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Action patterns of starch enzymes

Gene Muriel Wild
Iowa State College

NOTE TO USERS

ACTION PATTERNS OF STARCH ENZYMES

by

Gene Muriel Wild

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Signature was redacted for privacy.

Head of Major Department

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INTRODUCTION

The problem of the nature and structure of starch is one of long standing. As portions of the problem have gradually been solved, the way has been opened for work on new phases of the problem. The advances toward its ultimate solution have always waited for advances in the development of analytical tools and investigative methods.

Of the more recent advances, the development of the technique of filter paper chromatography has been one of which great use has been made. An increasing usefulness for paper chromatography will probably be seen, since there is no lack of variations and modifications in its application.

In this work, the use of paper chromatography has made possible a study of the action of several enzymes on low molecular weight starch degradation products. An attempt was made to determine the pattern of action of each of the several enzymes, and, at the same time, to learn something more of the structure of starch. The use of low molecular weight substrates offers distinct advantages. Defined compounds can be used; the reactions which occur can be more easily followed and evaluated. Some side reactions can be eliminated along with difficulties encountered because of the nature of high molecular weight compounds.

The enzymes studied include salivary amylase, the beta

amylases of soybeans and sweet potatoes, several plant phosphorylases, and the so-called Q- and R-enzymes which have branching and debranching actions respectively.

No doubt more decisive results could have been obtained had crystalline enzyme preparations been available for use. It is felt, however, that this work will at least serve as a useful guide for further study.

REVIEW OF PERTINENT LITERATURE

Starch

The observation by Kirchhoff (1) in 1815 that wheat gluten, or its aqueous extract, converts potato-starch paste into a sugar other than glucose probably marks the beginning of starch-enzyme studies as well as enzymology as a whole. This meager beginning was followed by the demonstration by Leuchs in 1831 (2) of the liquifying and sugar producing properties of saliva on starch. In 1833 Payen and Persoz (3) obtained what they called diastase from malt and showed that it also produced sugar from starch. That this malt sugar was different from glucose was rediscovered by Dubrunfaut (4) in 1847 who called it "maltose", and again by O'Sullivan (5) in 1872. The structures for glucose and maltose were definitely established by Haworth in 1928 (6). Through this work it was learned that the starch polymer was composed for the most part of glucopyranose residues united by alpha-1,4 linkages.

In 1879, it was found by O'Sullivan (7), and Brown and Heron (8) that only about 80% of the starch was converted into fermentable sugars by malt extracts. Maquenne and Roux (9) interpreted these results as signifying that starch was composed of two components. The 80% of the starch which stained with iodine and was degraded to maltose was termed "amylose". The 20% which was resistant that remained after fermentation

and did not stain with iodine was called "amylopectin", since it was supposed to bear a relationship to the pectins.

That starch probably consisted of more than one component was apparently widely anticipated, for many attempts were made by workers to demonstrate the two fractions of starch which occur. For the most part the fractions obtained consisted of mixtures of the two components: a lower molecular weight, more soluble mixture and a higher molecular weight, less soluble mixture.

A successful separation of one of the components was reported by Samec (10) in 1927, using the technique of electrodecantation. He said that "amylopectin" was deposited at the anode and "amyloamylose" was left in solution. He also reported (11) that the amyloamylose stained a deep blue with iodine and was wholly degraded to maltose by barley amylase, but the "amylopectin" stained a violet-red and was degraded only about 60% by the amylase.

It was not until 1940 that Meyer and coworkers (12) reported that it had been definitely established that ordinary starch consisted of two components. By using a fractionating scheme using warm water, 10-20% of a linear component and 80-90% of a highly branched component were separated. The term "amylose" was redefined by Meyer to represent a starch polysaccharide which was entirely linear. The term "amylo-

pectin" was redefined to represent a starch polysaccharide with a highly branched structure. Meyer's warm water soluble fraction had amylose-like properties, for it was completely degraded by beta amylase just like Samec's amyloamylose. This was confirmed chemically (13) by methylation end-group analyses along with molecular weight studies. On the other hand, the amylopectin preparations of both Meyer and Samec were shown to be similar, to be incompletely degraded by beta amylase, and to consist of greatly branched molecules.

It was in 1941 that Schoch (14) developed the best method for fractionating starch using butyl or amyl alcohol insoluble complex formation with the amylose from a starch sol. Schoch found that corn starch consisted of 21-23% amylose. The composition of starches from different sources varies from mostly amylose to all amylopectin. The "glutinous" starches like that from waxy maize are wholly amylopectin (15,16).

Staudinger and Husemann (17) proposed a structure consisting of a main chain with simple side branches along the chain to explain the branched structure for amylopectin. However, Meyer and Bernfeld (18) proposed a model of structure consisting of a chain of glucose units with many long branches which were themselves highly branched in a bush-like manner. They reasoned that this type of structure best explained the physical properties of amylopectin and its derivatives as well

as its behavior with beta amylase. Haworth and associates (19) did not feel that it was necessary to propose such a complicated structure to explain the facts, so they proposed the so-called laminated schematic formulation. This consisted of a chain which had one branch which in turn had one branch, etc., repeated many times. Myrbäck (20) preferred Meyer's formulation, as do most recent investigators as will be shown.

That amylose may not be entirely linear has been suggested by Peat and coworkers (21,22,23) who reported what they call Z-enzyme in crude preparations of soybean beta amylase. It appears to serve the function of a beta-glucosidase. Apparently the total degradation of most amyloses by pure beta amylases is not possible without the action of Z-enzyme. The beta-glucosidase of sweet almonds is said to simulate the action of the Z-enzyme.

Additional evidence that some amyloses may contain branches is given by Kerr and Cleveland (24). Using the amyloglucosidase of Aspergillus niger or crystalline sweet potato beta amylase, they show that the rates of production of glucose or of maltose, respectively, from potato and tapioca amyloses is two to three times that of glucose or maltose produced from crystalline corn amylose. They conclude that these amyloses must contain an average of 2 or 3 branches per molecule. The nature of the branching was not described. Whether normal alpha-1,6 branch linkages are responsible, or the type

of branch labile to Z-enzyme has not been determined.

Amylases

It was not until the latter part of the nineteenth century that it was suspected that the action of malt might be due to more than one enzyme. In 1870, Schwarzer (25) showed that the maltose producing power of a malt extract is greatly lessened on heating, but that its dextrinizing ability is little changed. Wijsman showed that two enzymes were present (26) by observing their different rates of diffusion from a drop of malt extract placed in the center of a starch containing gelatine plate. After treating with an iodine solution, there appeared a colorless spot surrounded by a red-violet ring, the rest of the plate staining blue. The two concentric areas of staining and non-staining material showed the presence of two distinct enzyme actions.

In 1922, Ohlsson (26,27) again demonstrated that there were two distinct enzymes in malt extract. He showed that one carried the hydrolysis only to the dextrin stage; the other hydrolyzes starch to maltase. From this he called them "dextrinogenase" and "saccharogenase". He selectively destroyed the saccharogenic amylase with heat, the dextrinogenic amylase at a low pH.

Kuhn (28,29) reported an interesting observation in 1924

concerning the products of action of the two types of amylases. It was previously known that "malt amylase" liberated beta maltose by its action, but Kuhn showed that pancreatic amylase formed downward mutarotating products. From this he named them beta and alpha amylases respectively.

In 1930, Ohlsson (30) demonstrated that beta maltose was produced by the saccharifying amylase and alpha maltose by the dextrin-forming amylase of malt. Thus the saccharogenic amylases are called beta amylases, the dextrinogenic amylases, alpha amylases. Animal amylases are alpha amylases; most plant amylases are beta amylases, although several plant alpha amylases occur.

Several investigators concluded that the production of alpha and beta maltose bore a relationship to starch structure, not realizing that the difference was in the modes of action of the different enzymes in the breaking of a given linkage.

Many investigators had tried to demonstrate in addition to alpha and beta amylases that a special liquefying amylase was present in many preparations. However, liquefying action was definitely shown in 1941 to be a property of the alpha amylases (31).

More definite characterization of amylase action had been rather slow in developing due to the difficulty in analysis of the products.

The demonstration by Samec and Waldschmidt-Leitz (11) in 1931 that their amyloamylose was completely degraded into maltose by beta amylase gave an indication of the action of the amylase as well as the structure of the amyloamylose. Their report that the amylopectin fraction was only 60% converted to maltose by beta amylase and van Klinkenberg's report (32) that potato starch was 64% converted to maltose by beta amylase began to shed light on both the nature of the amylase and the substrates. Klinkenberg named the beta amylase residual dextrin "erythrogranulose".

Since only maltose could be detected as the low molecular weight hydrolytic product from the action of beta amylase, the experiments of Myrbäck and associates (32,33) were sufficient to prove its method of attack. Since oxidation of the reducing group had no effect on the action of the enzyme, it could be asserted that the enzyme attacked the substrate at the non-reducing terminals splitting off successive maltose units. That this continues to complete degradation with an unobstructed chain of glucose units bonded with alpha-1,4 linkages was seen by the experiments of Samec and Waldschmidt-Leitz (11) on amyloamylose (amylose). However, upon coming to an obstruction (branch-point) in an amylopectin molecule its action was stopped (34). Meyer and coworkers determined that the limits of conversion to maltose of potato and corn amylopectins by beta amylase were respectively 59% and 62% (35). How near a

1,4 bond may be to a branch point and still be broken by beta amylase is still a point for study.

Myrbäck and associates determined with low molecular weight hydrolysis products of starch that those with an even number of glucose units were converted completely to maltose by beta amylase. Those with an odd number of glucose units were reported to give maltose plus one amylotriose molecule per molecule. No glucose was produced (36). French and coworkers (37) also found that from amyloheptaose only maltose and amylotriose were formed. Bailey, Whelan and Peat (38) have indicated that beta amylase slowly hydrolyzes amylotriose to maltose and glucose.

Alpha amylase preparations have been shown by many investigators to rapidly form dextrins from starch along with some maltose (30,39). Samec and Klemen claimed to have identified a trisaccharide among the dextrins and maltose produced (40). Various ones have suggested that glucose was also produced. Myrbäck and Lundberg (41) demonstrated its formation in 1943. They reported that it appeared along with the dextrins and the maltose, but was not formed from the maltose. In 1938, Myrbäck again showed a trisaccharide to be among the products of alpha amylase action (42). This was definitely proven by Wolfrom and coworkers when they isolated amylotriose (maltotriose) by making its crystalline acetate from an alpha amylase digest of waxy maize starch (43,44).

Soon after the alpha and beta amylases were separated it was obvious that the alpha amylase had a much different specificity than the beta amylase. The beta amylase gave a high molecular weight residual dextrin with starch that alpha amylase did not give (30). It was shown also that alpha amylase degrades this limit dextrin (45,46). If beta amylase was stopped by the branch points, it was obvious that alpha amylase either hydrolyzed them or by-passed them (34). But in 1940 Myrbäck and coworkers concluded that in the residual dextrins, after alpha amylase action, were included some linkages other than alpha-1,4 linkages (47). That same year they showed the presence of an alpha-1,6 linkage in a trisaccharide dextrin (48). In connection with the fact that 1,6 linkages were shown to be stable to alpha amylase (49), it was easy to conclude that the enzyme by-passed the linkages and hydrolyzed internal chains. Further evidence of the fact that the primary branch points in starch consisted of alpha-1,6 linkages came with the isolation by Freudenberg and Boppel of 2,3 dimethyl glucose from hydrolyzed methylated starch (50). Unequivocal proof was given by Wolfrom and coworkers in 1949 and 1950 by their isolation of crystalline isomaltose octaacetate from acetylated dilute acid hydrolyzates of glycogen and amylopectin (51,52).

The isolation by Ahlborg and Myrbäck (53) in 1941 and by Montgomery and associates (54) in 1949 of isomaltose from

taka-amylase digests of starch could not be relied upon as proof that the branch points in starch consisted of alpha-1,6 linkages. The isolation of panose (55), 4-α-isomaltosyl glucose (56,57), from starch digests treated with Aspergillus niger enzyme extracts, and the demonstration by Pazur and French (58,59) of a transglucosidase in A. oryzae showed the reason. The transglucosidase from A. oryzae (the source of taka-amylase) was capable of forming isomaltose, panose and various other sugars containing one or more 1,6 bonds from maltose.

As for the mode of alpha amylase attack on starch, Meyer and Bernfeld (60) postulated a random attack on the molecule except for the terminal linkages which they said hydrolyzed at a slower rate. This was their reasoning for the slow breakdown of amylotriose by malt alpha amylase into glucose and maltose, which they reported. Alfin and Caldwell also decided that pancreatic amylase hydrolyzed in a random fashion (61). The results of Dimler and associates (62) seem to substantiate these claims, for at the achromic point they found oligosaccharides up to 10 glucose units in size in their digests using malt alpha amylase. However, Dimler's work was carried out with digests at 70° C., and French, Knapp and Pazur (63) using that temperature have shown with salivary amylase that the nature of the action is different at that temperature than it is at ordinary temperatures. Using a temperature of 70° C. or using a digest at pH 10 at ordinary temperatures they found

results much like Dimler's group. But under normal conditions, paper chromatographic studies showed that the initial products of salivary amylase action on a linear starch substrate were maltose, amylotriose and amylotetraose, with only very small amounts of amylopentaose and even less of the higher oligosaccharides (63,64). After the high molecular weight material was substantially all hydrolyzed, the amylotetraose was hydrolyzed to maltose at a somewhat slower rate, with the trisaccharide being broken down to maltose and glucose at an even slower rate. French's group reasoned that under normal conditions, once the enzyme molecule and the substrate molecule had diffused close enough together for an enzyme-substrate complex to form, the substrate molecule was attacked several to many times in succession before the molecules had time to diffuse apart again.

