STUDIES ON THE STRUCTURE OF POLYSACCHARIDES

II. DEGRADATION OF POLYSACCHARIDES BY ENZYMES*

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Starch and related polysaccharides are degraded by two different types of enzymes, the amylases and phosphorylases. An investigation of some of the properties of the phosphorylases involved the use of polysaccharides hydrolyzed by α- and β-amylase and led to a number of observations about the action of these enzymes which are recorded in this paper. It appears from these experiments that the action of α-amylase on amylopectin and glycogen is less random than its action on amylose, that β-amylase degrades one polysaccharide chain completely before transferring its activity to another, and that potato and muscle phosphorylases differ in their action on branch points.

α-Amylases—It is known that α-amylases from different sources differ in the details of their action on starch (1). Therefore all interpretations are meant to apply only to the enzyme actually used.

The α-amylases of both plants and animals are characterized by their dextrinogenic action. Starch is rapidly degraded to fragments showing progressively reddening iodine colors until the color reaction entirely disappears. Reducing dextrins, maltose, and small amounts of glucose account for the reducing power at each stage. No high molecular weight residue remains when the reaction has reached apparent completion. It has been suggested that the primary action may be the splitting off of fragments about 6 units long, with subsequent formation of maltose from these small dextrins (2). It seems that the 1,6 bonds at the point of branching cannot be hydrolyzed by the enzyme. Suggestive evidence on this point is the finding of a small amount of an apparently resistant residue from starch treated with α-amylase. The exact nature of this material has not been ascertained, but it is presumed to consist of tri- or tetrasaccharides

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containing a 1,6 linkage (3). In most of these investigations either native or some variety of soluble starch has been used.

In this work salivary amylase from one person was used throughout, and the substrates were liver glycogen and purified fractions of corn-starch. The saliva was collected fresh on the day used, diluted with water, and centrifuged to remove mucus. Repeated tests showed that it was of consistent amylolytic power. A slight trace of maltase activity was detectable only on long incubation (over 24 hours).

In order to test the ability of α-amylase to split 1,6 linkages, dextran was used as the substrate. To 100 mg. per cent solutions of dextran and starch respectively were added equal amounts of enzyme. In less than 2 minutes the starch was completely achromic to iodine and gave a positive reduction test with Benedict's solution. Even after 24 hours incubation at 30° the dextran showed no sign of reducing power. However, this negative result does not necessarily show that the enyzme is incapable of attacking a 1,6 linkage as it occurs in amylopectin, since the whole structure of the dextran may be such as to inhibit the enzyme. For example, there is no doubt that α-amylase can hydrolyze 1,4 linkages, but those in the Schardinger dextrins are completely resistant.

If the 1,6 linkages are not split, then amylopectin and glycogen should leave an unhydrolyzed residue of significantly greater chain length than that left by amylose. Samples of these three polysaccharides were dissolved in 3 N KOH and neutralized with HCl; then 20 ml. of 0.1 M citrate, pH 6.5, 20 ml. of 2 per cent NaCl, and water to make 100 ml. were added. These solutions were incubated with α-amylase for 40 hours, and then reducing power was determined before and after acid hydrolysis.[1] The average chain length in glucose units was found to be 2.0 for amylose, 3.7 for amylopectin, and 2.9 for glycogen. Since the formation of a small amount of glucose has not been excluded, the apparently complete conversion of amylose to maltose may be fortuitous, but, with this as a standard of comparison, it may be seen that the residues from the branched polysaccharides are significantly longer. This suggests that α-amylase does not split the linkage at the branch point.

An attempt was made to obtain evidence for the removal of fragments of uniform size. Polysaccharides were incubated with α-amylase at 30°, and samples were taken at intervals. The enzyme action was stopped by heat; then 1 aliquot of each sample was used for a determination of reducing power in order to calculate the per cent hydrolysis, while another aliquot

[1] The average chain length was calculated from the differences in reducing power before and after acid hydrolysis. The copper reagent used (see Paper I of this series) showed a reducing power per mg. of maltose hydrate which was one-half that of glucose.

was dialyzed 20 hours against 4 volumes of distilled water in the refrigerator. Dialysis was carried out in cellophane sacs obtained from the Visking

TABLE I

Separation of Products of Hydrolysis of Glycogen Digested by α-Amylase

The samples were dialyzed in cellophane sacs for 20 hours against 4 volumes of H_2O at 2°.

