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Action of [Beta]-amylase on branched oligosaccharides

Russell E. Summer
Iowa State College

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134 ACTION OF β -AMYLASE ON BRANCHED OLIGOSACCHARIDES

by

Russell E. Sumner

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Head of Major Department

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Dean of Graduate College

Iowa State College

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INTRODUCTION

The great interest and activity in the study of starch stems from its biological as well as its industrial importance. It can be considered as a reservoir of energy for plants and animals alike. Therefore, its constitution, breakdown and synthesis are important biological problems. In the industrial field, starch has many diversified applications. To name just a few, it is used in the sizing of paper and textiles. It is also used as an adhesive, and also finds applications in many different food products. Starches from various sources differ considerably in their composition. Therefore, the source must be taken into consideration in many of the applications of starch. Furthermore, industry has modified starch both chemically and enzymatically in attempts to obtain products which better meet the requirements of farm, home and technology. A study of the structure and modification of starch is of great importance in all of these applications.

The structure of amylopectin, the branched component of starch, has been studied by both chemical and enzymatic methods. Glycogen, sometimes called animal starch, is very similar in structure to amylopectin, and has been studied in much the same way. A helpful method in the investigation of both of these structures has been partial degradation by β -amylase.

It is the purpose of this thesis to investigate the action of β -amylase as it approaches the α -1,6 linkages in an amylopectin type molecule. It is impossible to follow the detailed action on the high molecular weight natural products, and to circumvent this obstacle, a series of low molecular weight branched oligosaccharides were synthesized. These so-called model substrates were analogous to certain of the branch points found in the natural molecule, and their size made the study of β -amylase action much simpler. It should be kept in mind that the enzyme may act somewhat differently on amylopectin and glycogen than on the model substrates. However, bonds which are apparently resistant in the low molecular weight compounds would certainly be resistant in the natural molecules. Any differences would probably be concerned with the rate of enzyme action, rather than with the specificity.

REVIEW OF LITERATURE

Amylopectin and Glycogen

Amylopectin as one of the constituents of starch has been the subject of many investigators in the past fifty years. Since only about 80 percent of starch is converted into fermentable sugars by malt extracts, Maquenne and Roux¹ in 1906 suggested that there might be two components in starch. The resistant material which remained after fermentation was designated as "amylopectin". This interpretation was widely criticized, and as Meyer² pointed out, the possibility of partial degradation was ignored. Therefore, the discovery of the heterogenous nature of starch should not be ascribed to Maquenne and Roux.

Karrer and Kraus³ attempted to separate the components of starch by centrifugation of pastes, but no structural differences could be found in their fractions. Ling and Nanji⁴ described a similar method, but when Hirst et al.⁵ subjected fractions obtained by this method to acetylation and methylation experiments, an average chain length of 24 was obtained for both fractions. Samec⁶ was able to separate the components of starch by electrodecantation, and found that the component which moved to the anode ("amylopectin") was degraded to the extent of 60 percent by β -amylase. The component which remained in solution was called "amylo-amylose" and was completely degraded by

β -amylase. Meyer⁷ was successful in separating starch by extracting the amylose from starch with warm water. Meyer found that the soluble fraction was linear, and had a degree of polymerization of about 250, while the insoluble component consisted of a high molecular weight branched polysaccharide as established by methylation studies. Although both of these methods were imperfect, the heterogeneity of starch was definitely established. Freudenberg,⁸ using the methylation procedure, was unable to find any differences in the fractions separated by Samec's method.

The best methods of separation of starch are those employing specific precipitating or complexing agents as described by Schoch.^{9,10} It was found that various alcohols such as butanol were capable of complexing with the amylose fraction, and that the resulting complex precipitated from solution.

More recently Meyer and Gibbons¹¹ have fractionated amylopectin from potato starch by electro dialysis in the presence of iodine. These fractions, however, do not show any distinguishable chemical differences, and have degrees of polymerization ranging from 1,200 to 1,600. Meyer and Gibbons¹² have also attempted to remove small amounts of amylose which occur in amylopectin by adsorption on solid stearic acid. Gilbert et al.¹³ indicate that no selective precipitation of amylose occurs with stearic acid, and that the apparent purification noted by Meyer was due to retention of stearic acid by amylose. Cellulose was also found to be ineffective for the removal of residual

amylose.

In 1814 Kirchoff¹⁴ crystallized a new sugar, maltose, from a digest of starch with malt extracts. The structure of maltose was definitely established by Haworth.¹⁵ This led to the general belief that in starch there is a polysaccharide made up of glucose units linked together in maltose linkages. Using the methylation technique, Haworth found an end group for every 20 glucose units in starch,¹⁶ and an end group for every 12 glucose units in glycogen.¹⁷

Staudinger¹⁸ took up the idea of a branched structure for starch which was first conceived by Meyer.² Staudinger proposed a structure in which outer chains were attached to one main chain at various intervals, as in Fig. 1. Haworth¹⁹ proposed a laminated type of structure for both amylopectin and glycogen, Fig. 2.

Kalsell et al.²⁰ determined the end groups in glycogen by measuring the amount of formic acid produced on oxidation with potassium periodate. They found that most glycogens contain 12 glucose residues per end group. Potter and Hassid²¹ used a similar method on amylopectin, and found 22 to 27 glucose residues per end group for various amylopectins. Meyer and Rathgeb²² carefully investigated the necessary conditions for achieving best results when using the periodate oxidation on polysaccharides.

Hyrdöck and Ahlberg²³ found that α -1,6 bonds occur in the low molecular weight dextrans obtained from the α -amylase digest of

starch and glycogen. Gibbens and Blossomes²⁴ were able to demonstrate that amylopectin and glycogen contain less than one bond linked in position two or three, for 40 bonds linked in position six. Wolfson and co-workers²⁵ acetylated a hydrolyzate of glycogen, and isolated a number of acetates by chromatography. β -Isomaltose octaacetate was isolated in crystalline form, thereby putting the α -1,6 linkage on a more definitive basis. Bell and Manners²⁶ subjected various glycogen samples to prolonged periodate oxidation followed by acid hydrolysis. Since no significant amount of glucose was detected, they concluded that these glycogens contained only α -1,4 and α -1,6 linkages. Peat *et al.*²⁷ have been able to detect only α -1,4 and α -1,6 bonds in yeast glycogen by the isolation of the products of partial acid hydrolysis on charcoal.

According to Meyer,² amylopectin has a high degree of polymerization, and varies with the source from 250 to 1,850. Greenwood²⁸ indicates that glycogens are polydisperse with degrees of polymerization ranging from 1,000 to 130,000. The degree of polymerization is dependent on the source, the method of extraction, and the method of measurement. Recently Meyer and Settele²⁹ report finding low degree of polymerization amylopectin fractions in waxy maize and tapioca. By the use of β -amylase degradation and periodate oxidation, the outer branches of waxy maize were found to range in average chain length from 9 to 17 for these various fractions. The inner chain length, meanwhile, remained fairly constant at (7.5 ± 1.5) . The

determination of the average degree of polymerization of polysaccharides by the action of 3,5-dinitrosalicylic acid has recently been restudied by Meyer *et al.*³⁰ They found that the results from the determination give a degree of polymerization which can only be considered as a lower limit, and the error involved in the determination increases with an increase in the real degree of polymerization.

The use of enzymes for investigation of the structures of amylopectin and glycogen was found to be extremely helpful by Meyer and Bernfeld.³¹ By using β -amylase they obtained a β -dextrin which was degraded to the extent of 65 percent, but which still contained all the branch points and end groups.³² They also found an enzyme which was able to break the α -1,6 bonds in the β -dextrin. After the action of the enzyme, it was possible to get further degradation by β -amylase. It was concluded from this work that the branch points constitute blocks to the action of β -amylase, and furthermore that a highly ramified structure is the only one which will explain all the facts concerning amylopectin, Fig. 3.

In a similar investigation of glycogen, Meyer³³ found that glycogen contains one end group for every 11 glucose units, while after the degradation by β -amylase to the extent of 47 percent, only one end group for every five and one half glucose units is found. From his work on glycogen Meyer concluded that there are six to seven glucose units in the outer chains of glycogen, and three glucose units in the inner chains.

In 1939 Morris and Morris³⁴ reported a water soluble polysaccharide found in a variety of golden bantam corn which has properties identical with those of the glycogens from animal sources. Dvornch and Whistler³⁵ investigated this polysaccharide and also found it to be glycogen-like, but suggested that it be considered an amylopectin to avoid confusion.

Cori and Larnar³⁶ described a method for the degradation of branched polysaccharides in animal tissue by the successive action of phosphorylase and a special glucosidase specific for α -1,6 links. The branched polysaccharide is degraded simultaneously with phosphorylase and α -1,6 glucosidase. The ratio of the glucose produced to the free glucose plus phosphorylated glucose is a measure of the degree of branching. By successive action of phosphorylase and glucosidase, Illingworth et al.³⁷ were able to investigate the internal as well as the external structure of glycogens and amylopectins. These investigations substantiated the highly ramified structures of both glycogen and amylopectin originally proposed by Meyer.

Bell and Manners³⁸ have investigated the action of crystalline β -amylase on glycogen with a chain length of 12. With the crystalline sweet-potato enzyme the glycogen was degraded to the extent of 45 percent, with only maltose and a limit dextrin produced. Their evidence indicates an irregularly branched structure for glycogen with average exterior chains of seven glucose units and average interior chains of four glucose units. Manners³⁹ also reports that glycogen does not contain 2-labile linkages (i.e. β -D-glucopyranoside

groups).

Peat and co-workers⁴⁰ isolated the β -limit dextrin of waxy maize starch and treated it with R-enzyme. An isolation of the products of the dialyzable portion of this digest gave a molar ratio of 1.45 maltose to 1.00 maltotriose plus maltohexaose and higher maltodextrins. The total yield of maltose plus maltotriose indicates, that of the proposed structures for amylopectin, only Meyer's fits this data. Whelan⁴¹ degraded glycogen and amylopectin with α -amylase, and then treated the digest with R-enzyme. He interpreted the number of free reducing groups liberated by this treatment as being equal to the number of 1,6 linkages in the original polysaccharide. Peat et al.⁴² found that R-enzyme debranches amylopectin, but does not exert a similar effect on glycogen from oysters or rabbit liver. It was suggested that this effect was due to the high degree of branching or more compact structure of glycogen. It was also suggested that this difference in the action of R-enzyme could be used as a simple practical method for distinguishing between amylopectin and glycogen.

In an extension of Peat's work with R-enzyme, Hirst and Manners⁴³ were able to obtain a quantitative estimate of the degree of multiple branching in amylopectin. If one defines the A, B, and C chains as follows, it is possible to get an estimate of the ratio of A:B chains by using this method:

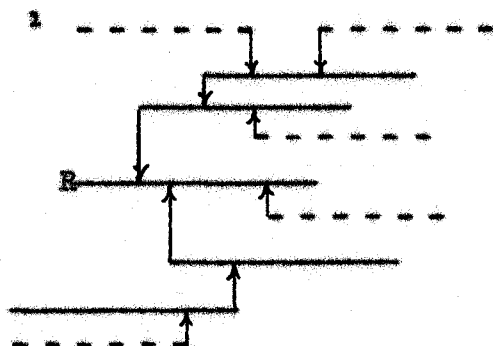


Fig. 1. Meyer's structure

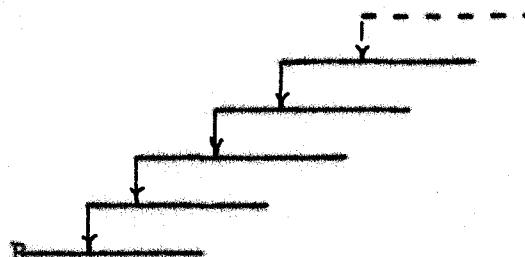


Fig. 2. Haworth's structure

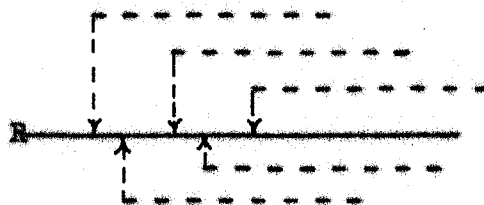


Fig. 3. Staudinger's structure

A = - - - - -
 B = _____
 C = R_____

R = reducing end group
 - and - = α -1,4 linked
 glucose units
 ↓ = α -1,6 linkage

The observed ratio of A:B chains in waxy maize amylopectin is approximately 1:4. This would indicate that only one unit-chain in every five contains more than one branch point. The structure then would be similar to Meyer's concept, but more elongated and less like a compact "tree" structure. It is also suggested that there is a

fundamental difference in the arrangement of the unit-chains in glycogen and amylopectin which may be due to different degrees of multiple branching.

β -Amylase

In 1877 Märker⁴⁴ reported that by varying the temperature of malt digests of starch, two different types of action were evident. In the first case mainly maltose was produced, along with a lesser amount of dextrin; while in the second case mainly dextrin was produced with a lesser amount of maltose. From these observations Märker deduced that there were two enzymes present in malt. Wijsman⁴⁵ was able to demonstrate the presence of these two enzymes by a unique method. A drop of malt extract was placed on a starch-containing gelatin plate, and the surface of the plate was then washed with an iodine solution. At the point where the malt was applied, a colorless circle was formed with a red staining ring encircling the colorless area. The rest of the plate stained dark blue. Due to the different rates of diffusion of the two enzymes, Wijsman was able to demonstrate the presence of two different enzymes in malt.

Kuhn⁴⁶ called the two enzymes α - and β -amylases. The first enzyme formed maltose which mutarotated downward, or maltose in the α configuration, while the second formed maltose which mutarotated upward, or maltose in the β configuration. Ohlsson⁴⁷ was able to

separate the two enzymes, and came to the conclusion that β -amylase was able to act from the non-reducing end of a chain, splitting off maltose units in the β configuration. Hanes⁴⁸ also supported this view concerning the mechanism of action of β -amylase.

In 1946 Ballis and co-workers⁴⁹ were successful in obtaining the first crystalline amylase, which was the β -amylase from sweet potatoes. In 1951 Meyer *et al.*^{50,51} were successful in purifying and crystallizing β -amylase from malt. The purification and crystallization of β -amylase from wheat flour was reported by Meyer and co-workers⁵² in 1953. Peat *et al.*⁵³ were able to obtain a purified soy bean β -amylase as an amorphous powder which was virtually free from α -enzyme as well as α -amylase and maltase. It was also found that for purposes of structural analysis the purified soy bean β -amylase was exactly equivalent to crystalline sweet potato β -amylase.

England and Singer⁵⁴ reported that eight times recrystallized sweet potato β -amylase indicated only one component on electrophoresis, but that 3 percent of an impurity was detected by ultracentrifugation. The molecular weight of sweet potato β -amylase, as determined by measurements of the sedimentation velocity and diffusion rate, is $152,000 \pm 10$ percent. Meyer *et al.*⁵² found only one component on electrophoresis of crystalline wheat β -amylase. This enzyme was also found to be free from sugars, polysaccharides, α -amylases, maltase, and β -glucosidase.

England and co-workers⁵⁵ have shown that chemical alteration of the sulphydryl groups of sweet potato β -amylase by specific reagents results in complete loss of enzymatic activity. Oxidative inactivation is thought to proceed by an intramolecular mechanism. Manners⁵⁹ lists the following reagents as inhibiting the action of β -amylase: heparin, sodium decedecyl sulphate, sodium cyanide, and ascorbic acid.

In 1931 Samec and Waldschmidt-Leitz⁵⁶ found that "amylomylase" is quantitatively degraded to maltose by β -amylase. Later, Meyer et al.² confirmed the earlier work on β -amylase stating that it does, indeed, act from the non-reducing chain end, and completely degrades amylose to maltose. It was Meyer's feeling that the finding of incomplete degradation of amylose by various workers was due to the use of impure samples of amylose. Whelan and co-workers⁵⁷ separated the products of acid hydrolysis of potato amylose on a carbon column, and subjected the products to the action of crystalline sweet potato β -amylase. Maltose was not degraded, maltotetraose formed maltose, maltopentaose formed maltose and maltotriose, while maltohexaose formed maltose. Bailey et al.⁵⁸ found that maltotriose was attacked by β -amylase, but at a very slow rate. Whelan and Bailey⁵⁹ reported that maltotriose was not attacked in the rapid phase of β -amylolysis, but was attacked slowly to form one mole of maltose and one mole of glucose. Later Whelan and Roberts⁶⁰ reported that studies of the action of β -amylase on various maltodextrins were in complete accord with the postulated course of action of β -amylase. Whistler and

Hickson⁶¹ isolated maltotetraose from corn sirup by column chromatography, and characterized this dextrin by various chemical methods. They found that β -amylase hydrolyzed maltotetraose to maltose to the extent of 87 percent. Later, Whistler and Duffy⁶² isolated and rigidly characterized maltopentaose from corn sirup. Maltopentaose was hydrolyzed by β -amylase to 34.3 percent maltose and 52.9 percent maltotriose.

In 1952 Peat and co-workers⁶³ found that crystalline sweet potato β -amylase converted potato amylose to the extent of 70 percent into maltose. This was in direct contrast to crude β -amylase which was capable of complete conversion. It was deduced that crude β -amylase must contain a so-called Z-enzyme which was capable of hydrolyzing an anomalous linkage in amylose. Later Peat et al.⁶⁴ were able to obtain Z-enzyme from soy bean as a freeze-dried powder. This preparation was free from α - and β -amylases, and was shown to be a β -glucosidase. Hopkins and Bird,⁶⁵ in an examination of Peat's evidence, suggested that the Z-enzyme might really be an α -amylase. Peat and Whelan⁶⁶ answered these arguments, and presented further evidence that Z-enzyme does have a β -glucosidic action.

Meyer and co-workers^{31,32,67} investigated the action of β -amylase on the branched component of starch, amylopectin. Amylopectin was reported as being degraded to the extent of 65 percent by β -amylase. The products of this degradation were maltose and a high molecular

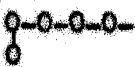

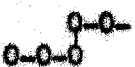
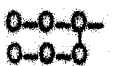
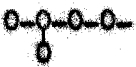


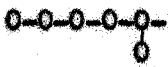
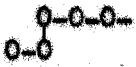


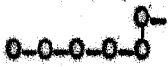

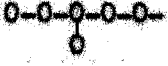
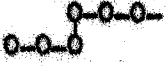

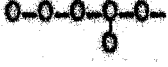
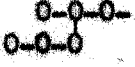

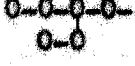
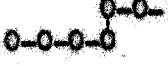



weight residual or limit dextrin. This dextrin was reported to be resistant to further attack by β -amylase, and thought to consist of 45 percent of the original amylopectin. Furthermore, by using the methylation technique, Meyer reported that the residual dextrin contained all the end groups and consequently all the branch points found in the original amylopectin molecule. Meyer therefore concluded that the branch points, or α -1,6 linkages, constitute hindrances to the action of β -amylase, and that β -amylase is only able to attack the longer chains. If the residual dextrin is degraded with an α -glucosidase (maltase from yeast), glucose is formed along with a dextrin of high molecular weight. This dextrin is no longer resistant to β -amylase, and can be degraded further with the production of maltose and a second limit or residual dextrin. The action of β -amylase on glycogen is similar to the action on amylopectin except that native glycogen is degraded only to the extent of 45 percent by β -amylase. From this work Meyer concluded that on the average, the stub left in a β -limit dextrin of either amylopectin or glycogen would contain 1.5 glucose units. According to Meyer³³ and Bernfeld⁶⁸ the end groups of the β -amylase limit dextrin of both glycogen and amylopectin must be of the four following types:



¹ O- signifies a glucose unit with its reducing group.
 ...O-O... signifies two glucose units bonded with an α -1,4 linkage.