Roberts and Whelan (65) have been unable to demonstrate the cleavage of amylotriose by salivary amylase, but the evidence for its occurrence appears to be unmistakeable.

When an alpha amylase is allowed to act upon amylopectin or glycogen or beta amylase limit dextrin, branched dextrins are formed (47,48). After a slight action of alpha amylase on beta amylase limit dextrin, it is again subject to the action of beta amylase because some inner chains are exposed (45,46,66). The exact nature of the branched sugars formed by a given alpha amylase has not been reported. Whelan and

Roberts (67) have described in a preliminary report a branched pentasaccharide which they are investigating from a salivary amylase digest of glycogen. They report it to be the lowest molecular weight branched dextrin formed.

The crystallization of amylolytic enzymes was a great step toward the learning of the true nature of action of these enzymes. The report of the crystallization of hog pancreatic amylase by Caldwell and coworkers (68) in 1930 was followed by the more detailed report of Meyer's group (69,70) in 1947. The first beta amylase to be crystallized was that of sweet potatoes by Balls, Thompson and Walden (71,72) in 1946. The alpha amylase of saliva was first crystallized in 1947 by Meyer's group (73,74,75) followed by human pancreatic amylase in 1948 (76). Meyer and coworkers concluded that the human salivary and pancreatic amylases were identical chemical substances.

Starch Synthesizing Enzymes

Evidence of phosphorolytic action was observed by Bodnar (77) in 1925, for he reported that pea meal placed in a solution of phosphate buffer was able to convert the inorganic phosphate into an organic form. Since this action did not occur with heated or methanol treated meal, he concluded it was an enzymic reaction. In 1935, Parnas and Baranowski (78) wrote of the esterification by free phosphate of glycogen by

an autolyzed, dialyzed extract of muscle to produce a monophosphoric ester. Ostern and associates (79) showed that this ester was glucose-6-phosphate. It was also shown by Ostern's group (80) that the same product could be obtained from the phosphorylation of starch by these muscle extracts.

It was demonstrated by Cori's group (81,82), however, that the formation of glucose-6-phosphate was the result of two enzymes, and that glucose-1-phosphate, (Cori ester), probably having the alpha configuration, was formed as an intermediate.

Mystkowski (83,84) reported in 1937, as did Cori, Colowick and Cori (85) in 1939, that phosphorolysis of glycogen was a process independent of the amylase in the system. Glucose-1-phosphate was assumed to be formed by the entrance of the inorganic phosphate at the maltosidic linkages of the glycogen (86) in a manner analogous to hydrolysis.

In 1938 Lehmann reported that glucose inhibited this phosphorolytic reaction (87). This was confirmed by Cori's group (85) in 1939, who also showed that small amounts of phlorizin or phloretin almost completely inhibited the reaction. It was soon after this that the reversibility of the reaction was discovered and the synthetic action of muscle phosphorylase demonstrated by the Coris and coworkers (88,89). From purified enzyme preparations, a blue staining polysac-

charide was formed, unlike glycogen. Schäffner demonstrated at about the same time the synthesis of glycogen by yeast from glucose-1-phosphate (90). The phosphorylase from muscle was shown to have no effect on galactose-1-phosphate or mannose-1-phosphate (88). It was later proven in 1943 that the glucose-1-phosphate formed had the alpha configuration when Wolfrom and associates (91,92) synthesized the beta isomer and showed that phosphorylase did not act upon it. Maltose-1-phosphate and xylose-1-phosphate were not acted upon either (93). Upon further purification of the muscle phosphorylase preparations it was noticed by the Coris (89,94) that added glycogen was necessary before synthesis took place.

In 1940 Hanes (95) reported finding phosphorylase activity in an extract from peas which also had reversible action. He postulated that the glucose-1-phosphate was formed by direct phosphorolytic cleavage of the terminal glucose units from the non-aldehydic ends of the chains. Soon after, Hanes (96,97) found that the white potato was a good source of phosphorylase, and was easier to purify. Added starch served as an activator for both of these enzymes. Hanes put forth the idea that possibly the activator entered into the reaction. The synthesized polysaccharide formed by purified potato phosphorylase stained a deep blue with iodine and was almost completely degraded by beta amylase, like amylose.

Phosphorylases from various plants have been studied.

Among them are sugar beets (98), soy, castor and broad beans (99,100), jack beans (101) and corn (102).

Meyer and Bernfeld (103) reported in 1942 that potato phosphorylase acts reversibly on 1,4 links but not on 1,6 linkages, hence does not act upon beta amylase limit dextrin.

Muscle phosphorylase was obtained crystalline first by Green, Cori and Cori (104) in 1942. Early studies on the nature of the action of muscle phosphorylase indicated the enzyme could by-pass or break 1,6 bonds, thus giving nearly total phosphorolysis of glycogen to glucose-1-phosphate. This finding was confirmed by Swanson (105) using the crystalline phosphorylase. It was found by Hestrin (106), however, that after a sufficient number of recrystallizations the enzyme preparation lost this property, and gave a limit dextrin from glycogen similar to that from potato phosphorylase or beta amylase. Swanson (105) showed that potato phosphorylase limit dextrin could be degraded further by beta amylase. Hestrin (106) found similar results for the muscle phosphorylase limit dextrin. The maltose removed amounted to about 1 mole for every end group. Investigation of the polysaccharide synthesized by muscle phosphorylase (107) showed it to have properties very similar to those of amylose and the synthetic starch from potato phosphorylase.

That there was a difference in the requirement of the two

enzymes for activators was quite apparent. Amylose, amylopectin and their partial acid and alpha and beta amylase hydrolytic products were good primers of potato phosphorylase. Swanson (108) showed oligosaccharides as small as 4 or 5 glucose units were good primers. Weibull and Tiselius (109) claimed amylotriose was a good primer. Bailey, Whelan and Peat (38) and Wild (110) established in 1950 that amylotriose was a weak primer, and that amylotetraose and higher oligosaccharides are very good primers. It was also shown by Wild (110) that the lack of priming by salivary amylase digests containing these oligosaccharides was due to the formation of branched oligosaccharides which inhibited potato phosphorylase action.

Muscle phosphorylase was primed best by high molecular weight primers like amylopectin and glycogen. More than slight degradation of these resulted in substantial decreases in priming ability (108). Apparently a high molecular weight, highly branched molecule is necessary before the enzyme substrate complex can form, however, it can be seen by comparing limit dextrins that "stubs" of only several glucose units must serve as priming points.

Another interesting phosphorylase of a different type is the sucrose phosphorylase isolated from Pseudomonas saccharophila and investigated by Doudoroff, Kaplan and Hassid (111,112). It has the ability to catalyze the reversible phosphorolysis

of sucrose to glucose-1-phosphate and fructose. During their studies with this enzyme (113) it was discovered that arsenate could take the place of phosphate in the degradation of sucrose, but that apparently glucose-1-arsenate was spontaneously hydrolyzed to glucose and arsenate. Using this system of "arsenolysis" complete degradation could be made to occur without the equilibrating reverse reaction. Hassid's group (114,115) also showed that arsenolysis could occur with potato phosphorylase. Meyer and coworkers (116) have shown that the limits of degradation of amylopectin by wheat beta amylase and by potato phosphorylase arsenolysis are the same.

In the impure state, phosphorylases from muscle and potato were noticed to be able to synthesize a polysaccharide which gave a red or violet stain with iodine rather than a blue stain characteristic of the synthetic product of the purified enzymes. In 1943, the Coris (117) working with animal tissues, and in 1944 Hanes (118) showed with potato preparations that a second enzyme was responsible. That this enzyme-type catalyzed the formation of branches was shown by the amylopectin properties of the polysaccharide synthesized by the potato preparation.

In 1945 Peat and associates (119,120) described this branch forming enzyme, which they called "Q-enzyme", as a non-amylolytic enzyme. They put forth the hypothesis that it took small amylose chains (pseudoamylose) of about 20 glucose

units and formed them into amylopectin by synthesizing 1,6 bonds. The pseudoamylose was to have come from phosphorylase synthesis and/or from hydrolytic cleavage of amylose by the Q-enzyme. In further studies, however, it was seen that amylopectin was actually not formed from a pseudoamylose-type polysaccharide, but larger amylose molecules were required (121,122,123).

In 1950 Peat and his group (124,125) showed that the action of Q-enzyme did not involve phosphate, but acted in the manner of a transglucosidase. Further studies (126) substantiated this finding. Hosoya (127) also stated its action was that of a transglucosidase.

Peat and his associates (128) demonstrated that with various mixtures of phosphorylase and Q-enzyme, different polysaccharides could be synthesized varying from those with amylose properties to those with amylopectin properties.

Gilbert and Patrick (129,130) obtained the Q-enzyme of potatoes in a crystalline form in 1950.

The broad bean has been found to be a good source of Q-enzyme. It is also present in peas (100). Sugar beets (98) have been reported to contain a Q-enzyme. A Q-enzyme from the organism, Polytomella coeca, has also been studied (131, 132,133).

Nussenbaum and Hassid reported in 1952 (134) that linear dextrins of average chain length up to 42 glucose units were not converted by potato Q-enzyme into amylopectin, but dextrins averaging 116 glucose units were formed into amylopectin. Bailey, Peat and Whelan (135) reported that Q-enzyme failed to act upon dextrins averaging 25 glucose units in size. It acted very slowly on dextrins averaging 30 glucose units, and more rapidly on dextrins with an average length of 58 glucose units.

Bernfeld and Meutemedian (136,137,138,139) claimed the discovery of an "isophosphorylase" in potatoes. Its action was described as phosphorolysis of and synthesis of alpha-1,6 glucosidic branch points. However, these claims have been disputed by Bailey and Whelan (140).

Debranching Enzymes

It was reported in 1950 that the enzyme responsible for allowing the complete phosphorolysis of amylopectin by crude plant extracts was not the same as the Q-enzyme.

Hobson, Whelan and Peat (141,142) demonstrated the presence of a separate debranching enzyme which they called "R-enzyme". They described its hydrolytic action on the branch points of amylopectin and beta amylase limit dextrin. They followed this action by the change in the color of the

iodine stain, which was toward the blue color of amylose.

Cori and Larner (143,144) reported also in 1950, on the action of a debranching enzyme from muscle, which they called "amylo-1,6-glucosidase". This enzyme was reported to hydrolyze off the glucose branch points remaining on muscle phosphorylase limit dextrin. After the action of amylo-1,6-glucosidase, the phosphorylase could continue phosphorolysis to the next branch point. From the action of beta amylase on muscle phosphorylase limit dextrin, and from the products of the action of amylo-1,6-glucosidase, they postulated that the glucose units of the periphery of the limit dextrin must be unevenly distributed, the branch consisting of one glucose unit, the chain extending beyond the branch point consisting of 5 glucose units.

Cori's group have given good evidence (145,146) for the Meyer formulation for amylopectin and glycogen. That these molecules must be irregularly and multiply branched is indicated by the fact "tiers" of branches can be shown to be degraded by the successive actions of muscle phosphorylase and amylo-1,6-glucosidase on these molecules.

Additional evidence in favor of Meyer's representation for amylopectin has been presented by Peat, Whelan and Thomas (147). Following the action of R-enzyme on beta amylase limit dextrin and observing the kind and amount of oligasaccharides

produced, they came to their conclusions. Nearly equal amounts of maltose and amylotriose "stubs" were split off in amounts intermediate between the minimal amount expected from the Haworth formula and the maximal amount that would be given by the Staudinger model. Other oligosaccharides split off, indicative of the nature of the secondary branches of the limit dextrin, began at 6 glucose units and increased in size, the 4 and 5 membered sugars being absent.

As early as 1940, Meyer (148) used what he called alpha glucosidase from yeast to remove the branch points of beta amylase limit dextrin. The beta amylase could again hydrolyze off maltose from the treated limit dextrin.

The "limit dextrinase" described by Back, Stark and Scalf (149) may be a similar enzyme.

Nishimura (150,151) reported in 1930 on an enzyme from yeast which he named "amylosynthease". The enzyme was given this name from its apparent synthetic activity as measured by the shift of iodine color from red to blue of the polysaccharides treated by it. Preparations of "amylosynthease" were made from rice and potato by Minagawa in 1932 (152). All these preparations had "synthetic activity" on branched substrates only. A renewed study of this enzyme from yeast was made in 1949 by Maruo and Kobayashi (153,154,155) and they came to the conclusion by 1951 that the enzyme was identical

with the alpha glucosidase of yeast used by Meyer (148) in 1940 and may be the same as R-enzyme of Hobson, Whelan and Peat (142). They have suggested it be called "isoamylase".

In 1947, Petrova (156,157,158) claimed to have isolated an enzyme from muscle which on acting along with phosphorylase could reversibly catalyze the formation and degradation of glycogen. "Amylose isomerase", as it was called, was most likely a mixture of branching and debranching enzymes.

MATERIALS AND METHODS

Chromatographic Technique

Filter paper

The filter paper predominantly used was Eaton and Dikeman 613, which was obtained in 1000 ft. rolls eight inches wide. The desired lengths were cut and used as such. To obtain uniform chromatograms a length of 8-5/8 inches was used consistently, for that length of paper could be used nicely in the containers which were available.

E. and D. 613 filter paper contains an amount of starch which in some instances is undesirable. In such instances Whatman No. 1 filter paper was available for use.