Sample No.	Hydrolysis	Apparent chain lengths	
		Dialyzed solution	Dialysate
	per cent	*glucose units*	*glucose units*
1	1.6	87	4.0
2	2.5	72	2.4
3	5.8		4.7
4	7.1	24.4	3.7
5	14.7	16.3	3.3
6	21.2	12.5	3.6
7	23.7	9.2	3.7
8	25.0	8.4	3.6
9	26.2	8.0	3.7
10	28.8	7.0	

TABLE II

Separation of Products of Hydrolysis of Amylose Digested by α-Amylase

The samples were dialyzed in cellophane sacs for 20 hours against 4 volumes of H_2O at 2°. B. = blue, P. = purple, R. = red.

Sample No.	Hydrolysis	Dialyzed solution		Dialysate	
		Chain length	Iodine color	Chain length	Iodine color
	per cent	*glucose units*		*glucose units*	
1	3.2	21.2	B.-P.	8.6	Faint P.
2	7.8	17.7	P.	6.2	R.
3	10.3	15.3	"	5.7	"
4	12.2	10.5	R.-P.	5.6	"
5	13.5	9.8	"	4.5	"
6	16.2	8.3	R.	4.4	"
7	17.2	7.3	"	4.0	Faint R.
8	23.2	6.2	"	3.4	
9	28.8	4.3		2.9	
10	32.8	3.4		2.7	
11	40.5	2.7		2.6	

Corporation. The reducing power of the inner and outer fluids was determined both before and after acid hydrolysis in order to calculate the chain length. From Tables I and II it can be seen that the dialyzable

material obtained from glycogen was remarkably uniform in size over a fairly wide range of hydrolysis, while the dialyzable fragments from amylose showed no such uniformity. Furthermore, the undialyzable fragments of amylose showed a much greater reduction in size for a given degree of hydrolysis than those of glycogen. These results might be ascribed to differences in structure. The linear polymer, having a uniform and easily accessible structure, is hydrolyzed by α-amylase in a more random manner than the branched polysaccharide.

β-Amylase—This enzyme, found only in plants, has attracted much attention because of the peculiarities of its action. It is characterized as saccharogenic, since large amounts of reducing sugar and no detectable intermediates are formed. The generally accepted view of its action is that postulated by Hanes (4), which is that the enzyme successively removes maltose units from the non-aldehyde end of the chain until the action is blocked by some anomaly in structure. This block is now thought to be the 1,6 linkage at the branch point, since the unbranched fraction of starch is completely broken down.

Meyer and Bernfeld's (5) kinetic prediction that the rate of hydrolysis should remain constant because the number of end-groups remains the same implies that all the chains are shortened at a uniform rate. If this were true, then short chain intermediates should be obtainable from amylose partly digested by β-amylase.

Our β-amylase was prepared from ungerminated wheat by the method of Ballou and Luck (6). It showed no trace of maltase activity. Although the optimum pH is 4.8, it was very active for long periods of time at pH 6.5, which was the pH used for all but the preliminary experiments. A sample of amylose was dissolved in about 1 ml. of 3 N KOH and neutralized with HCl; then 20 ml. of 0.1 M citrate at pH 6.5 were added and enough water to make 100 ml. When the amylose concentration was below 200 mg. per cent, this amount of salt was sufficient to keep it from retrograding before complete enzymatic hydrolysis was achieved. About 0.5 mg. of enzyme powder in 1 ml. of citrate buffer was added, the digest incubated at 30°, and a sample taken at intervals. As with the α-amylase, the enzyme action was halted by heating at 100° for 10 minutes. 1 portion of each sample was used to determine the reducing power, which was calculated as maltose. Another portion was dialyzed in cellophane sacs against 50 volumes of water for 20 hours at 3°; as shown in Table II, fragments of an average chain length of 9 glucose units can be removed by dialysis. The material which was recovered from inside the sacs had no reducing power and stained blue with iodine; it was completely hydrolyzed in acid and determined as glucose.

