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Action pattern of salivary amylase

Philip Nordin
Iowa State College

NOTE TO USERS

ACTION PATTERN OF SALIVARY AMYLASE

by

Philip Nordin

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In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

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I. INTRODUCTION

In the past fifty years a tremendous amount of work has been reported on problems related to starch. Not only is its structure very complicated, but biological problems are involved as well. Many of these problems are of great interest to commercial concerns engaged in the utilization of starch.

The many and varied enzymes associated with the breakdown and synthesis of starch have been thoroughly studied. This has led in many cases to a precise working knowledge of the enzyme mechanism. Such systems have in turn been of great value in revealing structural details of starch itself, supplementing the classical methods of organic chemistry.

Salivary amylase is a well known hydrolytic starch enzyme. However, there is as yet no universal agreement as to its mechanism. Clearly more information is required before agreement can be attained. It would be of value in formulating a mechanism if more were known about the specificity of the enzyme toward the various bonds present.

It was the primary purpose of these studies on salivary amylase to isolate and characterize some of the intermediary hydrolytic products, particularly the branched limit dextrins. The formation of such intermediary hydrolytic fragments demonstrates that some bonds are more resistant because of their location in the starch chain. The experiments with radioactive oligosaccharides also yield information as to the relative specificity of the enzyme toward glucosidic bonds that differ only with respect to their location in the chain.

II. REVIEW OF THE LITERATURE

A. Starch

Starch, being one of the basic foodstuffs and of very wide occurrence, has been the subject of considerable investigation. Early research based largely on methods of organic chemistry (methylation, periodate oxidation) was successful in elucidating the main features of its chemical structure. Accounts of these studies can now be found in text books (1, 2, 3). In addition, reviews on starch have appeared from time to time in the chemical literature (4, 5).

Starch generally is found to consist of amylose and amylopectin. Glycogen is an amylopectin-like material derived from animal sources. Amylose has been successfully separated from amylopectin by electrodecantation (6) and warm water extraction (7). The most useful method, however, is the butanol precipitation technique of Schoch (8). The amylose fraction consists of long chain polymers of D-glucose united in α-1,4 bonds. Recently some evidence has appeared that this simple picture may not be altogether correct. Peat and co-workers (9, 10, 11) have isolated an enzyme

(Z-enzyme) from β-amylase which appears to hydrolyze beta linkages. Their purified β-amylase only partially hydrolyzes amylose. When Z-enzyme is added, however, the reaction goes to completion. The beta linkages wherever they occurred in the chain, would halt the normal endwise attack of β-amylase from the non-reducing end. This is in conflict with Meyer et al. who report that crystalline malt β-amylase completely hydrolyzes amylose (12).

The amylopectin fraction has a highly branched structure. Some species of plants produce amylopectin which is free of amylose, for example, waxy maize. Amylopectin contains in addition to α-1,4 links, some α-1,6 links. The α-1,6 links are the sites of branches. The number of glucose units between branches may vary from species to species and also with the age of the plant (13). The average number of glucose units in outer and inner branches of various glycogens and amylopectins has been determined by an enzymatic method (14). The average number of glucose units for mature corn was found to be; outer branches - 6.9, inner branches - 5.0. Some starch fractions, classified as amyloses, actually have a low degree of branching (15).

By following the rate of glucose production by the enzyme amylo-glucosidase (see Section II, C), Kerr *et al*. could distinguish between slightly branched amylose and mixtures of amylose and amylopectin. The conclusion was reached that potato and tapioca amyloses contain a large proportion of slightly branched molecules.

Branching at sites other than position 6 in glycogen has been claimed (16). Complete methylation of glycogen followed by hydrolysis should give dimethyl glucose from the branching points and tetramethyl glucose from the end groups. The dimethyl glucose fraction isolated by these workers was found to consist mainly of 2,6-dimethyl glucose with some 2,3- and 3,6-dimethyl glucose. The authors were thus led to believe that branching occurred primarily at position 3. This work has since been checked with glycogen and amylopectin, using the periodate oxidation technique (17). A glucose moiety containing branches at positions 3 or 2 would be unattacked by periodate. Estimation of the glucose produced convinced the authors that branching at these sites is insignificant in both glycogen and amylopectin. Indeed, the traces of glucose could very well have come from incomplete oxidation.

B. Alpha Amylases

The literature on the starch enzymes has been thoroughly reviewed by Wild (18). It would be desirable, however, to restate some of the pertinent facts regarding α-amylases. All α-amylases have one feature in common, the ability to hydrolyze the internal α-1,4 linkages of starch leading to the formation of dextrins. As a class of enzymes their reaction mechanisms are distinctly different from those of β-amylase and amyloglucosidase, which operate from the non-reducing end. The latter enzymes yield maltose and glucose respectively. Human salivary amylase was successfully crystallized by Meyer et al. (19) in 1948. Crystallization of swine pancreatic amylase (20, 21, 22) and of human pancreatic amylase (23, 24) was also achieved. It is interesting that human pancreatic amylase and human salivary amylase not only had the same activity, but appeared to be identical chemically. Swine pancreatic amylase was different chemically; however, it seemed to have identical hydrolytic action. Recently a more rapid method for the crystallization of swine pancreatic amylase has been reported (25). Some other α-amylases now reported to have been crystallized, are malt amylase

from germinated barley (26) and a bacterial amylase from Bacillus subtilus (27).

Early investigators came to the conclusion that α-amylases operate by a random type mechanism, gradually breaking the starch chains into shorter and shorter dextrins (28, 29). Ohlsson's experiments were performed with malt α-amylase and his results demonstrated an increase in non-dializable particles during the early phase of the reaction. Similar conclusions were reached by Freeman and Hopkins (30) using malt α-amylase and pancreatic amylase. Alcohol fractionation of the partially hydrolyzed material yielded samples which contained maltose but must have contained dextrins because of their low reducing values.

More recently Bernfeld et al. (31, 32, 33) reported on the degradation of amylose by malt α-amylase. They concluded that all α-1,4 bonds were hydrolyzed with equal facility except the terminal bonds. Again Caldwell et al. (34, 35, 36) using pancreatic amylase observed that there were produced in the early stages of the digest, quantities of low molecular weight sugars which were hydrolyzed much more slowly. They felt that

the reaction was probably random in the early stages but departed from it as the reaction progressed. No attempt was made to identify the low molecular weight sugars that were formed. This is a characteristic weakness of most of the articles appearing on α-amylases. Using paper chromatography French et al. (37, 38) have conclusively demonstrated that the products formed by the action of salivary amylase on amylodextrin are not in accordance with what would be expected from random hydrolysis. In the early stages maltose (G_2), amylotriose (G_3) and amylotetraose (G_4) are the chief products formed. There were only traces of oligosaccharides in the G_5 and higher range. These are certainly produced in the random hydrolysis by acid. The enzyme, however, can be made to operate by random hydrolysis if conditions of unfavorable temperature and pH prevail. The authors held that the action was entirely explainable by an enzyme cage effect resulting in multiple scission in a localized area of the molecule.

There is evidence that the various α-amylases may differ in their mode of reaction. Meyer and Gonon (39) studied the hydrolysis of amylose from potato and from corn with crystalline swine pancreatic amylase and crystalline

malt α-amylase. The affinity for oligosaccharides of intermediate length is considerably less than for amylose in the case of malt α-amylase. With pancreatic amylase only a slight difference could be observed. Both enzymes hydrolyze amylotriose slowly to yield glucose and maltose. Salivary amylase hydrolyzes chains of intermediate length almost as fast as amylose itself (40). Whelan et al. (41, 42, 43, 44) have obtained results in agreement with the random hydrolysis concept of Meyer and Bernfeld (33). If the concept were true then at the end of hydrolysis one should find only maltose and amylotriose. These must resist the enzyme since they contain only terminal linkages. The ratio of maltose to amylotriose can be calculated on the basis of this theory to be 2.35:1. Their experiments consisted of allowing salivary amylase to react with amylose until no further increase in reducing power could be demonstrated. The products were then separated on a carbon column. They found that indeed maltose and amylotriose were the sole products and were in the correct ratio predicted by theory. They were unable to confirm the hydrolysis of amylotriose. Again the nature of the products present at early stages of the reaction does not appear to have

been investigated. They were successful in isolating by carbon chromatography members of the amylose series from G_3 to G_7 (from a partial acid hydrolysis of amylose). These were subjected to salivary amylase and the end products were separated by carbon chromatography and were found to consist only of maltose and amylotriose in the correct ratio. Amylotetraose yielded only maltose; this was also demonstrated by Pazur (45).

It should be stated at this point that if one were to start with an equimolar mixture of G_2, G_3 and G_4, which are the intermediate products formed (38), and were to allow it to react with salivary amylase until the G_4 was completely converted to maltose one would obtain very nearly the same ratio as predicted by Meyer's theory. G_3 is hydrolyzed so slowly that no detectable amount of glucose and maltose could come from this source at this stage of hydrolysis. It would thus seem that the terminal linkages are indeed much more resistant to hydrolysis than internal linkages, but hydrolysis of internal bonds cannot be a simple random type phenomenon.

It has long been recognized that amylopectin is

not completely degraded by α-amylase. It was noted in 1937 (46, 47) that after extended action of malt there remain the limit dextrins, oligosaccharides which contain the "anomalous" links of the starch chain. Possible anomalies considered at that time were links other than α-1,4 and substitution by phosphoric acid. It is now recognized that substitution by phosphoric acid in the 6 position as esters, occurs in root and tuber starches (48). The malt limit dextrins have since been shown by Myrbäck and Ahlborg to contain an average of one α-1,6 bond per molecule (49). Two thirds of the dextrins had an average chain length of six. Tetra- and trisaccharides were present. The methylated trisaccharide was shown to have the α-1,6 link. Pancreatic and salivary amylase yield limit dextrins with somewhat longer average chain length (50). This is another instance of a difference between malt amylase and pancreatic and salivary amylases.

The dextrins themselves are slowly attacked by the α-amylases. Extensive hydrolysis of amylopectin by crystalline malt amylase or crystalline pancreatic amylase yields as end products glucose and isomaltose (51). This was accomplished if maltose was removed by

fermentation since it inhibits the enzyme. However pure maltose was degraded by both enzymes whereas isomaltose was completely unaffected. The hydrolysis of the limit dextrins themselves is extremely slow and it is not surprising that workers in this field have regarded them as completely resistant end products. Roberts and Whelan (52) thus reported that a branched pentasaccharide is the lowest molecular weight limit dextrin formed by the action of salivary amylase on amylopectin. These authors speculated on the constitution of the dextrins. They regarded the three links adjacent to the α-1,6 bond as completely resistant to attack by the enzyme by virtue of their location. The structure of the pentasaccharide they postulated to be as follows:  . Higher molecular weight dextrins are formed by adding one glucose unit to the various ends of this basic structure, leading to dextrins containing from five to eight glucose units. The central location of the α-1,6 bond was also favored by Myrbäck (53). He suggested

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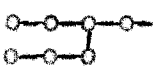
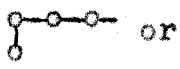
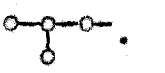
*In this type of structural representation, the circles refer to glucose units, the horizontal bars between circles are α-1,4 bonds, while the vertical bars are α-1,6 bonds. It is customary to indicate the reducing glucose unit with a short bar and to place that unit at the right hand side of the diagram.

that further degradation by amylase results most probably in the removal of maltose from the non-reducing ends. Wild (18) has examined the limit dextrins by paper chromatography at various stages in a salivary amylase digest. At early stages there are four singly branched limit dextrins designated as B_6, B_7, B_8 and B_9 corresponding to the number of glucose units. Shortly after the hydrolysis of G_4 a B_5 appears and B_9 has been hydrolyzed. This stage is accompanied by a great decrease in the rate of the overall reaction, as measured by reducing sugar value, and may be the one to which Whelan and Roberts (52) have carried their digests. These compounds are in turn degraded into a set of B_4, B_5, B_6 and B_7 dextrins which are a good deal more stable. At least one other series of dextrins is present at this stage corresponding in mobility to a doubly branched set of compounds from 9 to 12 glucose units. In addition some other high molecular weight dextrins could be discerned containing more than 13 glucose units. Wild isolated a few hundred milligrams of the various singly branched dextrins by paper chromatography and carried out structure determinations on them. R enzyme action on B_7 and the oxidized B_7

demonstrated that its structure was either  or  , with evidence favoring the latter. The use of R enzyme on the other dextrins gave less conclusive results because of the slow rate of reaction. Partial acid hydrolysis of B_4 and oxidized B_4 yielded results indicating the structures  or  .