Manipulation of samples

The samples of carbohydrates or enzyme digests were applied to the paper 2 cm. from the bottom edge. When spaced 2 cm. apart, nine spots could conveniently be put on each chromatogram. However, spots could be placed as close as 1.7 cm. and still give satisfactory results. The samples were applied with a platinum loop which had a normal capacity of 0.002 ml. When necessary, to obtain a high enough concentration of material, successive loops of solution were applied to the same spot after the preceding application had dried. In

cases where chromatographic fractionation was being carried out for isolation purposes, the solution was applied with a fine-tipped pipet made by drawing 8 mm. glass tubing down to a small diameter using a small flame. By using a pipet with an opening of appropriate size, the solution could be applied to the paper in a very uniform streak.

Containers and developing solution

Ascending type chromatography was used consistently, for no material is lost from the chromatogram and only very simple equipment is required. The containers used were wide mouthed gallon jars with screw lids or brown wide mouthed reagent jars.

After the spots or streaks were applied to the paper and dried, the paper was stapled to form a cylinder with the spots near the bottom edge. The stapled edges were not allowed to touch. The adhesive on the staples did not interfere with development, since the staples were nearly 2 cm. away from the path of the nearest spot. From 50-65 ml. of developing solution was used in each jar. The chromatogram was allowed to stand in the solution until the solution had reached the top of the paper by capillary action. The chromatogram was then taken out, dried and returned to the jar for another ascent if desired. A chromatogram was given as many ascents as necessary to achieve the desired result.

The solution used for all carbohydrate chromatography

consisted of three parts of water, four parts of pyridine and six parts of 1-butanol by volume (3-4-6) (159). Practical pyridine redistilled in the presence of barium oxide was used.

After the solution in a jar had been used for four ascents, its effectiveness in moving and resolving substances on the chromatogram was significantly reduced. This was no doubt due to an alteration in the composition of the solvent. Because of this, the solution was seldom used for more than four ascents.

Since pyridine is known to cause ketonization and epimerization of aldoses at elevated temperatures, the chromatograms were always dried at room temperature.

It required five to six hours for the 3-4-6 to reach the top of the 8-5/8 inch lengths of E. and D. 613 filter paper after the initial ascent, which took a little longer.

Color reagents

Several different color reagents were used. The principle reagent used to detect the reducing carbohydrates was the alkaline copper reagent. It was prepared by dissolving 7.5 g. of copper sulfate in 100 ml. of water and adding that with stirring to a solution of 25 g. of Rochelle salt and 40 g. of anhydrous sodium carbonate in 300 ml. of water. To this mixture was added 500 ml. of methanol, and the whole solution was

diluted to 1 liter with water. This solution was sprayed on a chromatogram until uniformly moistened using a DeVilbiss No. 31 atomizer with a continuous air stream. The chromatogram was then heated in a 100-110° C. oven for approximately five minutes. After this the chromatogram was sprayed with a phosphomolybdic acid solution (160), which brought out as blue spots the areas where reduction of the copper by the sugars had occurred. Oligosaccharides with a reducing terminal maltose unit (including maltose) appeared to give spots which had a greenish blue color. This combination of reagents could be used to detect as little as one microgram of glucose, two micrograms of amylotriose and about five micrograms of amylotetraose.

A very useful method for determining whether or not a given carbohydrate would serve as a primer for potato phosphorylase, and also for detecting very small amounts of priming oligosaccharides involved the use of a potato phosphorylase-glucose-1-phosphate spray (110). The procedure varied somewhat depending upon the activity of the phosphorylase used.

A mixture of 0.6 ml. of the phosphorylase preparation described later, 0.5 ml. of a 0.5 M. solution of dipotassium glucose-1-phosphate (brought to pH 7 with 1:1 HCl) and 0.7 ml. of water, was adequate for spraying one half of a chromatogram. The solutions could not be mixed together until immediately before spraying onto a chromatogram, for a sufficient amount

of priming carbohydrate was present to allow rapid synthesis of amylose. Immediately after a chromatogram was sprayed it was hung in a moist chamber consisting of a 16 liter cylindrical jar with a shallow layer of water in the bottom and covered with a glass plate. After ten minutes it was taken out, dried in an oven and sprayed with 0.01 N. I_2 in KI solution to bring out the areas where starch synthesis or inhibition had occurred. Inhibiting materials give a light spot against a bluish background. Weak primers like amylotriose give a blue or red-centered blue spot depending on the concentration. Good primers give a blue spot at very low concentrations, a red-centered blue spot at concentrations barely detectable by other reagents, and a spot shading from yellow at the center through red to blue at the edge for higher concentrations. A spot of amylotetraose containing down to as little as 0.05 microgram could be detected with the potato phosphorylase spray. A red-centered spot is given with 2 micrograms of amylotetraose, a yellow-centered spot by 20 micrograms. About 1 microgram of amylotriose can be detected with this method.

Ammoniacal silver nitrate solution (161) was used in some instances for reducing sugars and in others for detecting aldonic acids. It was prepared by adding little more than enough concentrated ammonium hydroxide to 8.5% silver nitrate to give a clear solution. After spraying onto a chromatogram,

it was heated in a 100° C. oven for several minutes or allowed to stand at room temperature for several hours. When allowed to stand at room temperature, a better looking chromatogram resulted. The limits of sensitivity for glucose, amylotriose and amylotetraose are about 0.1 microgram, 0.2 microgram and 0.5 microgram, respectively.

Spots of ketose containing sugars were brought out by spraying on a solution of 0.1 g. of phloroglucinol in 50 ml. of 1-butanol with 10 ml. concentrated HCl added, then heating in a 100° C. oven for several minutes. Overheating had to be avoided for the spots diminished in intensity when heated too long. This alcoholic reagent was much superior to the corresponding aqueous reagent for comparatively little background color tended to develop. The sensitivity of this reagent is comparable to that of the copper-molybdenum reagents for reducing sugars.

The hydroxylamine reagent followed by a ferric chloride spray was used to a limited extent for the detection of lactones (162).

Enzymes

Potato Q-enzyme

The procedure of Barker, Bourne and Peat (122) was used for the preparation of potato Q-enzyme. The juice (860 ml.) from 1.3 kg. of peeled potatoes was used. The preparation

scheme, including lead acetate precipitation followed by bicarbonate-CO_2 elution and precipitation of the enzyme by bringing to 19 g./100 ml. ammonium sulfate, was exactly followed to give a Q_1-enzyme preparation. This was dissolved in 85 ml. of 0.2M., pH 6.5 citrate buffer and freeze-dried.

Broad bean Q-enzyme

Broad beans were ground in a ball mill to pass at least a 50 mesh sieve. The flour was then extracted with diethyl ether in a Soxhlet extractor until no more color was imparted to the ether. The preparation procedure used for Q-enzyme from broad beans was that of Hobson, Whelan and Peat (100) and was very similar to that used in the preparation of the enzyme from potatoes. For the preparation used in this work, 150 g. of the ether extracted broad bean flour was extracted with 600 ml. of water. The centrifuged and filtered extract was treated in the same manner as the potato juice. The resulting Q_1-enzyme preparation was also freeze-dried in citrate buffer.

Broad bean R-enzyme

The R-enzyme preparation used was prepared following the directions of Hobson, Whelan and Peat (142). A Q_1-enzyme preparation (before freeze-drying) is treated in the cold with potato starch in a solution containing 1% sodium sulfate and 25% ethanol. The R-enzyme stays in solution, but substantially all of the Q-enzyme is adsorbed on the starch or

inactivated. R-enzyme powder is prepared by precipitation with cold acetone.

Potato phosphorylase

Potato phosphorylase solutions prepared according to two different procedures were used. One procedure was that used by Green and Stumpf (163). The other procedure was that used by Barker, Bourne and Peat (122) in connection with the preparation of Q-enzyme.

The method of Green and Stumpf was used to make a phosphorylase preparation thought to contain less Q-enzyme as an impurity, because of the heat treatment used. The juice from 2.25 kg. of potatoes was heated to 50° C. for five minutes. The precipitate formed plus that precipitated by bringing the solution to 15 g./100 ml. in ammonium sulfate was discarded. The precipitate formed by bringing the concentration of ammonium sulfate to 35 g./100 ml. was the fraction saved. This precipitate was dissolved in 0.2 M., pH 7 citrate buffer to give 100 ml. of potato phosphorylase solution.

The potato phosphorylase preparation made in connection with Q-enzyme utilized the supernatant obtained from the 19 g./100 ml. ammonium sulfate precipitation. The concentration of ammonium sulfate was increased to 35 g./100 ml., the precipitate centrifuged off and dissolved in just sufficient 0.2 M., pH 7 citrate buffer to dissolve it. This was the

enzyme solution used for the phosphorylase-glucose-1-phosphate chromatogram spray.

Broad bean phosphorylase

The broad bean phosphorylase solution used was made according to the procedure of Hobson, Whelan and Peat (100) in connection with the preparation of Q-enzyme. The procedure was essentially the same as that for the preparation of potato phosphorylase.

Other phosphorylases

Aqueous extracts of ground meal from lima beans, wrinkled peas, jack beans and corn were used to test for the type of phosphorylase activity in each.

Salivary amylase

The salivary amylase used was in the form of crude, unconcentrated saliva which had either been filtered or centrifuged, sometimes after diluting with an equal volume of water.

Soybean beta amylase

The soybean beta amylase used was a concentrated enzyme powder made by Newton (164) over twelve years ago. It has retained most of its activity. It has been stored in the refrigerator.

Sweet potato beta amylase

The sweet potato beta amylase used was a crystalline preparation kindly supplied through the courtesy of Dr. A. K. Balls (71,72).

Carbohydrates

Linear starch oligosaccharides

The solution of linear starch oligosaccharides used for a reference on the chromatograms was prepared by the acid hydrolysis of a 30% solution of amylodextrin. The average molecular size of the mixture was about 3 glucose units. The solution was stored in the refrigerator with thymol as a preservative.

Waxy maize salivary amylase limit dextrin

A 3% solution was made from 100 g. of waxy maize starch by adding a slurry of the starch to boiling water with rapid stirring. After cooling to room temperature, filtered saliva was added. Several hundred ml. of filtered or centrifuged saliva were added over a period of several weeks. The digest was preserved with toluene. The progress of the digest was followed by paper chromatography. When substantially all of the amylotriose initially produced had been converted to maltose and glucose (after about 20 weeks under the conditions used), the digest was filtered to remove precipitated material

(from the saliva), and evaporated to a syrup. Chromatograms showed the presence of glucose and maltose in large quantities, very small amounts of amylotriose and branched trisaccharide, and significant amounts of branched sugars of 4 glucose units and larger. A fractionation was carried out using methanol, acetone and toluene to attempt to separate some of the glucose and maltose from the mixture. The syrup was of a viscous consistency, but it could be stirred without difficulty. Methanol was added slowly with stirring until additional methanol caused no more precipitation. The solution was decanted from the precipitate, and acetone was added to the methanol solution until no more precipitate was produced. This solution was again decanted from the precipitate and toluene was added until additional precipitate no longer formed. The methanol and acetone precipitates still contained much glucose and maltose and substantially all of the branched material. The toluene precipitate and remaining solution contained considerable amounts of glucose and maltose and insignificant amounts of the branched sugars. In fact, the maltose crystallized spontaneously from the last precipitate and from the syrup which remained after evaporation of the supernatant. This fractionation procedure was repeated twice more on the combined methanol and acetone precipitates, and probably removed more than 50% of the glucose and maltose.

Waxy maize beta amylase limit dextrin

Waxy maize starch, 50 g., was dispersed in 0.5 N. NaOH, brought to pH 4.5 with acetic acid and diluted to 1%. Sixty mg. of a suspension of sweet potato beta amylase crystals was added, and the digest incubated at 40° for four days with toluene as a preservative. The limit dextrin was precipitated by bringing the digest to 60% in ethanol. The precipitate was allowed to settle in the cold. The solution was decanted and the precipitate pulverized in 95% ethanol. This procedure was repeated for a second digestion using proportional amounts of reagents. Five grams of the resulting limit dextrin was given a third digestion with 60 mg. of the crystalline beta amylase suspension at room temperature for two days. The overall yield for the three digestions was 36% of limit dextrin. This signified slightly less than 64% conversion of the waxy maize starch to maltose, taking into consideration slight losses during recovery.

Waxy maize potato phosphorylase limit dextrins

One hundred grams of waxy maize starch was dispersed in boiling water; one mole of KH_2PO_4 and one mole of K_2HPO_4 were added and the solution diluted to about 8 liters. When the solution had cooled to room temperature, 40 ml. of the phosphorylase solution prepared according to the method of Green and Stumpf (163) was added. Toluene was used as a preserva-

tive. After two days, 20 ml. more of phosphorylase was added. Six days after the digest was begun, the limit dextrin was precipitated by adding 95% ethanol to 50% by volume. (Glucose-1-phosphate was recovered from the solution by the method of McCready and Hassid (165).) The gummy precipitate was dispersed in 3 moles of NaOH in 4 liters of solution. Phosphoric acid was added to bring the solution to pH 7. The solution was diluted to 8 liters and 40 ml. of phosphorylase preparation added. After standing one day 20 ml. more of phosphorylase was added. One week after beginning the digest, the limit dextrin was again precipitated with a concentration of 50% ethanol. The resulting precipitate was pulverized in a Waring blendor in the presence of butanol and acetone. Ten grams of the phosphorylase limit dextrin was treated a third time with the enzyme. It was dispersed in 250 ml. of 1 M. KOH and neutralized with 85% H_3PO_4 to give a pH of 6. To the solution was added 45 ml. of phosphorylase solution. Toluene was used as preservative. After ten days the digest was boiled, filtered and dialyzed against distilled water until only a faint precipitate was given with silver nitrate. The solution was evaporated to 250 ml. in a hot air stream, and two volumes of 95% ethanol were added. The precipitate was allowed to settle over night. It was pulverized in a Waring blendor with butanol and acetone. A yield of 5.15 g. of the limit dextrin was obtained. Considerable material was lost during dialysis.

