ENZYMATIC SYNTHESIS OF AMYLOPECTIN*

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The problem of branching in glycogen and starch has been the subject of interest ever since Cori and Cori (1, 2) isolated the phosphorolytic enzyme from animal tissues and Hanes (3) found a similar enzyme in plants. Examination of polysaccharides produced by these enzymes when glucose-1-phosphate was used as a substrate showed that, similar to amylose, they had a linear configuration in which the glucose units are combined through 1,4-glucosidic linkages only (4, 5). However, naturally occurring glycogen and most starches also contain 1,6 linkages. It was therefore assumed that synthesis in vivo of glycogen and of the amylpectin starch component requires two enzymes and that in the process of preparation of the muscle or potato phosphorylase the enzyme producing the 1,6 linkages is eliminated. Proceeding on this assumption, Cori and Cori (6) produced evidence for the existence of a branching factor in liver and heart, capable of synthesizing 1,6 linkages. The combined action of the branching enzyme and crystalline muscle phosphorylase resulted in the formation of a polysaccharide which closely resembled glycogen. Later, Haworth, Peat, and Bourne (7) reported the isolation from potatoes of an enzyme, the so called Q enzyme, which, in association with potato phosphorylase, catalyzed the synthesis of amylpectin from glucose-1-phosphate.

In a series of papers, Peat and his collaborators (8-14) presented evidence that the Q enzyme converts linear amylose into branched amylpectin. Since this reaction proceeds without the presence of inorganic phosphate, they hold the view that Q enzyme functions as a non-phosphorolytic enzyme. Their evidence is based chiefly on the following experimental facts. When Q enzyme is allowed to act upon amylose, a product is produced that gives a purple-red color with iodine; the synthetic material is soluble in water, and does not retrograde from solution; it is attacked by β-amylase with the liberation of maltose, but hydrolysis is arrested before conversion is completed; end-group analysis by the methylation method indicates the existence of chains of about 20 glucose units in average length (8).

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However, Beckmann and Roger (15) were not able to duplicate these results. They claim that the material obtained by the English investigators is not amylopectin, but is an artifact. According to their findings, potato preparations contain fatty acids which form a complex with amylose, possessing many of the properties of amylopectin. They therefore concluded that these properties have been erroneously used by Haworth, Peat, and their associates as evidence to prove that their product is amylopectin.

Recently Bernfeld and Meutenédián (16–19) reported the isolation from potatoes of an enzyme “isophosphorylase,” which appeared to resemble the Q enzyme, inasmuch as it presumably played a part in the formation of amylopectin from both amylose and glucose-1-phosphate. There is, however, a difference between the action of the two enzymes. Whereas inorganic phosphate is not involved in the formation of branched polysaccharide by the Q enzyme, the Swiss investigators claim that phosphate constitutes an integral part of the branching mechanism. They believe that isophosphorylase catalyzes reversibly the formation of 1,6 bonds in a reaction analogous to that of the formation of 1,4 bonds by phosphorylase.

The evidence offered by Bernfeld and Meutenédián for the synthesis of amylopectin is as follows: When a mixture of isophosphorylase and phosphorylase acts on amylose in the presence of inorganic phosphate, a product is formed which gives a purple color with iodine and is degraded by β-amylase to the extent of 65 per cent, while amylose is degraded by this enzyme to the extent of 100 per cent. A similar product is obtained when a mixture of these enzymes acts on glucose-1-phosphate. Isolation and further characterization of the synthetic product were not undertaken.

In view of these conflicting results, it was of interest to reinvestigate the problem of enzymatic synthesis of amylopectin. The conversion of amylose to amylopectin with Q enzyme, reported by Peat and his associates (8–14), was successfully repeated, and further chemical and biochemical evidence was obtained showing that the synthetic product is branched. Like amylopectin, the synthetic material is precipitated with dilute alcohol, produces a purple color with iodine, and gives a very small reducing value. On treatment with β-amylase a hydrolysis limit of 51 per cent is obtained. End-group determination by periodate oxidation shows an average of 21 glucose residues per end-group, while with an enzymatic method a value of 20 is obtained. Estimation of the molecular weight by osmotic pressure measurements of the acetylated product indicates a value of 54,000, calculated as the deacetylated product. In contrast to amylose, the synthetic material is as effective a “primer” of muscle phosphorylase as corn amylopectin.
The synthesis of amylopectin with Bernfeld and Meutémédian's (16-19) "isophosphorylase" could not be repeated. Treatment of amylose with a potato preparation made according to these authors resulted in a degradation product which gave a purple color with iodine, but which on further analysis proved to be a mixture of short chain linear dextrins.