The aim of this experiment was to calculate the amount of unhydrolyzed

amylose (*a*) from the amount of undialyzable carbohydrate and (*b*) from the amount of maltose formed, the argument being that the formation of intermediate dextrins during the action of β-amylase should give rise to a discrepancy between these two values. The results are shown in Fig. 1, where the values for (*a*) and (*b*) are plotted as the per cent of the total amylose originally present. The two curves coincide up to a point where only 30 per cent of amylose is left unhydrolyzed; hence fragments other than maltose were apparently not formed in significant amounts. The divergence of the curves after more complete hydrolysis is due to the formation of a small amount of glucose, as is shown by the separate determination of maltose and glucose by differential fermentation with yeast. This makes

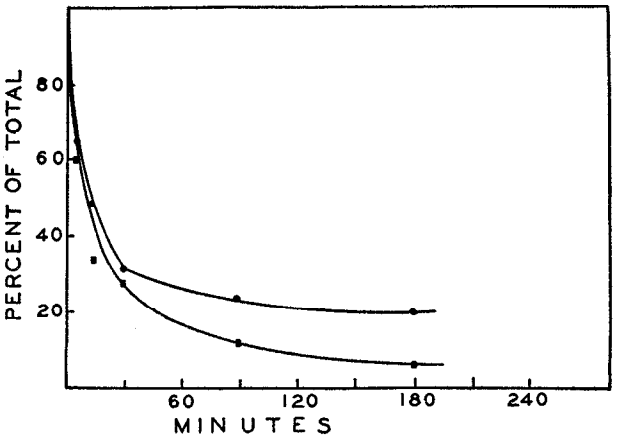

FIG. 1. Comparison of amount of amylose left unhydrolyzed after various intervals of digestion by β-amylase; ●, calculated from undialyzable carbohydrate; ■, calculated from determinations of reducing power as maltose.

the determination of the reducing power calculated as maltose too high and hence the hydrolysis of amylose appears more complete than it actually is. Had intermediate dextrins been present, the reducing power would have been determined too low, and the two curves would have diverged in the opposite direction.

The lack of short chain intermediates between amylose and maltose, the evidence from phosphorylase activation (Paper III) and from iodine colors (Paper IV), suggests that the action of β-amylase, at least on amylose, consists in complete scission of one whole chain to maltose before another chain is attacked. The same apparently applies to the action of β-amylase on amylopectin, since tests on the activation of phosphorylase show a decrease in the number of end-groups from the beginning of the reaction (Paper III). The action here is apparently the removal of one whole terminal chain as

maltose before another is attacked, and not a shortening of all the outside chains at the same rate.

The question is sometimes raised whether or not the amylases can attack very short chains. When partial hydrolysates of α-Schardinger dextrin were treated with either α- or β-amylase, there was a marked rise in the reducing power of the solution, and practically all of the reducing power could be removed by fermentation with fresh yeast. Since the unopened rings were not attacked at all, this shows that both enzymes are able to split very short chains. A very small amount of non-fermentable reducing material which remained has not been investigated further.

Degradation by Phosphorylases—In an early study of mammalian phosphorylases Cori, Colowick, and Cori (7) showed that 95 per cent of the glycogen which disappeared could be accounted for as glucose-1-phosphate. It was also noted that, if an excess of phosphate was added, all the glycogen was degraded.[2] These findings suggest a resemblance to the end-wise attack of β-amylase, with the rather surprising additional ability to by-pass the 1,6 linkages at the branch points, or perhaps even to break them. The action of crystalline muscle phosphorylase on purified glycogen and amylopectin was studied, and the results which had been obtained with crude muscle extracts were confirmed.