A waxy maize phosphorylase limit dextrin prepared by arsenolysis was made from 25 g. of the starch. The waxy maize was dispersed in KOH and neutralized to pH 6 with H_3AsO_4. The final solution of two liters was 0.05 M. with respect to arsenate. Thymol was used as a preservative. After standing eight and one-half months, the digest was concentrated and the limit dextrin precipitated with ethanol. The precipitate (12.2 g.) was redispersed in NaOH, neutralized with HCl and dialyzed against distilled water until only a faint turbidity was given with silver nitrate. The solution was precipitated with two volumes of 95% ethanol. Ten grams of this arsenolysis limit dextrin was given a treatment with phosphorylase and phosphate in exactly the same way that the third treatment was given to the regular phosphorylase limit dextrin. A low yield of 4.45 g. was obtained. A substantial amount of material was lost in the final dialysis.

Glycogen beta amylase limit dextrin

A digest of a partial beta amylase limit dextrin of glycogen was made by dissolving 0.5 g. of the material in 10 ml. of boiling water, and adding 18 mg. of soybean beta amylase powder to the cooled solution. Thymol was used as a preservative. After six days at room temperature the digest was inactivated by boiling. It was evaporated to dryness in a warm air stream. The dried material was dissolved in 2 ml. of water and precipitated with 10 ml. of methanol, the supernatant

decanted, and the precipitation repeated. The third time the precipitate was dissolved in 0.1 N. NaOH, heated in a boiling water bath for 30 minutes to destroy the reducing sugars still present, neutralized with HCl and evaporated to dryness. The residue was dissolved and reprecipitated as above, redissolved, treated with a small amount of charcoal, filtered and evaporated to dryness. The limit dextrin was given a fourth precipitation, filtered, washed with butanol and dried.

Amyloheptaose

The amyloheptaose used was prepared from Schardinger's beta dextrin by light acid hydrolysis, using the procedure given by French, Levine and Pazur (166).

Amylotetraose

The amylotetraose used was prepared by Mr. Philip Nordin from a salivary amylase digest of amylodextrin by charcoal column chromatography (167).

Amylotriose

The preparation of amylotriose used was made by Mr. Philip Nordin from a salivary amylase digest of amylodextrin. The hydrolysis of amylotetraose was complete; the glucose and maltose were removed by fermentation. It was purified on a charcoal column.

Panose

The crystalline panose available for use was kindly supplied by Dr. S. C. Pan.

Isomaltose

The isomaltose used was a preparation made available by Dr. M. L. Wolfrom.

Panose coupled-product

The panose coupled-product was made by Dr. D. French (168) by coupling Schardinger's alpha dextrin and panose by macerans amylase.

EXPERIMENTAL RESULTS

Action of Potato Phosphorylase

The synthetic and degrading actions of potato phosphorylase were investigated. The natures of the overall synthetic and degrading processes of potato phosphorylase are fairly well known, but the limits for these processes are not so well known. The kind of carbohydrates which will initiate carbohydrate synthesis, and the carbohydrates remaining after the limit of degradation were studied. Carbohydrates which inhibit the action of potato phosphorylase were also investigated.

The priming action of various members of the starch oligosaccharide series was determined by using a technique involving the enzyme reaction on a paper chromatogram. A paper chromatogram of the oligosaccharide series with five ascents in the developing solvent was satisfactory for the determination (Table 1). The designation G_1, G_2, G_3, etc. is used to indicate the number of glucose units in each oligosaccharide.

By using the potato phosphorylase-glucose-1-phosphate mixture as described above, it was readily seen (110) that glucose and maltose had no priming effect with the phosphorylase. Amylotriose was a weak primer and amylotetraose and higher linear sugars were very good primers. A weak or poor

Table 1

Mobility* Values for
Linear Starch Oligosaccharides

(5 ascents)

Mol. size	Mobility	Mol. size	Mobility
G_1	0.95	G_5	0.35
G_2	0.84	G_6	0.25
G_3	0.68	G_7	0.18
G_4	0.49	G_8	0.13

*Apparent R_f

Table 2

R_f Values for
Linear Starch Oligosaccharides

(1 ascent)

Mol. size	Mobility	Mol. size	Mobility
G_1	0.40	G_3	0.21
G_2	0.29	G_4	0.15

primer gives a spot which stains blue or blue with a red center with iodine. A good primer will give, for the same concentration, a spot which stains blue at the edge and a color gradation through red and brown to yellow in the center. Subsequent to this finding, this method was used often for

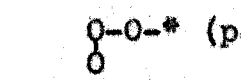
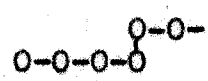
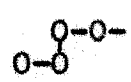
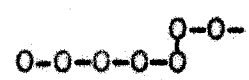
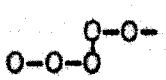
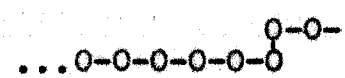
the detection of priming sugars as well as for the determination of the priming ability of various sugars.

By determining the priming action of various sugars obtained through the coupling reaction of macerans amylase (168), it was learned that a chain containing a minimum of three alpha-1,4 glucose units was necessary for weak priming. Whether the chain was free amylotriose or whether it was bonded to another molecule at its reducing end seemed to make little difference. The corresponding molecules containing a linear chain of four or more glucose units were excellent primers. (By "linear chain" is meant an unbranched chain of alpha-1,4 glucose units.) This point is illustrated by observing the action of phosphorylase on the principle components of the panose coupled-product. The five membered sugar was a weak primer; the six and higher membered sugars were excellent primers.

There occurred an interesting pattern of priming action with the components of the salivary amylase digest of amylopectin (See Figure 2). The glucose and maltose, of course, had no priming action; the amylotriose had a weak priming action. The amylotetraose, which diminished rapidly after the initial stages of the hydrolysis, was a good primer. The branched tetrasaccharides (B_4) had no priming action, nor did the branched pentasaccharides (B_5). The spot corresponding to the branched hexasaccharides (B_6) had a very weak priming

Table 3

Phosphorylase Priming action of the
Principle Components of the Panose Coupled-product

	Priming action		Priming action
 (panose)	none		good
	none		good
	weak		good

* O- signifies a glucose unit (g.u.) with its reducing group.
...O-O... signifies 2 g.u. bonded with an alpha-1,4 linkage.
 signifies 2 g.u. bonded with an alpha-1,6 linkage.

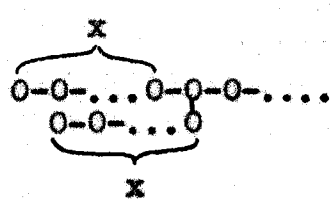
activity which started to diminish toward the late stages of the digest. The heptasaccharide (B_7) spot contained a very strong inhibitor. The branched 8, 9 and higher membered sugars were good primers in the initial stages of the digest, but were degraded. The doubly branched saccharides which appeared in the later stages were inhibitors of phosphorylase.

Among the partial acid hydrolysis products of starch were the linear oligosaccharides already discussed and a series of branched compounds. Chromatographic evidence indicated that the first weak priming activity occurred at the branched tetrasaccharide spot, with spots of all higher branched

compounds showing priming activity. No spots of inhibition could be observed.

The investigations of the phosphorylase limit dextrins of waxy maize, obtained by phosphorolysis and arsenolysis, were carried out using two methods. The methods involved the determination of the products formed by light acid hydrolysis and by action of R-enzyme.

If one subjected a dextrin of this sort to a very low degree of acid hydrolysis, it would be expected that from branches containing $\underline{x}$ glucose units, molecules from G_1 to G_x would be formed; only very small amounts of G_{x+1} would be formed. Molecules from G_{x+2} to G_{2x} would be formed in small amounts, but G_{2x+1} should be produced in somewhat larger amounts.



The molecules G_1 to G_x and G_{2x+1} could be formed by hydrolyzing only one bond, whereas the other molecules would require the hydrolysis of more than one bond. The phosphorylase limit dextrins were subjected to degrees of hydrolysis of 0.005 and 0.01, but inconclusive results were obtained. No significant variation in the amounts of the different

oligosaccharides produced could be observed. An explanation might be that the phosphorylase preparation used contained a small amount of alpha amylase. A significant increase in the solubility of the limit dextrin was noted after the last phosphorylase treatment. Possibly amylase action liberated enough reducing ends which after partial hydrolysis would have made the results less clear cut. This could be tested by oxidizing the reducing groups and repeating the experiment.

The R-enzyme experiments were more satisfactory, although they were not as good as desired because of the lack of purity of the enzyme. The products formed from the arsenolysis and phosphorolysis limit dextrins by R-enzyme were glucose, maltose, amylotriose and amylotetraose. The significance of the maltose and glucose formed is questionable, for they may have been formed by enzyme impurities from the amylotriose and amylotetraose. Besides the four above oligosaccharides, there appeared to have been produced smaller amounts of branched penta-, hexa-, and heptasaccharides and higher molecular weight compounds.

Action of Broad Bean Phosphorylase

The first time broad bean phosphorylase was used in place of potato phosphorylase for a chromatogram spray, it was assumed that the activities of the two phosphorylases would be the same. This, however, was not the case. It appeared

that the broad bean phosphorylase required oligosaccharides of one less glucose unit to give similar results to that of potato phosphorylase. Amylotetraose served as an excellent primer for broad bean phosphorylase; amylotriose was slightly less effective. Maltose was a very weak primer. On the chromatograms glucose gave an anomolous effect with the enzyme. The center of the spot was similar in color to that of the background, but there was a slightly darker ring around the spot indicative of a low degree of priming. The branched saccharides of the salivary amylase digest of waxy maize starch showed a corresponding difference in effect. The branched heptasaccharide spot inhibited the enzyme, but so did the branched hexasaccharide spot. The branched pentasaccharide spot was a weak primer; however, it had no effect on the potato phosphorylase.

Whether this difference in the action of broad bean phosphorylase is a true difference in the enzyme's action pattern, or whether it occurrs because of the co-action of some other enzyme present can not be definitely stated.

Action of Other Phosphorylases

The priming abilities of the linear starch oligosaccharides were determined for the phosphorylases present in the aqueous extracts of lima beans, wrinkled peas, jack beans, and corn. It was found that the actions of the phosphorylase in

lima beans, peas, and jack beans followed the broad bean pattern, but the action of the corn phosphorylase resembled the action of potato phosphorylase.

Action of Salivary Amylase

In connection with the preparation of the waxy maize salivary amylase limit dextrins, a smaller digest of waxy maize starch was carried out with a much larger proportion of saliva. Some micororganisms are known to grow in the presence of toluene. To check that possibility it was thought desirable to ascertain whether or not the products from the main digests were the same as those from the smaller digests, which were hydrolyzed at a much faster rate. As far as could be seen there was no difference in the products.

The small waxy maize digest was made by dispersing 10 g. of the starch in 50 ml. of 1 N. NaOH. After adding 50 ml. of water, the dispersion was neutralized with 6 N. HCl to the phenolphthalein end point. To 52 ml. of this solution was added 42 ml. of centrifuged saliva. This gave a 5% starch digest. This was kept at room temperature. The progress of the digest was followed by paper chromatograms (see Figures 1 and 2).

Similar small digests were made with potato starch and ordinary corn starch to see whether there was a noticeable difference in the action of salivary amylase on these starches.

These digests were followed by chromatograms, and no differences could be detected.