C. Amylo-glucosidases

The amylo-glucosidases are able to hydrolyze α-glucosidic linkages producing glucose. Members of this class of enzymes which are able to operate on polysaccharides were recognized by Kerr and co-workers (54, 55). An amylo-glucosidase from Aspergillus niger N.R.R.L. - 336 #1 produces exceptionally large yields of glucose (56). A careful study of this enzyme by Kerr et al. (57) indicated that it operates from the non-reducing end of the starch molecule like β-amylase, but splitting out glucose. The enzyme was freed from α-amylases by treatment at pH = 2.2 at 5°C for seven days. Reaction kinetics approximated first order which would be expected if the enzyme operated only by removal of non-reducing end groups. Digests examined by paper chromatography showed glucose as the only lower sugar

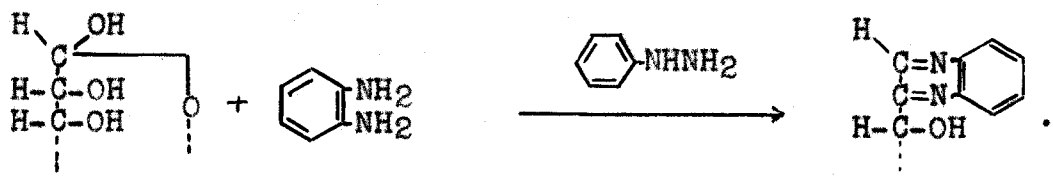
formed. The unconverted amylose residue at different times during the first half of the hydrolysis was found not to change materially in degree of polymerization as measured by iodine spectrophotometry. The initial rate for corn amylopectin was 4.5 times the initial rate for corn amylose, but the rate for amylopectin decreased abruptly to a lower value at about 60% hydrolysis. These results were interpreted that the enzyme must operate terminal-wise by a single chain mechanism. Somewhat like β-amylase, branching points introduce barriers over which the enzyme can cross with difficulty. The amylo-glucosidase of Clostridium acetobutylicum (58) appears to operate by a multi-chain mechanism. It degrades amyloheptaose in successive stages to G_6, G_5, G_4, G_3, G_2 and finally to glucose.

The amylo-glucosidase from Rhizopus delemar has been purified and studied (59, 60). Referred to as gluc amylase by these authors the enzyme is similar to that of the amylo-glucosidase already described. The authors found that it degraded both amylose and amylopectin very completely. There was no evidence for the formation of a limit dextrin with amylopectin and reducing values corresponded to 90% glucose. Low

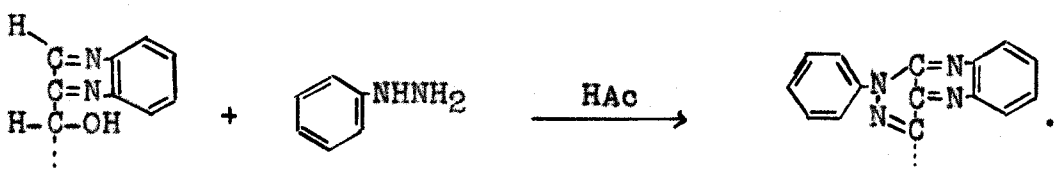
molecular weight sugars other than glucose, not identified, appeared only near the end of the reaction. Apparently the enzyme can either by-pass the α-1,6 link or hydrolyze it. No conclusive evidence for either was presented except that the enzyme had no action on isomaltose or dextran.

D. 1-Phenyl Flavazole Derivatives

1-Phenyl flavazole derivatives of reducing sugars were first described by Ohle (61, 62, 63, 64, 65). The flavazole reaction is a condensation process whereby a reducing sugar condenses with o-phenylene diamine to a quinoxaline derivative in the presence of a dehydrogenating substance.



In acetic acid solution containing phenylhydrazine a further condensation occurs as follows:

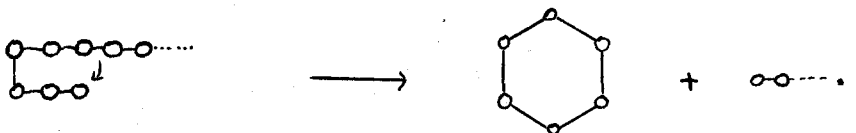


The resulting yellowish orange compound is a pyrazolo-3',4':2,3-quinoxaline sugar derivative and is called a flavazole. The phenylhydrazine, in addition to becoming part of the flavazole molecule, serves as the condensing agent, being reduced to aniline and ammonia. Hence an excess must be used.

The flavazoles are useful derivatives for structure determination of unknown carbohydrates. If positions 4 and 5 of the sugar are unsubstituted, periodate oxidation will yield 1-phenyl flavazole aldehyde, a crystalline compound readily identified by its melting point (62, 66, 67).

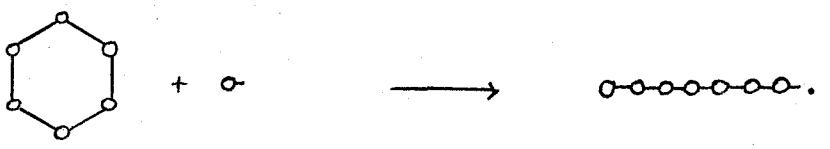
E. Macerans Amylase

The formation of the Schardinger dextrins by the organism Bacillus macerans was attributed by Cori to a glucosidic exchange reaction (68). He postulated that the α-1,4 glucosidic bonds of starch or other linear glucosidic dextrins are transferred by macerans amylase in such a manner that a cyclic dextrin is formed, as follows:

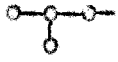
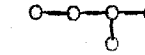
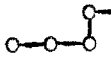


starch α-dextrin

Due to the small energy changes involved it is not surprising that the reaction was found to be reversible (69). α-Dextrin and a co-substrate such as glucose, methyl glucose, maltose, sucrose, cellobiose, maltobionic acid or panose were treated with the enzyme and linear dextrins were formed by coupling with the co-substrate. The initial reaction is illustrated with glucose:



The linear dextrins could also undergo coupling leading to a redistribution of chain length (70). Electrophoretic studies indicated that two molecules of G_7 react to produce G_4 and G_{10} as follows:

Other redistributions of the same type (termed homologizing reactions) soon produce a whole array of oligosaccharides from G_1 up. If the co-substrate is panose two coupling sites are available (71). Oligosaccharides of the following type are produced:  ;  ,  ;  ,  ,  ; etc.

III. MATERIALS AND METHODS

A. Chromatography

The paper chromatographic techniques and materials are those described by Wild (18). One other developing solvent was found to be useful for the flavazoles. This was saturated methyl ethyl ketone, prepared by simply shaking a container of water and methyl ethyl ketone. After the aqueous phase settled out the upper organic phase was decanted and used as required.

The carbon chromatographic procedure was essentially that of Whistler and Durso (72). A much larger column (7 cms. diameter containing 400 gms. of 50:50 celite - Norit) was found to function satisfactorily. For some experiments reported here, a particularly large volume of solution had to be passed through the column. The flow was speeded up to two drops per second by maintaining a hydrostatic pressure of about three feet. The eluting solution in an overhead container was fed to the column by tubing through an air tight stopper.

B. Analytical

Reducing sugars were determined by the iodometric oxidation method of Caldwell et al. (73).

C. Carbohydrates

1. Amylodextrin

Amylodextrin was prepared according to the procedure of Nägeli (74), with some modifications. Twelve pounds of potato starch and fifteen liters of 15% sulfuric acid were allowed to stand in a stoppered container for three and one half months. During this time the contents were frequently agitated. After this time, the material was washed free of sulfuric acid and dissolved in eight volumes of hot 50% ethyl alcohol. Norit was added and the solution was filtered through celite and a No.1 filter pad. The crystals which deposited were collected and a portion was recrystallized, washed with n-butanol and dried. The experiments reported here are with the twice crystallized material. The average chain length was twenty-five glucose units as determined by iodometric oxidation (73).

2. Maltose

Maltose was obtained by the action of β-amylase on potato starch. Three pounds of potato starch were gelatinized in eighteen liters of water, sterilized, cooled and adjusted to pH = 4.6 (phosphate buffer). Then ten grams of ether extracted soybean meal were stirred in. The digest was covered with toluene and allowed to stand for two weeks. The limit dextrins were then precipitated with one and one half volumes of methanol. The filtered solution was concentrated to about two liters and ethanol was added until the solution became cloudy, whereupon a small amount of syrup separated. To the decanted clear supernatant, three volumes of ethanol were added. Crystallization was complete in about one week. Recrystallization yielded two hundred grams of maltose. A 10% solution of this material when spotted on a chromatogram, showed no trace of other reducing or priming sugars:

$[\alpha]_D^{20^\circ} = 111.7^\circ$, theoretical for maltose.$H_2O = 112^\circ$.

3. Waxy maize salivary amylase limit dextrins

Large quantities of waxy maize limit dextrins were available from the experiments of Wild (18). These contained glucose and maltose in addition to the

dextrins. The singly branched dextrins consisted of compounds from B_4 to B_7.

4. Amyloheptaose

The amyloheptaose used was a stock reagent, prepared in this laboratory by controlled acid hydrolysis of β-dextrin (75).

5. Radioglucose

The radioglucose was supplied by Dr. S. Aronoff.

6. Schardinger dextrins

Cyclohexaamylose (α-dextrin), cycloheptaamylose (β-dextrin) and cyclooctaamylose (γ-dextrin) were available. The preparation of these has been described (76).

D. Enzymes

1. Salivary amylase

The salivary amylase utilized in all experiments was freshly collected and filtered each time.

2. Amylo-glucosidase

The amylo-glucosidase used was a commercial preparation "dextrinase", obtained from the Delta Chemical Works, Inc.

IV. EXPERIMENTAL

A. 1-Phenyl Flavazole Derivatives

The flavazoles were used in this research for tagging the reducing end of the oligosaccharides obtained from salivary amylase digests. Upon degradation of the flavazole derivatives by enzymes or acid, fragments containing the flavazole unit are produced. The identification of these fragments and the reducing sugars produced was of value in formulating the structures of the original carbohydrates.

The preparation consisted simply of heating the reaction mixture in a closed tube at 100°C for five to eight hours. The following proportions of reagents were employed: one mole of sugar, four moles of acetic acid, one mole of o-phenylene diamine, five moles of phenylhydrazine hydrochloride, ten liters of water. In some experiments flavazoles were prepared with as little as 2 mgs. of carbohydrate. In such cases it became very difficult to determine the exact amounts, and generally an excess of reagent was used.