Of the two mechanisms proposed for amylopectin synthesis, that based on the work of Peat and his collaborators appears to be well substantiated. The Q enzyme is apparently capable of splitting 1,4-glucosidic linkages in the amylose chain and exchanging them for 1,6 linkages without the mediation of phosphate, thus forming branches. Like the enzymes present in Pseudomonas saccharophila (20), Leuconostoc mesenteroides (21), and Neisseria perflava (22), the Q enzyme can thus be regarded as belonging to the class of trans-glycosidases.

EXPERIMENTAL

Preparation of Q Enzyme—The enzyme preparations were made according to the procedure described by Barker, Bourne, and Peat (11). Batches of approximately 5 kilos of potatoes yielded 2 liters of crude juice. The method involves precipitation from potato juice of Q enzyme and other proteins by the addition of lead acetate at pH 7.25, elution of the lead-protein complex with sodium hydrogen carbonate solution, through which a stream of carbon dioxide is passed, and precipitation of the enzyme from the supernatant liquid with neutral ammonium sulfate. The final solution was diluted to 400 ml. and kept in the refrigerator at 2°. The preparation was analyzed for inorganic phosphate (23) and was found to contain 0.0035 mg. per ml. of solution.

Conversion of Amylose to Amylopectin—A 3 gm. sample of corn amylose was dissolved in 0.5 N sodium hydroxide, diluted to 1300 ml., and neutralized with 0.5 N sulfuric acid. Then 500 ml. of Q enzyme and 500 ml. of 1 M acetate buffer, pH 7.0, were added, and the mixture was diluted to 3000 ml. The digest was allowed to remain at room temperature for 24 hours, and during the incubation period aliquot portions were analyzed for reducing sugars by the method of Hassid et al. (24, 25). The reducing values of a control experiment in which amylose was omitted were simultaneously determined. The difference between the reducing values of the two digests was considered a measure of the reducing groups liberated by the action of the enzyme preparation on amylose. Different preparations differed in the amount of reducing substances formed, but none showed an increase in 24 hours greater than 1.5 per cent (calculated as maltose) of the amylose added.

The digest was heated, the coagulated proteins removed by filtration,
and the solution dialyzed for several days until the dialysate was practically free of iodine-staining material\(^1\) and inorganic impurities. The solution was then concentrated \textit{in vacuo} at 60\(^\circ\) and the material precipitated with ethanol. It was dried in a vacuum oven at 50\(^\circ\) and extracted with methanol in a Soxhlet extractor for 12 hours to eliminate fatty acid impurities and dried again. A yield of 55 per cent of synthetic amylopectin was obtained, based on the original amylose. The product produced a purple-red color when treated with iodine and was soluble in water.

\textbf{Hydrolysis with \(\beta\)-Amylase}—The method used for hydrolysis of the synthetic polysaccharide with \(\beta\)-amylase was essentially that of Bernfeld and Gürtler (26), except that the maltose produced was estimated by oxidation with ferricyanide (24, 25). The crystalline \(\beta\)-amylase (27) was obtained from Dr. A. K. Balls of Western Regional Research Laboratory.

A 25 mg. sample of the polysaccharide was dissolved in 20 ml. of water. A solution containing an excess of \(\beta\)-amylase, approximately 500 units (28), and 6 ml. of acetic acid-sodium acetate buffer, pH 4.8, was added. The mixture was diluted to 50 ml. and 5 ml. aliquots were periodically drawn during 24 hours for determination of reducing values. It was found that the synthetic amylopectin was hydrolyzed to the extent of 51 per cent.

A 20 mg. sample of amylose originally used for the amylopectin synthesis was dissolved in 2 ml. of 2 N sodium hydroxide, neutralized with hydrochloric acid, diluted to 20 ml., and similarly treated with \(\beta\)-amylase. The hydrolysis limit of the amylose with this enzyme was 90 per cent. Further addition of \(\beta\)-amylase did not increase the extent of hydrolysis of either the synthetic amylopectin or the amylose.

\textbf{Fatty Acid Content of Synthetic Polysaccharide}—A 0.2 gm. sample of the material was hydrolyzed completely by boiling under a reflux condenser with 15 ml. of 3 N sulfuric acid for 1 hour. The solution was extracted for 24 hours with ether in a Soxhlet extractor and the ether extract was shaken twice with 5 ml. portions of water to extract traces of sulfuric acid. The ether solution was then evaporated, and the residue taken up in ethyl alcohol and titrated with 0.01 N sodium hydroxide. No acid could be detected. In a control experiment, in which 10 mg. of sodium oleate were added to 0.2 gm. of amylose and hydrolyzed with sulfuric acid as described, the oleic acid could be recovered quantitatively.