$\begin{array}{c} \text{O} \\ | \\ \text{---O} \end{array}$... signifies two glucose units bonded with an α -1,6 linkage.

Table 1. Dextrins from acid hydrolysis

Group 1	Group 2	Group 3	Group 4
			
			
			
			
			
			
			

Myrbäck and Sihlbom⁶⁹ arrived at somewhat different conclusions by examining the action of β -amylase on the low molecular weight oligosaccharides of an acid hydrolysis of starch found in Table 1. The oligosaccharides were separated into fractions of varying degrees of polymerization by alcohol precipitation. It was concluded from this work that β -amylase acts only from the non-reducing end of the chain, and the isomaltose linkage constitutes a block or hindrance to the enzyme. Myrbäck and Sihlbom suggested that no maltose is

years considerable discussion has arisen concerning the possibility of either the single-chain or multi-chain mechanism of action. In the single-chain mechanism the enzyme would act by completely degrading one substrate molecule at a time, while in the multi-chain process there would be a simultaneous attack of all chains.

Swanson⁷⁰ followed the action of β -amylase (wheat) on amylose by examining the products formed at intermediate stages of hydrolysis. No short chain intermediates could be found, and this observation led to the suggestion that the mechanism of β -amylase action was single-chain. In a later paper Swanson⁷¹ reported the change in spectra of the iodine complex during the course of a digestion of amylose with β -amylase. It was found that the height of the absorption curve steadily decreased, but did not shift its peak from 650 millimicrons. This observation also suggested a single-chain mechanism. Cleveland and Kerr,^{72,73} and Kerr⁷⁴ in similar experiments also supported the single-chain mechanism.

Hopkins and co-workers,⁷⁵ in a study of the kinetics of the action of β -amylase on potato amylose, suggested that the longer chains were attacked preferentially and more rapidly. Since the hydrolysis occurred at a steadily declining rate, multi-chain action was indicated. Bourne and Whelan⁷⁶ reviewed the work of Swanson, Cleveland and Kerr, as well as that of Hopkins et al., and came to the conclusion that the multi-chain mechanism was the more probable mode of attack. French and

co-workers⁷⁷ attacked the problem by following the action of β -amylase (soy bean) on maltoheptaose prepared from the β -Schardinger dextrin. Under conditions of temperature and pH which were favorable to the enzyme, only maltose and maltotriose were produced. At no time during the course of hydrolysis was any great amount of maltopentaose observed. This seemed to imply a single-chain mechanism, but French *et al.*⁷⁸ found that by using unfavorable conditions of temperature or pH the initial products of a β -amylase digest of maltoheptaose were maltose and maltopentaose. Eventually, the maltopentaose disappeared, forming maltose and maltotriose. Recently, Hopkins and Jelinek⁷⁹ have determined the Michaelis constants for β -amylase on potato amylose and its short chain fission products. It was found that they are of the same order of magnitude, indicating that β -amylase does not really have a greater affinity for either short or long chains. Bird and Hopkins⁸⁰ studied the action of β -amylase on amylose fission products ranging in degree of polymerization from 16 to 30. The results of this work were consistent with the multi-chain mechanism. It was also found that maltohexaose was attacked by β -amylase with the formation of maltose and maltotetraose. The maltotetraose accumulated until all of the maltohexaose was gone, and then it was hydrolyzed to maltose. It was reported in this work that β -amylase slowly hydrolyzed amylotriase.

A new method for the determination of multi-chain or single-chain action was reported by Whelan.⁴¹ One of the problems involved

in the differentiation of these two modes of attack, was the preparation of a homogeneous amylose-type substrate. This difficulty was overcome by preparing the substrate through the action of phosphorylase on glucose-1-phosphate using maltopentaose as a primer. It was found that these preparations did not vary widely from the average in their distribution of molecular weights. A 49 unit polysaccharide was degraded with β -amylase at varying conditions of temperature. The digest was followed by the change in the peak wave length (λ maximum) of the iodine stain at various intervals. If a strictly single-chain mechanism is followed, one would expect λ maximum to remain constant, for at any one time only unchanged substrate and maltose would be present. If a multi-chain mechanism is the case, λ maximum would change gradually during the course of digestion. It was concluded from these observations that the action is never of one type, but varies continuously as the temperature changes. As originally suggested by French, it appears that the reaction is most nearly multi-chain at extreme temperatures.

Recently Bailey and French⁵¹ have worked out a novel procedure for demonstrating the multiple attack of β -amylase. An amylose-type substrate having an average chain length of 43.7 glucose units was synthesized from glucose-1-phosphate and maltoheptaose using potato phosphorylase. This polysaccharide was then used as a "primer" with radioactive glucose-1-phosphate until an average of 0.22 more glucose units had been added to each molecule. It was calculated that this

degree of substitution gave a product with 80.25 percent of the chains containing no radioactive glucose, 17.62 percent containing one radioactive glucose unit in the terminal position, and 1.94 percent containing two radioactive glucose units. The distribution of more highly substituted chains fell off sharply. This tagged 44 unit chain was then subjected to the action of crystalline sweet potato β -amylase. The specific activity of the maltose formed at various degrees of conversion was determined. From this data and an analysis of the kinetics of β -amylase action, it was found that 4.3 maltose units were removed for each effective encounter of enzyme and substrate.

There are two alternative explanations for this action: (1) multiple successive reactions per encounter, or (2) multiple active centers per enzyme molecule. In order to distinguish between these two possibilities, a 35.0 unit polysaccharide was prepared which was labeled in the eighth glucose unit from the reducing end. When this polysaccharide was subjected to the action of β -amylase to the extent of 2.23 percent conversion, it was found that the maltose formed had a specific activity of 0.146 times that of the remaining polysaccharide. A theoretical value calculated on the basis of a multiple attack per encounter was 0.1446. The theoretical value for the hypothesis of multiple active centers was 0.0043. Therefore, this agreement between the observed and calculated value for the ratio of specific activity of maltose to that of polysaccharide substantiates the hypothesis of

multiple attack per encounter.

Macerans Amylase

Schardinger⁸² in 1908 isolated an organism capable of producing crystalline dextrans from starch, and named it Bacillus macerans. It was found that the dextrans from various starches were the same, and a procedure for separating two different crystalline dextrans was developed. The two dextrans were named α and β , and Schardinger found that the dextrans formed rather unusual crystalline iodine complexes.

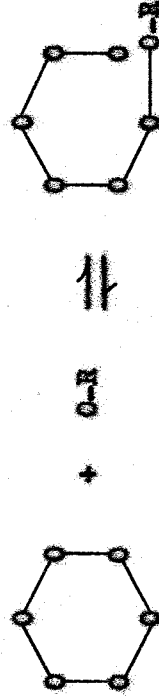
Freudenberg⁸³ in studies on the structure of the "Schardinger dextrans" came to the conclusion that the α -dextrin was an α -1,4 linked cyclic glucose polymer, containing five glucose units. The β -dextrin was also thought to be a cyclic glucose polymer, but containing six glucose units. French and Rundle⁸⁴ found, by using x-ray diffraction and crystal density measurements, that the α - and β -dextrans contained six and seven glucose units respectively, rather than five and six as originally reported. Freudenberg⁸⁵ at first bitterly opposed this work, and presented various arguments to refute the evidence of French and Rundle. However, Freudenberg⁸⁶ eventually presented evidence which substantiated the findings of French and Rundle.

Tilden and Hudson⁸⁷ obtained a fluid from a culture of Bacillus

macerans which contained an enzyme capable of producing the dextrins without the simultaneous formation of glucose, maltose, or other reducing sugars. It was inferred from this work that the dextrins were not products of synthetic metabolism of the organism, but were either true components of starch or closely related to such true components. Tilden and Hudson also devised a microscopic test for the determination of enzyme activity which was based on the production of crystalline iodine complexes of the Schardinger dextrins. Horberg⁸⁸ reported a method for the preparation of a highly purified macerans amylase which was essentially free from hydrolytic activity. Schwimmer and Garbald⁸⁹ reported a method which gave a tenfold increase in enzyme as compared with previous methods. In studies on a highly purified sample of macerans amylase Schwimmer⁹⁰ found that 90 percent of the protein consisted of one enzyme component having a molecular weight in the vicinity of 100,000 and an isoelectric point at pH of 4.5.

McClenahan et al.⁹¹ reported that the enzyme acted on starch with a rapid decrease in viscosity, and a gradual decrease in optical rotation. In contrast to other amylases there was little if any increase in the reducing power of the digest. Barber and Bourne⁹² pointed out that the formation of cyclic dextrins is facilitated by the natural tendency of α -1,4 glucose units to assume a helical configuration. Cori⁹³ suggested that the formation of α - and β -dextrins was an example of a glucosidic exchange reaction rather than a

hydrolytic action. Since the change in free energy for such an exchange reaction would be small, French and co-workers⁹⁴ anticipated the reversibility of the reaction. By allowing macerans amylase to act on α -dextrin and maltose, the reaction was found to be reversible. It was also found that glucose, α -methylglucoside, sucrose, cellobiose, and maltobionic acid could act as co-substrates with α -dextrin. Mehre⁹⁵ pointed out that the restriction as to the R group in the co-substrate in French's work was not particularly confining.

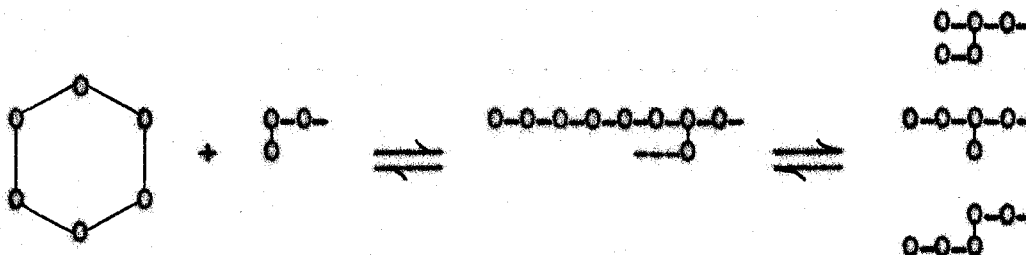


It was also shown by Nerberg and French⁹⁶ that the glucosidic exchange reactions are not limited to cyclic dextrans. Linear dextrans such as maltotriose and maltose are also capable of entering into reactions with each other. This action redistributes the glucose units between the various dextrans present, and is called a "homologizing" reaction. Typical examples are as follows: (1)



In 1951 Pan and co-workers⁹⁷ reported the isolation and crystallization of an unfermentable trisaccharide produced by Aspergillus niger. French⁹⁸ determined the structure as β -D- . This was later

confirmed by Wolfrom et al.⁹⁹ who named the sugar panose. French and co-workers¹⁰⁰ reported that panose acted as a co-substrate in a coupling reaction with α -dextrin. The initial product undergoes a "homologizing" reaction with the ultimate production of a mixture of low molecular weight branched oligosaccharides of varying degrees of polymerization.



The coupling reaction between α -dextrin and radioactive glucose has been used by Pazur¹⁰¹ and Nordin¹⁰² as a method for the preparation of linear oligosaccharides which are tagged in the reducing glucose unit.

R-Enzyme

Hobson et al.^{103,104} reported finding a debranching enzyme, R-enzyme, in broad beans and potatoes. R-enzyme acts on either amylopectin or the β -amylase limit dextrin of amylopectin to produce a material that has a greater iodine staining power than the original substrate. The ability of β -amylase to convert amylopectin to maltose is also increased after treatment with R-enzyme. R-enzyme is a purely hydrolytic enzyme, and has no link-synthesizing function.

It acts only on α -1,6 links, but does not attack isomaltose or bacterial dextran. An enzyme activity measurement can be made by observing the increase in iodine staining power of amylopectin treated with R-enzyme. Wild¹⁰⁵ reported that single glucose branches which occur in compounds such as isomaltose and panose were hydrolyzed very slowly. The rate of R-enzyme action increased with branches containing two or three glucose units, but dropped again with the longer branches.

Whelan and Roberts,¹⁰⁶ as well as Wild, used R-enzyme in determining the structures of various branched dextrans obtained from salivary amylase digests of amylopectin. Peat et al.⁴⁰ demonstrated multiple branching in waxy maize by treating the β -amylase limit dextrin with R-enzyme, and isolating the dialyzable portion of the digest. Hirst and Manners,⁴³ in an extension of Peat's observations, obtained a quantitative estimate of the degree of multiple branching in amylopectin. Peat and co-workers⁴² found that R-enzyme, although capable of debranching amylopectin, does not exert a similar action on glycogen. This lack of action is thought to be due to the compact nature of the glycogen molecule. A substrate may require a minimum number of α -1,4 linked glucose units near the branch point before the R-enzyme can act. This view is supported by the lack of action on isomaltose, panose, and dextran.

MATERIALS AND METHODS

Chromatographic Techniques

Paper chromatography of sugars

The techniques and materials used for ascending paper chromatography were essentially those described by Wild.¹⁰⁵ The solvent system used for developing the ascending chromatograms consisted of three parts water, four parts pyridine, and six parts 1-butanol by volume (3-4-6). Practical pyridine can be used, but for most purposes a distillation of practical pyridine in the presence of barium oxide is advisable. Eaton and Dickman 613 filter paper was used for all ascending chromatography. It was obtained in 1000 foot rolls eight inches wide.

The main color reagent used for reducing sugars was the alkaline copper reagent (A) followed by a phosphomolybdic acid solution (B).

The two solutions are made up as follows:

(A) Alkaline copper reagent

- (1) Dissolve 7.5 gm. of copper sulfate in 100 ml. of water.
- (2) Dissolve 25 gm. of Rochelle salt and 40 gm. of anhydrous sodium carbonate in 300 ml. of water.

(3) Add (1) to (2) with stirring, and make the solution up to one liter with water.

(B) Phosphomolybdic acid

(1) Dissolve 150 gm. molybdic acid (ammonia free) and 75 gm. of anhydrous sodium carbonate in 500 ml. of water, and heat until all the molybdic acid has dissolved.

(2) Filter if necessary. Then add 300 ml. of 85 percent phosphoric acid to the filtrate, and make up to a liter with water.

Reagent (A) was sprayed on the chromatogram, which was then heated in an oven at 100-110°C. for approximately five minutes. Next the chromatogram was sprayed with reagent (B) and allowed to air dry.

Densitometer recordings

All of the chromatograms developed as indicated were recorded with a photovolt densitometer, model 425. The instrument was equipped with a scanning stage for semi-automatic plotting, and a slit aperture 1 by 15 mm. was used in all cases. The control of background intensity on paper chromatograms proved extremely difficult, with the optical density of the background varying considerably. In each recording the background was set at an optical density of 1.0×10^{-1} . The recordings obtained were, in effect, plots of the distance from the application of the spot against the optical density. They were not

particularly helpful in a quantitative estimation of concentration, but were of value as a permanent non-fading recording.

N-(1-Naphthyl)-ethylenediamine derivatives

A second method of paper chromatography was used which was first reported by Nadman and co-workers.¹⁰⁷ This procedure was used for a quantitative as well as a qualitative estimation of reducing oligosaccharides. In the quantitative method the sugar solutions were applied to Whatman no. 1 filter paper with capillary pipettes. A solution of N-(1-naphthyl)-ethylenediamine dihydrochloride was then applied directly on top of the sugar spot with the same capillary pipette. The chromatogram was heated in an oven at 100°C. for 30 minutes. The resulting sugar derivatives which were fluorescent were then separated by descending paper chromatography. For a quantitative determination the individual spots were cut out, and the fluorescent derivative was eluted with a 1 percent solution of sodium phosphate. The samples were then made up to 5 ml. in volumetric flasks, and read in a fluorometer. Standard curves were made for the various sugars being determined.

Flavazole derivatives

In certain cases Flavazole derivatives of the reducing sugars were prepared and separated on paper as described by Kordín.¹⁰² The solvent system commonly used in this separation was water-saturated methyl ethyl ketone.

Column chromatography

The carbon column technique of Whistler and Durso¹⁰⁸ was used both in separating out fairly large quantities of sugars, and in purification of enzyme digests preparatory to paper chromatography.

Analytical

Reducing determinations

Reducing determinations were made by the Nelson¹⁰⁹ colorimetric method. A standard curve was made for maltose, and reducing values were calculated as maltose.

Polarimetric measurements

The polarimeter was used in determining the concentrations of the various branched oligosaccharides. A glass polarimeter tube 20 cm. in length was used throughout. The specific rotation for the various branched compounds was calculated using B₃ or panose as the basic structure, and adding the contributions of the remaining single glucose units. A sample calculation for the B₅ compounds follows:

$$\text{Molecular rotation} = [M] = [\alpha]_D \times \text{M.W.}$$

$$[M]_{B_5} = [M]_{B_3} + 2 \left(\frac{[M]_{\infty}}{\infty} \right) = 154 \times 504 + 2(200) \times (162) = 142,500$$

$$[\alpha]_{D,B_5} = \frac{[M]_{B_5}}{\text{N.W.}_{B_5}} = \frac{142,500}{828} = 172^\circ$$

Periodate oxidation

Periodate oxidation was used specifically in determining the structures of various flavanoses obtained from β -amylase digests of the branched oligosaccharides. Upon oxidation at room temperature, the production of flavanose aldehyde could be used in distinguishing between two alternative structures.

Carbohydrates

Penose

Crystalline penose used in these experiments was supplied by Dr. S. O. Pan.

Isomaltose

Isomaltose was prepared by the acid hydrolysis of dextran as described by Wolfson.¹¹⁰ The acid hydrolyzed mixture was separated on a carbon column, rather than by conversion to the acetate and subsequent separation on magnesium-celite. A 10 gm. sample of dextran was hydrolyzed, and added to the top of a carbon-celite column containing 300 gm. of adsorbent. The column was washed with about 4.5 liters of water, effectively removing the bulk of the glucose. About 1.5 liters of 7.5 percent ethanol contained the bulk of the isomaltose. A 12 percent yield of isomaltose was obtained which was suitably free from other reducing sugars as judged by paper chromatography.

α -Dextrin

The α -dextrin prepared by the action of macerans amylase on starch was available at this laboratory.

Panose coupled mixture

The panose coupling reaction was used to prepare a mixture of branched oligosaccharides of varying degrees of polymerization. This mixture was then separated into fractions having the same degree of polymerization by mass paper chromatography.