The method of purification depends upon the

molecular weight of the carbohydrate. Those derivatives of three glucose units and less precipitate out as the reaction progresses. They can be conveniently purified by recrystallization from n-butanol, glacial acetic acid or n-butanol - n-proponal - water. Those derivatives of more than three glucose units are soluble in the reaction medium. This factor made it necessary to purify them by paper chromatography. Two solvents were employed, saturated methyl ethyl ketone and 3:4:6 (3 parts water, 4 parts pyridine and 6 parts n-butanol). A tabulation of the R_f values of the various flavazoles prepared in the course of this research will be found in the appendix.

B. Amylotriose and Amylotetraose

According to the literature already cited (38) a salivary amylase - amylodextrin digest produces maltose, amylotriose and amylotetraose, and only traces of the higher members. A good source of amylotriose and amylotetraose was prepared by allowing the reaction to proceed only to the achromic point before inactivation by heating. The maltose was then removed by fermentation. When the salivary amylase reaction was allowed

to continue ten times as long the amylotetraose was completely hydrolyzed and preparations consisting essentially of amylotriose were obtained after fermentation. The amylotriose itself is hydrolyzed only to a small extent at this stage of hydrolysis. This is a convenient way to make amylotriose, but the shorter hydrolysis period is essential if one desires amylotetraose as well. Such digests invariably contain some high molecular weight materials. The concentration of these was considerably reduced by precipitation with 90% methanol. The methanol extract was then concentrated to a thick syrup and subjected to further resolution on a carbon column. The results obtained from two grams of syrup are recorded in Table 1. A total of eight grams of syrup was treated in the same manner. By combining the appropriate fractions 3.1 grams of trisaccharide and 1.6 grams of tetrasaccharide were obtained, as determined by iodometric oxidation (73).

Flavazole derivatives were prepared and crystallized from n-propanol - n-butanol - water. No reports of a crystalline derivative of amylotetraose could be found in the literature. The flavazole derivative appears as needles under the microscope, and a good

X-ray powder diffraction pattern was obtained from it by Mr. W. James. A solution of it is hydrolyzed by β-amylase to yield maltose and maltose flavazole as the only degradation products. These were identified by their characteristic R_f values.

Table 1

Chromatographic Separation of Amylotriose and Amylotetraose

Eluting agent % ethyl alcohol	Eluate collected liters	Description of eluate as shown by paper chromatography
12	1	Traces of maltose and inorganic materials -- discarded
12	3	Amylotriose
15	1	Amylotriose
15	1	Branched tetrasaccharide (traces)
18	1	Branched tetrasaccharide (traces)
18	3	Amylotetraose

C. Salivary Amylase Hydrolysis Rates for Some Carbohydrates

The general pattern of the degradation of amylodextrin and amylose has been shown by paper chromatography (38). G_2, G_3 and G_4 are produced in approximately equal amounts to the achromic point. Next G_4 is degraded at a somewhat slower rate. Finally G_3 is cleaved to glucose and maltose at a very much slower rate.

In order to provide quantitative measurements the following experiment was set up. Digests were made up each containing 2% carbohydrate and adjusted to 0.001 M in sodium chloride and phosphate buffer (pH = 7). Saliva in amounts as indicated in Table 2 was added. Total volume of each digest was 15 mls. One ml. samples were withdrawn at the indicated time intervals, and analyzed for reducing sugar by the method of Caldwell et al. (73). The values obtained are reported in Table 2. The results of Table 2 are shown graphically in Figure 1. Reducing values are plotted against log.ET, where E = mls. of saliva in the digest and T = time in minutes. A more even distribution of the data is obtained by plotting log.ET rather than ET.

Table 2

The Reducing Values of Salivary Amylase Digests as a Function of Time and Enzyme Concentration

Substrate	Saliva mls.	Reducing values in eqs. x 10^{-4} per ml. of digest: 5 mins.	10 mins.	15 mins.	20 mins.	30 mins.	50 mins.	75 mins.	100 mins.	175 mins.	200 mins.	470 mins.	1000 mins.	1880 mins.	2000 mins.
Amylo-dextrin	0.04	0.19	0.23	----	0.30	----	0.50	----	0.70	----	0.84	0.91	0.96	----	0.98
	0.40	----	----	----	----	----	0.95	----	0.97	----	0.98	1.02	1.10	----	1.16
	4.00	----	----	----	----	----	0.10?	----	1.04	----	1.07	1.15	1.21	----	1.24
Amylo-triose	0.40	0.76	0.76	----	0.76	----	0.77	----	0.76	----	0.75*	0.77*	0.79*	----	0.81*
	4.00	----	----	----	0.80	----	0.88	----	1.01	----	1.23	1.37	1.41	----	1.43*
Amylo-tetraose	0.04	0.62	0.63	----	0.64	----	0.68	----	----	----	0.71*	0.74*	----	----	----
	0.40	0.71	0.81	----	0.95	----	----	----	1.11	----	1.10	1.22	1.26	----	----
Amylo-heptaose	0.10	0.40	0.47	0.53	----	0.66	0.70	0.76	0.78	0.82	----	0.86	----	0.93	----

*These points are not plotted in Figure 1 since the enzyme had become inactivated.

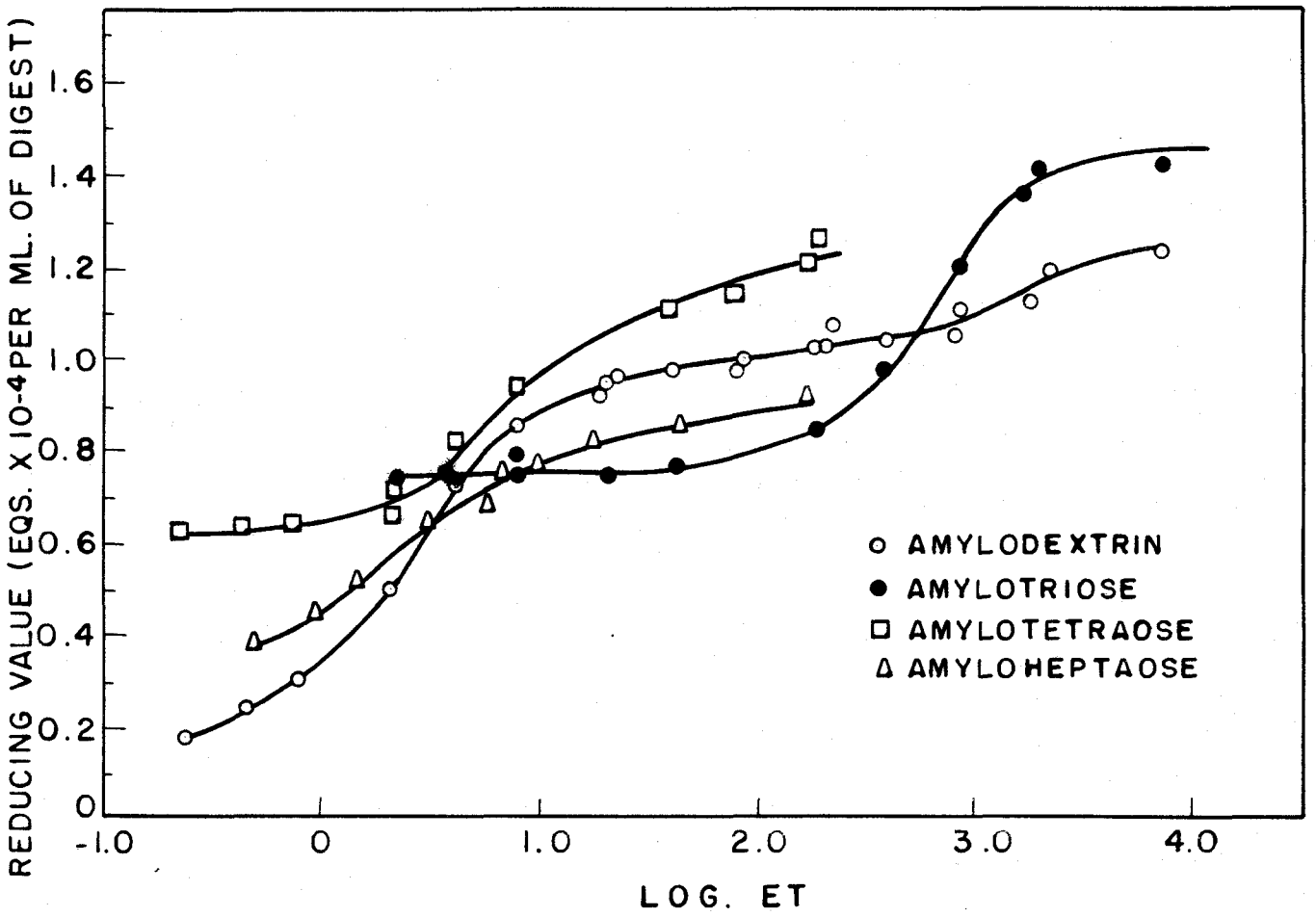


Fig. 1. Hydrolysis of Some Carbohydrates by Salivary Amylase.

The progress of the reactions was also followed by paper chromatography. With amylodextrin the main sugars present up to the achromic point are G_2, G_3 and G_4. The first plateau in Figure 1 coincides with the complete hydrolysis of amylotetraose. The second plateau coincides with the complete hydrolysis of amylotriose. The hydrolysis of pure amylotetraose was mainly to maltose, although traces of glucose and amylotriose could be seen. The hydrolysis of amylotriose yielded glucose and maltose. Amyloheptaose yielded, in the early stages of hydrolysis, maltose, amylotriose, amylotetraose and amylopentaose. The latter is hydrolyzed at about the same rate as amyloheptaose. Pure maltose is hydrolyzed by saliva but the rate is so slow as to be insignificant at even the most advanced stages of hydrolysis shown in Figure 1.

The rates of hydrolysis of the Schardinger dextrins, α, β and γ, by saliva were examined. γ-Dextrin was hydrolyzed at about the same rate as amylotriose, with glucose, maltose and amylotriose being visible on a chromatogram. α- and β-dextrins were extremely resistant. No significant reducing values could be obtained even after ET values one

hundred times greater than those required to completely hydrolyze γ-dextrin.

D. Radioactive Amylo-oligosaccharides

One characteristic of the reaction mechanism of macerans amylase is the fact that the co-substrate must appear only at the reducing end of the linear dextrins formed. The mechanism involves only the transfer of some glucose moieties from one glucose acceptor to another. There is no net synthesis or hydrolysis of bonds. Thus when radioglucose is used as a co-substrate oligosaccharides are produced which should be uniquely labeled at the reducing end. Such short chain oligosaccharides are interesting substrates for the study of salivary amylase. An examination of the activity of the degradation products at intermediate stages of hydrolysis will reveal what preference the enzyme may have for the various bonds present.

1. Preparation

Ten milligrams of α-dextrin and two milligrams of glucose containing some radioglucose were reacted with macerans amylase for fifty conversion periods (77).