\textbf{End-Group Determination}—A 0.2 gm. sample of the material was oxidized with 0.37 M sodium periodate at 2\(^\circ\) according to the method of Potter and Hassid (29). After 25 hours, the excess periodate was removed by the addition of ethylene glycol and the formic acid formed was estimated by titrating with 0.01 N barium hydroxide. For the blank titration, 0.2

\(^1\) The nature of this material is now being investigated.
gm. of synthetic amylopectin was added to the periodate, which had been previously reduced with ethylene glycol. Because of the slight alkalinity of the blank, 10 ml. of 0.01 N hydrochloric acid were added to both the blank and the sample, and a correction was made when the sample was titrated with the barium hydroxide. The average number of glucose residues per end-group for the synthetic amylopectin, calculated on the basis of 1 mole of formic acid produced per chain, was found to be 21. The chain length of the parent amylose (corn) determined by this method was 490 glucose residues per end-group.

**Determination of Molecular Weight**—A 1 gm. sample of the product was dispersed in formamide and acetylated at room temperature with acetic anhydride in the presence of pyridine (30). The molecular weight was determined on the acetylated product from osmotic pressure measurements, with chloroform as a solvent. The method employed was the same as that previously used for the determination of a variety of starch fractions (30). The osmotic pressure was measured at several different concentrations (Table I).

The intercept of the coordinate was determined by plotting \( \pi/C \) against \( C \), and the molecular weight was calculated by the van't Hoff equation. From the intercept of the ordinate a value of \( \pi/C \) was obtained equal to 0.262 for the acetylated product. This value corresponds to a number average molecular weight of 97,000 for the acetylated product. On this basis, the molecular weight of the deacetylated synthetic polysaccharide is 54,000, as compared to an average molecular weight of the parent amylose of about 100,000. This molecular weight is smaller than those obtained for amylopectins of natural starches (30), but its magnitude is nevertheless large enough to eliminate the possibility that the synthetic product consists of unbranched fragments of amylose, such as would be produced by the action of \( \alpha \)-amylase.

**Reducing Value of Synthetic Amylopectin**—The reducing value of a 30 mg. sample of synthetic amylopectin was determined (24) and compared...
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with the reducing values of the parent amylose and of a few natural starch fractions of known molecular weight. The soluble amylopectin samples (30 mg.) were dissolved in 5 ml. of water. The insoluble amylose was brought into solution with 2 N sodium hydroxide and neutralized with acid. The results are given in Table II.

The reducing value of the synthetic amylopectin appears to be of the same order or magnitude as that of the parent amylose or of an amylopectin subfraction of low molecular weight. The comparatively low reducing value of the synthetic product indicates that the synthetic polysaccharide cannot be degraded amylose.

Action of "Isophosphorylase" on Amylose—The experiments of Bernfeld and Meutémédian (16-19) were repeated with an enzyme preparation from potatoes, made according to their directions, with the following results.

A mixture consisting of amylose + phosphorylase + isophosphorylase + inorganic phosphate (phosphate buffer), originally giving a blue color with iodine, produced a purple color after 24 hours of incubation. When the inorganic phosphate was eliminated from the reaction by substituting veronal for phosphate buffer of the same pH, the reaction mixture stained blue with iodine after incubation. However, it was observed that, when the quantity of isophosphorylase was doubled or tripled, the presence of either phosphate or phosphorylase was not necessary to convert amylose into a purple-staining polysaccharide.

One possible interpretation of these results is that the supposed "isophosphorylase" preparation contains α-amylase and that the combined action of this enzyme with phosphorylase in the presence of inorganic phosphate is responsible for the conversion of the amylose into fragments that stain purple with iodine. The phosphorylase also contributes to the degradation of the molecule, owing to the small amount of α-amylase impurity.

TABLE II
Reducing Values and Molecular Weights of Synthetic Amylopectin and Related Polysaccharides

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mol. wt.</th>
<th>0.01 N ceric sulfate*</th>
<th>ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic amylopectin</td>
<td>54,000</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>Corn amylopectin</td>
<td>3,000,000</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>&quot; subfraction</td>
<td>290,000</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>&quot; amylose</td>
<td>128,000</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>&quot; amylodextrin</td>
<td>6,700</td>
<td>0.190</td>
<td></td>
</tr>
</tbody>
</table>

* 2.4 ml. of 0.01 N ceric sulfate solution are equivalent to 1 mg. of maltose.
However, phosphorylase alone at this phosphate concentration will not cause a change in iodine color when acting upon amylose. When the amount of the isophosphorylase preparation is increased, the concentration of α-amylase probably becomes sufficiently large to accomplish the degradation of amylose alone without the aid of phosphorylase.