A digest containing 1 gm. of panose, 1 gm. of α -dextrin, 45 units of macerans amylase, and a grain of thymol was incubated at 40°C. for five days. At this time 15 units more of macerans amylase was added to the digest, and incubation continued at 40°C. for 12 days. At the end of this period the solution was filtered, concentrated, and the sirup was added to the top of a carbon-celite column containing 50 gm. of adsorbent. The column was washed with 300 ml. of 10 percent ethanol and a second sample of 100 ml. of 10 percent ethanol. A paper chromatogram indicated that large amounts of the low molecular weight products including panose had been washed off the column. The column was then washed with 300 ml. of 25 percent ethanol and 350 ml. of 40 percent ethanol. A paper chromatogram indicated that the compounds of interest were contained in these fractions. The solution was concentrated in vacuo, and applied to 16 paper chromatograms by streaking the sirup across the paper with

a fine bore pipette. The chromatograms were given ten ascents in 3-4-6. Narrow strips were cut from the edges and the middle of each chromatogram, and developed with the copper reducing spray. In this way the positions of the compounds on the remaining filter paper were determined and cut out. The paper strips from the various chromatograms were grouped together and extracted in a Soxhlet extractor. The B₅ solution was then concentrated to a small volume and streaked on four paper chromatograms. The chromatograms were developed ten times in 3-4-6, and the B₅ compounds located and cut out as previously indicated. The B₅ compounds were eluted, and the resulting solution was read in the polarimeter to determine the concentration of B₅. The specific rotation of the B₅ fraction as previously calculated was 172°, and the observed rotation found was 1.412°.

$$\text{Since } [\alpha]_D = \frac{[\alpha] \text{ observed}}{\text{gm./ml.} \times 2}$$

$$\text{gm./ml.} = \frac{1.412}{304} = .0041 \text{ gm./ml. or } 4.1 \text{ mg./ml.}$$

The isolation of the B₆ and B₇ fractions was carried out in the same way, and the results are recorded in Table 2. Each fraction was found by paper chromatography to be fairly free of higher or lower homologues.

Isomaltose coupled products

A digest containing 1.2 gm. of isomaltose, 1.2 gm. of α-dextrin, 75 units of macerans amylase, and a grain of thymol was incubated at

Table 2. Concentrations of branched oligosaccharides

Fraction	Calculated specific rotation	Observed rotation	Concentration in mg./ml.
B ₅	172°	1.412	4.10
B ₆	176°	1.870	5.32
B ₇	180°	1.189	3.10

40°C. for 12 days. The digest was then filtered and concentrated, and the low molecular weight sugars were removed on a carbon column. The branched compounds of interest were eluted from the column with a 40 percent solution of ethanol. This solution was concentrated to a small volume and streaked on 30 paper chromatograms. The chromatograms were given eight ascents in 3-4-6, and the branched compounds were located and cut out as previously described for the panose products. The branched fractions B₄, B₅, and B₆ were fairly well separated by eight ascents, and it was not necessary to rechromatograph for further purification. The specific rotation was calculated for this series of compounds using panose as the basic structure. The results obtained for the isolated fractions are found in Table 3. The homogeneity of each fraction was checked by paper chromatography. It was found that, although the B₄ and B₅ fractions were fairly free from other members of the series, the B₆ fraction contained a substantial amount

Table 3. Concentrations of branched oligosaccharides

Fraction	Calculated specific rotation	Observed rotation	Concentration in mg./ml.
B ₄	165°	2.335	7.07
B ₅	172°	1.853	5.40
B ₆	176°	1.467	4.16

of B₅ and a somewhat smaller amount of B₇.

B₇ from action of salivary amylase on amylopectin

This B₇ was obtained from a mixture of salivary amylase limit dextrins prepared from waxy maize by Wild.¹⁰⁵ The B₇ was separated by the multiple ascent technique. Twenty chromatograms were developed 12 times each, and the B₆ and B₇ compounds were cut out together. The branched compounds were eluted, concentrated, and streaked on six pieces of filter paper. These chromatograms were developed 20 times, and the B₇ was extracted with boiling water. The concentration of B₇ was determined by optical rotation, and was found to be 2.90 mg./ml.

Sweet corn glycogen

Sweet corn glycogen was purified according to a method described by Hassid and McCready.¹¹¹ About 50 gm. of the water soluble mixture

obtained from sweet corn was dissolved in 500 ml. of water, and one liter of glacial acetic acid was added. This suspension was stored overnight in the refrigerator, and the insolubles were filtered off the next day. One-half volume of absolute ethanol was added to the filtrate, and this solution was again allowed to stand overnight in the refrigerator. This suspension was filtered, the precipitate dissolved in water, and reprecipitated with ethanol. This final precipitate was filtered, washed with absolute ethanol, and stored in a vacuum desiccator.

Maltoheptaose

The maltoheptaose used was prepared in this laboratory by partial acid hydrolysis of β -dextrin.¹¹²

Enzymes

Macerans amylase

The macerans amylase was prepared by a method similar to that described by Norberg.⁸⁸ Several 10 ml. portions of glucose broth were made up, autoclaved, cooled, and inoculated from a dried Bacillus macerans culture. These solutions were incubated at 40°C. until effervescence indicated active growth. This first step usually required about two days.

Several 50 ml. portions and an equal number of 500 ml. portions

of oatmeal medium were made up. In order to obtain the best yield of macerans amylase it was found that distilled water rather than tap water should be used in making up all the medium. The oatmeal medium was boiled, cooled overnight, autoclaved, cooled, and stored in the refrigerator until used.

The glucose broth cultures were used to inoculate the 50 ml. portions of oatmeal medium which were incubated at 40°C. until effervescence indicated active growth. At this time the 500 ml. portions of oatmeal medium were inoculated with the 50 ml. cultures, and incubated at 40°C. for eight to ten days. The cultures were then strained through cheese cloth, and filtered by suction using a number 0 filter pad. A small amount of thymol was added to the clear light brown culture filtrate, and it was stored in the refrigerator in this crude form.

The unit of activity for macerans amylase was defined by Tilden and Hudson⁸⁷ in the following way. A digest containing 30 mg. of soluble starch and 1 ml. of the solution to be assayed was made up to a total volume of 3 ml. and incubated at 40°C. At various intervals a drop of 0.1 N iodine in 0.1 M potassium iodide was mixed with three drops of the digest on a spot plate, and a portion of this mixture streaked on a microscope slide. At first one could observe blue hexagonal crystals of α -dextrin iodide forming at the edge of the streak as it dried. During the later stages of the digest, the blue

hexagons changed to a needle crystal structure. The appearance of the needles was taken as the assay end point. That amount of enzyme which will convert 30 mg. of starch to the end point in 30 minutes at 40°C. is defined as one unit of maceras amylase. A conversion period for maceras amylase as defined by French and co-workers¹¹³ is "that time of reaction between enzyme and substrate which, under the conditions, would be just sufficient to convert an equal weight of starch to the Tilden and Hudson end point".

The crude filtrate was found to have an activity of two units per ml., but it also had a certain amount of hydrolytic activity. One ml. of this filtrate was capable of producing reducing substances equivalent to 1.92 mg. of maltose in 60 conversion periods. This hydrolytic activity greatly reduced the yield of the higher branched oligosaccharides in the coupling reactions, and it was obvious that further purification of the enzyme was necessary.

The crude enzyme solution was purified in the following fashion, and the purified enzyme was used immediately after purification. The crude enzyme was cooled to 0°C. and an equal volume of 95 percent ethanol was cooled in a dry ice acetone bath to -20°C. The two solutions were mixed, and kept in the dry ice bath, keeping the temperature below 0°C. The precipitate was then centrifuged down, and the supernatant liquid was discarded. The precipitate was suspended in cold water with vigorous stirring, the volume of water being equal to one-tenth that of the original crude enzyme solution.

This suspension was allowed to stand overnight in the refrigerator, and then the remaining insoluble material was centrifuged and discarded. The solution was cooled down in a brine bath, and enough acetone was added to bring the concentration of acetone to 25 percent. Methanol extracted potato starch (1 gm./ 150 ml. crude enzyme) was added to the solution, and the suspension was stirred vigorously for one half hour. The suspension was centrifuged, and the enzyme was eluted from the starch with a 0.5 percent β -dextrin solution. The volume of the β -dextrin solution was one-tenth that of the original crude enzyme solution, and the elution was effected by stirring the suspension for one half hour while immersed in a brine bath. The suspension was centrifuged, and the supernatant was dialyzed against cold water in an attempt to remove the β -dextrin. The purified enzyme was now suitable for a coupling reaction. The final enzyme had a macerans amylase activity of five units per ml., and 1 ml. of the enzyme produced the equivalent of 0.15 mg. of maltose in 60 conversion periods.

Repeated attempts were made to improve the yield of macerans amylase according to the method of Schwimmer and Garibaldi.²⁹ The composition of the medium was changed, and the culture was aerated. All attempts, however, to improve the yield over the method previously described were unsuccessful.

Sweet potato β -amylase

The crystalline β -amylase used in this work was kindly supplied by Dr. S. Schwimmer of the Western Regional Research Laboratory. The determination of the activity of β -amylase was a modified version of the method reported by Bailey,¹¹⁴ and was carried out as follows:

The β -amylase solution was made up from the crystalline suspension at intervals as needed. The working solution was then assayed for activity. The enzyme unit was defined as that amount of β -amylase which would produce 1 mg. of maltose in 30 minutes under optimum conditions. Into each of two 100 ml. volumetric flasks, 15 ml. of 1 percent starch, 2 ml. of acetate buffer (pH = 4.82, M = 0.2), and 10 ml. of water were pipetted. The flasks were then incubated at 35°C., and when the contents had reached that temperature, 0.1 ml. of the β -amylase to be assayed was added to one of the flasks. Both samples were incubated at 35°C. for 30 minutes, and at that time they were made up to 100 ml. with distilled water. One ml. aliquot samples were immediately analyzed for reducing power by the Nelson method and calculated as mg. of maltose.

R-Enzyme

The R-enzyme used was prepared by Dr. G. M. Wild from broad bean.

Salivary amylase

The salivary amylase was freshly collected and filtered before use.

EXPERIMENTAL

Action of β -Amylase on Panose Coupled Products

The action of β -amylase on the individual fractions of B_5 , B_6 , and B_7 was qualitatively followed by paper chromatography. Each digest was incubated at 35°C ., and in each case a zero time sample was taken which indicated a suitably homogeneous substrate.

Table 4. β -Amylase Digest of B_5

Time in hours	0	2.5	9	24	42	56	68	80	104	116
Enzyme in units	12	24	36	24	24	24	120	120	120	--

A B_5 solution containing approximately 24 mg. of the branched oligosaccharides in 0.2 ml. of water was incubated with β -amylase. The enzyme was added periodically as indicated by Table 4. Samples were taken at various intervals during the digest, and a paper chromatogram was prepared. The chromatogram was given four ascents in 3-4-6, and then developed with the spray reagents (A) and (B) as previously described. The chromatogram was air dried in the hood, and recorded with the densitometer. The recording is reproduced in

Fig. 4.

A 0.1 ml. sample of B₆ containing approximately 40 mg. was incubated with β -amylase. Varying amounts of enzyme were added periodically as indicated in Table 5.

Table 5. β -amylase digest of B₆

Time in hours	0	6	18	36	50	62	74	98	110
Enzyme in units		12	24	24	24	24	120	120	--

A paper chromatogram was made, developed and recorded for the B₆ digest. The method was similar to that used for the B₅ fraction except that six ascents in the J-4-6 solvent system were used. The recording for the B₆ digest is reproduced in Fig. 5.

A 0.2 ml. solution of B₇ containing approximately 18 mg. of the branched fraction was incubated with β -amylase. Enzyme was added periodically as indicated in Table 6. A paper chromatogram was made and recorded for the B₇ digest in the same way as for the B₅ digest. The recording is reproduced in Fig. 6.

It was apparent from the chromatograms obtained during the early

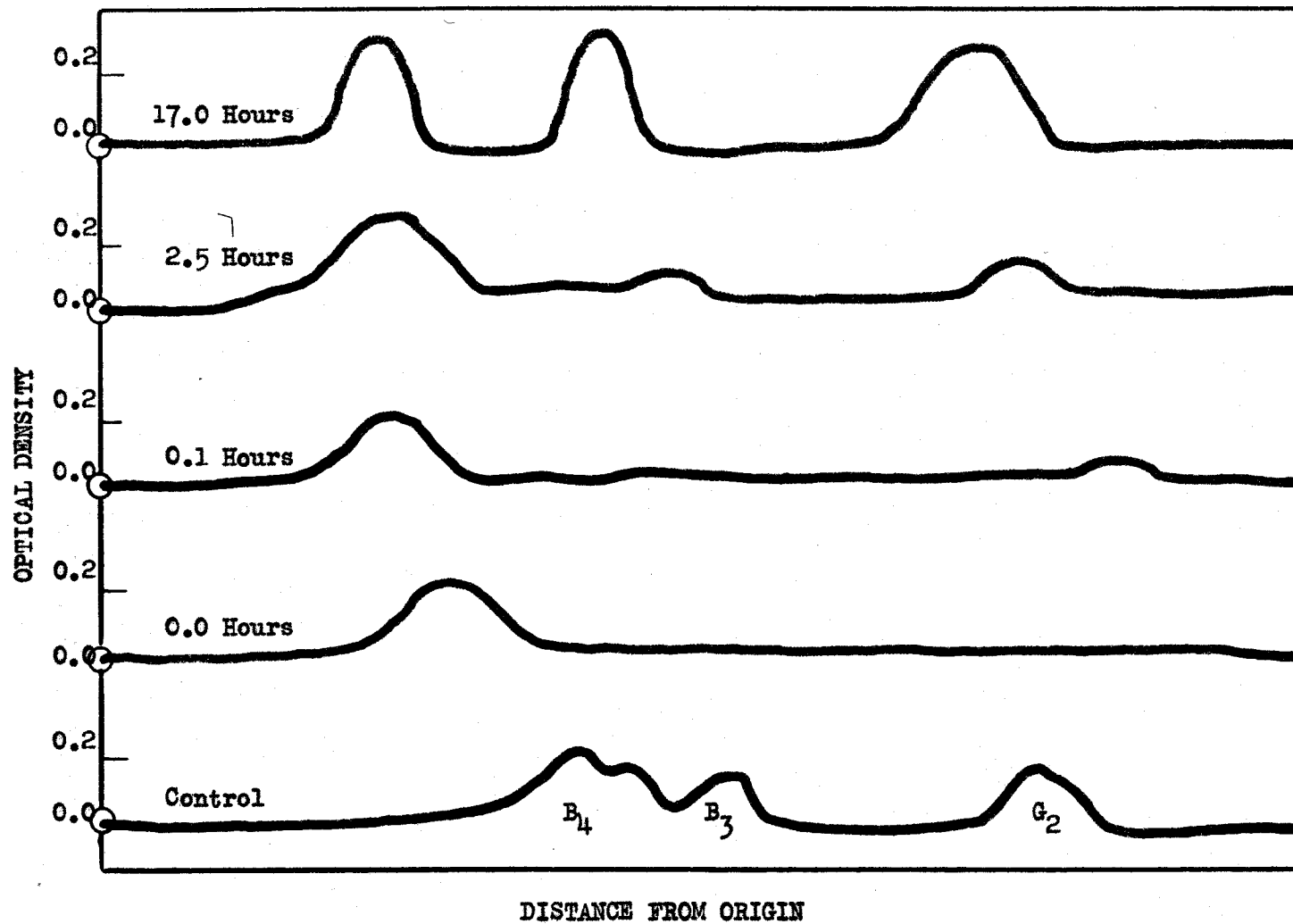
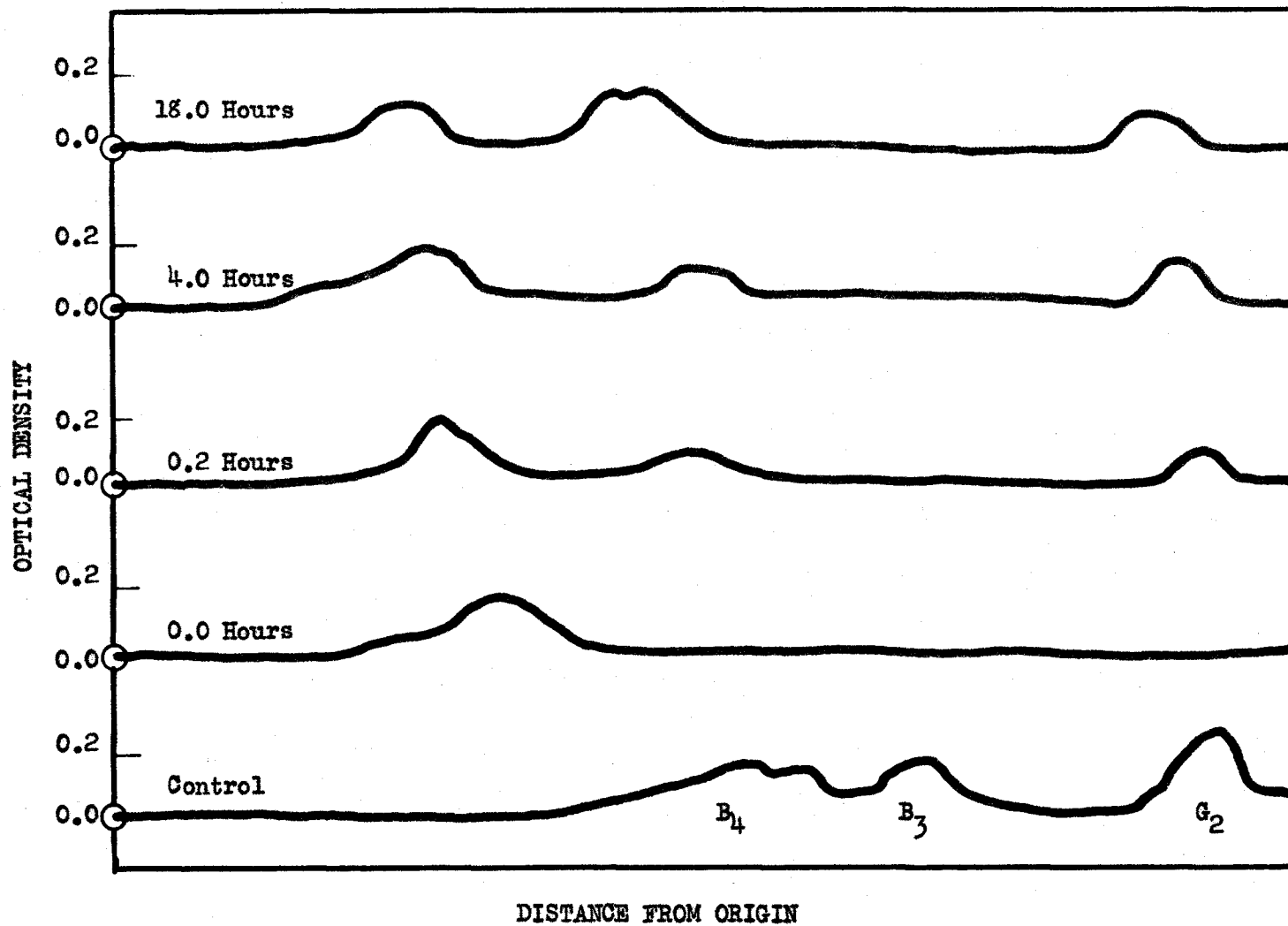


Fig. 4. Action of β -amylase on B₅ from panose



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Fig. 5. Action of β -amylase on B₆ from panose