The entire digest was applied in a streak near the bottom of a sheet of Whatman No.1 paper 35 cms. long and resolution of the individual oligosaccharides was accomplished in 3:4:6 solvent. After three passes a radioautograph was made, Figure 2. The positions of the dark bands correspond in R_f to members of the amylose series (78), as shown. The first seven members were located with the help of the radioautograph and the paper was sectioned. The sugars were eluted from the strips by a method based on the procedure of Dimler et al. (79), who report quantitative elution with a small volume of water. A convenient method of eluting the paper strips was to use two cover glasses fitted together horizontally, the upper one having a hole in its center through which water could be added. The paper strips were inserted between the cover glasses around the edges, and allowed to hang down. Small pieces of paraffin, having rounded depressions large enough to receive a few drops of eluate were used as receptacles. The whole apparatus was then covered by an air tight Bell jar. The paraffin containers permitted a more quantitative removal of contents and they could be used directly as vessels for carrying out

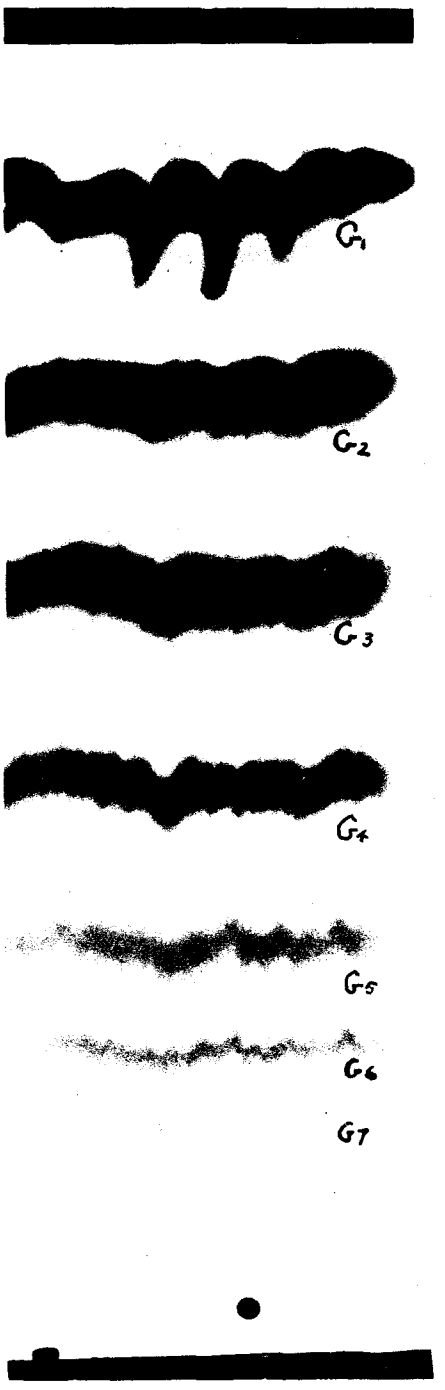


Fig. 2. Radioactive Amylo-oligosaccharides.

enzymatic reactions on the eluate.

2. Proof of terminal labeling

Radioactive amylotriose and amylopentaose were subjected to degradation by soybean β-amylase. Samples of the digests were then transferred to filter paper and chromatographed in 3:4:6 solvent. After three passes radioautographs were made. The results for amylotriose are shown in Figure 3(b). Those for amylopentaose are not shown but are similar; in both cases only the glucose and unconverted amylotriose are radioactive. On subsequent development of the paper with alkaline copper and phosphomolybdic acid, spots corresponding to glucose, maltose and amylotriose were revealed. The maltose that is produced contains no significant activity.

Radioactive maltose was converted into radioactive maltose flavazole. It was then hydrolyzed by acid and chromatographed. A radioautograph of the chromatogram showed active glucose flavazole. The glucose produced was inactive.

3. Reaction with salivary amylase

The individual oligosaccharides G_3, G_4, G_5, G_6 and

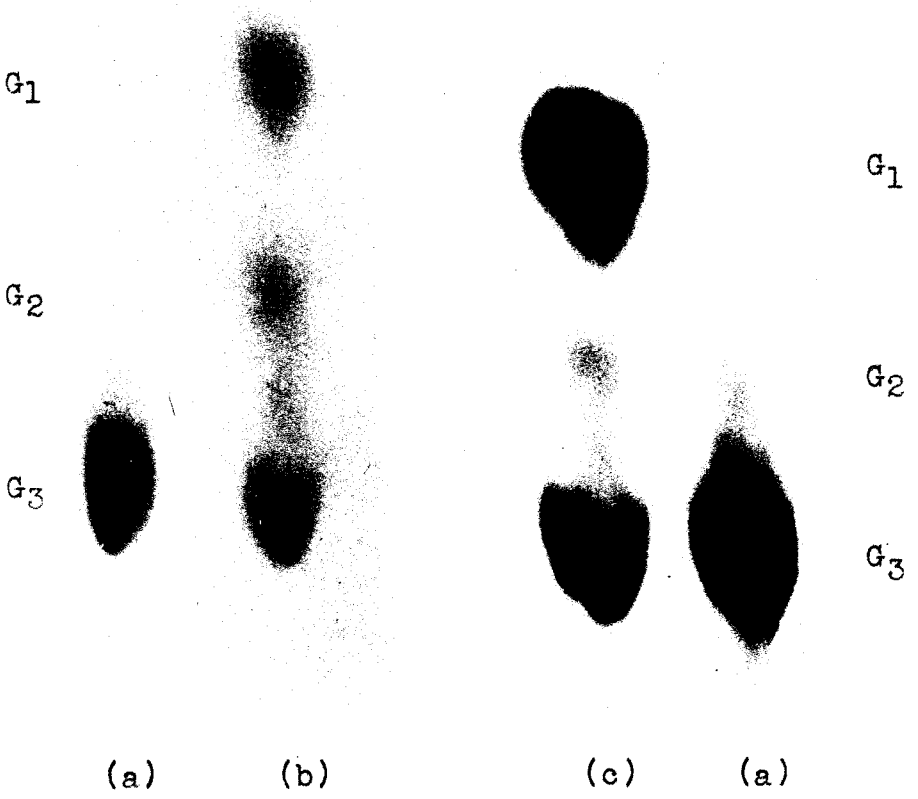


Fig. 3. Radioautograph of Chromatogram.
(a) Amylotriose.
(b) Amylotriose partially hydrolyzed by salivary amylase.
(c) Amylotriose partially hydrolyzed by β-amylase.

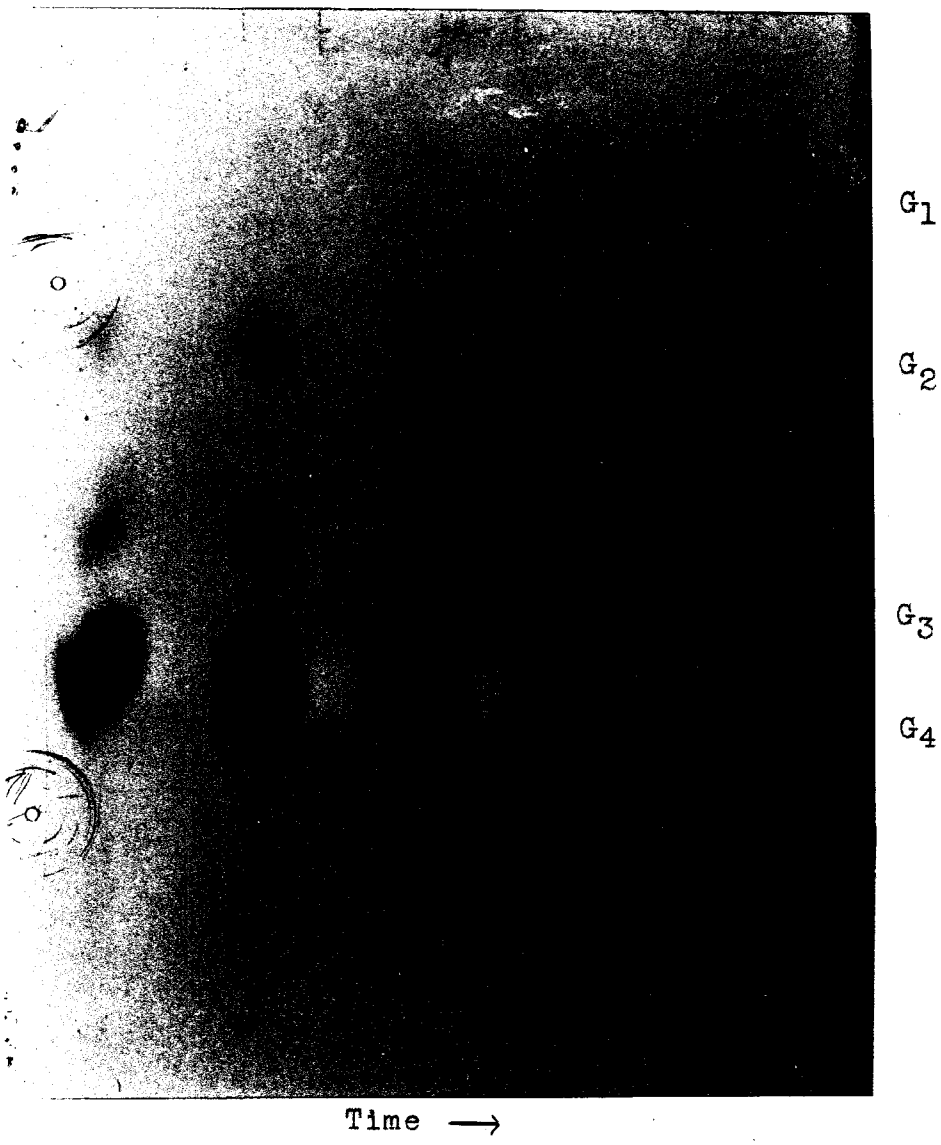


Fig. 4. Hydrolysis of Amylotetraose by Salivary Amylase as a Function of Time.

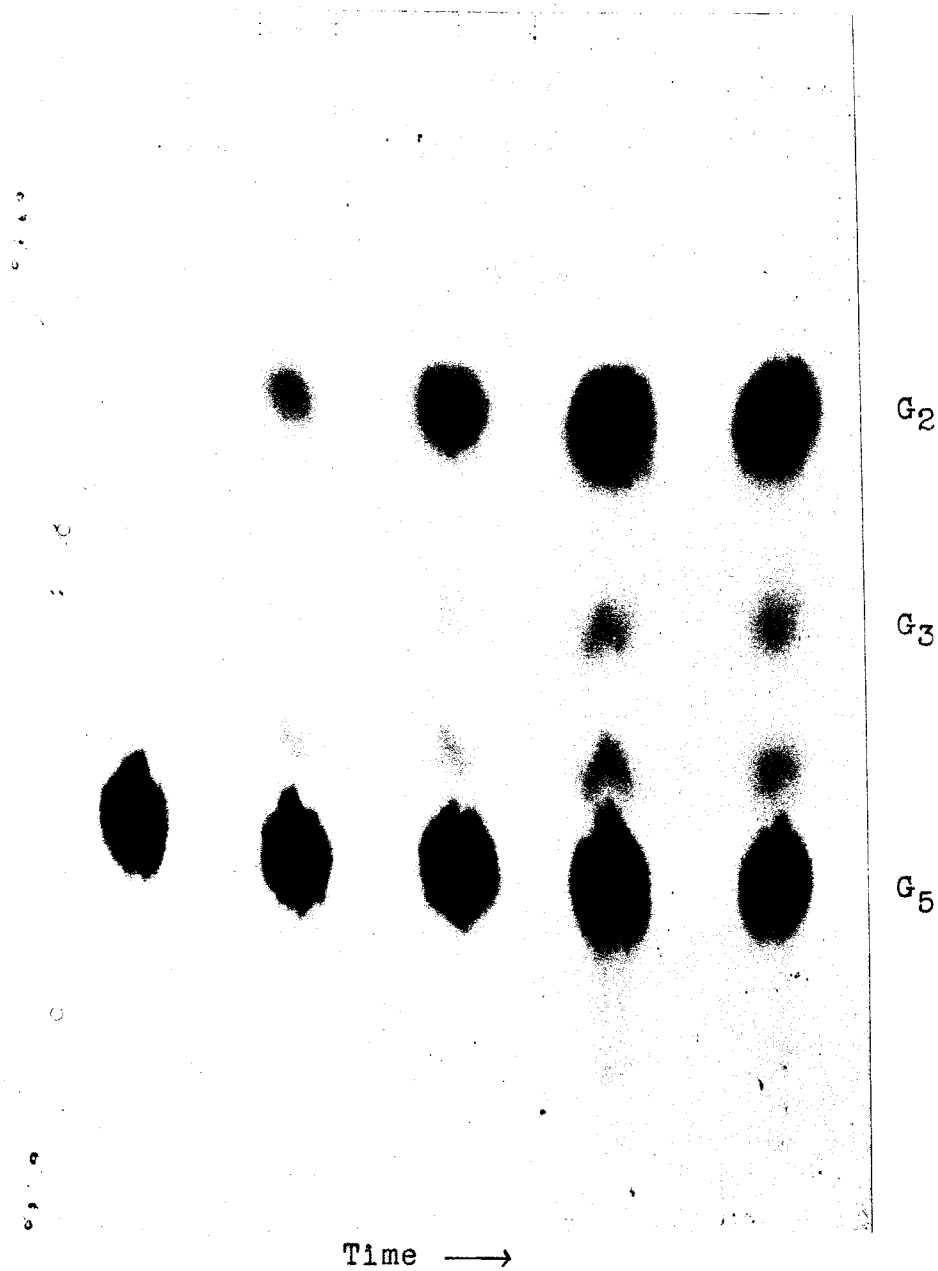


Fig. 5. Hydrolysis of Amylopentaose by Salivary Amylase as a Function of Time.