Reducing Value of Product of “Isophosphorylase” Action—Samples containing 10 mg. of amylose, phosphorylase, phosphate buffer, and “isophosphorylase” were mixed and allowed to digest for 24 hours. After deproteinization by heating, filtering, and washing, it was found that the sample possessed a reducing value which was considerably greater than that of the samples in which the “isophosphorylase” or amylose was left out. The increase amounted to 1.2 ml. of 0.01 N ceric sulfate titration (equivalent to 0.5 mg. of maltose) per 10 mg. of amylose. This value is of the order of magnitude that would be expected if the amylose were degraded to chains of approximately 40 glucose units in average length by an α-amylase.

Identification of Breakdown Products of Starch with “Isophosphorylase” —A 0.5 gm. sample of amylose was converted with “isophosphorylase” preparation and the resulting material precipitated and dried. The material possessed the following properties. (1) It was soluble in water, but retrograded after remaining at room temperature for 3 or 4 days. (2) When dissolved in hot water and cooled, it did not precipitate when methyl or ethyl alcohol was added in 50 per cent concentration. Amounts of this material formed from 50 mg. of amylose did not produce any cloudiness in 50 per cent alcohol. Less than 1 mg. of amylopectin or the synthetic branched polysaccharide in the same volume will produce a definite turbidity. (3) Acetone was found to precipitate the polysaccharide. (4) When the material was dialyzed against distilled water, most of the purple-staining material was lost, indicating that the molecules are small.

The properties of this product are associated with those of amylose degradation products, rather than with properties of branched polysaccharide. On the basis of these experiments, the mechanism of Bernfeld and Meutémédian for amylopectin formation involving “isophosphorylase” cannot be accepted.

Note on the Properties of Synthetic Amylopectin

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Enzymatic End-Group Determination—The method (31) is based on the degradation of the polysaccharide by phosphorylase plus amylo-1,6-glu-
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cosidase (debrancher). The latter enzyme is specific for hydrolytic scission of the 1,6 linkages. The ratio of phosphorylated plus free glucose to free glucose is determined. Control experiments were run simultaneously with a sample of the amylose from which the amylopectin had been synthesized and with three branched polysaccharides (corn amylopectin, rabbit liver glycogen, and dog liver glycogen), the end-groups of which had also been determined by the periodate oxidation method (31). End-group values pertaining to the dog liver glycogen were also available from methylation experiments (32). The results are summarized in Table III.

Three separate experiments carried out with the synthetic amylopectin showed good agreement. An end-group of 5.1 per cent was obtained, corresponding to an average chain length of approximately 20 glucose units. This value closely agrees with that of 21 units obtained by the periodate oxidation method. The 1.7 per cent end-group found for amylose is probably due to amylopectin impurity. Amylopectin is rapidly attacked by muscle phosphorylase, while the action of this enzyme on amylose is very slow. This is shown by the fact that only 13 per cent of the polysaccharide was digested. If this assumption is correct, the amylose would contain approximately 5 per cent amylopectin.

Phosphorolysis of Synthetic Amylopectin—The synthetic material was subjected to the action of muscle phosphorylase (ninth crystallization) in the presence of an excess of inorganic phosphate, and the limit of phosphorolysis was compared with that of rabbit liver glycogen and of natural

### Table III

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Polysaccharide</th>
<th>Time of incubation</th>
<th>Digested</th>
<th>End-group</th>
<th>No. of glucose residues per end-group</th>
<th>Glucosidase units used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corn amylose</td>
<td>1 hr.</td>
<td>13</td>
<td>1.7</td>
<td></td>
<td>2250</td>
</tr>
<tr>
<td>2</td>
<td>Q enzyme synthesized</td>
<td>1</td>
<td>61</td>
<td>5.3</td>
<td>19</td>
<td>2250</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>2</td>
<td>66</td>
<td>5.0</td>
<td>20</td>
<td>1900</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>1</td>
<td>67</td>
<td></td>
<td></td>
<td>3850</td>
</tr>
<tr>
<td>5</td>
<td>Corn amylopectin</td>
<td>2</td>
<td>82</td>
<td>5.1</td>
<td>20</td>
<td>3850</td>
</tr>
<tr>
<td>6</td>
<td>Rabbit liver glycogen</td>
<td>1</td>
<td>103</td>
<td>6.7</td>
<td>15</td>
<td>2250</td>
</tr>
<tr>
<td>7</td>
<td>Dog liver glycogen</td>
<td>2</td>
<td>100</td>
<td>8.3</td>
<td>12</td>
<td>3850</td>
</tr>
</tbody>
</table>
amylopectins. The per cent degradation was as follows: glycogen 37, corn amylopectin 53, wheat amylopectin 44, synthetic amylopectin 34. The outer branches of the synthetic polysaccharide are relatively short and are comparable to those of glycogen.