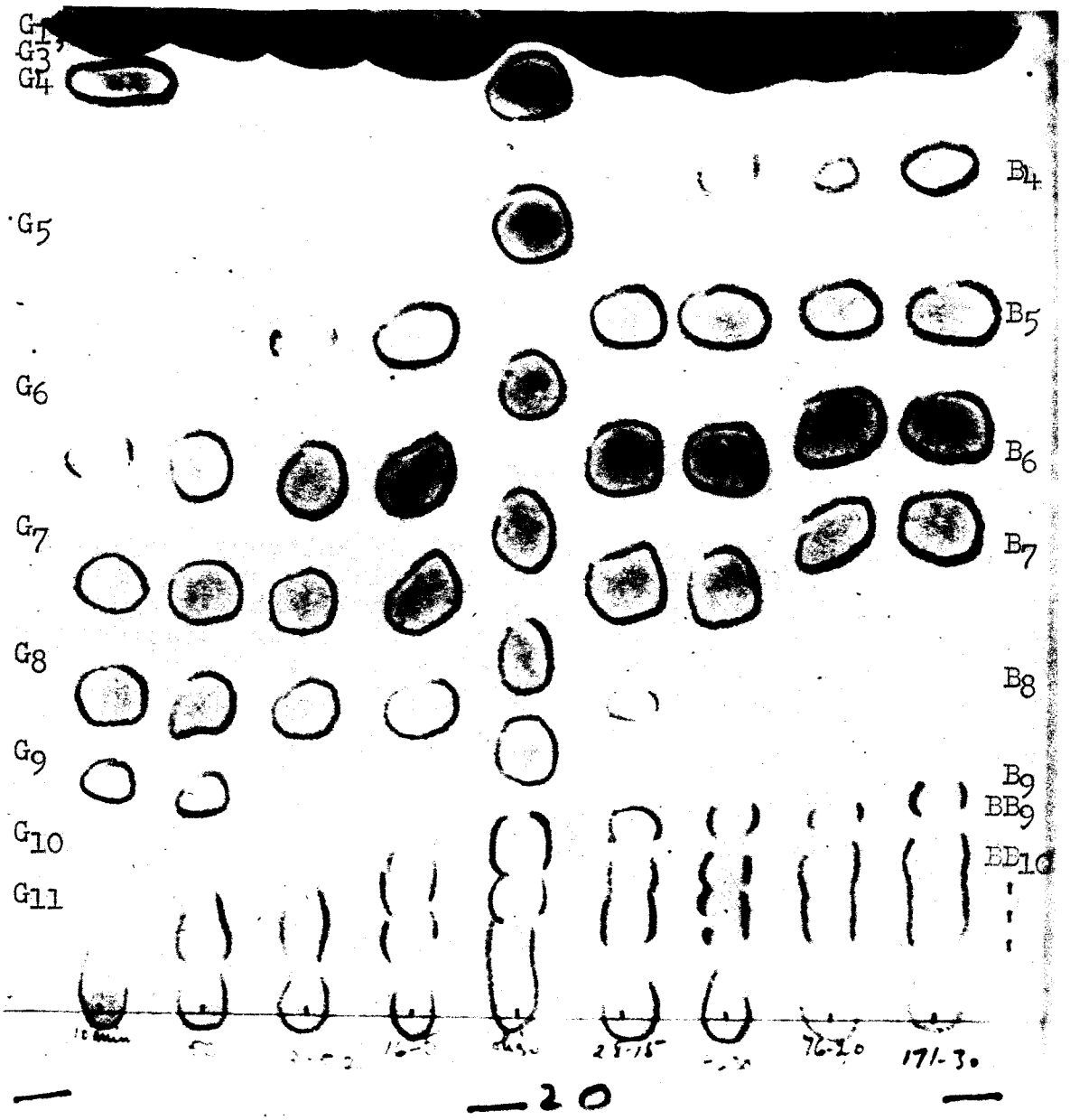
The extent of action of salivary amylase can be described in terms of conversion periods (169). One unit of salivary amylase acting upon 1 mg. of substrate for 1 minute is defined as 1 conversion period (1 c.p.). A unit of salivary amylase activity will convert 1 mg. of soluble starch to the red-brown end point in 1 minute at 40° C. The end point determination is carried out by observing the color produced by 1 drop of 0.1 N. I-KI solution in 3 drops of a 2% soluble starch digest. The saliva used (the author's) had an activity of about 100 units per ml. (40° C.) or the equivalent of 33 units/ml. at room temperature.

After the initial very rapid hydrolysis of starch into maltose, amylotriose and amylotetraose (2.7 c.p.), it can be seen in Figure 2 that only diminishing traces of amylopentaose (G_5), amylohexaose (G_6) and amyloheptaose (G_7) are present. Significant quantities of simple branched compounds are already present. The 6, 7, 8, 9, 10 and 11 membered branched sugars appear to be formed initially, the 7 and 8 membered sugars being present in the largest quantities. As the hydrolysis proceeds, the larger branched sugars tend to be degraded into a fairly stable series of sugars composed of branched 4, 5, 6 and 7 membered sugars, designated as B_4, B_5, B_6 and B_7.

When the symbols, B_3, B_4, etc., are used, it is not necessarily meant that they are single compounds, although they may be. Also when a B_n is used in one instance it is not necessarily meant that when used again it refers to the same compound or mixture of compounds.

Knowing something of the nature of the carbohydrate, it is often possible to obtain evidence for some features of structure from papergram mobility (170). It has been observed that with sugars of the same molecular size, but with one 1,6 linkage in place of one 1,4 linkage, a consistently lower papergram mobility is observed for the sugar containing the 1,6 bond. From these considerations it could be recognized that the series of four sugars resistant to salivary amylase contained one branch per molecule. In Figures 1 and 2 can be seen evidence for another series of sugars which most likely contain 2 branches per molecule (BB series). A chromatogram of the doubly branched material only, obtained by the papergram fractionation of a salivary digest described later, showed a small spot of BB_7, an intermediate sized spot of BB_8, and larger spots of BB_9, BB_{10} and BB_{11}, with lesser amounts of components up to about BB_{13}. Between that series and the origin is observed a discontinuity which very likely signifies that additional larger dextrins are present which contain three and probably more branches per molecule. Comparative mobilities of the linear, singly, and doubly branched sugars are

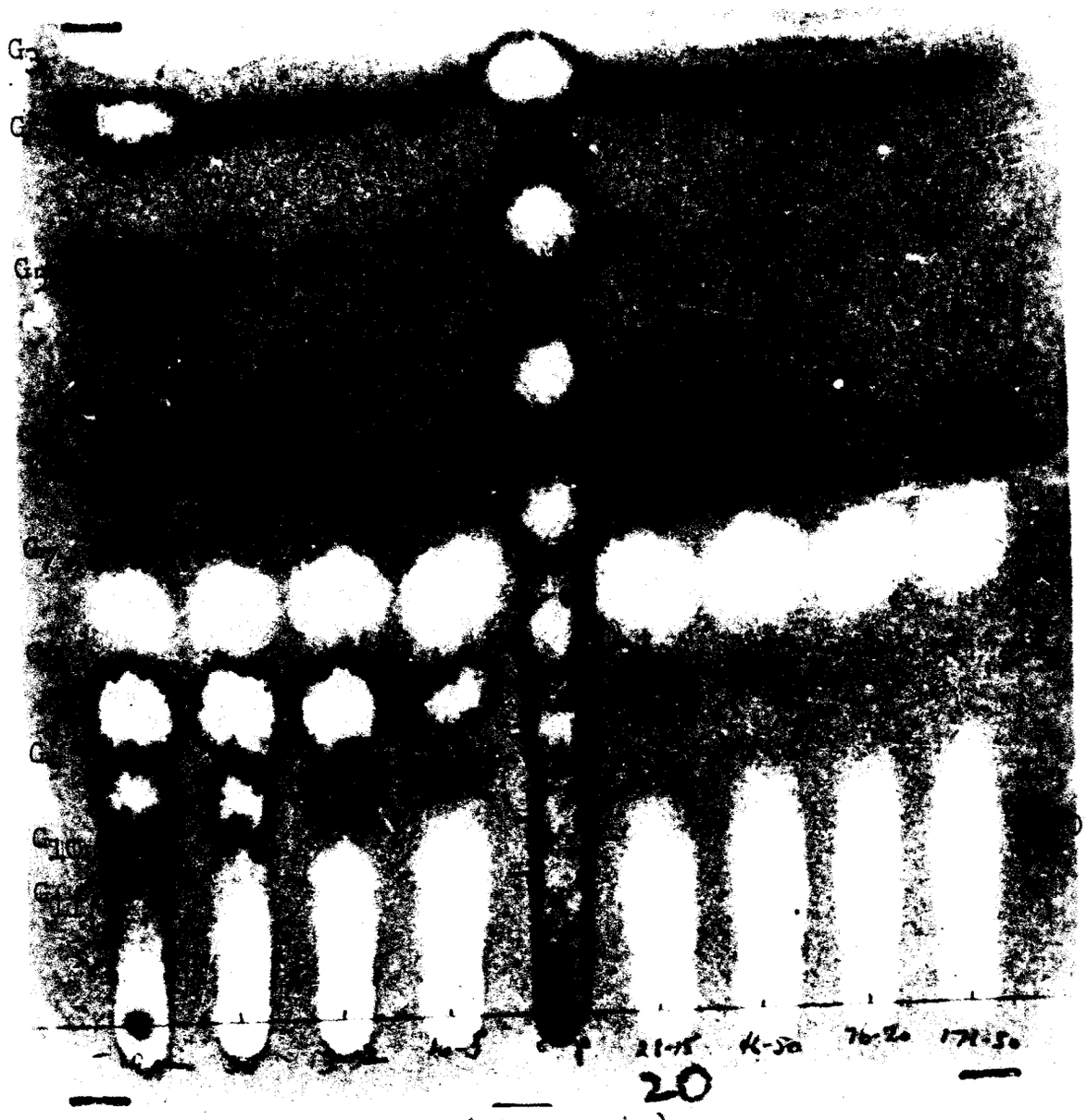
Fig. 1. Digest of Waxy Maize Starch by Salivary Amylase. Paper chromatogram showing reducing sugars. (a) 2.7 c.p.(conversion periods). (b) 14 c.p. (c) 65 c.p. (d) 270 c.p. (e) Linear oligosaccharide reference series. (f) 450 c.p. (g) 775 c.p. (h) 1250 c.p. (i) 2800 c.p.



(20 ascents)

(a) (b) (c) (d) (e) (f) (g) (h) (i)

Fig. 2. Digest of Waxy Maize Starch by Salivary Amylase. Paper chromatogram showing potato phosphorylase priming sugars. (a) 2.7 c.p.(conversion periods). (b) 14 c.p. (c) 65 c.p. (d) 270 c.p. (e) Linear oligosaccharide reference series. (f) 450 c.p. (g) 775 c.p. (h) 1250 c.p. (i) 2800 c.p.



(20 ascents)

(a) (b) (c) (d) (e) (f) (g) (h) (i)

given in Tables 4 and 5.

An examination of the small digests after about six months showed that practically all of the branched material had been degraded. A small amount of branched trisaccharide was present.

Chromatograms of the products of salivary action on a partial beta amylase limit dextrin of glycogen showed that maltose, amylotriose and amylotetraose were produced in nearly equal amounts at 0.12 to 0.25 conversion periods. (Amylotetraose probably would not have been formed in significant quantities with a true beta amylase limit dextrin of glycogen.) The amylotetraose had mostly disappeared by 38 conversion periods. Glucose formation was detectable at 12 conversion periods. The amylotriose had nearly all been degraded by 2500 conversion periods. The branched 6, 7, 8, and 9 membered sugars began to appear at 1.2 conversion periods. B_5 appeared at 12 conversion periods. B_8 had disappeared and B_4 had begun to appear at 370 conversion periods. A larger proportion of branched compounds and relatively larger amounts of the doubly branched compounds in comparison with the singly branched compounds were produced from the glycogen limit dextrin than from amylopectin (waxy maize).

Fractionation of the waxy maize salivary amylase limit dextrins was carried out on paper chromatograms as described

Table 4

Comparative Mobilities of Linear and Singly Branched Oligosaccharides

(7 ascents)

Mol. size	Mobility	Mol. size	Mobility	Mobility Difference
G_4	0.67	B_4	0.55	.12
G_5	0.50	B_5	0.41	.09
G_6	0.37	B_6	0.30	.07
G_7	0.27	B_7	0.22	.05
G_8	0.19	B_8	0.16	.03
G_9	0.13			

Table 5

Comparative Mobilities of Linear and Doubly Branched Oligosaccharides

(24 ascents)

Mol. size	Mobility	Mol. size	Mobility	Mobility Difference
G_7	0.52	BB_7	0.34	.18
G_8	0.38	BB_8	0.27	.11
G_9	0.28	BB_9	0.21	.07
G_{10}	0.20	BB_{10}	0.16	.04
G_{11}	0.14	BB_{11}	0.11	.03

above. After giving the chromatograms 8 ascents, strips were cut from them and the different sugars located. The chromatograms were then sectioned into G_1, G_2 and G_3, B_3, B_4, B_5, B_6, B_7 and BB fractions. Less than 100 mg. of B_4, about 325 mg. of B_7, somewhat more of B_5 and B_6 and approximately 1 g. of BB compounds were obtained by this method from a portion of the limit dextrin mixture.

The very extended action of saliva on pure maltose, panose, B_4 and B_7 was followed. The digests were made by dissolving the sugars in filtered saliva. Papergrams showed that at 4500 c.p. maltose had been about 5% hydrolyzed to glucose. It was seen by the phosphorylase spray that a perceptible trace of amylotriose appeared in the digest, which was not originally present. At 11,000 c.p. an estimated 10-15% hydrolysis to glucose had occurred by 21,000 c.p. At about 65,000 c.p. about 50% of the maltose had been hydrolyzed. The amylotriose had disappeared but small amounts of what appeared to be isomaltose (B_2) and B_3 were formed.

Saliva had only a very slight action on panose. At 12,000 c.p. only a trace of glucose could be detected. At 74,000 c.p. possibly 1% of glucose had been formed from panose. Faint spots corresponding to maltose and isomaltose could be seen, and a light spot was seen having the mobility expected for a tetrasaccharide containing two 1,6 bonds.

The action of saliva on B_4 was also only slight, but traces of glucose and B_3 could be detected after 12,000 c.p. At 74,000 c.p. about 1% of glucose had been formed from the B_4, and very light spots corresponding to maltose and B_3 were seen. In addition a very faint spot having a mobility of slightly less than that of a B_7 spot was observed. The B_4 compound inhibited potato phosphorylase slightly at high concentrations.

The extended action of saliva on the B_7 sugar (with a small amount of B_6 impurities) resulted in extensive degradation. At 7000 c.p. about half of the B_7 had been degraded. Mostly glucose and maltose were formed, a slight amount of amylotriose could be seen, a barely detectable trace of amylotetraose was noticed along with moderate amounts of B_4, B_5 and B_6. At 43,000 c.p. most of the B_7 had been degraded to glucose and maltose. A small amount of B_4 was present, along with traces of isomaltose, amylotriose, B_3, B_5 and B_6.

Action of Q-enzyme

In a preliminary examination of the action of the Q-enzyme preparation from potatoes, it was observed that amylose treated with the enzyme became purple-staining with iodine rather than blue staining. This information was already available (123). It was also observed that the action of the Q-enzyme on Nägeli's amylodextrin (171), an essentially linear

carbohydrate, caused a change in the iodine color from red-brown to reddish-purple. Also, amyloheptaose, which gives no iodine stain, gave a deep brown stain with iodine after treatment with the potato Q-enzyme preparation.