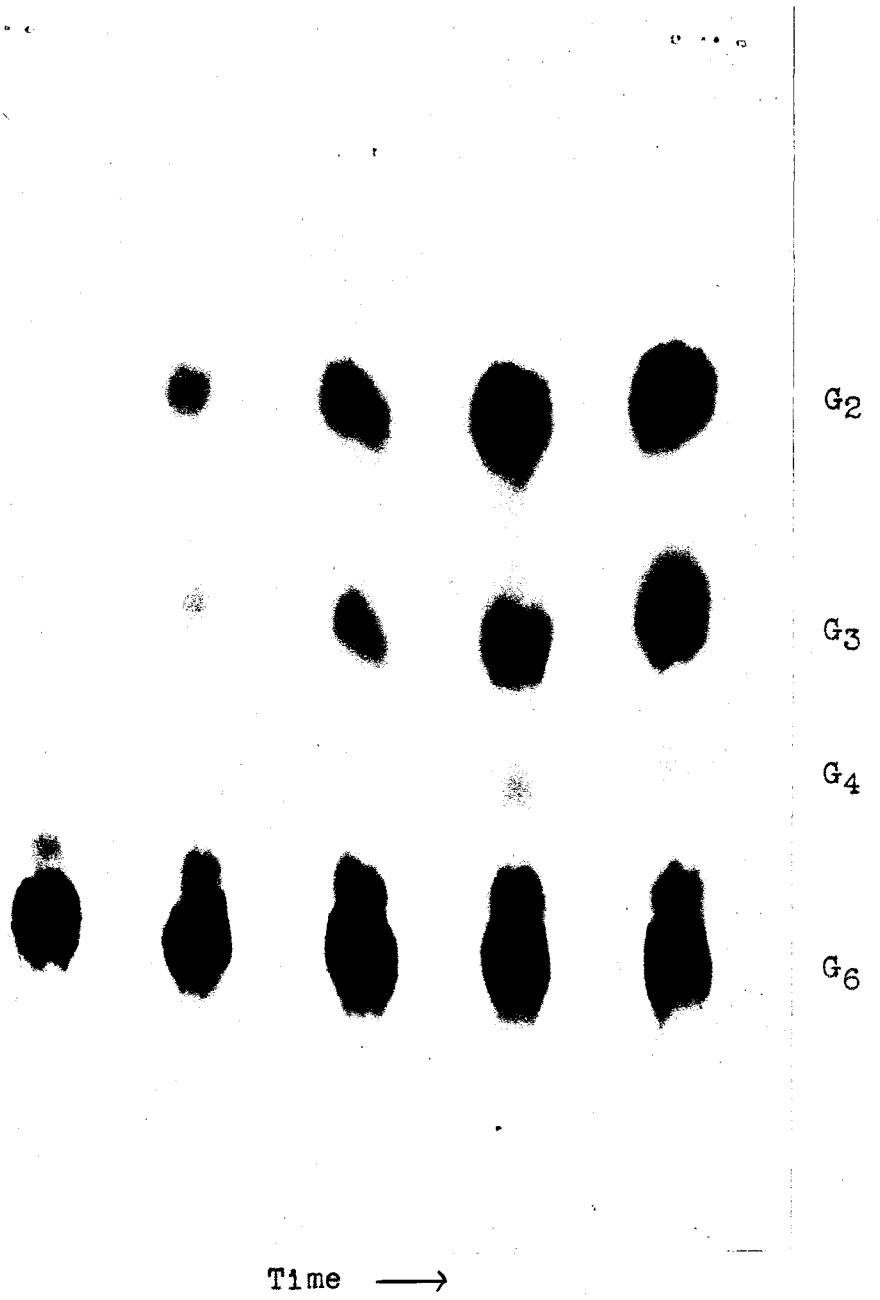


Fig. 6. Hydrolysis of Amylohexaose by Salivary Amylase as a Function of Time.

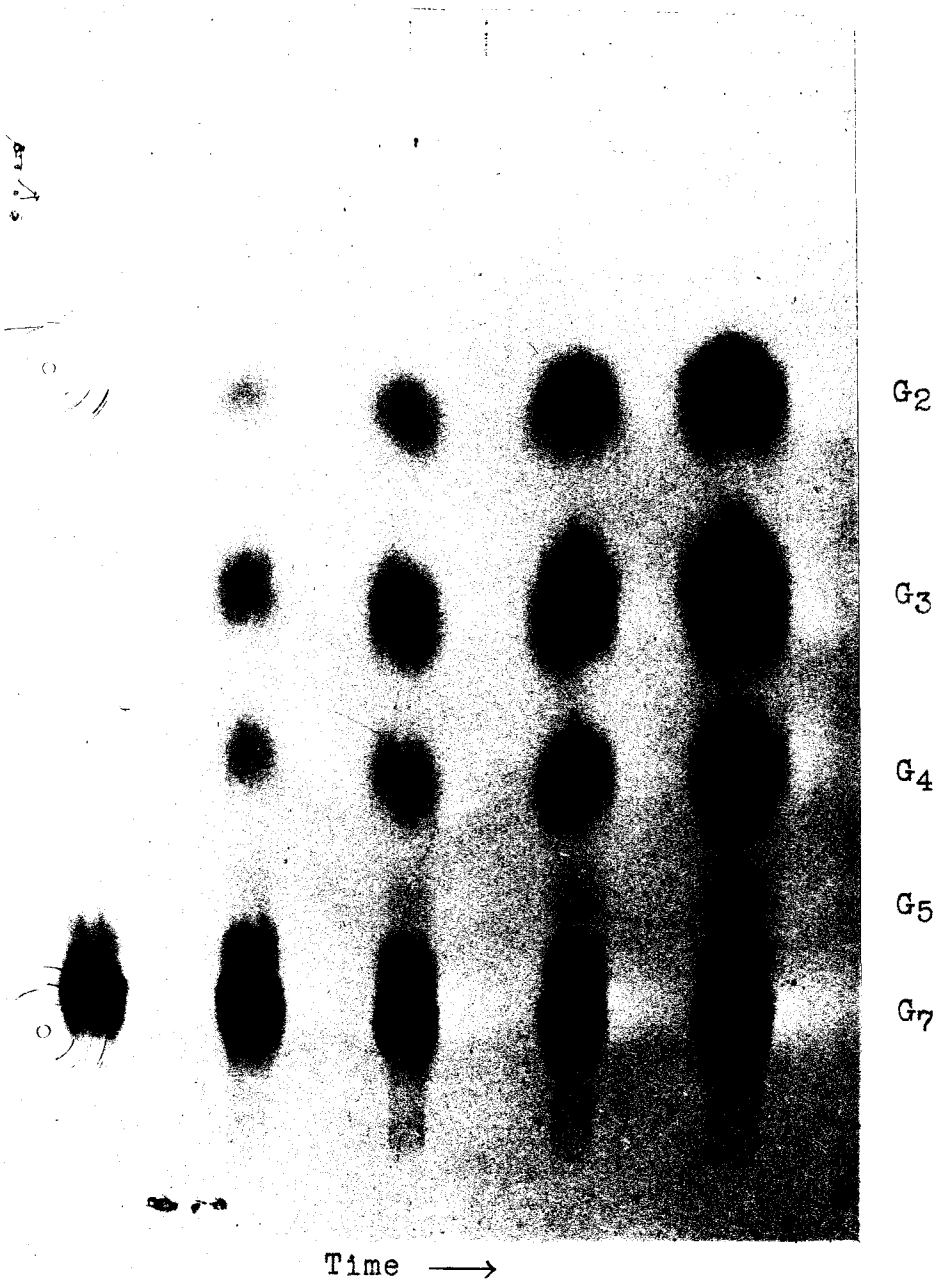


Fig. 7. Hydrolysis of Amyloheptaose by Salivary Amylase as a Function of Time.

G_7 were subjected to the action of salivary amylase. Samples were withdrawn with a micro pipet at various stages of the hydrolysis for paper chromatography. After three passes of the chromatograms in 3:4:6, radioautographs weremade. These are shown in Figures 3 to 7. Each chromatogram had a reference oligosaccharide series, prepared by partial acid hydrolysis of 30% amylodextrin -- average chain length about three glucose units. After the radioautographs had been prepared the filter paper was developed to bring out the reducing sugar spots. The results of this treatment showed that salivary amylase action on the oligosaccharides yielded the following sugars:

$G_3 \longrightarrow G_1 + G_2$,

$G_4 \longrightarrow G_2$ (traces of G_1 and G_3),

$G_5 \longrightarrow G_2 + G_3$,

$G_6 \longrightarrow G_2, G_3 + G_4$,

$G_7 \longrightarrow G_2, G_3, G_4 + G_5$.

These are marked on the chromatograms. It should be noted that in each case, the substrate contains a trace of the next lower homolog. This is due to incomplete resolution (Figure 2) and it becomes more marked with the higher homologs. It will be noted in the discussion

that these trace materials do not alter the final conclusions.

E. Salivary Amylase Limit Dextrins

1. Partial acid hydrolysis

The acid hydrolysis of starch can be treated as an approximate random hydrolysis of equivalent α-1,4 glucosidic bonds (80, 81, 82). As the hydrolysis progresses the reaction constant, based upon a first order reaction, increases slightly. These considerations led to the modification (83, 84) that the terminal linkage is hydrolyzed somewhat faster than internal linkages. This is also in agreement with the fact that maltose is hydrolyzed faster than starch. The α-1,6 bonds, however, are more resistant; isomaltose is hydrolyzed at a rate one quarter that of maltose (85). For a first order reaction and at low degrees of hydrolysis $\alpha = Nkt$, where α is the degree of hydrolysis of α-1,4 bonds, N is the molar concentration of acid, k is the reaction rate constant (0.067 at 100°C), and t is the time in minutes. The degree of hydrolysis for an α-1,6 bond will be $\alpha/4$. All partial acid hydrolysis experiments with oligosaccharides reported

here are to a degree α = 0.25 to 0.30. The reaction is treated as a random hydrolysis of all bonds except the α-1,6 and the α-1,4 adjacent to the flavazole unit. The products are identified by their R_f values. A comparison of the concentrations of the products is also made when it is obvious from the appearance of the chromatogram that large differences exist.