Separate and Consecutive Degradation of Synthetic Polysaccharide with Phosphorylase and Glucosidase—A sample weighing 37 mg. was submitted to exhaustive phosphorylase action, as described in the preceding paragraph. When the reaction had come to an end-point, 32 per cent of the polysaccharide had been converted to glucose-1-phosphate. The remaining polysaccharide (first limit dextrin) was isolated by several precipitations with alcohol and submitted to glucosidase action in the absence of phosphate. Glucose liberated was determined by a specific enzymatic method. It was found that 3.7 per cent of the limit dextrin had been converted to glucose, which indicates that one-half of the branch glucose residues present in the original material has been split off. The glucosidase was destroyed by heating and phosphorylase was again permitted to act on the remaining polysaccharide, with the result that 36 per cent of it was converted to glucose-1-phosphate. At this point, 58 per cent of the original material had been degraded. In addition, an aliquot of the first limit dextrin was submitted to an end-group determination; 7.7 per cent end-groups were found. From the end-group concentration of the original material (5.1 per cent) and the loss of 32 per cent by phosphorylase action, the calculated value is 7.5 per cent. These results, which are very similar to those recently obtained on glycogen and amylopectins (Larner et al. (33)), are compatible only with a tree-like structure of the synthetic polysaccharide, which therefore does not differ in this respect from naturally occurring polysaccharides.

Activation of Crystalline Muscle Phosphorylase—The “primer” action of synthetic amylopectin was compared with that of glycogen, natural amylopectin, and amylose in reaction mixtures constituted as follows: 12 mM initial concentration of glucose-1-phosphate, 40 mg. per cent of polysaccharide and muscle phosphorylase. The following ratios of activities were found: when no polysaccharide was added, 0; with glycogen, 100; with corn amylopectin, 63; with synthetic amylopectin, 60; and with amylose, 7. These figures vary somewhat with different polysaccharide-glucose-1-phosphate ratios. The conclusion can be drawn that the synthetic amylopectin possesses almost the same activating power as natural amylopectin.

Molecular Size of Synthetic Polysaccharide—A 1 per cent solution of the sample in water was examined in the Spinco analytical ultracentrifuge by Dr. John F. Taylor. At 59,700 r.p.m., a single boundary was observed, moving with a sedimentation constant (20°) of 3.1 S at the start and falling to 2.8 S at the end. The spreading of the boundary indicates that the
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material is polydisperse, as has been observed from other polysaccharides. The diffusion constant of this material has not been measured. If the frictional ratio, $f/f_0$, were assumed to be similar to that reported for glycogen (1.6 to 1.9), the “mean” molecular weight would be from 31,000 to 41,000.

SUMMARY

Linear amylose can be transformed with Q enzyme from potatoes to a branched polysaccharide possessing the chemical and biochemical properties of amylopectin. Its molecular weight, estimated from osmotic pressure measurements, is 54,000, which is less than that of naturally occurring amylopectins.

The synthetic product is soluble in water, does not retrograde from solution, and stains purple-red with iodine. Chain length determination by periodate oxidation shows an average of 21 glucose residues per end-group, while with an enzymatic method a value of 20 was obtained.

The synthetic polysaccharide is degraded by $\beta$-amylase to the extent of 51 per cent and by crystalline muscle phosphorylase to the extent of 33 per cent. An analysis of structure by means of enzymatic degradation shows close resemblance to the structure of natural amylopectins. As a “primer” substance for muscle phosphorylase, it is about as effective as naturally occurring corn amylopectin.

The claim that the synthetic amylopectin is in reality a combination of amylose with fatty acids, simulating the properties of an amylopectin, could not be confirmed. Neither could evidence be obtained for the existence of “isophosphorylase” in potatoes, capable of synthesizing amylopectin.

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