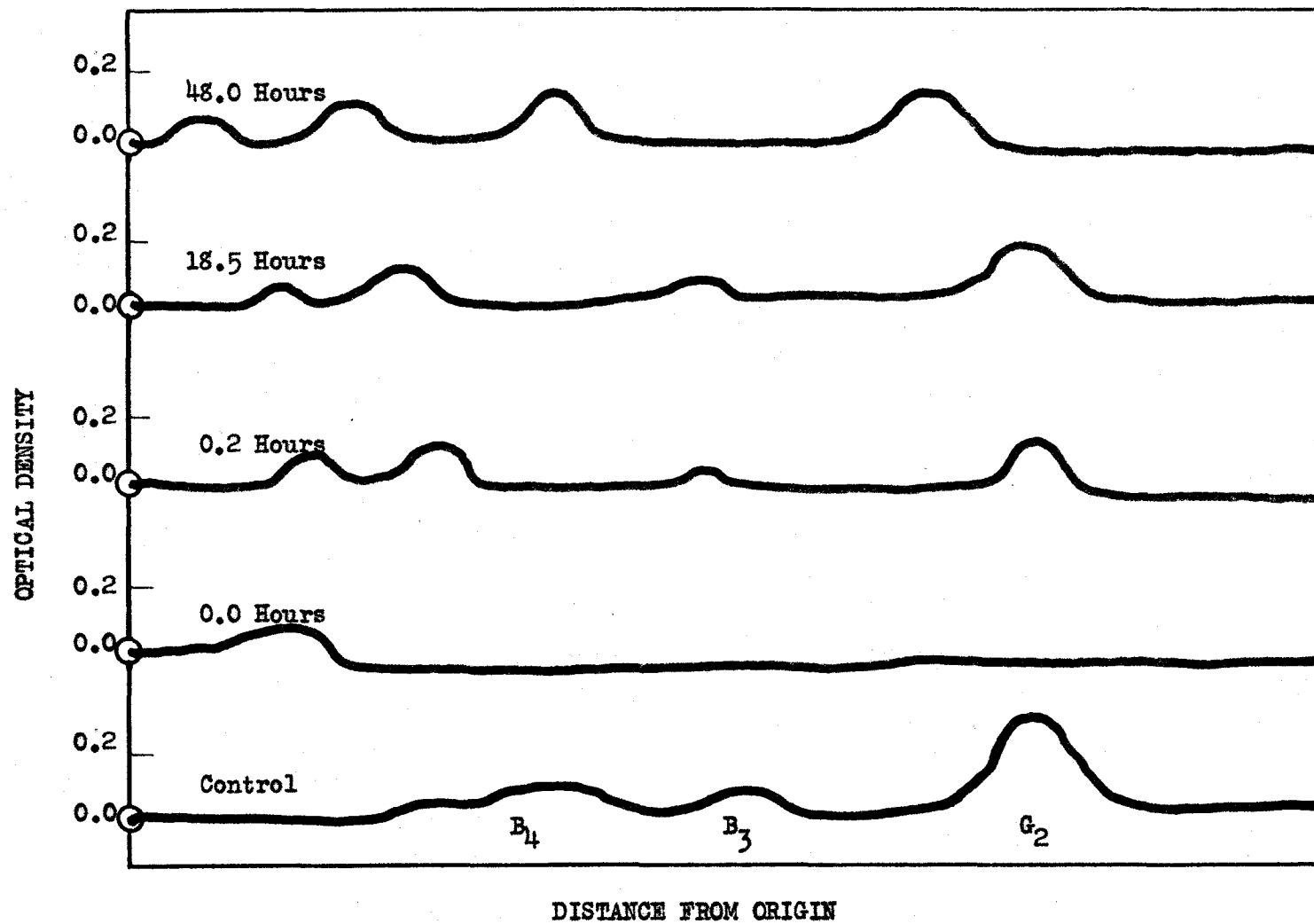


Fig. 6. Action of β -amylase on B₇ from panose

Table 6. β -amylase digest of B₇

Time in hours	0	18	48	66	80	92	104	128	140
Enzyme in units	12	24	24	24	24	120	120	120	-

stages of the digests that some impurity was interfering with the separation of the oligosaccharides. In order to remove the interfering materials, each of the digests was purified by adsorption on charcoal. A charcoal-celite column was made up, approximately 3 cm. in diameter, and contained 2 gm. of adsorbant. After the column was washed with distilled water, the B₅ digest was added to the top, and washed with 40 ml. of distilled water which was discarded. The sugars of the B₅ digest were then eluted with 150 ml. of 45 percent ethanol. The B₆ and B₇ digests were purified in a similar way. The three ethanol solutions containing the B₅, B₆, and B₇ digests were concentrated in vacuo to small volumes of several tenths of a ml. A chromatogram was made and recorded, as previously indicated, for the three purified digests. The chromatogram was given four ascents in 3-4-6, and is reproduced in Fig. 7. After the last chromatogram was made from the purified digests of branched oligosaccharides, the remainder of each digest was streaked onto a piece of filter paper. The chromatograms were developed, and the residual B₅ and B₆ were isolated. Both of these

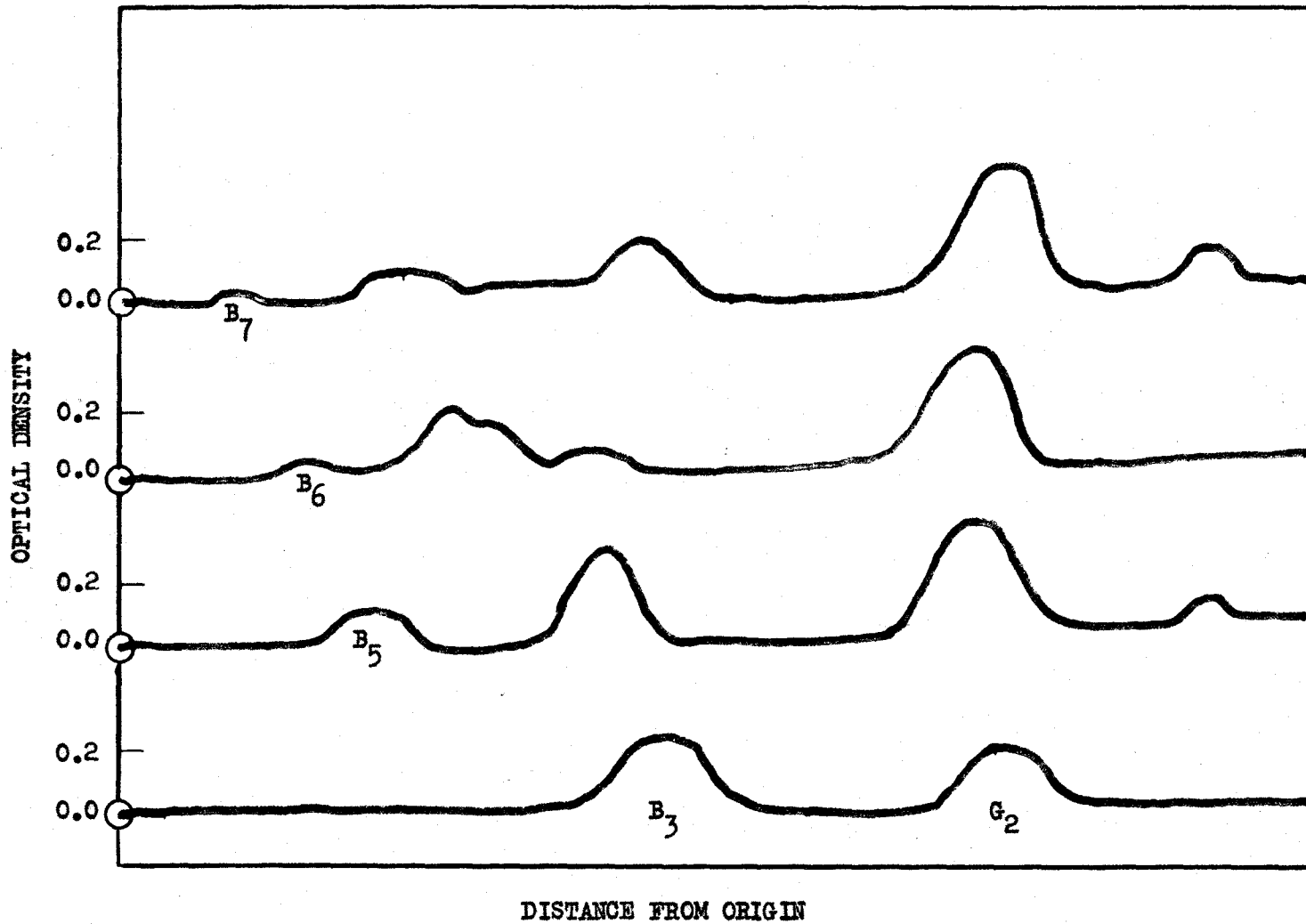
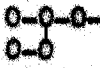
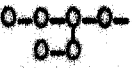
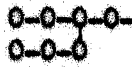




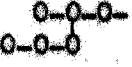
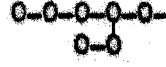

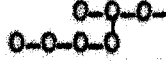
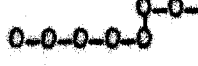


Fig. 7. Action of β -amylase on panose coupled products

Table 7. Panose coupled oligosaccharides

	B ₅	B ₆	B ₇
a			
b			
c			
d			
e			

fractions were subjected to further action by β -amylase, but no further hydrolysis was detectable.

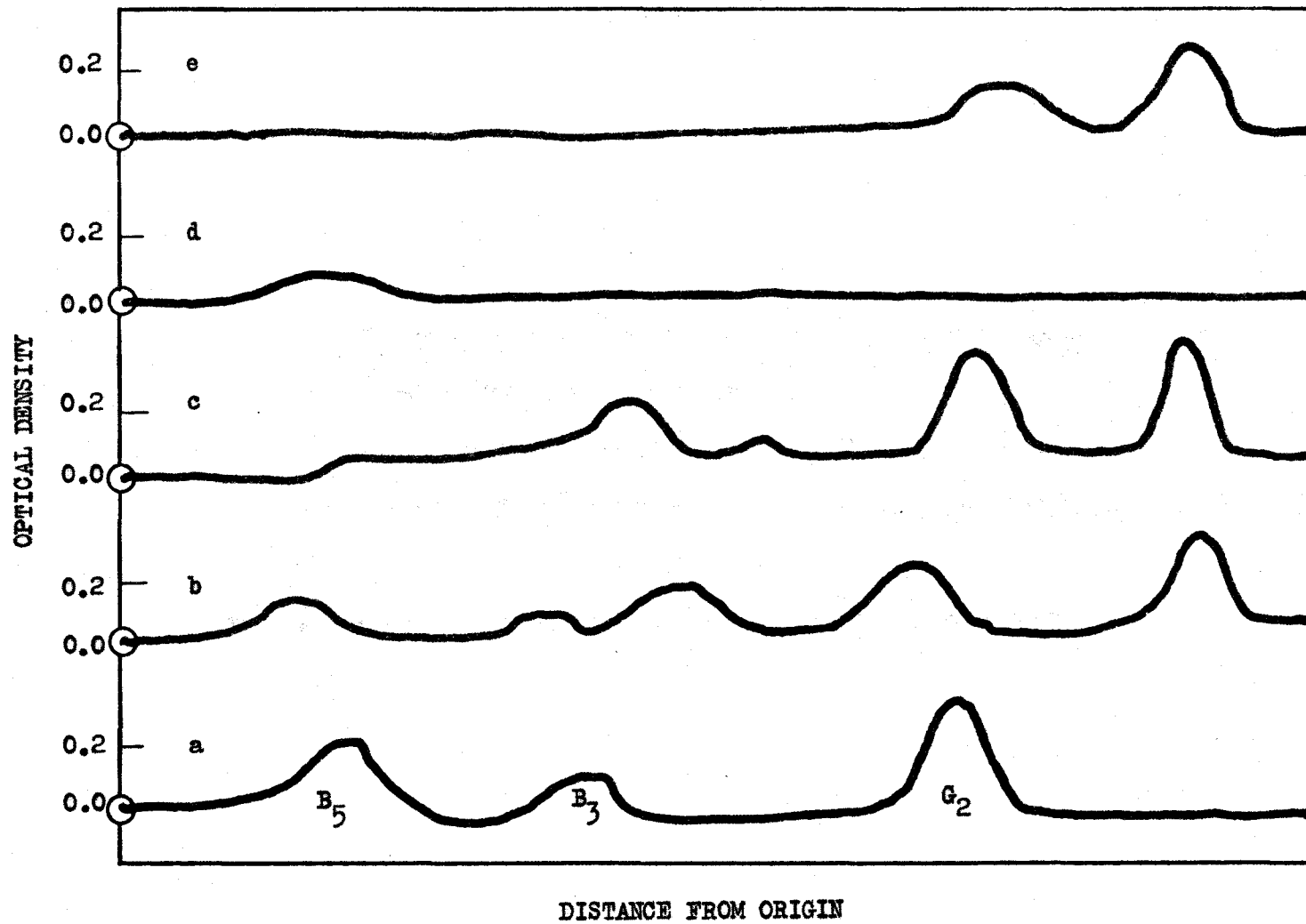
In Table 7 are found the possible isomers for the B₅, B₆, and B₇ fractions. There was a resistant B₅ and B₆, and there may have been a very small amount of a resistant B₇. The isomer B_{5a} was definitely resistant, and Wild¹⁰⁵ stated that B_{5c} was destroyed by β -amylase. From this information it was impossible to say whether B_{5b} was resistant or not. It was impossible to predict which of the B₆ or B₇ isomers might have been resistant to β -amylase.

A digest of R-enzyme and the B₅ fraction was incubated at room temperature. A sample was spotted on a paper chromatogram. Next, the products of this digest were treated with β-amylase at 35°C., and a second sample was spotted on a chromatogram. The chromatogram was recorded and reproduced in Fig. 8. After R-enzyme action, B₃, G₃, G₂, and G₁ were produced with a small amount of B₅ remaining resistant. After having been digested with β-amylase, nearly all the remaining resistant B₅ disappeared, with the production of more G₂ and B₃. Wild¹⁰⁵ indicated that a number of branched compounds with a single glucose branch were fairly resistant to the action of R-enzyme. This would mean that B_{5b} should be the most resistant to the action of R-enzyme. Since the R-enzyme resistant B₅ was attacked by β-amylase, it seemed reasonable that B_{5b} was attacked by β-amylase, and that B_{5a} was the only β-resistant isomer of the B₅ group.

The β-resistant B₆ compound was digested with R-enzyme at room temperature, with the production of a small amount of G₃ and fairly large amounts of G₂ and G₁. This experiment was repeated in order to get a chromatogram suitable for a recording which is reproduced in Fig. 8. In the second experiment, only glucose and maltose were formed with all of the B₆ being hydrolyzed. It was apparent from this experiment, as well as from others, that there is enzymic activity present in the R-preparation other than that of the R-enzyme itself. It may be due to an amylase or a glucosidase. The glucose and maltose were present in nearly equimolar quantities. It appeared that the

Fig. 8. Action of R-enzyme on various branched oligosaccharides

- a. Control
- b. Action of R-enzyme on B_5 from panose coupled products
- c. Action of β -amylase on R-enzyme digest of B_5
- d. B_6 from panose coupled products
- e. Action of R-enzyme on B_6 from panose coupled products



glucose and maltose were derived from the breakdown of maltotriose. If this was the case, the only resistant B₆ compound was B_{6c}.

Action of β -Amylase on Isomaltose Coupled Products

The qualitative action of β -amylase on the B₄, B₅, and B₆ compounds obtained from the isomaltose coupling reaction was followed in the same way as that of the panose coupled products. All the digests were carried out at 35°C., and a zero time spot was taken in each digest.

A 0.3 ml. sample containing 18 mg. of the B₄ fraction was digested with 520 units of β -amylase. Samples were spotted periodically, and the resulting paper chromatogram was given two ascents in 3-4-6. The chromatogram was developed with reagents (A) and (B), and recorded with the densitometer. The recording is reproduced in Fig. 9.

A 0.5 ml. sample containing 25 mg. of the B₅ fraction was incubated with 400 units of β -amylase. Samples were spotted periodically, and the chromatogram was given four ascents in 3-4-6. It was developed and recorded as previously described. The recording is reproduced in Fig. 10.

A sample of 32 mg. of B₆ in 0.3 ml. was digested with 750 units of β -amylase. Samples were spotted periodically, and the resulting chromatogram given four ascents in 3-4-6. The chromatogram was developed, recorded, and is reproduced in Fig. 11.

