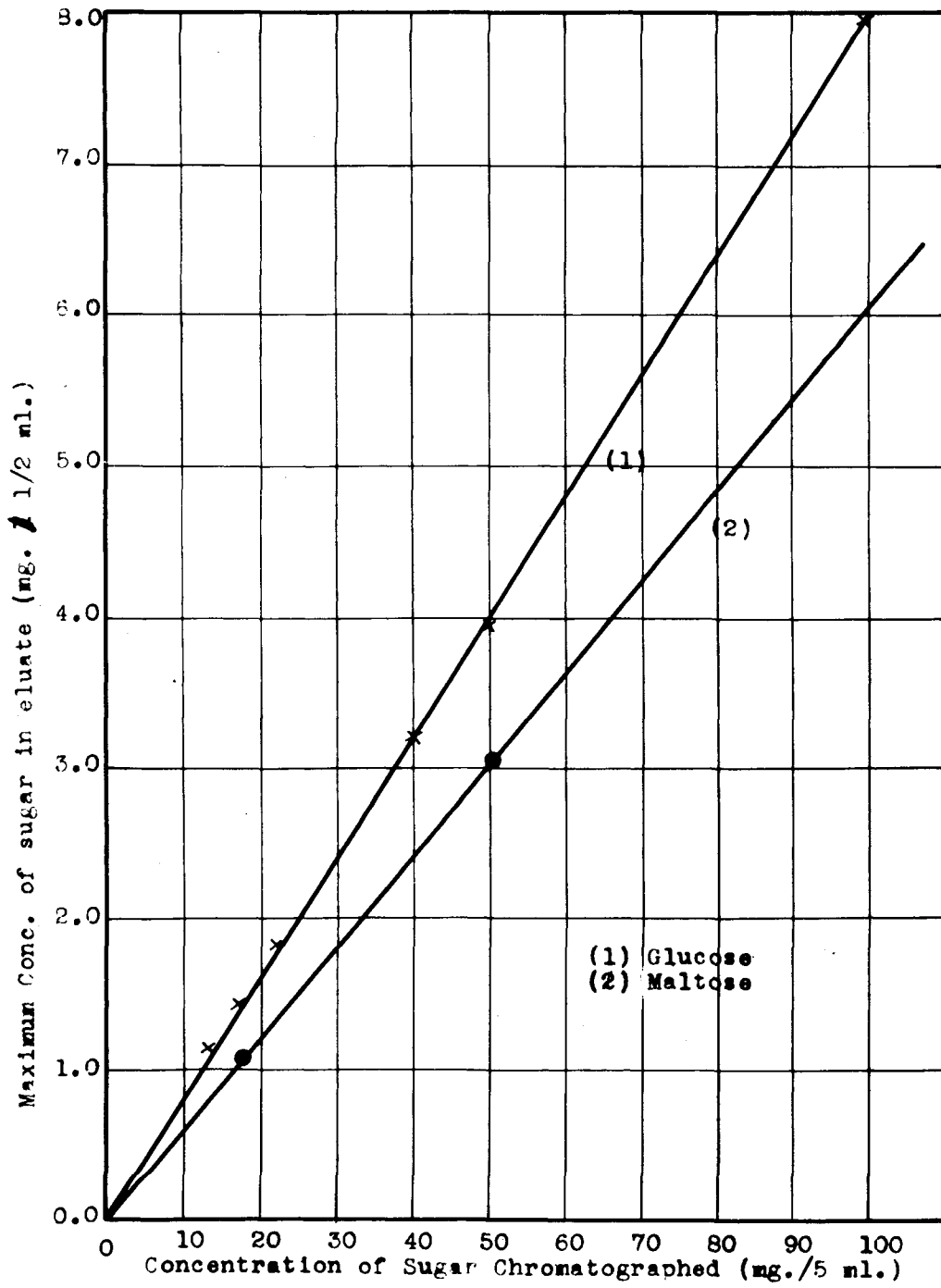


Fig. 8. Relationship between maximum conc. of sugar in eluate and original conc.