

*The Enzymic Synthesis and Degradation of Starch. Part XIX.**
The Action of R-Enzyme on Glycogen.

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It is shown that R-enzyme, which debranches amylopectin, does not exert a similar hydrolytic action on the glycogen from oysters or from rabbit liver. Evidence is adduced to support the view that this lack of action is due to the higher degree of branching of the glycogen and consequently to the greater compactness of the molecule which makes the majority of the branch links inaccessible to the enzyme.

It is worthy of note that the action of R-enzyme provides a simple practical method of distinguishing amylopectins from glycogens.

R-ENZYME, found in the potato and the broad bean, is described as a debranching enzyme inasmuch as it catalyses the hydrolysis of the branch-forming (α -1 : 6-)linkages of amylopectin (Part XIV, *J.*, 1951, 1451). The debranched product, compared with the original amylopectin, is stained more intensely by iodine and is more susceptible to β -amylolysis. Further it reduces copper reagents under conditions in which amylopectin does not. The purposes of the present communication are to record the striking fact that glycogen, a polysaccharide closely related to amylopectin in structure, appears not to be debranched by R-enzyme, and to attempt to elucidate the reasons for this difference.

When glycogen (from oysters or from rabbit liver) was incubated with R-enzyme, no significant change occurred in iodine-staining power, in β -amylolysis limit or in reducing power. In a control experiment, a portion of the digest (R-enzyme + glycogen) was added to a solution of β -dextrin (the limit dextrin derived from amylopectin by β -amylolysis). The usual increase in iodine-absorption value occurred, demonstrating that the lack of action on glycogen was not due to inactivation of the enzyme.

The work of Wolfrom, Lassettre, and O'Neill (*J. Amer. Chem. Soc.*, 1951, **73**, 595) proves that the chain-forming (α -1 : 4-) and the branch-forming (α -1 : 6-)links are the same in rabbit-liver glycogen and amylopectin. Each polysaccharide possesses a highly ramified structure, which may be pictured as constituted of congeries of short "basal" chains of α -1 : 4-linked glucose residues, the chains being mutually joined by α -1 : 6-linkages. The structural differences between amylopectin and glycogen are differences of degree of branching and not of type of linkage. End-group assay shows that the *average* basal chain-length of amylopectin (*ca.* 24 glucose units) is twice that of glycogen (*ca.* 12) and as the number of branch-points in a large molecule is the same as the number of non-reducing end groups, it follows that the ratio of branch- to chain-linkages in amylopectin is only half that in glycogen. In both polysaccharides, multiple branching occurs, as first postulated by Meyer and Bernfeld (*Helv. Chim. Acta*, 1940, **23**, 875) and established experimentally for amylopectin by Peat, Whelan, and Thomas (*J.*, 1952, 4546) and for glycogen by Lerner, Illingworth, Cori, and Cori (*J. Biol. Chem.*, 1952, **199**, 641). Further, it is shown in the Experimental section that the limit of conversion of rabbit-liver glycogen by β -amylase is the same as the conversion limit of amylopectin, namely *ca.* 50%. Otherwise expressed, in these two polysaccharides the branch points are randomly distributed with respect both to the number of branches attached to each basal chain and to the position of attachment of a branch to a basal chain. With such random distribution it is to be expected, therefore, that the distance (measured in glucose units) between two adjacent branch-points in glycogen will, on average, be half the distance in amylopectin, and it is reasonable to suppose that the lack of action of R-enzyme on glycogen is attributable to the higher degree of branching of the latter.