The solution of polysaccharide (250 mg.) and enzyme was placed in a small dialyzing sac, suspended in a large volume of 0.1 M phosphate-cysteine buffer, pH 7.2, and incubated at 30°. A drop of toluene was added to the contents of the sac to inhibit bacterial growth. At intervals the outer solution was renewed so that glucose-1-phosphate was continually removed from the system. A control with citrate-cysteine buffer instead of phosphate was run simultaneously to check on the possible presence of amylases, and to correct for the effects of osmosis which tended to increase the concentration of glycogen. Samples were removed at intervals from both digests, the glycogen (or amylopectin) twice precipitated with alcohol, hydrolyzed in acid, and determined as glucose. The results are shown in Fig. 2, where the amount of polysaccharide remaining is plotted as per cent of that found in the control.

In order to find possible intermediates too small to be precipitated in alcohol, duplicate samples were hydrolyzed directly without precipitation. Identical amounts of glucose were found in both precipitated and non-precipitated samples. Even at the level of 80 per cent degradation the residue showed all the properties of the original glycogen; it was non-dialyzable, stained red with iodine, formed translucent solutions in water, and was precipitated on the addition of 2 volumes of ethanol. This pre-

[2] Cori, G. T., unpublished.

cipitate was redissolved, insoluble protein and cystine removed by centrifugation, and the material made up to 1 per cent solution and the experiment repeated. It was attacked by the phosphorylase at the same rate as the original glycogen.

The results in Fig. 2 indicate that muscle phosphorylase can degrade glycogen and amylopectin beyond the branch points. As a further check the limit dextrin of glycogen was prepared by prolonged action of β-amylase. This material was incubated for 24 hours with a phosphate-phosphorylase-phosphoglucomutase system. (The latter enzyme was added in order to

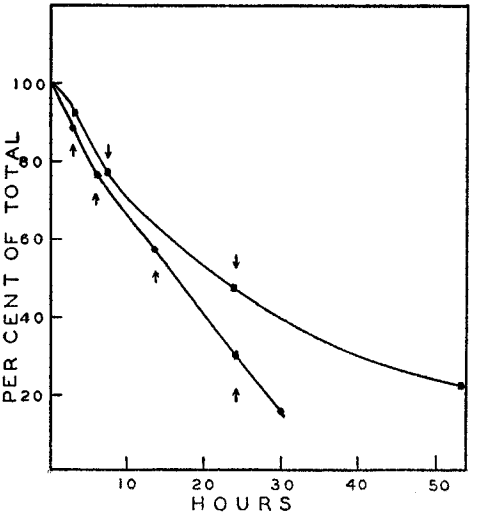

FIG. 2. Degradation of branched polysaccharides by crystalline muscle phosphorylase. Value plotted as a fraction of the amount of carbohydrate found in the control; ●, glycogen; ■, amylopectin; the arrows represent the addition of more enzyme.

convert the glucose-1-phosphate formed by phosphorylase to glucose-6-phosphate and thereby to shift the equilibrium away from glycogen.) The barium salts of the phosphate esters were isolated in good yield and analyzed for total and easily hydrolyzable phosphate. In three such experiments the easily hydrolyzed phosphate averaged 5 per cent of the total esterified phosphate. This is the proportion found when phosphoglucomutase acts on a solution of pure glucose-1-phosphate. Since the limit dextrin was used, the formation of phosphate esters indicates that muscle phosphorylase can by-pass 1,6 linkages.

The question whether phosphorylase can actually break 1,6 linkages has not been settled. When dextran was treated with muscle phosphorylase, no uptake of phosphate was found even on prolonged incubation with an

amount of enzyme which completely degraded a similar amount of glycogen in 3 hours. However, as in the case of α-amylase, this negative result is open to the criticism that the structure of the dextran may be so different from that of glycogen as to be unavailable to attack. Interestingly enough, dextran is able to activate phosphorylase for polysaccharide synthesis (Paper III).