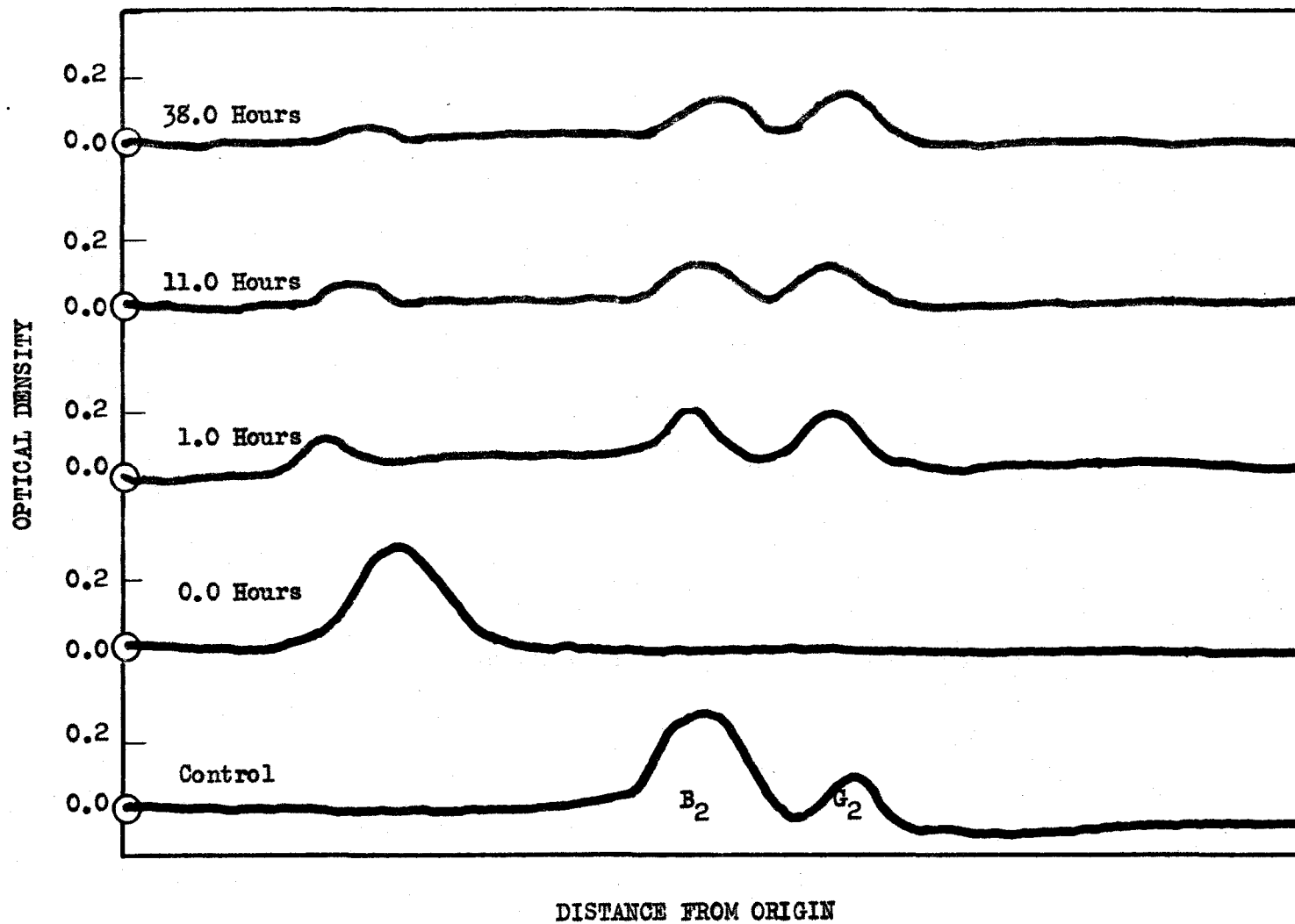


Fig. 9. Action of β -amylase on B₁ from isomaltose

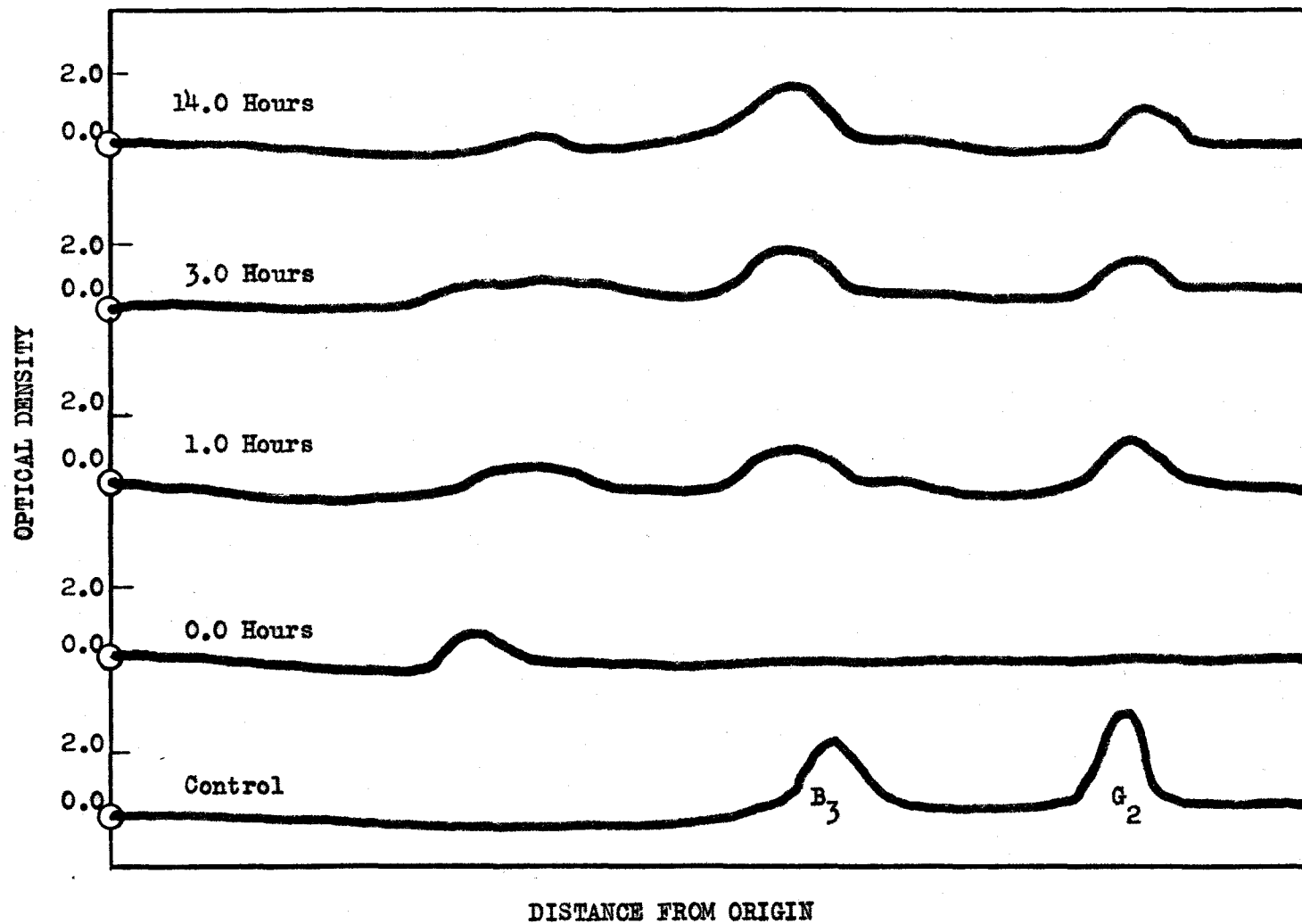


Fig. 10. Action of β -amylase on B₅ from isomaltose

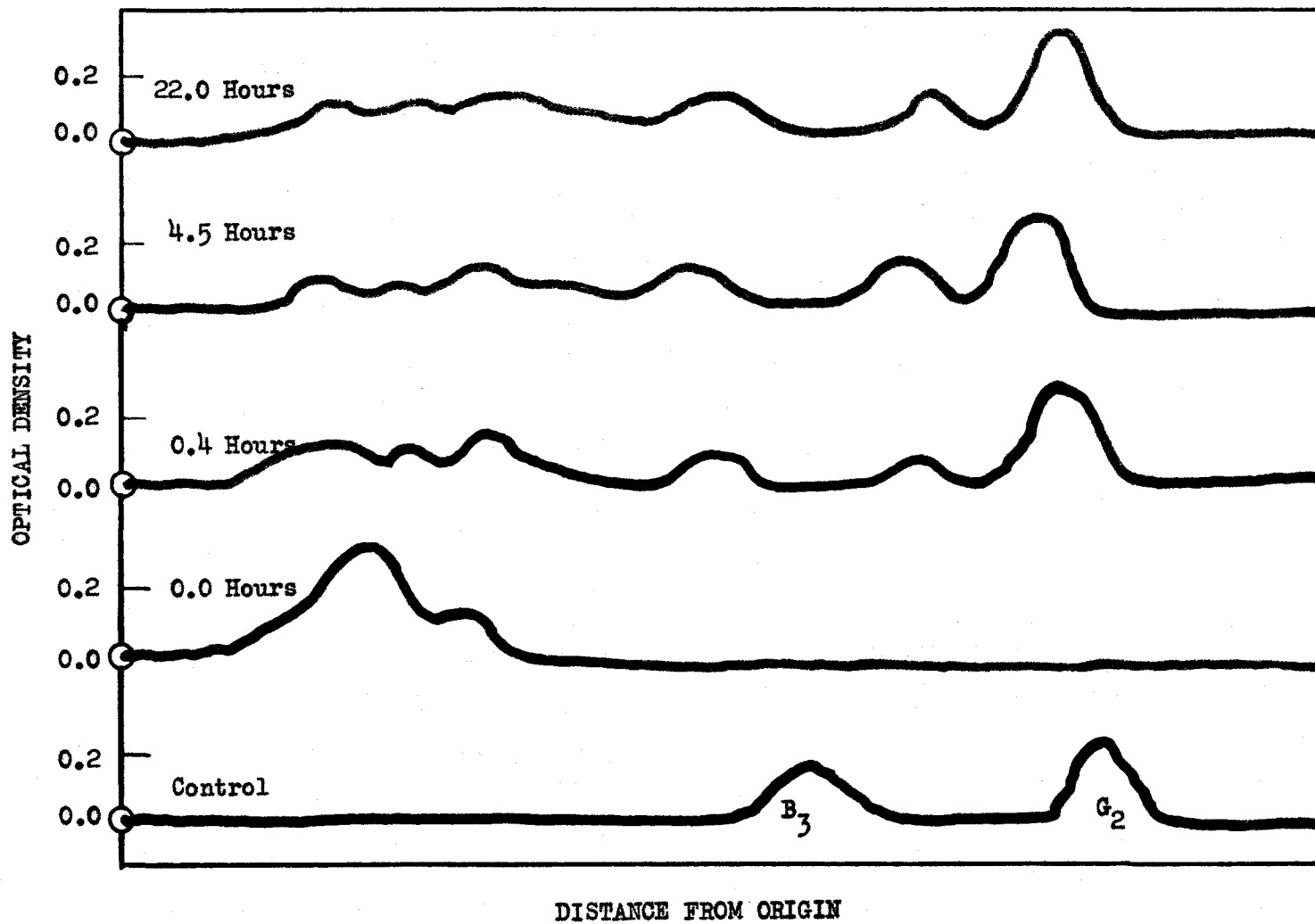





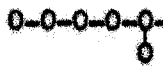


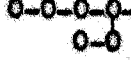

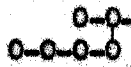
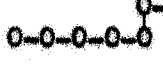


Fig. 11. Action of β -amylase on B₆ from isomaltose

The B₄, B₅, and B₆ digests were then streaked on filter paper, and the resistant B₄, B₅, and B₆ were isolated. The B₄ fraction was subjected to further action of β -amylase, and was found to be resistant to the prolonged action of large amounts of enzyme.

Table 8. Isomaltose coupled oligosaccharides

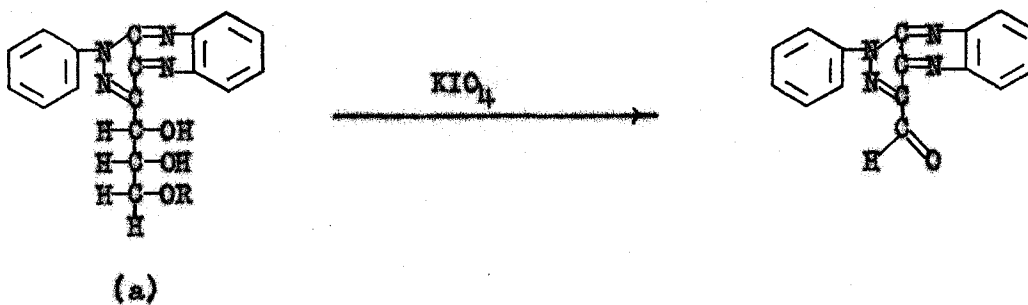
	B ₄	B ₅	B ₆
a			
b			
c			
d			
e			

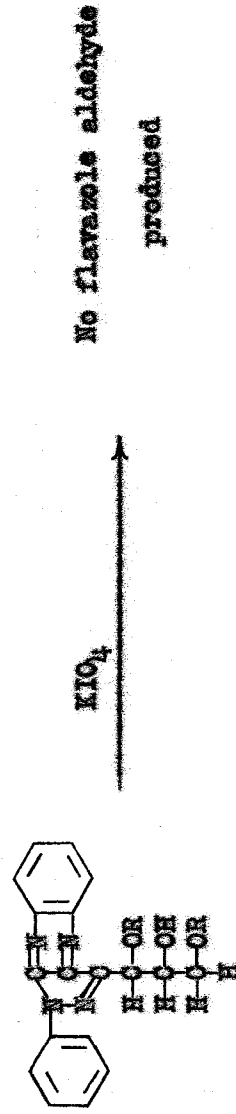
In Table 8 are found the possible isomers for the B₄, B₅, and B₆ fractions obtained from the isomaltose coupling reaction.

Some of the chromatograms of the isomaltose coupled products seemed to indicate that coupling may occur exclusively or preferentially at one of the two positions available. In order to





investigate this possibility, the B_3 fraction from the β -amylase digest of B_5 was isolated. A flavazole derivative was made from the B_3 fraction and streaked onto one filter paper. The chromatogram was given two ascents in water saturated methyl ethyl ketone, and two B_3 flavazoles were evident. However, the rapidly moving flavazole from B_{3a} had a much stronger band than the slower moving flavazole from B_{3b} . The individual strips were cut out, and the flavazoles were eluted with 10 percent methanol. Each of the eluates was made up to 5 ml. volume, and the optical density was read in the Klett-Summerson photoelectric colorimeter. The purple filter no. 42 was used, and the instrument was zeroed with 10 percent methanol. The flavazole from B_{3a} had a Klett reading of 225, and the one from B_{3b} had a reading of 110. This indicated a 2:1 ratio of B_{3a} to B_{3b} .

According to Nordin,¹⁰² structure (a) will form flavazole aldehyde when oxidized with potassium periodate at room temperature, and structure (b) will not form the flavazole aldehyde.





(b)

Of the two possible B_3 isomers, one has the (a) type structure , and one has the (b) type structure . Upon treatment of B_{3a} flavazolinol with potassium periodate at room temperature for 30 minutes, flavazolinol aldehyde was produced which was identified by its chromatographic mobility in water saturated methyl ethyl ketone. The same treatment of B_{3b} flavazolinol did not produce flavazolinol aldehyde in any significant quantities. Therefore, the structure of B_{3a} must be  and that of B_{3b} .

A flavazolinol derivative was prepared from the raw B_4 fraction, as well as from the β -amylase resistant B_4 fraction. Paper chromatography of the raw B_4 flavazolinol indicated at least two flavazolinols and possibly a third, but the resistant B_4 flavazolinol moved as a single spot. Both of these B_4 flavazolinols were oxidized with potassium periodate. The raw B_4 mixture produced flavazolinol aldehyde, but the β -amylase resistant B_4 did not. The B_{4a} was certainly resistant to the action of β -amylase, and the B_{4b} was definitely hydrolyzed by β -amylase. Since only one B_4 flavazolinol was evident in the resistant material, and only a very small amount of the B_4 fraction was resistant to the action

of β -amylase, it appeared that B_{4b} was also hydrolyzed by β -amylase.

The resistant B_4 , which probably consisted of the single compound B_{4a} , was digested with R-enzyme. It was felt that maltose might have been obtained from the B_{4a} , but R-enzyme had no action on the β -amylase resistant B_4 .

The β -amylase resistant B_5 was subjected to the prolonged action of large amounts of salivary amylase. The products of the digest were derivatized as flavazoles. A paper chromatogram of the flavazole mixture indicated substantial amounts of a B_3 flavazole which corresponded in mobility to B_{3b} . A very faint spot of a second B_3 flavazole corresponding to B_{3a} was also evident. Furthermore, a small amount of B_5 was apparently resistant or only slowly hydrolyzed by salivary amylase.

Since B_{3a} and B_{3b} were produced from the B_5 fraction, it was apparent that B_{5b} and B_{5d} must have been attacked by β -amylase. In addition, the above experiment indicated that both B_{5a} and B_{5c} were resistant to β -amylase.

The resistant B_6 compound was digested with R-enzyme at room temperature. The products of the digest were found to be G_1 , G_2 , and G_3 . A small amount of B_6 remained unattacked, but apparently the major portion of the resistant B_6 was B_{6a} .

Action of β -Amylase on Salivary Amylase Limit B_7

The B_7 obtained from the later stages of a salivary amylase digest of amylopectin has been reported by Nordin¹⁰² to have the following structure: $\begin{matrix} 0-0-0-0- \\ | \\ 0-0-0 \end{matrix}$. This branched compound, in contrast to those obtained from the coupling reactions, consisted of a single known structure.

To 0.3 ml. of a B_7 solution containing 11.6 mg., 520 units of β -amylase were added. The digest was incubated at 35°C., and a chromatogram was spotted at various intervals. The chromatogram was given two ascents in 3-4-6, and no G_2 or B_5 was produced even after 35 hours of incubation. Although this B_7 was apparently completely resistant to the action of β -amylase, it was slowly attacked by salivary amylase to produce G_2 and B_5 .

Rate of β -Amylase Action on Branched Oligosaccharides

Each of the branched fractions was incubated with a carefully measured amount of β -amylase at 35°C. Samples were withdrawn periodically and analyzed by the Nelson method for reducing power.

Panose coupled products

Two ml. aliquots of the B_5 fraction (8.2 mg.) were pipetted into three 25 ml. volumetric flasks. In addition, 1 ml. of buffer (pH = 4.82, 0.2 M acetate) was added to each flask. The flask was

then filled nearly to the mark, and pre-incubated at 35°C. After the flasks and contents had reached 35°C, 475 units of β -amylase was added to each of the first two flasks, and 950 units to the third. Each solution was made up to the mark with distilled water, mixed, and incubated at 35°C. At intervals, duplicate 1 ml. aliquots were removed and immediately pipetted into 1 ml. of alkaline copper solution. Each sample was analyzed for reducing value by the Nelson method.

Two ml. aliquots of the B₆ fraction (10.7 mg.) were pipetted into three 25 ml. volumetric flasks. Two ml. of buffer (pH = 4.82, 0.2 M acetate) were added to each flask. Each of the flasks was made up nearly to the mark with distilled water, and pre-incubated at 35°C. until the enzyme was added. Three different levels of enzyme were added to the flasks: 35, 700, and 3,500 units respectively. The digests were then made up to volume, and duplicate 1 ml. aliquots were withdrawn at intervals for analysis by the Nelson method.

The B₇ digest was made up in the same way as the B₆ digest. Each of the three digests contained 6.2 mg. of B₇, and the levels of enzyme were 26, 502, and 5,050 units respectively. This digest was followed by the reducing determination just as the others. A semi-logarithmic plot for the B₅, B₆, and B₇ digests is found in Fig. 12. A plot of the initial rate of each of these digests is found in Fig. 13.