Two explanations suggest themselves. First, the branch-linkage may be inaccessible to the operative centres of the enzyme molecule because of the compact

* Part XVIII, *J.*, 1953, 1422.

character of the glycogen structure as compared with the more open network of amylopectin. Secondly, it may be that a substrate susceptible to the debranching action of R-enzyme must contain a minimum number of α -1 : 4-linked glucose units in the vicinity of an α -1 : 6-linkage before the latter can undergo scission. This implies the formation of an enzyme-substrate complex in which the α -1 : 4-linked glucose units in the neighbourhood of a branch-link are directly involved. The number of such units actually participating is not known but it might be suggested that the average number between two adjacent branch points in glycogen is too few for complex formation to occur. That a minimum number of α -1 : 4-linked units is involved can be inferred from the observation that R-enzyme exerts no hydrolytic action on isomaltose or dextran (Part XIV, *loc. cit.*), or on panose, a trisaccharide containing an α -1 : 4-link and an α -1 : 6-link.

The fact that glycogen becomes susceptible to the debranching action of R-enzyme when it is partially hydrolysed by α -amylase provides a means of deciding between these alternative hypotheses. α -Amylolysis of rabbit-liver glycogen was carried to the stage of *ca.* 40% conversion (in terms of maltose) and, after inactivation of the enzyme, portions of the product were treated separately with (i) R-enzyme, (ii) β -amylase, and (iii) a mixture of the two enzymes. The results are shown in the Table which also records the results of parallel experiments in which the substrate was limit β -dextrin (from waxy maize amylopectin) instead of glycogen. Whereas β -amylase acting alone on α -amylolysed glycogen effected a small increase in the number of reducing groups, R-enzyme brought about a substantial increase in reducing power (from 37.5 to 59.3%, as maltose). This increase is

Action of α -amylase, R-enzyme, and β -amylase on glycogen and amylopectin.

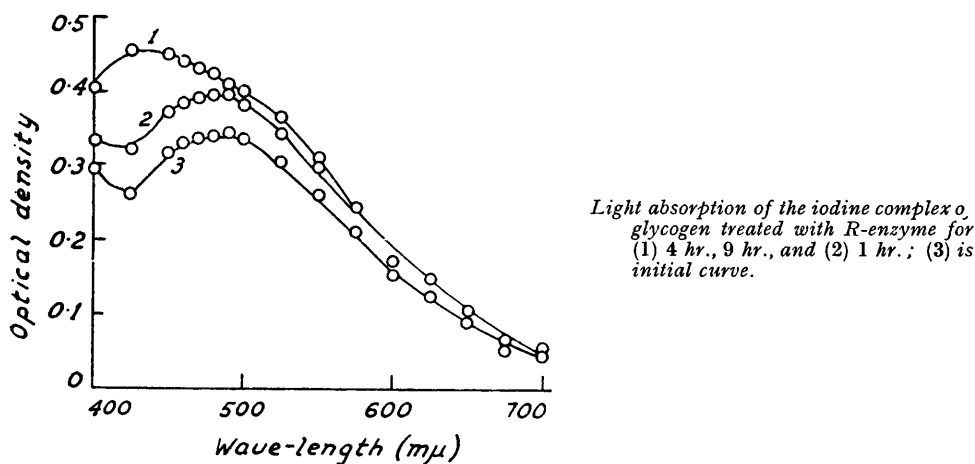
	Reducing power developed (as % theor. maltose)	
	Glycogen	β -Dextrin
α -Amylase alone	37.5	39.0
α -Amylase followed by : (i) β -amylase	44.5	43.8
(ii) R-enzyme	59.3	61.0
(iii) R-enzyme + β -amylase	78.8	80.5

clearly due to the scission of the branch links which are inaccessible to R-enzyme in the original glycogen, inasmuch as the presence of β -amylase during the action of R-enzyme brought about the hydrolysis of *ca.* 80% of the glycogen (Table). The sequence of events thus appears to be (i) fragmentation of the glycogen structure by the random α -amyinolysis of α -1 : 4-links, (ii) scission of the now accessible α -1 : 6-links by R-enzyme, and (iii) β -amyinolysis of the linear chains thus liberated by the debranching enzyme. The demonstration that under these conditions the branch linkages can be hydrolysed establishes the validity of the hypothesis that the compact conformation of the glycogen molecule prevents a sufficiently close approach of the R-enzyme molecule for scission of the branch links. The alternative hypothesis of the availability of too few unsubstituted α -1 : 4-linked glucose units between points of branching becomes untenable because α -amylase, acting by scission of the chains between the branch points actually diminishes, rather than increases, the number of such 1 : 4-linked units.