Digests were set up to examine for the action of Q-enzyme on various substrates, using a weight of substrate approximately twice that of the enzyme powder used. The digests were carried out at room temperature on a 0.5 ml. scale with digests of about 2% substrate concentration. Thymol was used as a preservative.

The effects of Q-enzyme preparations from both potato (Q_p) and broad beans (Q_b) were observed on pure maltose. After two days glucose and a branched trisaccharide could be detected. After eleven days the glucose and B_3 spots were heavier and a trace of amylotriose was detected by the phosphorylase spray. At the end of two months the Q_b-enzyme digest contained much glucose, still much maltose, significant amounts of isomaltose, B_3, and amylotriose. A small amount of amylotetraose was found. In the Q_p-enzyme digest were found less glucose and more maltose than in the Q_b-enzyme digest. Less isomaltose and B_3 were found, but comparable amounts of G_3 and G_4 and traces of G_5 and G_6 were found. The digests were treated with salivary amylase. Papergrams of the salivary digests showed large amounts of glucose and maltose and moderate amounts of isomaltose and B_3. The Q_b-enzyme preparation

appeared to be somewhat more active than the Q_p-preparation, but also contained more hydrolytic activity than the Q_p-enzyme preparation.

The initial action of Q_p-enzyme on amylotriose gave glucose, amylopentaose and amyloheptaose as primary products. After twelve hours glucose, a small amount of maltose, amylotriose, amylotetraose and larger linear sugars were present, with a small amount of branched compounds apparently consisting of B_5 and larger branched compounds.

The products of the action of Q_b-enzyme on amylotriose were very similar, except that the linear compounds larger than amylopentaose were present only in very small amounts. The maltose was present in larger quantities, which probably signifies that an amylolytic activity is present.

The initial action of Q_p-enzyme on amylotetraose gave glucose, slightly less amylotriose, small amounts of amylopentaose and amyloheptaose. After 24 hours the digest contained glucose, a very small amount of maltose, a small amount of amylotriose, amylotetraose, and substantial amounts of larger linear sugars. It appeared that a small amount of B_5 and larger branched sugars were present.

The action of the Q_p- and Q_b-enzymes on amyloheptaose followed much the same pattern as with amylotriose and amylotetraose. After the initial action of the Q-enzyme prepara-

tions, all of the compounds from glucose to amylohexaose were present in small quantities. Amyloheptaose and larger compounds were present in much greater quantities. As the digest progressed, it appeared that in the Q_p-enzyme digest the concentration of B_5 and several higher branched sugars increased somewhat. In the Q_b-enzyme digest, however, the higher molecular weight linear compounds soon disappeared, and the digest contained glucose to amylopentaose and isomaltose, B_3 and small amounts of some higher molecular weight branched compounds. No doubt the R-enzyme content of the Q_b-enzyme preparation was responsible for the lack of higher branched compounds. It appears likely a certain amount of amylase activity is responsible for some of the degradation.

The Q-enzyme digests were treated with soybean beta amylase and salivary amylase. Isomaltose, B_3, B_4 and small amounts of several higher molecular weight branched sugars were shown to be present in the Q_p- and Q_b-salivary digests. A beta amylase digest of a Q_p-enzyme digest gave a B_3 spot and indications of small amounts of larger branched sugars on the chromatograms.

Kneen's inhibitor (172), prepared from wheat, is known to strongly inhibit salivary and several other amylases. Its possible effect on the amylase, the R-enzyme or the Q-enzyme in the Q_b-enzyme preparation was investigated. It had no apparent inhibiting effect at all on any of these enzymes, but

instead, added more enzyme to the system. Its presence hastened the disappearance of the larger linear oligosaccharides initially formed in Q_b-enzyme-amyloheptaose digests, but did not significantly change the final composition of the digest. A digest containing only amyloheptaose and Kneen's inhibitor very slowly gave approximately one molecule of amylotriose to two molecules of maltose, and a little glucose. This indicates a wheat beta amylase impurity in the Kneen's inhibitor.

The possibility that the homologization of the linear sugars by the Q-enzyme preparations was due to a phosphorylase impurity was checked by adding some Schardinger alpha dextrin to an amyloheptaose digest. The alpha dextrin is a very potent inhibitor of phosphorylase. The presence of the alpha dextrin had no apparent effect on the digest.

Isomaltose was treated with the Q_b-enzyme preparation under conditions similar to those of the before mentioned digests. After 48 hours a chromatogram showed the presence of glucose, a large quantity of unchanged isomaltose, dextran trisaccharide and dextran tetrasaccharide. No products were formed which activated potato phosphorylase.

The products from the action of the Q_b-enzyme preparation on panose (4-isomaltosyl glucose) after one week gave heavy spots of glucose, maltose and panose, a weak isomaltose spot, a spot of a material having the mobility expected for

4-dextrantriosyl glucose, and a fainter spot having the mobility of 4-dextrantetraosyl glucose. No products were formed which activated potato phosphorylase.

After the action of the Q_b-enzyme preparation on sucrose for seven days, a fructose spot and an erlose spot were present besides the sucrose. Erlose is a trisaccharide having one maltose bond and one sucrose bond. It is formed in macerans amylase coupling digests with sucrose (110) and by honey invertase from sucrose (173). The only reducing sugar present was fructose, which indicated the absence of invertase activity from the preparation. A digest including both maltose and glucose, gave after four days glucose, fructose, sucrose, maltose, amylotriose, B_3, erlose and the erlose isomer containing one isomaltose bond and one sucrose bond. The amount of fructose produced was much less than in the digest containing only fructose, which may be an indication the enzyme transfers glucose units from maltose with greater ease than from sucrose.

An experiment to determine whether glucose can serve as the acceptor for a transferred glucose unit was set up using radioactive glucose. The radioactive glucose was supplied by Dr. S. Aronoff of the botany department. About 0.2 mg. of radioactive glucose, which gave 2370 counts/min./sq. cm. on a chromatogram, and 0.25 mg. of maltose were combined in a 0.16 ml. digest containing 0.3 mg. of Q_b-enzyme powder and a crystal

of thymol. The container used was a paraffin block with a depression in it. The container was covered with a microscope cover glass. After one week, the digest was partially evaporated and all applied to one spot on a paper chromatogram. A radioautograph was made and developed after three and one half weeks. It showed a large, intense glucose spot, a maltose spot and an amylotriose spot. The presence of an isomaltose spot could not be definitely shown, since it would not be well resolved from the amylotriose. If present, it was formed in only small amounts.

Action of R-enzyme

The maximum of the I-KI absorption spectrum of waxy maize beta amylase limit dextrin shifted toward the blue end of the spectrum upon treating with the broad bean R-enzyme preparation used in these experiments. This phenomenon is used as an activity index for the enzyme by its discoverers (142). After circumventing difficulties preventing satisfactory resolution of the components of R-enzyme digests, the enzyme action on various substrates was investigated. It was discovered that citrate buffer, which was used in the first digests, interfered with the proper resolution of the carbohydrates on paper in the solvent being used.

Unless otherwise stated, the digests of R-enzyme were made up much the same as with the Q-enzymes. About twice as

much substrate as enzyme powder, on a weight basis, was used. The digests were generally 0.5 ml. in volume and about 2% in substrate concentration.

The action of R-enzyme on isomaltose was extremely slow, but glucose was formed in significant amounts after a sufficient time.

Panose was cleaved by R-enzyme at a little faster rate than isomaltose. The only products formed were glucose and maltose, as would be expected from a hydrolysis of the 1,6 bond in panose.

After an extended period of action of R-enzyme on isomaltose and panose, higher molecular weight compounds could be detected. This observation was also made on digests of other sugars after extended action. The fact that the products formed were the same as those formed from isomaltose and panose in the Q-enzyme digests gave evidence for a Q-enzyme impurity. Isomaltose, B_3, B_4, and higher branched sugars were also detected in the salivary amylase digest of an amyloheptaose digest by R-enzyme. The presence of amylase activity was also detected, for the R-enzyme preparation slowly hydrolyzed amyloheptaose to amylopentaose, amylotriose and maltose. Amylotetraose was formed only in small amounts, which signified a predominance of beta-type amylolytic activity. But the fact that the viscosity of beta amylase and phosphorylase

limit dextrins was rapidly decreased by the R-enzyme preparation gave evidence for the possible presence of small amounts of alpha-type activity as well. It was seen then that products formed as a result of the extended action of the R-enzyme preparation could not necessarily be relied upon to be a result of the action of R-enzyme itself.

The action of the R-enzyme preparation was observed on a mixture containing B_4, B_5, B_6 and B_7 obtained from chromatographic fractionation of the salivary amylase limit dextrins of waxy maize. Glucose, maltose, amylotriose and amylotetraose were formed. The action of R-enzyme on the doubly branched mixture gave as products, glucose, maltose, G_3, G_4, small amounts of G_5 and G_6, somewhat more G_7 and lesser amounts of higher sugars. There was no apparent formation of smaller branched compounds like B_4, B_5, B_6, etc.

The action of the R-enzyme preparation on the individual branched fractions obtained from the salivary amylase limit dextrin of waxy maize was investigated. With B_4 very little action occurred even over an extended period of time. A small amount of glucose was formed, but only traces of maltose and amylotriose were ever detected. The glucose formed was probably of significance, but the fate of the corresponding G_3 expected was undetermined.

The products of R-enzyme action on B_5 were maltose and

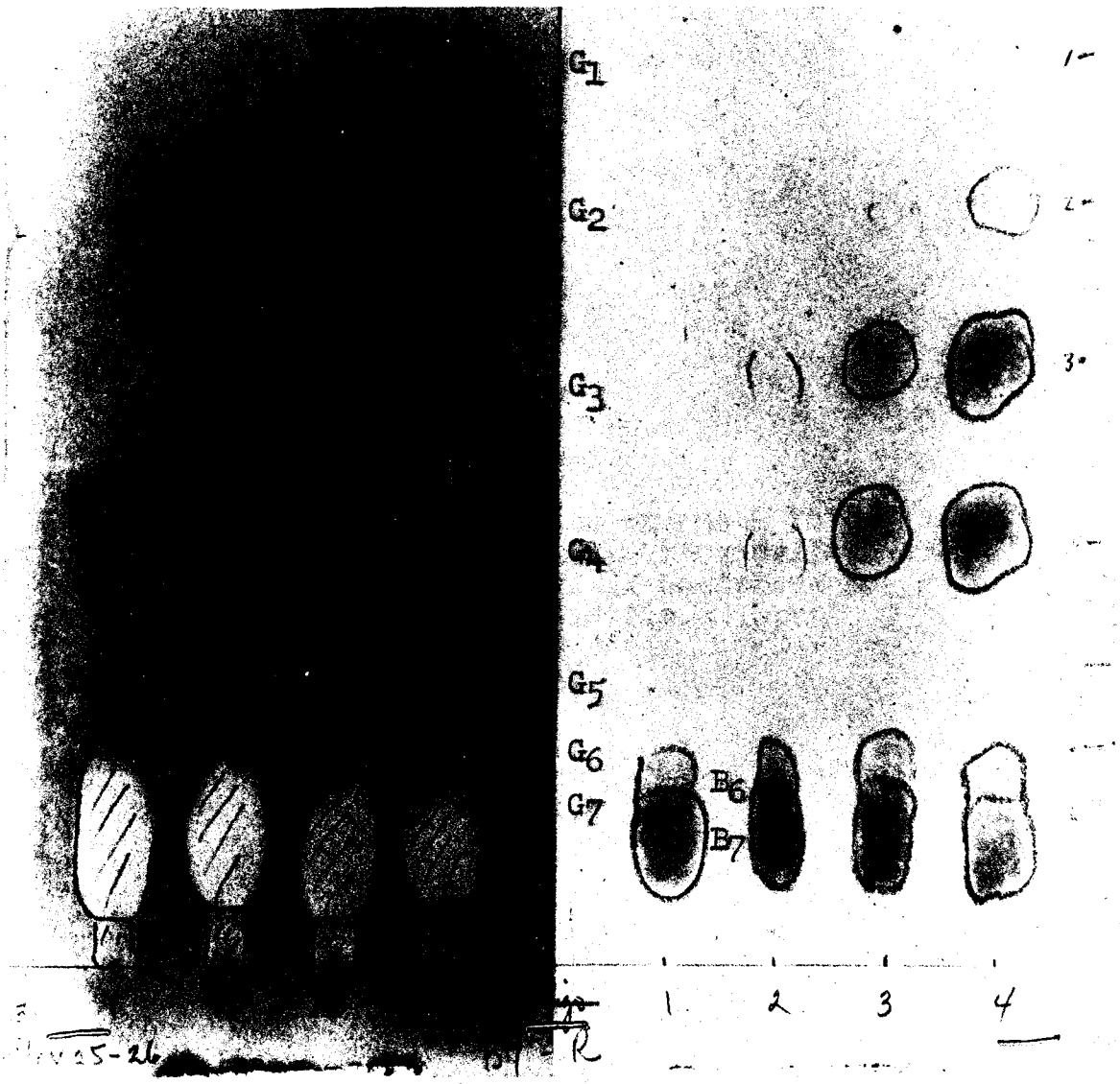
G_3, which were produced rapidly. Traces of glucose and G_4 were formed, the G_4 probably coming from the B_6 impurities in the B_5. Only a little over one half of the B_5 was hydrolyzed, the rest remained for an extended period.

From B_6 by R-action were rapidly obtained maltose and G_4. Small amounts of glucose and G_3 were produced. At least part of the G_3 came from B_5 and B_7 impurities. Whether enzymic action in the preparation causes significant formation of G_3 from G_4 was not certain. About 25% of the B_6 was not readily attacked by the R-enzyme.