With acid hydrolysis of flavazole derivatives it might be expected that the α-1,4 bond to the glucose moiety containing the flavazole would be hydrolyzed at a different rate than the rest of the α-1,4 bonds. To test this, amylotriose flavazole (o—o—oF) was subjected to partial acid hydrolysis and the products were examined by paper chromatography. The concentration of glucose was very much greater than that of maltose and the concentration of maltose flavazole was much greater than that of glucose flavazole. This difference in the rate of hydrolysis was noted with all the oligosaccharide flavazoles examined.

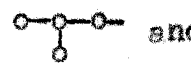
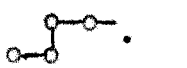
2. The panose coupled oligosaccharides

The panose coupled oligosaccharides derived from macerans amylase action on α-dextrin and panose have

been described (page 18). These are interesting compounds for study because they contain the α-1,6 bond as do the salivary amylase limit dextrins.

One half gram of α-dextrin, one half gram of panose and 20 μ macerans amylase were incubated for one week and the products were then subjected to carbon chromatography. Panose was eluted with 12% ethanol and the branched tetrasaccharides with 15% ethanol. The branched tetrasaccharides were eluted together and were obtained essentially free of the other homologous members. Thirty per cent ethanol was then passed through the column to elute the higher oligosaccharides, no attempt being made to resolve the individual members.

The two branched tetrasaccharides have slightly different R_f's in 3:4:6. Since these tetrasaccharides are used for identification purposes with the salivary amylase limit dextrins, it became necessary to distinguish between them. The branched tetrasaccharides were separated into their individual components by paper chromatography and the paper was sectioned to yield a few milligrams of each. Each isomer was then partially hydrolyzed with sulfuric acid, neutralized

with barium carbonate, filtered, concentrated and chromatographed. The more mobile isomer, termed B_{4a}, yielded glucose, branched trisaccharide and traces of maltose, isomaltose and amylotriose. The latter was identified by its ability to prime phosphorylase. The less mobile tetrasaccharide, B_{4b}, yielded no amylotriose but yielded more maltose than did B_{4a}. Thus B_{4a} has the structure  and B_{4b} has the structure  .

3. Dextrinase

The action of dextrinase on linear oligosaccharides results in rapid degradation to amylotriose, maltose and glucose. Amylotriose is degraded at a somewhat slower rate and finally maltose is cleaved to glucose. At the latter stages traces of isomaltose and a B_3 appear, no doubt due to transglucosidase action. Pure maltose is hydrolyzed to glucose, again with the production of traces of isomaltose and B_3. It is also capable of carrying out reversion of glucose to maltose if the glucose concentration is very high, isomaltose and B_3 again appearing as with maltose.

Branched oligosaccharides of the panose coupled series are rapidly degraded to panose and glucose.

Panose is very resistant but it is slowly hydrolyzed largely to glucose, although small amounts of isomaltose and maltose are formed. Panose is hydrolyzed much more slowly than amylotriose, no doubt because of the α-1,6 link which imposes a barrier to the enzyme. When the flavazole derivatives of panose and amylotriose are compared, the same preference is shown. The maltose flavazole which is produced in both cases, however, is resistant to further action by the enzyme.

4. Structure of the singly branched dextrins

The waxy maize salivary amylase limit dextrins were freed of glucose and maltose and applied to a carbon column in an attempt to obtain homogeneous species. The results for 2 gms. of limit dextrin are shown in Table 3. As with the panose coupled series a B_4 fraction essentially homogeneous was obtained. The B_5 was less homogeneous. With increase in molecular weight, the separation became increasingly poorer.

Flavazole derivatives were prepared from the most homogeneous fractions. This procedure offered many advantages:

(a) They could be chromatographed in the same

Table 3

Fractionation of the Salivary Amylase Limit Dextrins by Means of a Carbon Column

Eluting agent % ethyl alcohol	Volume of eluate liters	Description of eluate as shown by paper chromatography
10	2	Maltose
12	2	Maltose (traces)
12	2	Amylotriose (traces)
15	2	B_4
15	2	B_4 (trace of B_5)
18	2	B_5 (trace of B_6)
18	2	B_5, B_6
21	2	B_6, B_7 (trace of B_5)
21	2	B_6, B_7

solvent 3:4:6, used for the sugars. The R_f's are very much greater, however, and one pass was sufficient to clearly separate the various components, hereafter referred to as B_4^F, B_5^F, B_6^F and B_7^F. The structural features of the carbohydrate part of the derivative still exert the same influence on the R_f. Thus the dextrin flavazoles migrate on the paper in the same

order as do the dextrins themselves, but about five or six times as far in one pass. The influence of the α-1,6 link is still the same. Panose flavazole moves just behind amylotriose flavazole just as the sugars themselves. Similarly, B_4^F for example, moves just behind G_4^F. As with the sugars, isomeric sugar derivatives would be expected to have similar R_f's and may not be resolved.

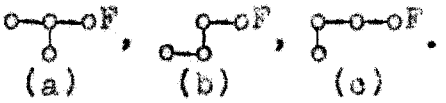
(b) The flavazoles are colored and they could be observed without the use of developing strips. They fluoresce under ultraviolet light revealing any trace materials. After one pass the various components could be isolated by sectioning the paper and eluting it with methanol and water.

(c) Dark colored impurities from the reaction mixture move to the top of the paper and are thus eliminated.

B_4^F yielded a B_3^F with dextrinase, in the early phases of the reaction. On extensive treatment a G_2^F appeared. The reaction was halted at this stage and the residual B_4^F was isolated and retreated with fresh enzyme. This material was found to be more resistant than the starting material and when treated extensively only G_2^F was produced (no B_3^F). B_4^F is thus apparently

composed of two isomers, one which readily yields a B_3^F with dextrinase and one, the major component, which is resistant to the enzyme.

The resistant isomer upon partial acid hydrolysis yields glucose, isomaltose (B_2) and a B_3 in relative concentrations as follows: $B_2 > G_1 >> B_3$. Of the various possible structures only the following could yield isomaltose:



(a) (b) (c)

Only (c) could yield more B_2 than G_1. The relative concentrations of flavazoles produced were as follows: $G_2^F > G_1^F$, G_3^F, again consistent only with structure (c).

The B_3^F produced from the other isomer yielded upon partial hydrolysis, glucose and isomaltose as the only reducing sugars. There are three possible isomeric B_3^F's:

panose flavazole

Only panose flavazole could yield isomaltose. The B_4^F isomer which produces B_3^F must therefore have the

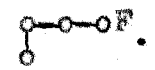
structure (a) or (b).

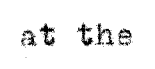
B_5^F, B_6^F and B_7^F are all completely hydrolyzed by dextrinase yielding a resistant B_4^F in each case. With B_6^F and B_7^F all the intermediate stages are visible. The reaction for B_7^F is shown in Figure 8. The reactions can be represented as follows:

$B_5^F \longrightarrow B_4^F$ + 1 glucose,

$B_6^F \longrightarrow B_5^F \longrightarrow B_4^F$ + 2 glucose,

$B_7^F \longrightarrow B_6^F \longrightarrow B_5^F \longrightarrow B_4^F$ + 3 glucose.

Glucose was the only reducing sugar produced and the resulting B_4^F in each case was identical with the major component of the salivary B_4^F, namely  . This was concluded from the products obtained by partial acid hydrolysis and its reaction with dextrinase.

The structures for the limit dextrins are, with the exception of the isomeric B_4, thus limited to those containing the B_{4c} structure () at the reducing end. These structures are presented in Table 4.

The B_5^F upon partial acid hydrolysis yields B_4^F, G_2^F and traces of G_1^F. The reducing sugars formed are G_1, B_3 and traces of B_4, B_2 and G_2. Considering the

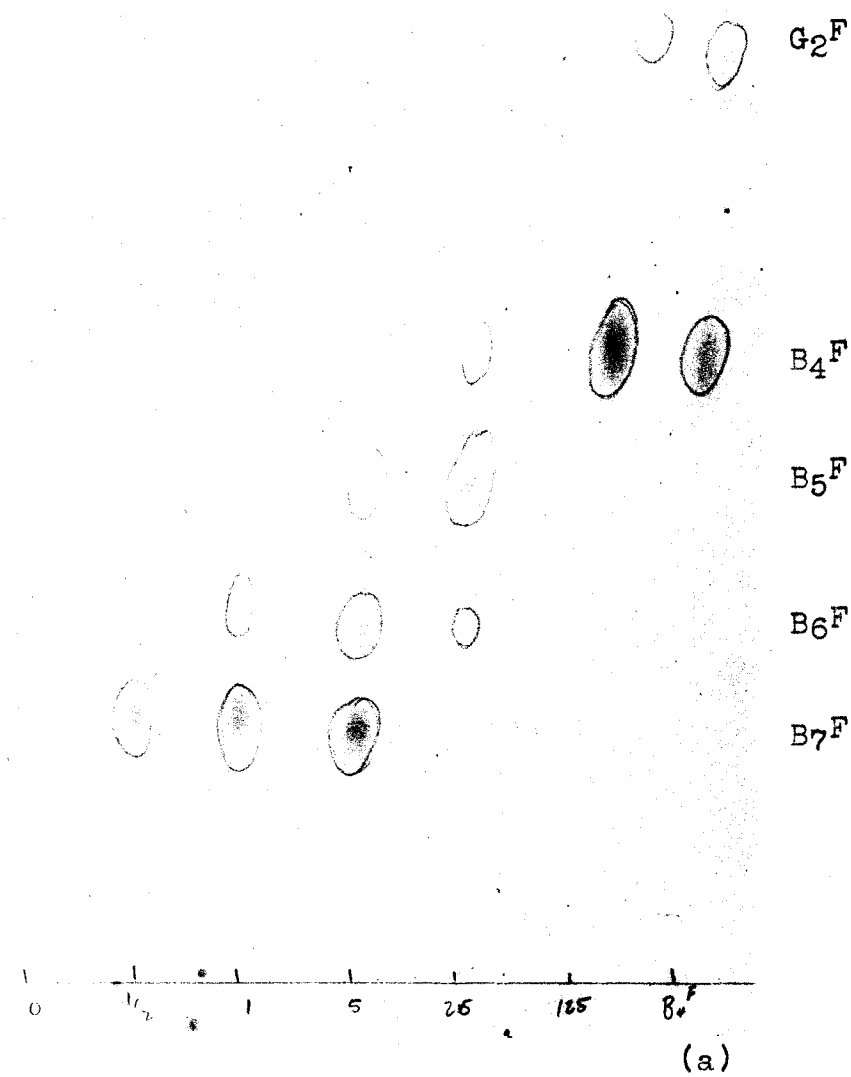
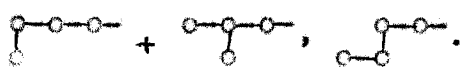
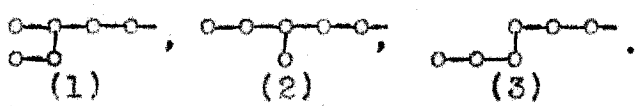
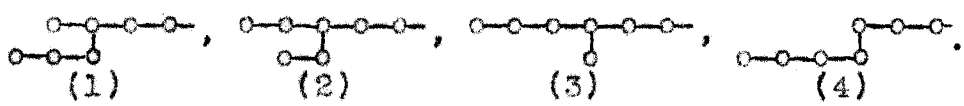
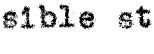
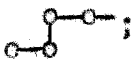


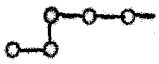
Fig. 8. Hydrolysis of B_7^F by Dextrinase as a Function of Time in Minutes.
(a) Reference spot of B_4^F partially hydrolyzed by dextrinase.