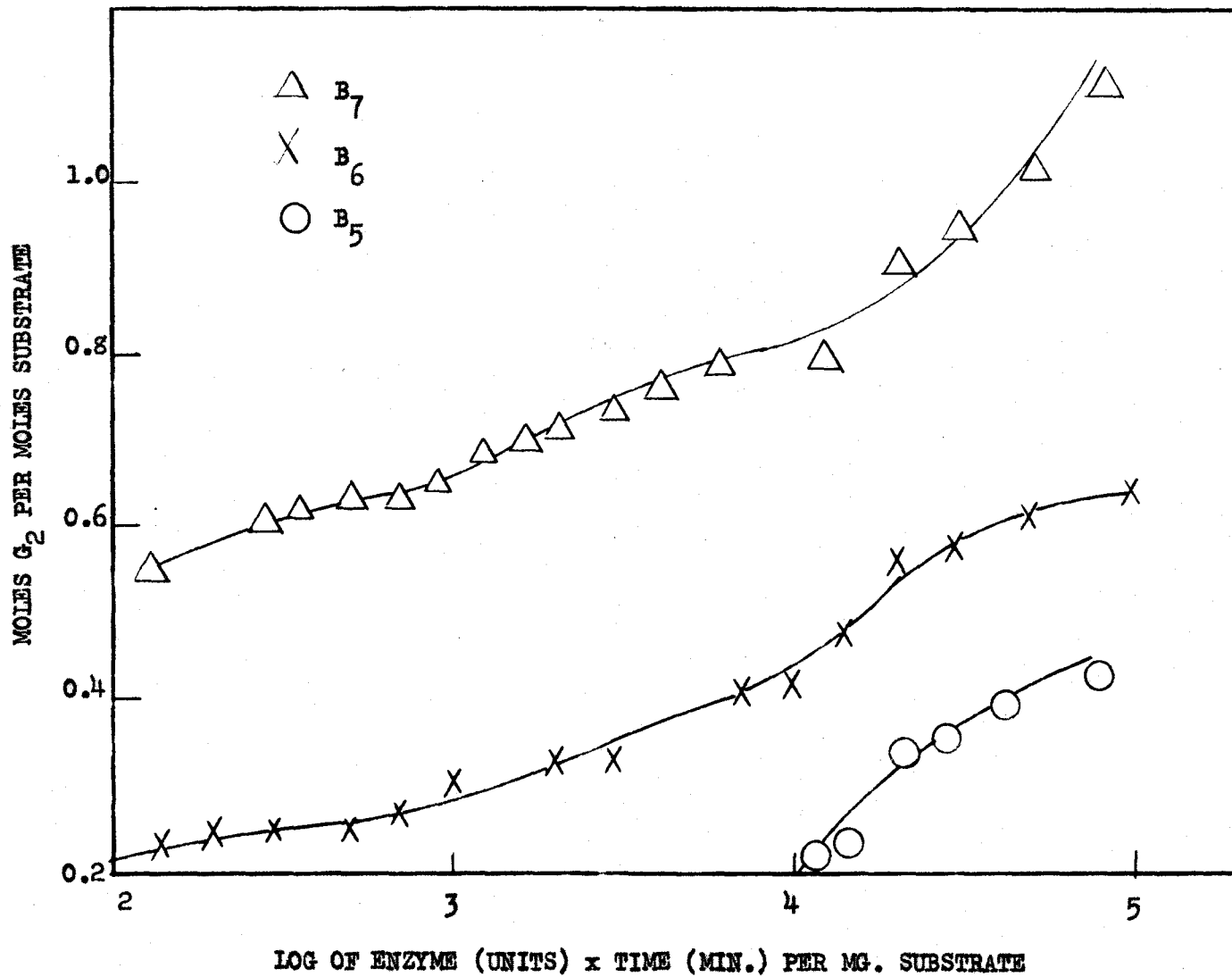


Fig. 12. Action of β -amylase on panose coupled products

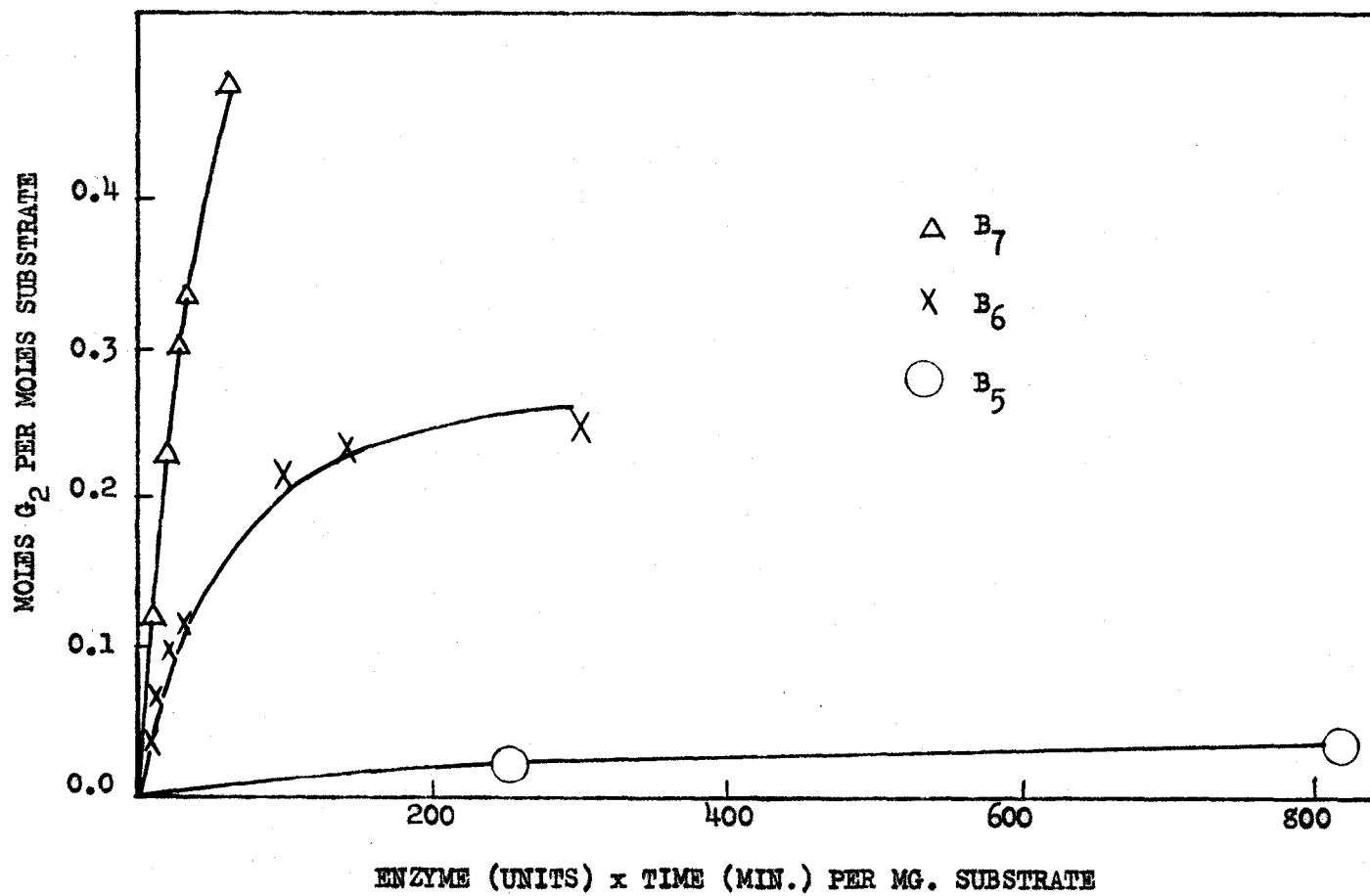


Fig. 13. Action of β -amylase on panose coupled products

Isomaltose coupled products

The β -amylase digests of the B_4 fractions were made up in two 25 ml. volumetric flasks each containing 7 mg. of B_4 and 2 ml. buffer (pH = 4.82, 0.2 N acetate). The first digest contained 160 units of β -amylase, and the second contained 2,000 units of β -amylase.

Two B_5 digests were made up in the same way. Each flask contained 5.4 mg. of B_5 ; the first digest contained 80 units of enzyme while the second contained 2,000 units of β -amylase.

The two B_6 digests also were made up in the same way. The first flask contained 4.1 mg. of B_6 and 36 units of enzyme, while the second contained 4.1 mg. of B_6 and 3,600 units of β -amylase. A semi-logarithmic plot for each of these digests is found in Fig. 14. A plot of the initial rate of β -amylase on each of the three branched compounds is found in Fig. 15.

Sweet corn glycogen

Three digests of glycogen were carried out. Each contained 10 mg. of glycogen and 2 ml. of buffer in a 25 ml. volumetric flask. Three levels of β -amylase were used: 52, 600 and 2,360 units respectively. The reducing value was determined periodically as previously described. A semi-logarithmic plot of this digest is found in Fig. 16, and a plot of the initial rate of β -amylase is found in Fig. 17.

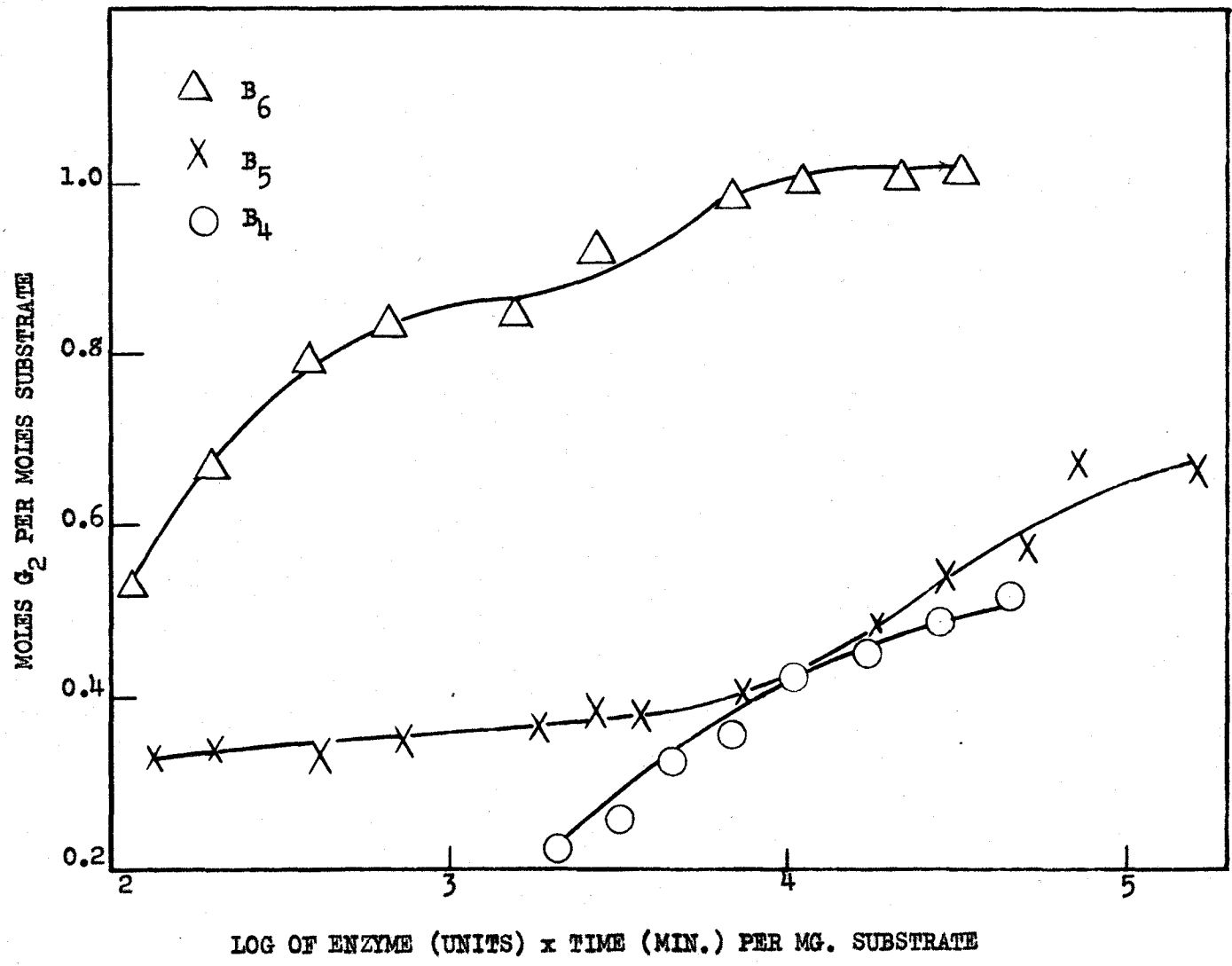


Fig. 14. Action of β -amylase on isomaltose coupled products

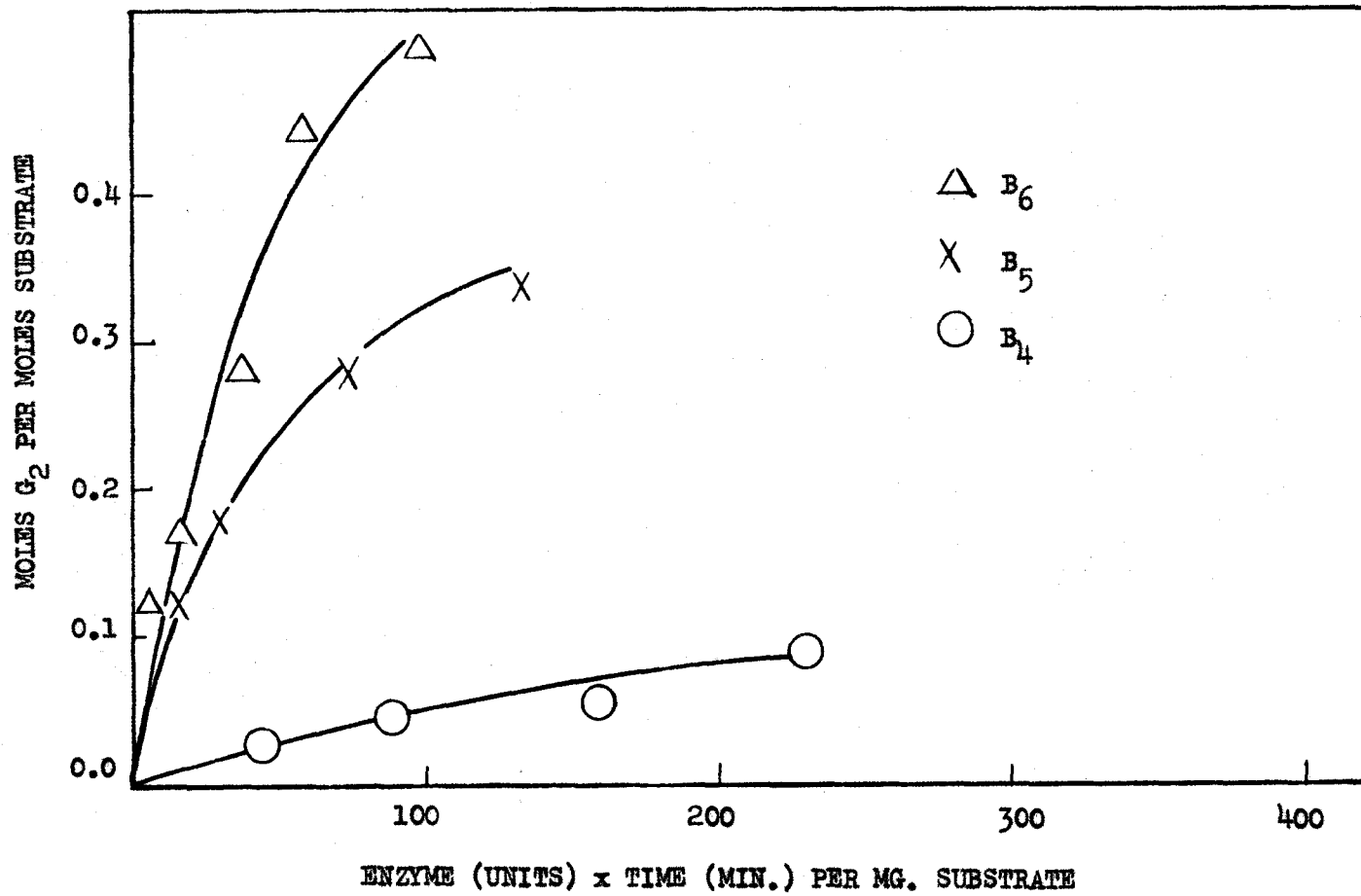


Fig. 15. Action of β -amylase on isomaltose coupled products

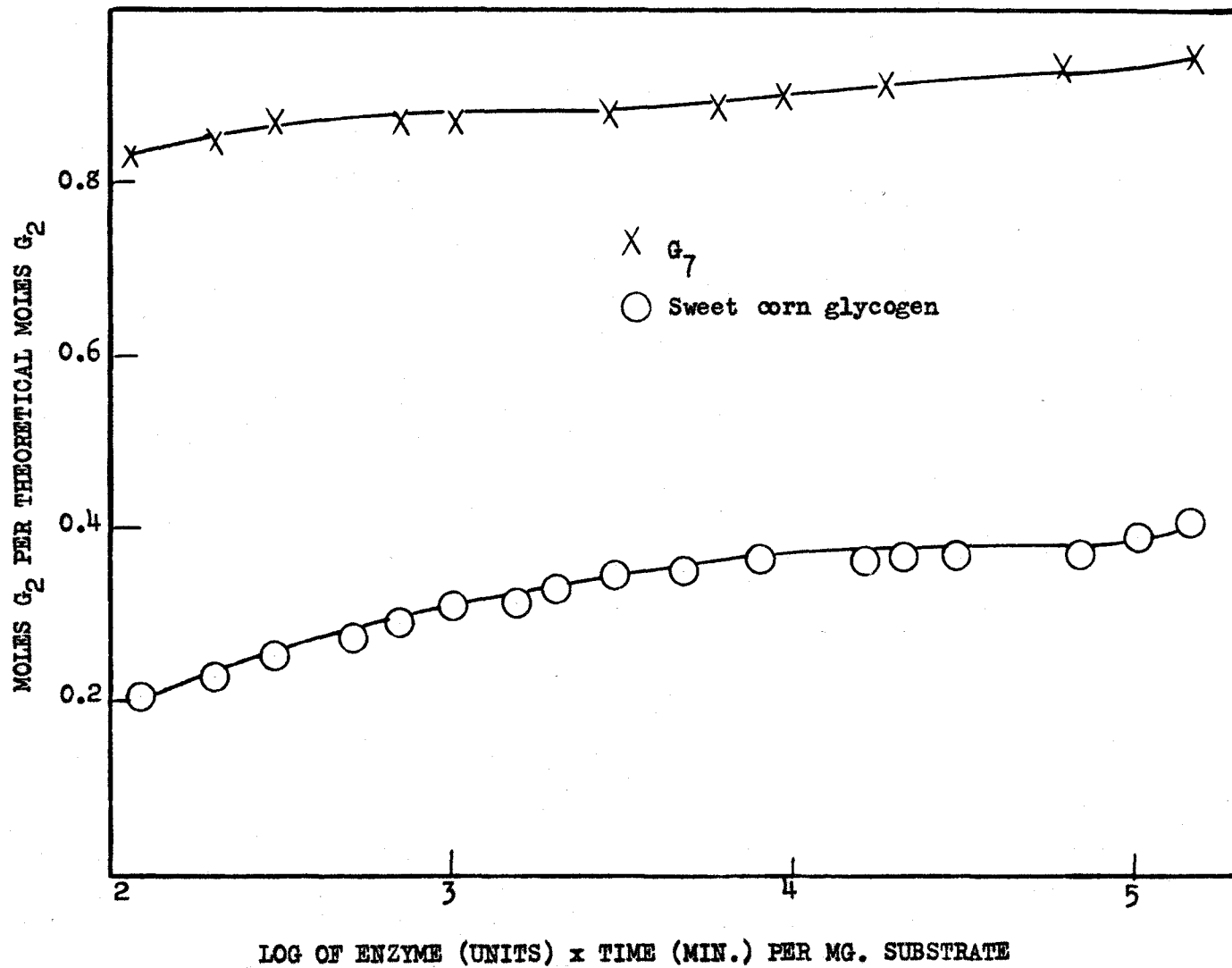


Fig. 16. Action of β -amylase on sweet corn glycogen and maltoheptaose

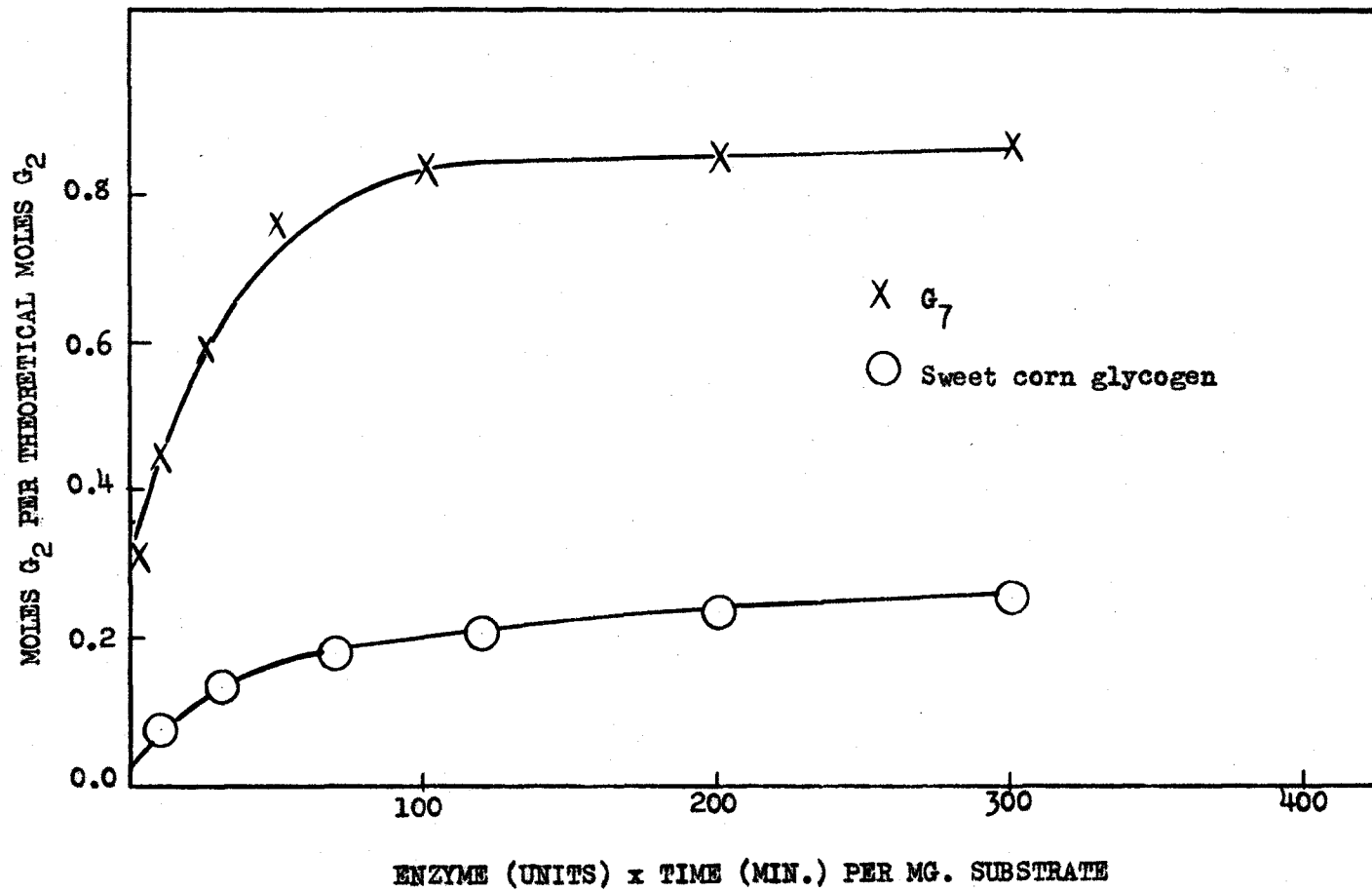


Fig. 17. Action of β -amylase on sweet corn glycogen and maltoheptaose

Rate of β -Amylase Action on Maltoheptaose

Two digests of G_7 were made up, each containing 10 mg. of G_7 and 2 ml. of buffer. The digests were incubated in 25 ml. volumetric flasks, with the first containing 50 units of β -amylase, and the second containing 1,800 units. The semi-logarithmic plot of this digest is found in Fig. 16, and the plot of the initial rate of the reaction is found in Fig. 17.