Potato phosphorylase is similar to β-amylase in being unable to pass the branch point. Meyer and Bernfeld (8) have shown that the limit dextrin from amylopectin is not attacked by potato phosphorylase.

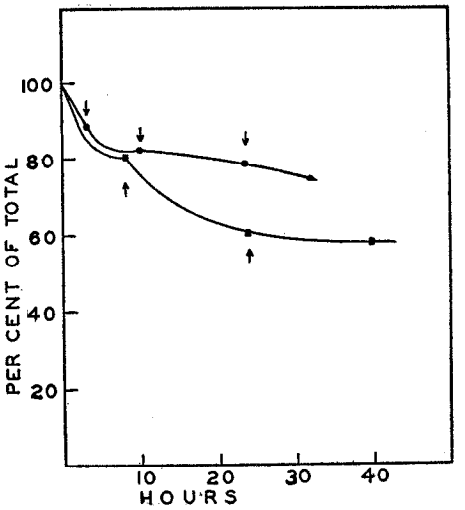

FIG. 3. Degradation of branched polysaccharides by potato phosphorylase. ●, glycogen; ■, amylopectin; the arrows represent the addition of more enzyme.

When amylopectin and the potato enzyme were dialyzed against a phosphate buffer, as described for the muscle enzyme, the degradation did not proceed beyond 40 per cent, as compared to a degradation of 60 per cent with β-amylase. The ability of the potato enzyme to degrade glycogen was even more limited (see Fig. 3).

The following experiment was designed to characterize the limit dextrin formed from amylopectin by potato phosphorylase. About 250 mg. of amylopectin and a concentrated solution of potato phosphorylase were dialyzed for 40 hours against several changes of phosphate buffer, and then the residual polysaccharides precipitated with methanol. A portion of the dried sample was redissolved and treated with phosphorylase for a further 40 hours. This second residue was also precipitated and dried. The two fractions were tested for their ability to activate potato phosphorylase in

the synthesis of polysaccharide and for their susceptibility to attack by β-amylase. The results are shown in Table III. It is seen that even at the limit of degradation with potato phosphorylase some branches remain which can be removed by the β-amylase. These remaining branches, perhaps 5 to 6 units long if the branches were shortened uniformly, are not very effective in activating phosphorylase in the synthetic reaction. The limit dextrin formed from amylopectin by β-amylase does not activate potato phosphorylase.

TABLE III

Properties of Residue from Amylopectin Degraded with Potato Phosphorylase

Residue I, obtained after 40 hours of incubation with potato phosphorylase, was isolated and incubated with the enzyme for another 40 hours, giving Residue II. Both residues were tested for their ability to activate potato phosphorylase for polysaccharide synthesis with glucose-1-phosphate as the substrate. An amount of residue was used corresponding to an original amylopectin concentration of 35 mg. per cent and was compared with the activating effect of 500 mg. per cent of soluble starch. Both residues were also subjected to the action of β-amylase.

Sample	Time of degradation	Activating effect for phosphorylase	Degradation by β-amylase
	hrs.	*per cent*	*per cent*
Amylopectin	0	70	60
Residue I	40	62	30
" II	80	38	19

SUMMARY

The degradation of polysaccharides by four enzymes has been investigated. The action of salivary α-amylase on amylose, a linear polysaccharide, appears to be more random than its action on glycogen, a branched polysaccharide. During the action of β-amylase of wheat on amylose, intermediates of short chain length could not be detected. This observation, as well as others, suggests that this enzyme degrades one whole amylose molecule completely to maltose before attacking a new chain. Both α- and β-amylase hydrolyzed the short saccharide chains obtained by partial hydrolysis of the Schardinger dextrins.

Muscle phosphorylase can degrade glycogen and amylopectin beyond the branch points. Potato phosphorylase is similar to β-amylase in being unable to pass branch points, but differs from β-amylase in removing the terminal chains of amylopectin less completely. No evidence has been obtained that any of these enzymes can break the α-1,6 linkages at the branch point. They do not act on dextran, a polysaccharide consisting of α-1,6 linkages.

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