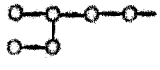
Table 4

Possible Dextrins

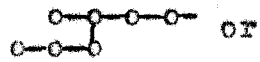
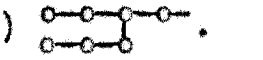
B_4	
B_5	 (1) (2)
B_6	 (1) (2) (3)
B_7	 (1) (2) (3) (4)

two possible B_5^F structures (Table 4) the B_4 produced could have only two possible structures: B_{4a},  or B_{4b},  ; namely, the panose coupling tetrasaccharides. Since the B_4 was produced only in traces from B_5^F a concentrated sample was obtained as follows. About 20 mgs. of B_5^F was partially hydrolyzed and the whole digest was applied in a streak at the bottom of each of four pieces of filter paper. After one pass the papers were sectioned and the portions corresponding to B_4 were eluted by the techniques already described. The eluate

was concentrated under an air stream and applied to one spot on a chromatogram. The chromatogram contained a reference spot of the panose coupled tetrasaccharides. After seven passes in 3:4:6 (to obtain nearly maximum resolution of the tetrasaccharides) the chromatogram was sprayed to bring out the reducing sugars. The B_4 was found to correspond in R_f with B_{4b} and contained none of the other isomer. Hence B_5 of the salivary limit dextrins must have the structure  .

B_6^F upon partial acid hydrolysis yields B_5^F and G_2^F with traces of G_1^F and B_4^F. The reducing sugars produced are G_1 and B_4 with traces of maltose and B_5. On examination of the possible structures for B_6 (Table 4), (2) and (3) yield B_4^F as readily as B_5^F, for these can be obtained by a single scission. Calculations indicate that the yield of B_4^F should be slightly greater than B_5^F for both these structures. Structure (1) however, can yield B_4^F only through multiple scission. Calculated yields made on this structure are $B_5^F:B_4^F \approx 5$. Thus at least the major component of B_6 must be  , although the presence of the other two structures cannot be ruled out.

Confirmatory evidence for the presence of structures (2) or (3) was obtained by treating B_3^F with saliva. The reaction proceeded with the formation of maltose and B_4^F. Under more extensive degradation, G_1, B_4, B_5 and G_2 were formed. The latter set of products was formed at a much reduced rate. The initial formation of maltose suggests that isomers (2) or (3) are degraded more readily and that isomer (1) is degraded finally by scission of three possible α-1,4 bonds.

Wild's (18) findings with B_7 indicated that it had one of two structures: (1)  or (2)  . Since the action of dextrinase on its flavazole yields B_4^F, having the structure  , the only possible structure for B_7 is (1).

5. Structure of the doubly branched dextrins

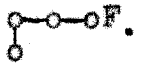
The doubly branched dextrins present with the singly branched dextrins consist of compounds in the range BB_9 to BB_{12}, the numerals indicating the number of glucose units. These dextrins were examined with the view of determining the number of glucose units between branches. Due to their large molecular weight, resolution of the individual members was not attempted.

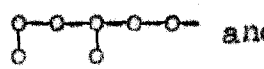
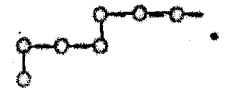
It was decided instead to examine the products obtained after reaction with dextrinase. Dextrinase would be expected to degrade the series of compounds down to the first branch point.

Waxy maize salivary amylase limit dextrin was treated with dextrinase and samples were spotted periodically for chromatographic analysis. As the reaction progressed the singly branched dextrins were gradually converted into a singly branched B_4. At the same time small amounts of B_3 accumulated in the same manner as when derivitized as the flavazole. The doubly branched dextrins were converted at the same rate into two partly resolved compounds having the mobility of doubly branched dextrins with seven glucose units (BB_7). The triply branched dextrins were converted into one or more compounds of ten to twelve glucose units.

A digest carried to the latter stage of hydrolysis with dextrinase was resolved on the carbon column. Glucose, maltose and B_3 were eluted with 12% ethyl alcohol. B_4 was eluted with 15% ethyl alcohol and BB_7 was eluted with 21% ethyl alcohol.

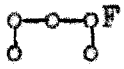
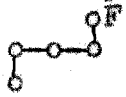
BB_7 was converted into the flavazole and subjected

to further extensive degradation by dextrinase. The reaction proceeded very slowly, but BB_7^F was eventually completely converted into B_4^F without the formation of any discernable intermediate compounds. The B_4^F was in turn hydrolyzed at about the same rate into G_2^F, again without the apparent formation of an intermediate compound. The B_4^F was further partially hydrolyzed with acid and the products indicated it to be identical with salivary B_4^F, namely  .

Since the enzyme hydrolyzes α-1,6 links with difficulty, once these have been cut, the resulting exposed glucose unit now contains a bond readily susceptible to action of the enzyme. Hence the degradation proceeds to the next branch point at such a fast rate that intermediary products between B_4^F and BB_7^F do not accumulate in sufficient concentration to be seen on the chromatogram. The same considerations would apply to B_4^F. The only reducing sugar produced is glucose. On this basis two structures for the BB_7 seem reasonable: (1)  and (2)  .

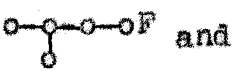
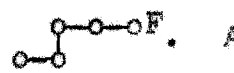
BB_7^F was partially hydrolyzed with acid and the products were examined by paper chromatography. There

were produced large quantities of B_5^F, B_4^F and G_2^F, and lesser amounts of G_1^F. In addition traces of a compound having the correct R_f for G_3^F were produced. The reducing sugars were G_1, B_2, B_3 and a compound corresponding to BB_5. In addition some B_4 was produced. The formation of these compounds is consistent with the formulae shown. G_3^F does indicate at least that (2) must be present.

The BB_5 was isolated by elution from the paper and converted into its flavazole derivative. When chromatographed in 3:4:6 this derivative was quite well resolved into two spots. The more mobile fraction (BB_{5a}^F) reacted slowly with dextrinase to yield a B_3^F which was slowly converted into a B_2^F or G_2^F. The less mobile fraction (BB_{5b}^F) reacted slowly with dextrinase to produce B_2^F or G_2^F with the formation of only traces of B_3^F. These are compatible with the structures  for BB_{5a}^F and  for BB_{5b}^F, since the first contains the maltose flavazole link known to be resistant to the enzyme.

However, on treatment with neutral periodate for thirty minutes, both compounds were extensively degraded.

In each case, apparently the oxidation proceeded to the flavazole aldehyde. This was shown by the complete solubility in benzene and high mobility in 3:4:6 and methyl ethyl ketone. This is the expected result for the structure shown for BB_{5b}^{F}, but not for that shown for BB_{5a}^{F}.

In addition, the B_5^F isolated from BB_7^F was partially hydrolyzed with a view to obtaining the B_4 sugar and comparing it with the panose tetrasaccharide, as described under the singly branched dextrins. Because of the small yields, the B_4 produced was not readily identified, but it was apparent that there were two partly resolved B_3 components in addition to glucose and isomaltose. If the original BB_7 was a mixture of the two isomers shown on page 57, then two isomeric B_5^F's should be produced on partial acid hydrolysis, namely,  and  . Although there was no evidence for more than one, it may be that they were not resolved. The above compounds would account for the formation of two B_3's on acid hydrolysis.

V. DISCUSSION OF RESULTS

A. Salivary Amylase Action on Linear Substrates

On examining the data of Figure 1 the hydrolysis rates of pure amylotetraose and pure amylotriose are observed to coincide with the rates for these substrates produced from the hydrolysis of amylodextrin. Amylotetraose and amylotriose approach a reducing value twice that of the starting material, corresponding to the hydrolysis of one bond. Amyloheptaose approaches a value three times that at zero time, corresponding to the hydrolysis of two bonds. Comparative initial rates of hydrolysis for the four substrates examined, are obtained by plotting reducing value versus ET (Figure 9). Straight lines are obtained for the first few points in each case. Relative rates can then be calculated by measuring the slopes. ET values for the trisaccharide are divided by 200 and for the tetrasaccharide by 4 in order to fit the data into a smaller space. The slopes as measured from Figure 9 are compared with that for amylodextrin to obtain the relative rates, reported in Table 5.

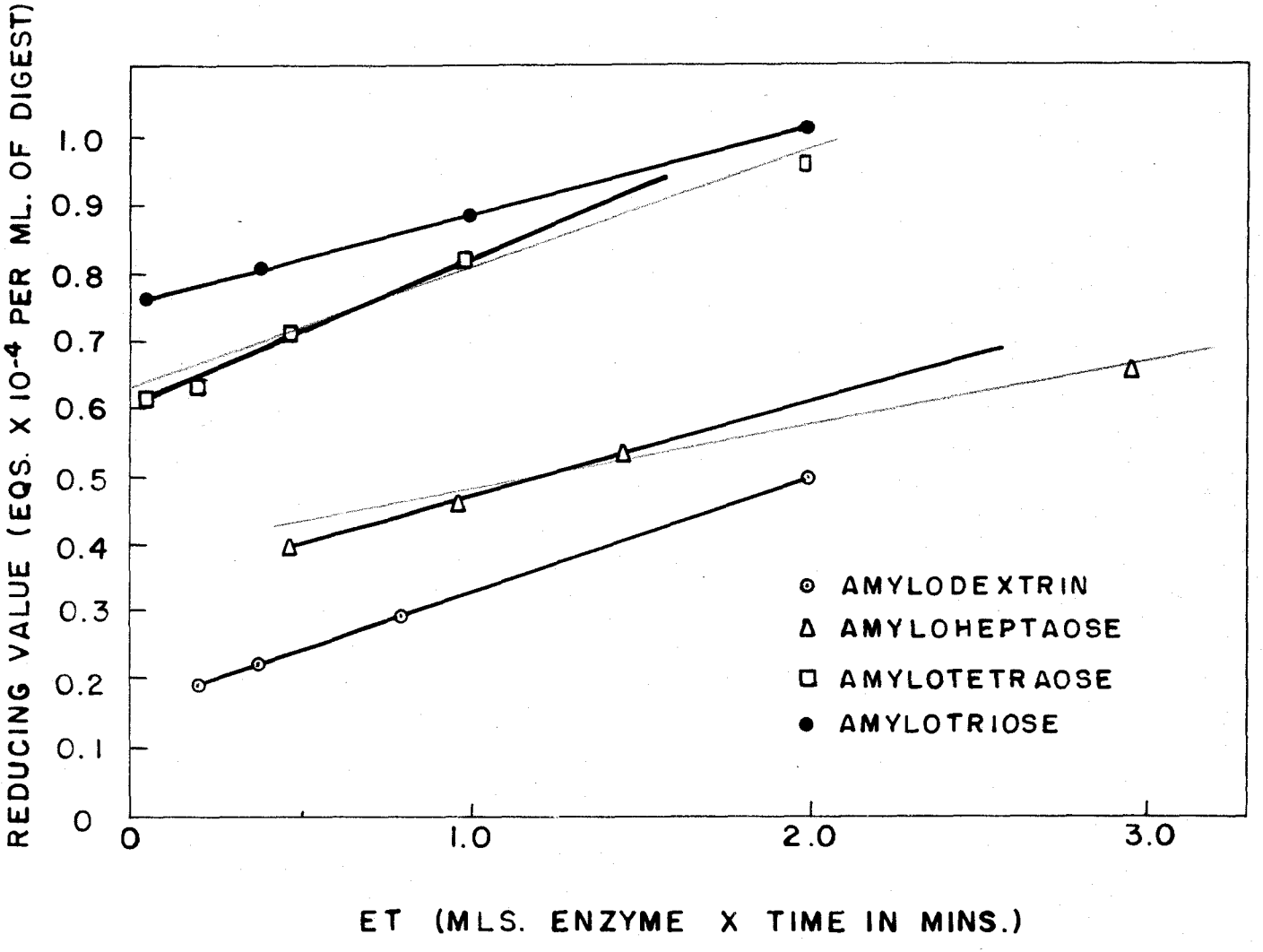


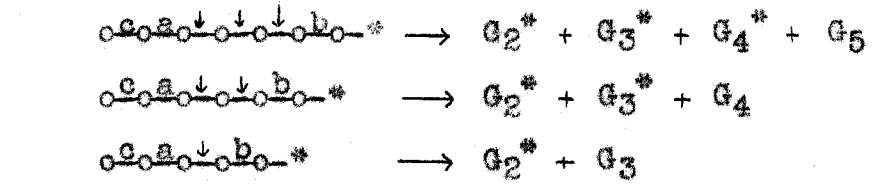
Fig. 9. Initial Rates of Hydrolysis of Various Substrates by Salivary Amylase.