DISCUSSION

Preparation of Model Substrates

The preparation and use of model substrates in the determination of enzyme action is a valuable tool in the study of any enzyme system. A well known example of this approach is found in the work of Bergmann and Fritton.¹¹⁵ The use of this technique in the study of amylase action has been employed by various workers, including Nordin¹⁰² and Wild¹⁰⁵ in this laboratory.

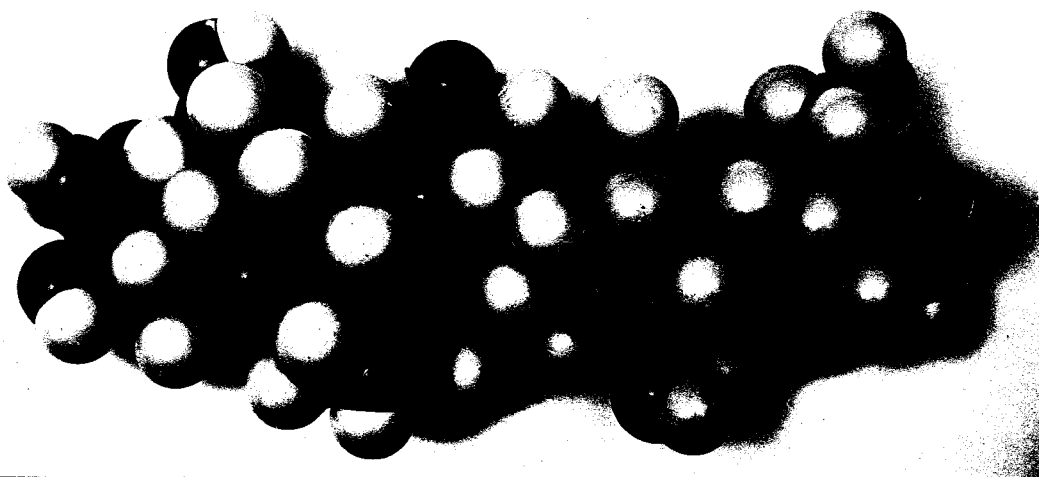
The preparation of suitable model substrates for investigation of β -amylase action presented certain difficulties. The most desirable method would have been a chemical synthesis of single compounds of known structure. However, the chemical synthesis of the type of oligosaccharide needed would become extremely complicated, and would not be practical. The macerans amylase coupling reaction provided a biological synthesis of oligosaccharides which was suitable for a study of this type. The great difficulty of this synthesis was in the nature of the products produced. Not only were compounds of varying degrees of polymerization produced, but also isomeric oligosaccharides. The problem of separating this mixture was a difficult one, but the technique of paper chromatographic separation provided at least a partial answer. It was a fairly simple project to separate these mixtures into fractions having the same degree of

polymerization. At the present there is no known method for separating the fractions into their individual isomeric compounds, with the exceptions of the B_1 compounds from the panose coupling reaction, and the B_3 compounds from the isomaltose coupling reaction.

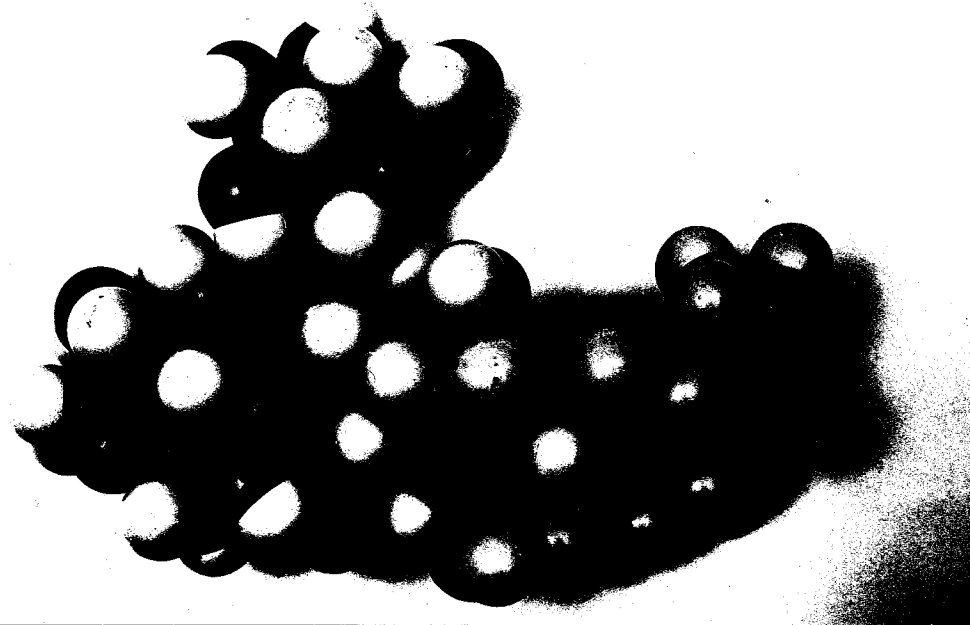
This method of preparation of a model substrate leaves something to be desired, in that the final product is not a single compound of known structure. However, each of these fractions containing from three to five isomers can be used to provide some interesting information concerning the action of β -amylase. The study of the action of β -amylase on any one particular fraction did not prove to be as helpful as the combination of facts obtained from studying the action on all of the fractions. Furthermore, in many fractions an oligosaccharide was apparently completely resistant to the action of the hydrolytic enzyme, and a determination of the structure of these residual oligosaccharides proved particularly helpful.

Qualitative Action of β -Amylase on Branched Oligosaccharides

In Fig. 15 are found photographs of Fischer-Hirschfelder-Taylor models of two oligosaccharides. Oligosaccharide (A) is linear, and contains five glucose units joined together by α -1,4 linkages, while (B) contains one branch or α -1,6 linkage, and is comprised of five glucose units. Oligosaccharide (B) is typical of the molecules studied in this work. The qualitative study of the action of β -amylase



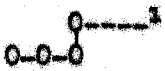
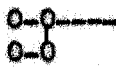

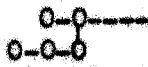

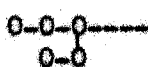
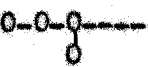
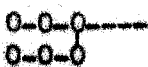
(A) Linear pentasaccharide



(B) Branched pentasaccharide

Fig. 18. Fischer-Hirschfelder-Taylor models of branched and linear pentasaccharides

Table 9. Action of β -amylase on branched oligosaccharides

	Non-resistant linkages	Resistant linkages
a		
b		
c		
d		

¹The end groups listed in Table 9 were arrived at by an examination of the action of β -amylase on all the various branched oligosaccharides. In certain cases (non-resistant d, resistant c), these conclusions were not supported by direct evidence. However, the questionable end groups do fit the over-all picture.

on the various oligosaccharides which are analagous to the branch points in amylopectin or glycogen led to the conclusions summarized in Table 9. It was apparent that one chain exerted an influence on the other in so far as the action of β -amylase was concerned. The presence of one glucose unit on the opposite chain appeared to make a resistant linkage out of one which was formerly non-resistant. It wasn't particularly surprising that this was the case, for the enzyme itself, according to England and Singer,⁵⁴ has a molecular weight of 152,000 \pm 10 percent. On examination of a Fischer-Hirschfelder-Taylor

model of the particular oligosaccharides involved, it seemed quite reasonable that an extra glucose unit might well have prevented the approach or attachment of the bulky enzyme.

A comparison between the action of salivary amylase and β -amylase in the vicinity of a branch point indicated that the two enzymes might really be quite similar. Salivary amylase was capable of hydrolyzing any links that β -amylase could and in some cases could act upon linkages which were resistant to β -amylase. Salivary amylase acted upon amylopectin to produce a number of branched oligosaccharides, including a B_7 with the following structure:

$$\begin{array}{c} \text{O-O-O-O-} \\ | \\ \text{O-O-O} \end{array} .$$
 This oligosaccharide was not completely resistant to the action of salivary amylase, but was acted upon so slowly that it accumulated in the latter stages of the digest. Upon isolation of this compound, it was found that although salivary amylase slowly broke it down, β -amylase was incapable of hydrolyzing it. This observation led to the suggestion that the difference between these two enzymes might be one of degree rather than a distinct qualitative difference.

A study of the action of β -amylase on these low molecular weight oligosaccharides suffered from at least two standpoints. The first of these is the fact that the substrates were mixtures, as has previously been discussed. The second was concerned with the relationship between these rather small molecules and the comparatively huge

amylpectin, Meyer concluded that the above structures represented resistant end groups. These structures certainly did represent the absolute limits as determined by the methylation study. However, there was no good evidence to indicate that β -amylase was capable of hydrolyzing bonds as close to the branch point as indicated. In fact Meyer indicated that further action of β -amylase on the isolated limit dextrans produced small amounts of maltose. He interpreted this as meaning that certain or all of 2, 3, and 4 were being hydrolyzed at an extremely slow rate. However, this result could also mean that the residual chains were really somewhat longer than indicated in the preceding structures.

Myrbäck and Sihlbom⁶⁹ indicated certain structures in Table 1 (page 16 of this thesis) which were resistant to β -amylase, and others that were not. These structures did not agree with methylation and periodate experiments which supported the view that no end groups were lost during the action of β -amylase. The test substrates in Table 1, which were prepared by acid hydrolysis and separated by alcohol precipitation, must have been obtained as rather crude mixtures. An attempt to follow the action of β -amylase on these mixtures could only result in an approximation of the true situation.

More recently Peat and co-workers⁴⁰ have subjected a β -amylase limit dextrin of amylopectin to the action of E-enzyme. The dialyzable portion of this digest consisted of maltose and maltotriose, and it was concluded that the average stub in the limit dextrin contained 2.5

glucose units. The maltose and maltotriose could be derived from the side chains only, and not from the main chains. These conclusions are valid only if the side chains do not contain any single glucose stubs. If this is the case, the resistant linkages as represented in Table 9 are entirely consistent with the R-enzyme experiments.

Quantitative Action of β -Amylase

The initial rates of β -amylase action on the various substrates are plotted in Figs. 13, 15, and 17. A determination of the slope was made from each of these curves, and is recorded along with the relative rate in Table 10. In the case of sweet corn glycogen, the moles of maltose per theoretical moles of maltose were converted to moles of maltose per exterior chain. The initial rate of β -amylase on G₇ was converted to the same basis as that used for the various branched oligosaccharides. If the rate of β -amylase on G₇ is considered to be one, then the relative rates for the other substrates can be calculated. The relative rates, as well as the figures, indicate that in the glycogen molecule, the exterior branches are being hydrolyzed at about 0.6 the rate of maltoheptaose. Furthermore, the rate on the various branched molecules becomes progressively slower as the α -1,6 linkage is approached. This observation indicates that quite possibly an absolute limit dextrin is a misleading term if not a practical impossibility. It would be far superior to define a limit dextrin according to the amount of enzyme used in the degradation

Table 10. Relative rates of β -amylase on glycogen, maltoheptaose, and the branched oligosaccharides

Substrate	Slope	Relative rate
Maltoheptaose	423×10^{-4}	1.
Sweet corn glycogen	262×10^{-4}	0.620
Panose coupled products B ₇	156×10^{-4}	0.370
Panose coupled products B ₆	40×10^{-4}	0.095
Panose coupled products B ₅	1×10^{-4}	0.002
Isomaltose coupled products B ₆	94.5×10^{-4}	0.223
Isomaltose coupled products B ₅	69.5×10^{-4}	0.164
Isomaltose coupled products B ₄	5.4×10^{-4}	0.013

multiplied by the time digested under certain specified conditions. An examination of Figs. 12, 14, and 16 adds support to this view. If a true absolute limit dextrin is formed, one would expect these curves to level off after extended periods of degradation. However, this is not the case, and it is impossible, by examining these curves, to say that a limit dextrin is ultimately produced in any of these digests.

Action of β -Amylase on a Natural Molecule Such as Amylopectin or Glycogen

Using low molecular weight model substrates to study the action of a particular enzyme on a high molecular weight natural product has certain limitations. In the case of a molecule having the amylopectin type structure, there are many sites where enzyme action can occur. It was evident from this work that one chain had an effect on another. In the simple cases studied, only two chains were available. In amylopectin or glycogen, where many chains were available, this effect might have been greatly exaggerated. Furthermore, the only type of branched compounds studied were those which would be found in the Meyer type structure. It is quite possible that branching, such as one would find in the Haworth or Staudinger structures, might also occur. An isolation and study of doubly branched dextrans might be helpful in answering a number of these questions.

The low molecular weight branched oligosaccharides which are resistant to the action of β -amylase do give some indication as to the type of end groups one could expect in a so-called limit dextrin. It seems reasonable that degradation under comparable conditions would proceed no farther, if as far, in the natural molecule. There are very definitely certain similarities in the curves found in Figs. 12, 14, and 16. In the curve for glycogen, there is apparently a slow hydrolytic action in the latter stages of the digest. The rate of this action is comparable to the rate of β -amylase on the panose

coupled E_5 and the isomaltose coupled E_4 .

Suggestions for Further Study

The use of a debranching enzyme, such as R-enzyme, in the study of the branched structure of amylopectin would be a fruitful area for investigation. The R-enzyme used in these experiments evidently contained an enzymic impurity which made it a less valuable tool. A study of the isolation, purification and action of R-enzyme would be of great help. The model substrates used in this work would be very helpful in the study of the specificity and kinetics of R-enzyme.

A highly purified sample of this enzyme would be a powerful analytical tool in the determination of the structure of low molecular weight branched oligosaccharides. However, the action of R-enzyme on the β -amylase limit dextrin of amylopectin might prove to be a more valuable method of investigation. This type of work again would require a highly purified R-enzyme, and an enzyme whose action is characterized. The action of a highly purified R-enzyme on amylopectin should result in a series of amylose oligosaccharides. Flavazole derivatives could be made of these oligosaccharides, and the resulting derivatives could be separated and identified on paper. The successive action of R-enzyme and α -1,6 glucosidase on the β -amylase limit dextrin of amylopectin could settle the question concerning the occurrence of single glucose stubs in the limit dextrin. It would again be necessary to purify and study carefully the action of the debranching enzyme

(α -1,6-glucosidase) before its use would be meaningful.

A study of the action of β -amylase on branched dextrins obtained by the limited action of salivary amylase or acid hydrolysis would offer some interesting information. Doubly branched compounds, and other dextrins which are more nearly analogous to the linkages in amylopectin than those investigated here, could be studied in this way. The problem in this type of investigation would be in the separation of the various branched dextrins. The answer to this question might be in the preparation of a dextrin derivative which would be more readily separable. The use of this technique would necessitate the removal of the derivatizing group, without degradation of the dextrin.

SUMMARY

1. Homologous panose coupled branched oligosaccharides have been prepared by the action of macerans amylase on α -dextrin and panose. The isomeric fractions B_5 , B_6 , and B_7 were separated by the multiple ascent technique of paper chromatography.
2. Isomaltose has been separated from the products of acid hydrolyzed dextran by carbon chromatography. Isomaltose coupled products were prepared by the use of the macerans amylase coupling reaction. The oligosaccharide fractions were separated by multiple ascent paper chromatography into B_4 , B_5 , and B_6 groups.
3. The action of β -amylase on the panose coupled oligosaccharides has been studied. Fractions of the B_5 and B_6 groups were resistant to the action of β -amylase. By the use of R-enzyme, it was concluded that the resistant B_5 was $\begin{matrix} \text{O-O-O-} \\ \text{O-O} \end{matrix}$, and the resistant B_6 was $\begin{matrix} \text{O-O-O-} \\ \text{O-O-O} \end{matrix}$. There might have been a small amount of a resistant B_7 with the probable structure $\begin{matrix} \text{O-O-O-O-} \\ \text{O-O-O} \end{matrix}$. A measure of the rate of β -amylase action on the various fractions has been made. The rate of enzyme action decreased considerably as the branch point (α -1,6 linkage) was approached.
4. The action of β -amylase on the isomaltose coupled oligosaccharides has been studied. Fractions of the B_4 , B_5 , and B_6

groups were resistant to the action of the enzyme. The resistant B_4 consisted mainly of $O-O-O-$ but could conceivably have contained $O-O-O-\overset{O}{\underset{O}{|}}$. The resistant B_5 contained both $O-O-O-$ and $O-O-O-\overset{O}{\underset{O}{|}}$. The resistant B_6 consisted of $O-O-O-\overset{O}{\underset{O}{|}}$. One chain had an effect on the other as far as the action of the enzyme was concerned. The presence of a single glucose unit on the opposite chain was enough to make a resistant bond out of one which was formerly non-resistant. The rate of β -amylase action on these fractions was also studied. Again the rate diminished as the branch point was approached.

5. B_7 from the salivary amylase hydrolysis of amylopectin was separated by paper chromatography. This preparation consisted of a single oligosaccharide of known structure, $O-O-O-O-$. Salivary amylase was capable of slowly hydrolyzing this B_7 to a B_5 and maltose, but the B_7 was apparently completely resistant to the action of β -amylase.