The B_7 fraction appeared to be completely hydrolyzed by R-enzyme to G_3 and G_4 (see Figure 3). Small amounts of glucose and maltose were produced, but probably were formed by hydrolysis of the G_3 and G_4.

The above results on B_4, B_5, B_6 and B_7 give evidence for the structures of some of the compounds. But the evidence is insufficient for any definite structure assignments for the compounds. Additional evidence for the structure of B_7, which is probably a single compound or at most a mixture of two compounds, was obtained by R-enzyme action on the aldonic acid of B_7. This aldonic acid was prepared by oxidation with alkaline I_2, using the procedure given by French (56), but substituting $Ba(OH)_2$ for KOH. The small portion of B_7 which remained unoxidized after the treatment was degraded by treating with

Fig. 3. Action of R-enzyme on the Branched Heptasaccharide from Salivary Amylase Limit Dextrins of Amylopectin. Digest contained 11.1 mg. of the sugar and 2.3 mg. of R-enzyme. Left half of chromatogram shows phosphorylase priming sugars; right half, reducing sugars. (1) 2 min. (2) 20 min. (3) 3 hr. (4) 18 hr.



(4 ascents)

0.1 N. $Ba(OH)_2$ in a boiling water bath for 30 minutes. The solution was acidified with H_2SO_4, the excess iodine extracted with petroleum ether, and the solution neutralized with $BaCO_3$. The solution was evaporated to dryness and the dry material was used to make up the oxidized B_7 (B_7Ox) digest with R-enzyme. In the digest G_3 was formed, but not G_4. This signified that the reducing group of B_7 was on the G_4 portion of the molecule. In line with its inhibiting action on phosphorylase and its relative resistance to saliva, it is likely that it is either O-O-O-O- or O-O-O-O- or possibly a mixture of both.

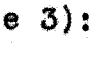
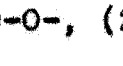
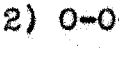
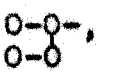
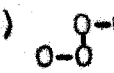
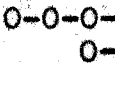
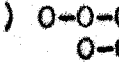
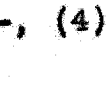
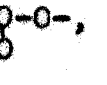
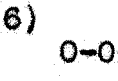
The action of R-enzyme was observed on the limit dextrin of glycogen formed by the extended action of soybean beta amylase and upon the sweet potato beta amylase limit dextrin of waxy maize starch. The fact that the glycogen limit dextrin had been given more extended beta amylase action than the waxy maize dextrin was reflected in the results. The action of R-enzyme produced glucose, maltose, G_3 and G_4 from both limit dextrins. Maltose was produced in the largest quantities. (No maltose could be detected in the original limit dextrins.) Following maltose in order of abundance were G_3, glucose and G_4. Very small amounts of G_4 were produced in both digests, less in the glycogen limit dextrin digest than in the waxy maize limit dextrin digest. The waxy maize limit dextrin digest contained a larger proportion of G_3, but the glycogen

limit dextrin digest contained a larger proportion of glucose.

The action of R-enzyme on potato phosphorylase limit dextrins has already been reported in the phosphorylase section.

The action of R-enzyme on waxy maize starch gave a small amount of glucose, a larger amount of maltose and diminishing amounts of oligosaccharides from G_3 - G_9 and higher.

Structure of Salivary Amylase Branched Limit Dextrins

An attempt to elucidate the structures of the B_4 and B_7 components was carried out, since it appeared likely that these were single compounds. The six possible structures for the branched tetrasaccharides are as follows (see footnote, Table 3): (1)  , (2)  , (3)  , (4)  , (5)  , (6)  . The six structures considered for the branched heptasaccharide are as follows: (1)  , (2)  , (3)  , (4)  , (5)  , (6)  .

The B_4 component was given a degree of hydrolysis of 0.38 by treating with 0.198 M. H_2SO_4 at the temperature of a boiling water bath for 40 minutes. The solution was neutralized with $BaCO_3$, filtered, evaporated to a small volume and

chromatographed. The composition of the hydrolysate included G_1, G_2, G_3, B_2, B_3 and B_4. Assuming B_4 was a single compound or essentially so, these results eliminated structures 5 and 6 from consideration, for neither could produce G_3 on partial acid hydrolysis. The corresponding hydrolysis of B_4 after being converted to the aldonic acid (B_4Ox) by alkaline I_2 produced only G_1, G_2, B_2 and B_3. (The aldonic acids have different mobilities and do not reduce the alkaline copper reagent.) This eliminated formulas 2 and 3, for upon oxidation and partial acid hydrolysis 2 and 3 would have produced no branched compounds, but 3 should have produced G_3. This left structures 1 and 4 for consideration. An effort was made to distinguish between the two possibilities by determining the products from the partial acid hydrolysis of the B_3 fraction, after oxidation, obtained from the partial acid hydrolysis of B_4-onic acid. The B_3 fraction was obtained from the B_4-onic acid hydrolysis mixture by separation on paper chromatograms in the way mentioned above. A chromatogram of the partially hydrolyzed oxidized B_3 fraction gave glucose and an uncertain indication of the presence of maltose and isomaltose, for the mixture was poorly resolved. The presence of these sugars would indicate that structure 1 was that of B_4.

From a consideration of the probabilities that any given bond will or will not be broken at a designated degree of hydrolysis, calculated values can be obtained for the

proportions of the different products formed from a given oligosaccharide at that degree of hydrolysis. Such values obtained for structures 1 and 4 are given in Table 6.

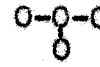
Hydrolysates of B_4 and B_4Ox with a degree of hydrolysis of 0.38 were chromatographed, and the relative intensities of the spots were visually estimated. The strongest spot was given a value of 10 and the others proportional values. The following values were obtained from the B_4 hydrolysate: G_1 - 10, G_2 - 3, G_3 - 1, B_2 - 5, B_3 - 3, and B_4 - 5. From the B_4Ox hydrolysate these values were obtained: G_1 - 10, G_2 - 1, B_2 - 10, and B_3 - 7. By comparing these sets of values with those from Table 6, it can be seen that formula 1 is favored. Further work is necessary before the structure for B_4 can be definitely established.

The structural evidence offered by the R-enzyme experiments on B_7 was backed up by the results obtained by partial acid hydrolysis studies. A solution of B_7 in 0.198 M. H_2SO_4 was kept at the temperature of a boiling water bath for 20 minutes to give it a degree of hydrolysis of 0.2. At the end of that time, the solution was neutralized with $BaCO_3$, filtered, evaporated to a small volume and chromatographed. The hydrolysis products included G_1 - G_4 and B_2 - B_7. No G_5 was produced. Branched compounds formed which primed potato phosphorylase were included in the B_4, B_5 and B_6 spots. It also appeared as if inhibiting components were included in

Table 6

Calculated Amounts of Products Formed by Partial Acid Hydrolysis of B_4 and B_4Ox

(degree of hydrolysis = 0.38)

(1) 				(4) 			
Before Oxidation							
G_1	0.66*	B_2	0.34	G_1	0.87	B_2	0.13
G_2	0.26	B_3	0.21	G_2	0.04	B_3	0.42
G_3	0.04	B_4	0.35	G_3	0.04	B_4	0.35
After Oxidation							
G_1	0.28	B_2	0.34	G_1	0.49	B_2	0.13
G_2	0.02	B_3	0.21	G_2	0.02	B_3	0.21

* Moles produced per mole hydrolyzed.

the B_5 and B_6 spots. The absence of G_5 from the hydrolysate ruled out structures 1, 2 and 3 for B_7. Also the fact that branched priming compounds were formed was additional evidence against structure 1 and probably structure 3. Structures 2 and 6 would be expected to prime phosphorylase, rather than act as inhibitors.

A hydrolysate of B_7Ox with a degree of hydrolysis of 0.2 contained G_1 - G_3 and B_2 - B_6. No G_4 was present. The same proportions of the phosphorylase priming components of B_4 and B_5 were present in both the B_7 and B_7Ox hydrolysates. The B_6

spot exhibited only inhibiting and no priming properties toward phosphorylase. The absence of G_4 in the hydrolysate stood along with the evidence given with R-enzyme, that the G_4 portion of the molecule held the reducing group. This eliminated B_7 structure 6, leaving only structures 4 and 5.

The calculated amounts of products which should occur in the B_7 and B_7Ox hydrolysates are given in Table 7. The estimated relative amounts which were formed, as judged by the intensity of the chromatogram spots were as follows for the B_7 hydrolysate (using a value of 10 for the most intense spot): G_1 - 10, G_2 - 5, G_3 and G_4 - less than 1, B_2 and B_3 - less than 1, B_4 - 1, B_5 - 2, B_6 - 3 and B_7 - 3. The relative intensities of the chromatogram spots from the B_7Ox hydrolysate were as follows: G_1 - 10, G_2 - 3, G_3 - 1, B_2 - 1, B_3 - 1, B_4 - 2, B_5 - 3 and B_6 - 2.

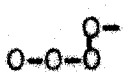
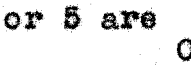
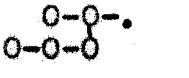
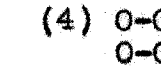
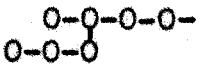
Upon comparing the above figures with Table 7, it can be seen that formula 5 most nearly fits the results. A bit of additional evidence in favor of structure 5 for B_7 is the fact that nearly the same relative amounts of the phosphorylase priming compounds of the B_4 and B_5 spots are present in both the B_7 and B_7Ox hydrolysates. The priming B_4 compound is most likely  . The likely B_5 priming sugars which could arise from either structure 4 or 5 are  and  .

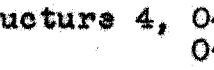
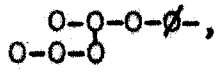
Table 7

Calculated Amounts of Products Formed by Partial Acid Hydrolysis of B_7 and B_7Ox

(degree of hydrolysis = 0.2)

(4) 				(5) 			
Before Oxidation							
G_1	0.69*	B_2	0.01				
G_2	0.34	B_3	0.04				
G_3	0.04	B_4	0.08	same as (4)			
G_4	0.03	B_5	0.25				
		B_6	0.23				
		B_7	0.31				
After Oxidation							
G_1	0.49	B_2	0.01	G_1	0.49	B_2	0.01
G_2	0.33	B_3	0.02	G_2	0.18	B_3	0.04
G_3	0.04	B_4	0.05	G_3	0.04	B_4	0.08
		B_5	0.04			B_5	0.14
		B_6	0.08			B_6	0.08

* Moles produced per mole hydrolyzed.

By examining structure 4,  , and structure 5,  , it can be seen that oxidation of the reducing group would not alter the amount of priming B_4 formed from either structure. The amount of priming B_5 formed from 4 would be changed by

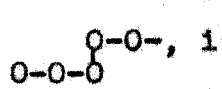
oxidizing the reducing group. The yield from 5 would be unaltered.

This structure evidence cannot be taken as proof for the structures suggested. It has been assumed that B_4 and B_7 were individual compounds. This has not been proven, and the results obtained may have arisen from more than one compound. Though its true significance cannot yet be evaluated, the evidence can still be used as a basis for further work on the problem.

Action of Beta Amylase

After the initial rapid action of beta amylase on linear oligosaccharides, there remains maltose and a small amount of amylotriose, one molecule for every oligosaccharide molecule containing an odd number of glucose units. Roberts and Whelan (65) have reported that amylotriose is hydrolyzed by beta amylase into glucose and maltose at a slow rate. This finding has been confirmed with a concentrated soybean beta amylase preparation. A digest containing 6.2 mg. of amylotriose and 3 mg. of the enzyme powder in 0.5 ml. of digest was prepared. Thymol was used as a preservative. After several minutes a slight hydrolysis was observed. After 12 hours at room temperature about 80% of the amylotriose had been converted to glucose and maltose. After two weeks evidence of transglucosidase action was present for spots corresponding to

isomaltose and B_3 could be seen.

To see whether the beta amylase enzyme preparation had any action on maltose, a 0.3 ml. digest containing 4.1 mg. of maltose and 2.1 mg. of beta amylase powder was set up with thymol as a preservative. After 16 hours at room temperature a slight conversion to glucose was seen, and a trace of B_3 was observed. After five days about 15% of the maltose had been hydrolyzed to glucose. A small B_3 spot was present. Acid hydrolysis studies, exactly like those carried out on the phosphorylase limit dextrins, were done on the beta amylase limit dextrins of glycogen and waxy maize. From both limit dextrins were produced glucose, maltose and amylotriose in significant quantities. Only traces of G_4 and G_5 were formed. These results, along with those already observed from the action of R-enzyme, show that the beta amylase can rapidly degrade branch ends to a length of 2 and 3 glucose units. Judged from the action of beta amylase on amylotriose, it is likely that one more maltose unit is slowly cleaved from the 3 glucose unit stubs. Additional evidence for such an occurrence is given by the action of beta amylase on the panose coupled-product of macerans amylase action. A phosphorylase priming B_5,  , is present which is destroyed by the action of beta amylase. Panose and maltose are probably formed.

That the soybean beta amylase preparation (and soybeans) probably contains a slight trace of alpha amylase activity was suggested by the observation of small amounts of amylotriose in the glycogen limit dextrin preparation digest.

DISCUSSION OF RESULTS

Action of Phosphorylase

From the iodine color of the synthesized carbohydrates in a priming spot on a paper chromatogram, one can draw conclusions relative to the nature of the kinetics of synthesis, for equilibrium is not attained in the short period allowed for reaction. With an amylotetraose spot, the color changes from blue at the edge through red and brown to yellow in the center. These color differences are a result of the gradation of primer concentration from the outside edge of the spot to the center of the spot. From the given amount of glucose-1-phosphate present, applied in the spray, enough is available for long chain synthesis in areas of low primer concentration. As the primer concentration increases, relatively less glucose-1-phosphate is available per priming unit. The yellow color at the center of the spot shows that the multichain mechanism occurs in synthesis with a good primer.