Table 5

Relative Rates of Hydrolysis for Amylodextrin, Amyloheptaose, Amylotetraose and Amylotriose

Substrate	Slope	Relative rate
Amylodextrin	174 x 10^{-7}	1
Amyloheptaose	154 x 10^{-7}	0.88
Amylotetraose	50 x 10^{-7}	0.32
Amylotriose	0.67 x 10^{-7}	0.0038

These rates should be borne in mind when examining the results obtained by salivary amylase on the radioactive amylo-oligosaccharides, Figures 3 to 7. Beginning with amyloheptaose it will be observed that there are three bonds readily susceptible to attack; those within two bonds from the non-reducing end and those within one bond from the reducing end. The same rule holds for amylohexaose and amylopentaose.



Bond a is hydrolyzed to a significant but much lesser

extent as evidenced by the low activity of G_5 from G_7, G_4 from G_6 and G_3 from G_5. No figures are available for the comparative rates of hydrolysis for these three substrates, but it has been observed that G_5 produced from G_7 disappears about as fast as the parent substrate (page 32). There is good reason to believe, therefore, that the rates for these three substrates are very similar.

On going to amylotetraose, the reaction rate is reduced considerably, and the bond most susceptible to attack is the center one, with minor hydrolysis of bond b. $o\underline{c}o\underline{a}o\underline{b}o^* \longrightarrow G_2^*$. Amylotetraose contains no linkages of the type present in the higher homologs shown to be more easily hydrolyzed. Since bond a is of a type more resistant to attack, the hydrolysis rate is naturally lower. Because of the presence of radioactive G_3 impurity in the amylotetraose, it cannot be determined whether bond c is hydrolyzed to any extent.

Amylotriose contains linkages of the type b and c only, and again the rate is reduced. Both linkages are hydrolyzed but the bond nearer the reducing end is preferred.

The presence of some G_4 impurity in G_5 does not change the conclusions regarding G_5 because the activity of G_3 produced from G_5 is very low compared to the activity of G_2. Similar considerations apply to amylohexaose and amyloheptaose.

One should not conclude, however, that the position of the bond is the only factor influencing its hydrolysis rate. Maltose for example contains a link of the type present in amylotriose, yet is hydrolyzed very much more slowly. Apparently the size of the molecule is also a factor. The extreme difference between the rates for β- and γ-dextrins may be due to configuration.

It has been held (86) that salivary amylase contains maltase, an impurity that was supposed to account for the hydrolysis of maltose and amylotriose. Such an enzyme operates by the removal of glucose units from the non-reducing end of amylose chains. It should yield inactive glucose with radioactive amylotriose. Salivary amylase, however, has a specificity for the reducing glucose unit. The view held by Meyer et al. (87, 19) and Koehler (88) that saliva contains only one enzyme is thus supported.

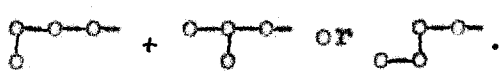
The radioactive experiments demonstrate that internal linkages are indeed more easily hydrolyzed than terminal linkages, but the area of preference is not as simply defined as Meyer and Bernfeld (33) and Whelan et al. (44) state. At least for the short chain dextrins examined, the area would be more correctly defined as within two bonds from the non-reducing end and within one bond from the reducing end. It is not certain either that within this area all bonds are hydrolyzed with equal ease. On examining Figure 7 the G_3 spot is more intense than either G_2 or G_4. Similarly in Figure 6 the G_2 spot is more intense than G_3.

There is also the fact that multiple attack appears to take place with the long chain oligosaccharides. It would seem that amyloheptaose has too short a chain for this factor to be significant. It is impossible to write any combination of scissions so as to yield the observed products. One must conclude that with these substrates the amylase functions largely by a single attack mechanism. Quantitative measurements of the activity and total carbohydrate of the hydrolytic products are necessary however to rule out multiple attack as a contributory factor. It may be that

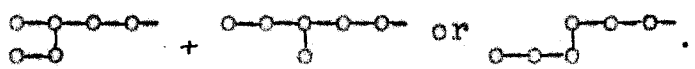
multiple attack gradually assumes more significance with increase in chain length.

B. Salivary Amylase Action on Branched Substrates

The structures of the salivary amylase limit dextrins reveal a rather striking specificity of the enzyme in the vicinity of the α-1,6 link. The structures of the singly branched dextrins were found to be:

B_4 -  .

B_5 -  .

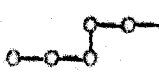
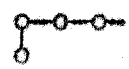
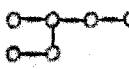
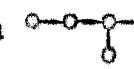
B_6 -  .

B_7 -  .

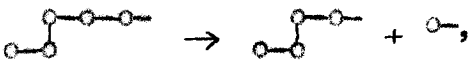
These dextrins have one feature in common with the exception of the minor component of B_4; they all have two glucose units to the right of the α-1,6 link. No dextrins were found with more than this number of glucose units in this position. To the left of the α-1,6 link the number of glucose units varies but the lower chain is more difficultly hydrolyzed than the upper chain (B_5, B_7). This is of course in disagreement

with the view of Whelan *et al.* (52) who regard the dextrins as more or less symmetrical about the α-1,6 link.

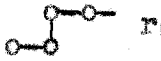
In discussing the origin of the dextrins, the structure of waxy maize starch should be recalled. The evidence of Cori (13, 14) indicates that it has a structure consisting of branch upon branch in a bushy type arrangement. The average number of glucose units between branches is 5.0. No doubt this figure varies a good deal because it has been shown that the doubly branched dextrins have only one glucose unit between branches. It is easy to see why such arrangements are resistant to the enzyme since on hydrolysis they must yield singly branched dextrins of less than two glucose units to the right of the α-1,6 link (page 66). Apparently then all arrangements greater than one glucose unit between branches are degraded to this uniform arrangement, shown on page 66. This at least must be the case for advanced stages of hydrolysis. It would be interesting to examine the dextrins B_6 to B_9 produced at early stages of hydrolysis to see if this same specificity extends to them.

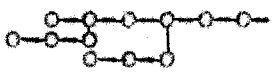
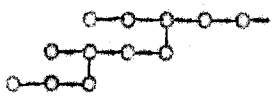
Variability of chain length at the non-reducing end of the dextrins may be due to the reason suggested by Myrbäck (53); namely, the enzyme prefers to remove maltose units rather than glucose units, much like β-amylase. The non-reducing end of the dextrin, unlike the reducing end may have its origin from two sources, peripheral chains or internal chains (between branches). As both these vary in length they may have a number of glucose units either even or odd. A structure such as  shown for B_6 is hydrolyzed by the removal of maltose to yield  , whereas the major component of B_6,  , is more resistant because hydrolysis must proceed by the removal of glucose units. This is an analogous situation to that found with the linear radioactive oligosaccharides. Removal of a glucose unit from the non-reducing end proceeds at a very slow rate. Why the upper non-reducing end should be hydrolyzed more readily than the lower one is not apparent, but it is undoubtedly the reason for the more or less homogeneous nature of the dextrins at advanced stages of hydrolysis. It seems logical therefore that the isomeric B_6 component has the structure  rather than  .

Uncertainty still remains as to the minor constituent of B_4. Two sources of it are suggested:

(1) hydrolysis of B_5, e.g.  ,

(2) hydrolysis of doubly branched dextrins.

The latter source should also yield a B_4 with the structure  rather than its isomer,  .

The doubly branched dextrins consisted of compounds from BB_9 to BB_{12}. The structure at the reducing end has been observed to be identical with that of the singly branched dextrins. It seems logical that the configuration at the non-reducing ends should also be similar. There are left therefore two glucose units for the third remaining site. A structure for BB_{12} is suggested as:  . This structure has the same configuration at the non-reducing ends as the singly branched B_7, and for that reason should be as stable to salivary amylase. The isomeric structure,  , should be less stable if this situation is truly analogous to the singly branched dextrins.

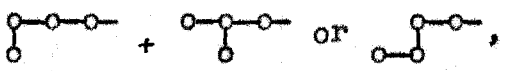
VI. SUMMARY

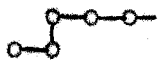
1. Amylotriose and amylotetraose have been separated from salivary amylase digests by carbon chromatography. Crystalline 1-phenyl flavazole derivatives of both compounds have been prepared.

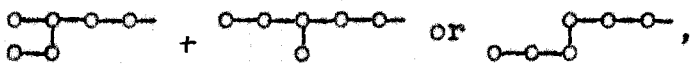
2. Comparative salivary amylase hydrolysis rates for amylotriose, amylotetraose, amyloheptaose and amylodextrin were determined.

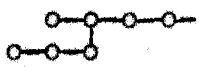
3. Linear radioactive oligosaccharides have been prepared by the coupling reaction of macerans amylase on α-dextrin and radioglucose. These have been shown to be labeled only at the reducing glucose unit. The reaction of salivary amylase on the members G_3 to G_7 has been studied by paper chromatography. Examination of the activity of the hydrolytic products has led to the following general definition of bond specificity by the enzyme: those bonds within two links of the non-reducing end and within one link of the reducing end are hydrolyzed much faster than the remaining three bonds. The two terminal links are extremely resistant and the link second from the non-reducing end is intermediate between these and internal linkages.

4. The singly branched limit dextrins obtained by the extensive action of salivary amylase on waxy maize starch have been found to have the following structures:

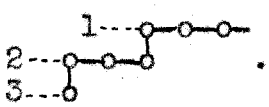
B_4 -  ,

B_5 -  ,

B_6 -  ,

B_7 -  .

5. Evidence has been presented that the doubly branched limit dextrins consist of oligosaccharides containing only one glucose unit between branch points and that a basic structure for these compounds is:



The dextrins consist of compounds having from two to six glucose units distributed among three possible positions, 1, 2 or 3, of this basic structure as indicated above.

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IX. APPENDIX

Table 6

R_f Values of 1-Phenyl Flavazole Derivatives

1-Phenyl flavazole derivative	R_f in sat. methyl ethyl ketone	R_f in 3:4:6
G_1^F	0.9	0.97
G_2^F	0.6	0.90
G_3^F	0.45	0.80
G_4^F	0.25	0.70
B_3^F	0.40	0.75
B_4^F	0.20	0.65
B_5^F	----	0.55
B_6^F	----	0.40
B_7^F	----	0.30