6. The rate of β -amylase on sweet corn glycogen and maltoheptaose was studied. Two distinct phases of hydrolysis were evident for both of these substrates. The slow phase of each was comparable in rate to that of β -amylase on B_5 from panose and B_4 from isomaltose. Evidence was presented which indicated the impracticality of attaining an absolute limit dextrin from a high molecular weight natural molecule such as glycogen or amylopectin.

BIBLIOGRAPHY

1. L. Maquenne et E. Roux. Recherches sur l'amidon et sa saccharification diastatique. Ann. Chim. Phys., (8) 2, 179 (1906).
2. K. H. Meyer. The past and present of starch chemistry. Experientia, 8, 405 (1952).
3. P. Karrer und E. v. Krauss. Beitrag zur physikalischen Struktur der Stärke. Helv. Chim. Acta, 12, 1114 (1929).
4. A. R. Ling and D. R. Nanji. Studies on starch. Part I. The nature of polymerised amylose and amylopectin. J. Chem. Soc., 123, 2666 (1923).
5. E. L. Hirst, M. M. T. Plant and M. D. Wilkinson. Polysaccharides. Part XIV. The molecular structure of amylose and of amylopectin. J. Chem. Soc., 2375 (1932).
6. M. Samec. Kolloidchemie der Stärke. Verlag Th. Steinkopf, Leipzig (1927).
7. K. H. Meyer. Recent developments in starch chemistry. Advances in Colloid Sci., 1, 143 (1942).
8. K. Freudenberg und H. Boppel. Methylierte Stärke. Ber., 71, 2505 (1938).
9. T. J. Schoch. Physical aspects of starch behavior. Cereal Chem., 18, 121 (1941).
10. T. J. Schoch. The fractionation of starch. Advances in Carbohydrate Chem., 1, 247 (1945).
11. K. H. Meyer et G. G. Gibbons. Fractionnement de l'amylopectine. Recherches sur l'amidon 47. Helv. Chim. Acta, 33, 213 (1950).
12. K. H. Meyer et G. G. Gibbons. Purification de l'amylopectine. Recherches sur l'amidon 46. Helv. Chim. Acta, 33, 210 (1950).
13. G. A. Gilbert, C. T. Greenwood and F. J. Hybart. An evaluation of methods using stearic acid and cellulose for the purification of amylopectin. J. Chem. Soc., 4454 (1954).

14. H. C. Kirchhof. Ueber die Zuckerbildung beim Malzen des Getreides, und beim Bebrühen seines Mehls mit kochendem Wasser. Schweigger's J. Chem. Phys., Nuremberg, 14, 389 (1815).
15. W. N. Haworth. The structure of carbohydrates. Helv. Chim. Acta, 11, 534 (1928).
16. W. N. Haworth. Molecular structure of cellulose and of amylose. Nature (London), 129, 365 (1932).
17. W. N. Haworth and E. G. V. Percival. Polysaccharides. Part XI. Molecular structure of glycogen. J. Chem. Soc., 2277 (1932).
18. H. Staudinger und E. Huseman. Über hochpolymere Verbindungen. Über die Konstitution der Stärke. Ann., 527, 195 (1936).
19. W. N. Haworth. The structure, function and synthesis of polysaccharides. Proc. Roy. Soc. (London), A 186, 1 (1946).
20. T. G. Halsall, E. L. Hirst and J. K. N. Jones. The structure of glycogen. Ratio of non-terminal to terminal glucose residues. J. Chem. Soc., 1399 (1947).
21. A. L. Potter and W. Z. Hassid. Starch. I. End-group determination of amylose and amylopectin by periodate oxidation. J. Am. Chem. Soc., 70, 3488 (1948).
22. K. H. Meyer et P. Rathgeb. Recherches sur l'amidon 45. Sur le dosage des groupes terminaux de polysaccharides et d'oligosaccharides au moyen de periodate. Helv. Chim. Acta, 32, 1102 (1949).
23. K. Myrbäck und K. Ahlberg. Die Konstitution eines Stärke-grenzedextrins. Nachweis α -glykosidischer 1,6-Bindungen in Dextrin und Stärke. Biochem. Z., 307, 69 (1940).
24. G. C. Gibbons et R. A. Boissomas. Nature de la liaison d'embranchement du glycogène et de l'amylopectine. Recherches sur l'amidon 48. Helv. Chim. Acta, 33, 1477 (1950).
25. M. L. Wolfrom, E. N. Lassetre and A. N. O'Neill. Degradation of glycogen to isomaltose. J. Am. Chem. Soc., 73, 595 (1951).
26. D. J. Bell and D. J. Manners. α -1,4-glucosans. Part I. The interchain linkages in glycogens. J. Chem. Soc., 1891 (1954).
27. S. Peat, W. J. Whelan and T. E. Edwards. Polysaccharides of baker's yeast. Part I. Glycogen. J. Chem. Soc., 355 (1955).

28. C. T. Greenwood. The size and shape of some polysaccharide molecules. Advances in Carbohydrate Chem., 7, 300 (1952).
29. K. H. Meyer et W. Settele. Sur l'inhomogénéité des amylopectines de tapioca et de waxy maize. Recherches sur l'amidon 52. Helv. Chim. Acta, 36, 197 (1953).
30. K. H. Meyer, A. J. A. van der Wyk et Chen-Piao Feng. Sur la détermination du poids moléculaire des polysaccharides. Recherches sur l'amidon 54. Helv. Chim. Acta, 37, 1619 (1954).
31. K. H. Meyer et P. Bernfeld. Recherches sur l'amidon. V. L'amylopectine. Helv. Chim. Acta, 23, 875 (1940).
32. K. H. Meyer, M. Wertheim et P. Bernfeld. Recherches sur l'amidon. XI. Sur la dextrine résiduelle de l'amidon de maïs (érythrogramlose). Helv. Chim. Acta, 24, 212 (1941).
33. K. H. Meyer. The chemistry of glycogen. Advances in Enzymol., 3, 109. (1943).
34. D. L. Morris and C. T. Morris. Glycogen in the seed of *Zea mays* (variety Golden Bantam). J. Biol. Chem., 130, 535 (1939).
35. W. Dvornch and R. L. Whistler. Water-soluble polysaccharides of sweet corn. J. Biol. Chem., 181, 889 (1949).
36. G. T. Cori and J. Lerner. Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. Biol. Chem., 188, 17 (1951).
37. B. Illingworth, J. Lerner and G. T. Cori. Structure of glycogens and amylopectins. I. Enzymatic determination of chain length. J. Biol. Chem., 199, 631 (1952).
38. D. J. Bell and D. J. Manners. The action of crystalline β -amylase on some glycogens. J. Chem. Soc., 3641 (1952).
39. D. J. Manners. The enzymic degradation of starch and glycogen. Ann. Repts. on Progr. Chem. (Chem. Soc. London), 50, 288 (1953).
40. S. Peat, W. J. Whelan and G. T. Thomas. Evidence of multiple branching in waxy maize starch. J. Chem. Soc., 4546 (1952).
41. W. J. Whelan. The enzymic breakdown of starch. Biochem. Soc. Symposia, 11, 17 (1953).

42. S. Peat, W. J. Whelan and P. N. Hobson. The enzymic synthesis and degradation of starch. Part XIX. The action of R-enzyme on glycogen. J. Chem. Soc., 4440 (1954).
43. E. L. Hirst and D. J. Manners. Multiple-branching in amylopectin. Chemistry and Industry, 224 (1954).
44. M. Märker. Ber., 10, 2234 (1877).
45. H. P. Wijsman, Jr. La diastase considérée comme un mélange de maltase et de dextrinase. Rec. Trav. Chim., 9, 1 (1890).
46. R. Kuhn. Der Wirkungsmechanismus der Amylasen, ein Beitrag zum Konfigurationsproblem der Stärke. Ann., 443, 1 (1925).
47. E. Ohlsson. Über die beiden Komponenten der Malzdiastase, besonders mit Rücksicht auf die Mutarotation der bei der Hydrolyse der Stärke gebildeten Produkte. Hoppe-Seyler's Z. Physiol. Chem., 189, 17 (1930).
48. C. S. Hanes. The action of amylases in relation to the structure of starch and its metabolism in the plant. New Phytologist, 36, 189 (1937).
49. A. K. Balls, M. E. Walden and R. R. Thompson. A crystalline β -amylase from sweet potatoes. J. Biol. Chem., 173, 9 (1948).
50. K. H. Meyer, Ed. H. Fischer et A. Piguet. Purification et cristallisation de la β -amylase de malt. Sur les enzymes amylolytiques XVI. Helv. Chim. Acta, 34, 316 (1951).
51. A. Piguet et Ed. H. Fischer. Propriétés de la β -amylase de malt. Sur les enzymes amylolytiques 21. Helv. Chim. Acta, 35, 257, (1952).
52. K. H. Meyer, P.-F. Spahr et Ed. H. Fischer. Purification, cristallisation et propriétés de la β -amylase de blé. Sur les enzymes amylolytiques 22. Helv. Chim. Acta, 36, 1924 (1953).
53. S. Peat, S. J. Pirt and W. J. Whelan. The enzymic synthesis and degradation of starch. Part XVI. The purification and properties of the β -amylase of soya bean. J. Chem. Soc., 714 (1952).
54. S. Englard and T. P. Singer. Physicochemical studies on β -amylase. J. Biol. Chem., 187, 213 (1950).

55. S. Englard, S. Sorof and T. P. Singer. Intramolecular nature of the oxidative inactivation of crystalline β -amylase. J. Biol. Chem., 189, 217 (1951).
56. M. Samec und E. Waldschmidt-Leitz. Über die enzymatische Spaltbarkeit der Amylo- und Erythrokörper aus Stärke. I. Mitteilung über enzymatische Amylolyse. Hoppe-Seyler's Z. Physiol. Chem., 203, 16 (1931).
57. W. J. Whelan, J. M. Bailey and P. J. P. Roberts. The mechanism of carbohydrase action. Part I. The preparation and properties of maltodextrin substrates. J. Chem. Soc., 1293 (1953).
58. J. M. Bailey, W. J. Whelan and S. Peat. Carbohydrate primers in the synthesis of starch. J. Chem. Soc., 3692 (1950).
59. W. J. Whelan and J. M. Bailey. The action pattern of potato phosphorylase. Biochem. J., 58, 560 (1954).
60. W. J. Whelan and J. G. Roberts. Products of the β -amylolysis of maltodextrins. Biochem. J., 58, 569 (1954).
61. R. L. Whistler and J. L. Hickson. Maltotetraose and crystalline pentadecaacetylmaltotetraitol. J. Am. Chem. Soc., 76, 1671 (1954).
62. R. L. Whistler and J. H. Duffy. Maltopentaose and crystalline octadeca-O-acetylmaltopentaitol. J. Am. Chem. Soc., 77, 1017 (1955).
63. S. Peat, S. J. Pirt and W. J. Whelan. Enzymic synthesis and degradation of starch. Part XV. β -amylase and the constitution of amylose. J. Chem. Soc., 705 (1952).
64. S. Peat, G. J. Thomas and W. J. Whelan. The enzymic synthesis and degradation of starch. XVII. Z-enzyme. J. Chem. Soc., 722 (1952).
65. R. H. Hopkins and R. Bird. The Z-enzyme in amylolysis. Nature (London), 172, 492 (1953).
66. S. Peat and W. J. Whelan. Nature (London), 172, 494 (1953).
67. K. H. Meyer, P. Bernfeld, P. Rathgeb et P. Görtler. Recherches sur l'amidon, 41. La dégradation de l'amylopectine par la β -amylase. Helv. Chim. Acta, 31, 1536 (1948).

68. P. Bernfeld. Enzymes of starch degradation and synthesis. Advances in Enzymol., 12, 379 (1951).
69. K. Myrbäck und E. Sjöbom. Wirkung der Malzamyblasen auf niedermolekulare Hydrolyseprodukte der Stärke. Arkiv Kemi, 1, 1 (1949).
70. M. A. Swanson. Studies on the structure of polysaccharides. II. Degradation of polysaccharides by enzymes. J. Biol. Chem., 172, 805 (1948).
71. M. A. Swanson. Studies on the structure of polysaccharides. IV. Relation of the iodine color to the structure. J. Biol. Chem., 172, 825 (1948).
72. F. C. Cleveland and R. W. Kerr. The action of beta-amylase on corn amylose. Cereal Chem., 25, 133 (1948).
73. R. W. Kerr and F. C. Cleveland. The action of crystalline β -amylase on corn crystalline amylose. J. Am. Chem. Soc., 73, 2421 (1951).
74. R. W. Kerr. Action of beta amylase on amylose. Nature (London), 164, 757 (1949).
75. R. H. Hopkins, B. Jelinek and L. E. Harrison. The action of β -amylase on potato amylose. Biochem. J., 43, 32 (1948).
76. E. J. Bourne and W. J. Whelan. Mechanism of the beta-amyolysis of amylose. Nature (London), 166, 258 (1950).
77. D. French, M. Levins, J. H. Pazur and E. Norberg. Studies on the Schardinger dextrans. IV. The action of soy bean beta amylase on amyloheptaose. J. Am. Chem. Soc., 72, 1746 (1950).
78. D. French, D. W. Knapp and J. H. Pazur. Amylase action under conditions of unfavorable temperature or hydrogen ion concentration. J. Am. Chem. Soc., 72, 1866 (1950).
79. R. H. Hopkins and B. Jelinek. The mechanism of β -amylase action. 1. 'Multichain' action on amylose. Biochem. J., 56, 136 (1954).
80. R. Bird and R. H. Hopkins. The mechanism of β -amylase action. 2. 'Multichain' action on amylose fission products. Biochem. J., 56, 140 (1954).
81. J. M. Bailey and D. French. Ames, Iowa. The concept of multiple attack in enzyme-polymer reactions. (Unpublished manuscript.) 1955.

82. Franz Schardinger. Ueber die Bildung kristallisierter, Fehlingsche Lösung nicht reduzierender Körper (polysaccharide) aus Stärke durch mikrobielle Tätigkeit. Zentr. Bakt. Parasitenk., II 22, 98 (1908-9).
83. K. Freudenberg. Polysaccharides and lignin. Ann. Rev. Biochem., 8, 81 (1939).
84. D. French and R. E. Rundle. The molecular weights of the Schardinger alpha and beta dextrans. J. Am. Chem. Soc., 64, 1651 (1942).
85. K. Freudenberg, E. Plankenhorn and H. Knauer. Schardinger's dextrans derived from starch. Chemistry and Industry, 731 (1947).
86. K. Freudenberg und F. Cramer. Über die Schardinger-Dextrine aus Stärke. Ber., 83, 296 (1950).
87. E. B. Tilden and G. S. Hudson. The conversion of starch to crystalline dextrans by the action of a new type of amylase separated from cultures of Aerobacillus macerans. J. Am. Chem. Soc., 61, 2900 (1939).
88. E. Norberg. Purification and characterization of macerans amylase. Unpublished Ph.D. thesis. Ames, Iowa, Iowa State College Library. 1949.
89. S. Schwimmer and J. A. Garibaldi. Further studies on the production, purification and properties of the Schardinger dextrinogenase of Bacillus macerans. Cereal Chem., 29, 108 (1952).
90. S. Schwimmer. Evidence for the purity of Schardinger dextrinogenase. Arch. Biochem. Biophys., 43, 108 (1953).
91. W. S. McClenahan, E. B. Tilden and G. S. Hudson. A study of the products obtained from starch by the action of the amylase of Bacillus macerans. J. Am. Chem. Soc., 64, 2139 (1942).
92. S. A. Barker and E. J. Bourne. Enzymic synthesis of polysaccharides. Quart. Reviews, 7, 56 (1953).
93. G. F. Cori. Symposium on the formation of disaccharides, polysaccharides and nucleosides. Federation Proc., 4, 226 (1945).
94. D. French, J. Pazur, M. L. Levine and E. Norberg. Reversible action of macerans amylase. J. Am. Chem. Soc., 70, 3145 (1948).

95. E. J. Hehre. Enzymic synthesis of polysaccharides; a biological type of polymerization. Advances in Enzymol., 11, 297 (1951).
96. E. Norberg and D. French. Studies on the Schardinger dextrans. III. Redistribution reactions of macerans amylase. J. Am. Chem. Soc., 72, 1202 (1950).
97. S. C. Pan, L. W. Nicholson and P. Kolachov. Isolation of a crystalline trisaccharide from the unfermentable carbohydrate produced enzymically from maltose. J. Am. Chem. Soc., 73, 2547 (1951).
98. D. French. Structure of Pan's crystalline trisaccharide. Science, 113, 352 (1951).
99. M. L. Wolfrom, A. Thompson and T. T. Galkowski. 4- α -isomaltopyranosyl-D-glucose. J. Am. Chem. Soc., 73, 4093 (1951).
100. D. French, M. L. Levine, E. Norberg, P. Nordin, J. K. Pazur and G. M. Wild. Studies on the Schardinger dextrans. VII. Co-substrate specificity in coupling reactions of macerans amylase. J. Am. Chem. Soc., 76, 2387 (1954).
101. J. H. Pazur. The preparation and characterization of 1-C¹⁴-amylotriase. J. Am. Chem. Soc., 77, 1015 (1955).
102. P. Nordin. Action pattern of salivary amylase. Unpublished Ph.D. thesis. Ames, Iowa, Iowa State College Library. 1953.
103. P. N. Hobson, W. J. Whelan and S. Peat. A 'de-branching' enzyme in bean and potato. Biochem. J., 47, xxxix (1950).
104. P. N. Hobson, W. J. Whelan and S. Peat. The enzymic synthesis and degradation of starch. Part XIV. R-enzymes. J. Chem. Soc., 1451 (1951).
105. G. M. Wild. Action patterns of starch enzymes. Unpublished Ph.D. thesis. Ames, Iowa, Iowa State College Library. 1953.
106. W. J. Whelan and P. J. P. Roberts. Action of salivary α -amylase on amylopectin and glycogen. Nature (London), 170, 748 (1952).
107. W. H. Wadman, G. J. Thomas and A. B. Pardee. Quantitative method using paper chromatography for estimation of reducing oligosaccharides. Anal. Chem., 26, 1192 (1954).

108. R. L. Whistler and D. F. Durso. Chromatographic separation of sugars on charcoal. J. Am. Chem. Soc., 72, 677 (1950).
109. N. Nelson. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153, 375 (1944).
110. M. L. Wolfrom, L. W. Georges and I. L. Miller. Crystalline derivatives of isomaltose. J. Am. Chem. Soc., 71, 125 (1949).
111. W. Z. Hassid and R. M. McCready. The molecular constitution of glycogen and starch from the seed of sweet corn (*Zea mays*). J. Am. Chem. Soc., 63, 1632 (1941).
112. D. French, M. L. Levine and J. H. Pazur. Studies on the Schardinger dextrans. II. Preparation and properties of amyloheptaase. J. Am. Chem. Soc., 71, 356 (1949).
113. D. French, M. L. Levine, J. H. Pazur and E. Norberg. Studies on the Schardinger dextrans. The preparation and solubility characteristics of alpha, beta and gamma dextrans. J. Am. Chem. Soc., 71, 353 (1949).
114. J. M. Bailey. Some starch metabolizing enzymes. Unpublished Ph.D. thesis. Bangor, Wales, University College of North Wales. (ca. 1951).
115. M. Bergmann and J. S. Fruton. The specificity of proteinases. Advances in Enzymol., 1, 63 (1941).

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