With a poor primer, like amylotriose, the color of the spot stays quite blue at ordinary concentrations. This signifies that the nature of the carbohydrate synthesized is not changed much by differences in amylotriose concentration. This suggests that with a poor primer, the synthesis kinetics approach the single chain mechanism where many successive glucose units are added to a given primer before any are added

to another priming unit. The reason for this is quite simple. After adding one glucose unit to amylotriose, the amylotetraose formed is a good primer. After a good priming molecule is once formed, phosphorylase continues to add glucose units to it in preference to a poor priming unit.

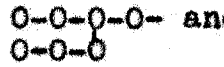
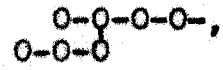
The structural feature which distinguishes a poor primer from a good primer is probably the size. Below a certain size, a molecule probably cannot form a sufficiently stable complex with enzyme to allow much chance of reaction. The same reasoning seems to apply to carbohydrate inhibitors of phosphorylase. If the carbohydrate contains a portion capable of good enzyme-substrate complex formation, but its priming function is blocked by a branch or by lack of a non-reducing end, it serves as a potent inhibitor. The B_7 from salivary digests and the Schardinger dextrins are good examples.

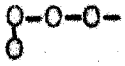
The priming of the phosphorylases obtained from members of the legume family followed a pattern different from that of potatoes and corn. Maltose served as a very weak primer. Whether the priming effect of maltose is characteristic of phosphorylase of these plants or whether it is a result of the simultaneous action of another enzyme has to be considered. The background color of the chromatograms on which the tests were run was noticeably more purple than the background obtained with potato phosphorylase. This suggested that a transglucosidase action may be forming G_3 from the maltose,

giving rise to a slight priming action. Maltose on a chromatogram was sprayed with the potato phosphorylase-glucose-1-phosphate spray to which had been added a substantial amount of potato Q-enzyme. No priming by the maltose was detected. This still does not rule out the possibility of Q-enzyme or some other enzyme participating. Other proportions of phosphorylase and Q-enzyme might have given the effect. Further purification of the phosphorylases would give an indication of the answer.

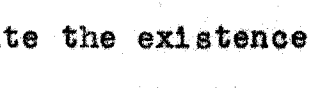
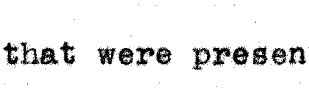
Actions of Saliva and R-enzyme

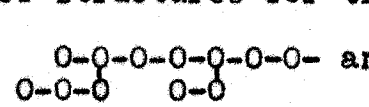
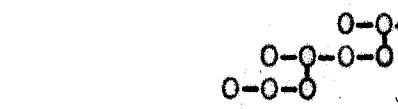
The structures for B_4 and B_7 sugars isolated from saliva digests of waxy maize may be different from those structures suggested by the evidence. Intuitively, the symmetrical structure would appear to be the most likely for B_4.

Of the two likely B_7 structures,  and  , the former should be produced in small amounts in salivary digests of the panose coupled-product. Phosphorylase inhibitors are present in small amounts in the B_7 position on chromatograms of these digests. Sugars of both structures would probably inhibit phosphorylase, though, so such evidence carries little weight.

The former B_7 structure together with the B_4 structure,  , would not appear to be compatible with any scheme for

the formation of the branched compounds. The other three possible combinations appear to be more likely. If the following structures for B_4 and B_7 are assumed: and , reasonable structures for B_5 would be  and . The corresponding structures for B_6 would be and . B_5 and B_6 are very likely mixtures containing at least two components each. Other sets of structures could be devised as readily from the other combinations. The type of structures given for B_5 account quite well for the results of the action of R-enzyme on the B_5 fraction. The G_2 and G_3 produced could come from the first structure. The half of B_5 which is not readily hydrolyzed could have a structure like the second model. Since it has been shown that R-enzyme splits off the glucose branch with difficulty from isomaltose, panose and B_4, it is not unreasonable to expect a similar behavior with the corresponding B_5 compound. If B_6 was composed of mostly the second model with a small amount of the first present, the rapid formation of G_2 and G_4 from the action of R-enzyme could be explained. However, there is little reason for believing that the rate of splitting of by R-enzyme would be noticeably different from  or . An explanation for the resistance of a portion of B_6 to the action of R-enzyme is not readily forthcoming.

One could postulate the existence of  in the B_6 fraction, but if that were present,  would probably be present. The failure to detect G_5 among the R-enzyme and partial acid hydrolysis products of B_7 discounts that possibility.

The products of R-enzyme action on the BB compounds give rise to interesting speculation. G_1 was formed in moderate amounts, G_2, G_3 and G_4 in fairly large amounts, and only traces of G_5 and G_6 were formed. A larger amount of B_7 was formed. Examples of possible types of structures for the doubly branched oligosaccharides are  and  . The first is suggestive of the Staudinger hypothesis of branching in amylopectin: the second resembles Haworth's model. The G_4 formed by the action of R-enzyme could have come only from the second type structure. The amount of G_7 formed could have been formed only from a structure of the first type structure. This demonstration of the existence of both types of doubly branched compounds is more evidence in support of the Meyer formulation for amylopectin.

The action of R-enzyme on waxy maize starch with the formation of oligosaccharides from G_1 to G_9 and larger molecules shows that the branch lengths in amylopectin range from 2

(possibly 1) to more than 9 glucose units. The diminishing yield of oligosaccharides of increasing size probably reflects the relative ease of hydrolyzing off the different branch lengths.

The significance of the glucose formed in the R-digests was difficult to ascertain. Glucose was produced in all instances, in larger quantities from some substrates than others. Some of the glucose formed is no doubt a result of the action of Q- or other enzymic impurities.

Whether the transglucosidase action of several samples of saliva is a result of the action of salivary amylase is subject to question. The saliva used was not purified, so other possible enzymes from saliva or autolyzed bacteria in the saliva would have a chance to act if present. The occurrence of a transglucosidase action for salivary amylase would not be entirely unexpected in view of the similar properties of other hydrolytic enzymes. It is felt by the author that the very slow hydrolysis of maltose to glucose by saliva is an action of the salivary amylase.

The observation that B_3 was produced from maltose by transglucosidase action in saliva, and the fact that B_3 was formed in salivary digests of amylopectin raised the question concerning the origin of the B_3 in the amylopectin digests. Whether it arose by hydrolytic action or by transglucosidase

action is at present unknown.

The detection of maltose in the salivary digest of panose and of traces of amylotriose and amylotetraose in the salivary digests of B_7 raises the question as to whether saliva contains any 1,6-amylase or R-activity. If so, this again could be due to other enzymes present.

Action of Q-enzyme

There is a possibility that the observed action of the Q-enzyme preparations on the low molecular weight oligosaccharides studied was that of another enzyme. However, in view of the fact that the action of the Q-enzyme preparations from both potato and broad beans appeared to be identical (except for hydrolytic activity in the broad bean preparation), it would seem that the action observed was actually due to Q-enzyme.

The rate of action on maltose was much slower than on G_3, G_4 or G_7, and more 1,6 bonds than 1,4 bonds were synthesized. With G_3, G_4 and G_7, though, Q-enzyme causes a more rapid redistribution of 1,4 bonds than formation of 1,6 bonds. With amylose, the formation of 1,6 bonds probably predominates. Whether a 1,4 or a 1,6 bond is formed probably depends upon the nature of the enzyme-substrate complex formed and the number of non-reducing ends present. The presence of relatively few non-reducing terminals in amylose no doubt greatly

favors the formation of 1,6 bonds by Q-enzyme. Since more end groups are present in amylopectin, during amylopectin synthesis the proportion of 1,4 bonds formed would probably increase. This would result in a homologizing of the outer branches.

By observing the relative amounts of products formed in initial stages of the reactions of G_3 and of G_4 with Q-enzyme, it can be seen that from G_3, maltose is transferred much more rapidly than glucose. With G_4, G_3 is transferred the most rapidly, glucose at a slower rate, and maltose at a very slow rate. It is possible that this could mean that Q-enzyme preferentially acts at terminal linkages.

That at least portions of Q-enzyme action are reversible is indicated by the homologizing reactions in which 1,4 bonds are redistributed. A corresponding behavior with 1,6 linkages is observed with isomaltose. That 1,6 bonds are formed from 1,4 bonds is well established. But the reverse reaction has not been demonstrated. This possibility could be tested with a Q-enzyme digest containing radioactive isomaltose and glucose. Any maltose formed could be easily resolved on paper and detected by a radioautograph.

The combined action of Q- and R- enzymes on some sugars would result in a simulated amylase action. This situation existed in the broad bean Q-enzyme preparation making interpretation of the action of the enzyme more difficult.

The action of Q-enzyme on sucrose demonstrates a possibility for the mechanism of synthesis of the initial phosphorylase priming materials needed for starch synthesis.

The action of Q-enzyme on isomaltose with the formation of dextran trisaccharide shows there is a possibility, although extremely unlikely, for the occurrence of successive 1,6 linkages in starch.

Action of Beta Amylase

The relation between the rates of the hydrolysis of amylotriose and of maltose by the soybean beta amylase preparation is comparable to the relation between the rates of hydrolysis of these sugars by saliva. As in the case of saliva, the transglucosidation reactions with maltose proceeded at a rate slower than the rate of hydrolysis. The possibility that these actions on maltose by the beta amylase preparation were a result of the action of enzymic impurities must be borne in mind. But it is not unreasonable to expect that beta amylase itself is responsible. The lack of previous recognition of these reactions may be due to their very slow rate and to the lack of specific sensitive methods for determination of the products.

Additional similarity in the limits of action of salivary amylase and beta amylase is shown by the similarity in the lengths of branches left after degradation. The salivary

amylase limit dextrins contain branches of one to three glucose units in length. The same is true of the beta amylase limit dextrin of amylopectin, as determined by R-enzyme and light acid hydrolysis.

SUMMARY AND CONCLUSIONS

1. Potato phosphorylase limit dextrins were prepared from waxy maize starch. The length of the longest peripheral branches of the limit dextrins was determined to be 4 glucose units by the action of R-enzyme.

2. Impure preparations of phosphorylase from seeds of several legumes were weakly primed by maltose. These phosphorylases were different from potato phosphorylase, which was not primed by maltose.

3. Q-enzyme catalyzed reversible transglucosidase action on 1,4 bonds of starch. This action caused the formation of homologous compounds of the linear series from a given linear starch oligosaccharide. Q-enzyme also catalyzed reversible transglucosidase action on 1,6 bonds giving homologous compounds from isomaltose. Q-enzyme catalyzed the transfer of 1,4 bonds over to 1,6 bonds. This reaction has not been shown to be reversible. Q-enzyme catalyzed the transfer of the glucose unit of sucrose to form 1,4 and 1,6 bonds.

4. Salivary amylase catalyzed the formation of several series of branched dextrins from amylopectin. These compounds were relatively stable in the presence of saliva. One series contained one branch per molecule, another two branches per molecule. Series containing more than two branches per molecule were no doubt formed.

5. The singly branched limit dextrins produced by saliva from amylopectin were shown to have a molecular size of 4, 5, 6 and 7 glucose units. Up to several hundred mg. of the different dextrins were isolated by paper chromatography. By partial acid hydrolysis of the branched tetrasaccharide and its aldonic acid, it was shown to consist of an amylotriose chain with a glucose branch on the second or third glucose unit. By the same type of acid hydrolysis studies and by the action of R-enzyme, the branched heptasaccharide was shown to consist of an amylotetraose chain with an amylotriose unit bonded to the second or third glucose unit. Two types of doubly branched compounds were demonstrated by the action of R-enzyme. One type consisted of a single chain with two branches. The other type consisted of a chain with one branch which in turn had a branch.

6. Saliva catalyzed the slow hydrolysis of amylotriose to maltose and glucose. Maltose was hydrolyzed also, but at a much slower rate. Saliva exhibited a weak transglucosidase action on maltose with the formation of linear and branched trisaccharides.

7. A soybean beta amylase limit dextrin of glycogen and a sweet potato beta amylase limit dextrin of waxy maize starch were prepared. Light acid hydrolysis and the action of R-enzyme demonstrated that the peripheral branches of both limit dextrins were predominantly two and three glucose units long.

8. R-enzyme catalyzed the cleavage of 1,6 bonds in starch degradation products and a limited amount of the 1,6 bonds in amylopectin. Single glucose branches as in isomaltose, panose and the branched tetrasaccharide from salivary amylase digests of amylopectin were hydrolyzed off slowly. Branches of two and three glucose units were hydrolyzed off more rapidly, but the rate diminished for the larger branches.

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VITA

Gene Muriel Wild was born in Saunders county, Nebraska, October 15, 1926, the first child of Marion (West) and Homer Wild. His early education was obtained in the public schools of Saunders county and Fremont, Nebraska. He entered the University of Nebraska in 1943 but was called to the Service in the Spring of 1945. While in the Service he was sent to Iowa State College. He continued there after being separated from the Service, and received the degree of Bachelor of Science in Chemistry in 1948. He undertook graduate studies at Iowa State College under Dr. Dexter French and received the degree of Master of Science in Plant Biochemistry in 1950. He continued his studies under Dr. Dexter French. In 1948 he married Betty Jane Gilbert of Freeport, Illinois. His two children, Rodney Bryan and Carolyn Joy, were born in 1950 and 1952.