Considerable interest attaches to a comparison of the behaviour of limit β -dextrin in these experiments with that of glycogen. The β -dextrin was partially hydrolysed by α -amylase to the same extent (deliberately) as was the glycogen, and the Table shows that the polysaccharides are virtually indistinguishable, even quantitatively, as substrates for these enzymes. In a structural sense also, β -dextrin and glycogen are extremely closely related. The action of β -amylase on amylopectin is to remove the outer chains (an outer chain is that part of a chain between a non-reducing end and a branch point) as maltose. Since about 50% conversion occurs (Part XV, *J.*, 1952, 705) it follows that the β -dextrin so produced has a basal chain length of 12 glucose units (confirmed by end-group assay). Further, it contains all the branch links of the original amylopectin and therefore has about the same proportion of branch links as has glycogen. Nevertheless, the branch linkages in β -dextrin are hydrolysable by R-enzyme (Part XIV, *loc. cit.*) whereas those in glycogen are not. The difference is attributable to the fact that while the distribution of branch points is random in the glycogen, the branch linkages in β -dextrin are peripherally arranged in

close proximity to the non-reducing chain ends, so that the molecule retains the open-lattice structure of the amylopectin from which it is derived. The difference between glycogen and β -dextrin is eliminated by α -amylolysis because the compact structure of the glycogen is destroyed thereby. From either glycogen or β -dextrin, fragments of relatively low molecular weight are obtained and these contain, on average, only one α -1 : 6-linkage per molecule (Whelan and Roberts, *Nature*, 1952, 170, 748).

It should be noted that if glycogen is treated first with R-enzyme and then with α -amylase the degradation effected is the same as that by α -amylase only. This clearly indicates the necessity of fragmentation of the glycogen molecule before debranching can occur. Support for this view came from a study of the action of β -amylase on glycogen. The fact that branch points act as barriers to this enzyme means that it operates by the stepwise shortening, two glucose units at a time, of the outer chains of amylopectin or glycogen until a branch point is approached when action ceases and only 2—3 glucose units, adjacent to a branch point, remain of an outer chain. We have seen that β -amylolysis of amylopectin does not alter the physical conformation of the molecule; the open reticulated structure persists in the β -dextrin which, in consequence, is hydrolysable



by R-enzyme. It is to be expected therefore that β -amylolysis of glycogen will yield a limit β -dextrin in which the tight interlocking persists. Experiment showed this inference to be correct; the branch links of glycogen β -dextrin are not attacked by R-enzyme. When the limit β -dextrin from oyster glycogen was incubated with R-enzyme no change was observed, and similarly when R-enzyme and β -amylase were allowed to act together on rabbit-liver glycogen the limiting conversion reached the same value as when β -amylase acted alone.

It should be noted, incidentally, that these experiments prove the specimens of R-enzyme and β -amylase to be entirely free from α -amylase.

It is now necessary to examine a little more closely one of the criteria of R-enzyme activity, namely, the increase in intensity of iodine staining. Measurements were carried out under conditions approximating to those in the determination of blue value (Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924) and in particular at a wave-length of 680 m μ . It was observed that while the iodine-glycogen complex shows very weak light absorption at any wave-length, its absorption at 480 m μ is relatively much stronger than at 680 m μ (see Figure). The staining properties of R-treated glycogen were therefore re-examined over the spectral range 400—700 m μ , with concentrations such that the light absorption was 2.5 times that encountered in B.V. determinations. The results, plotted in the Figure, show that R-enzyme does in fact bring about an appreciable increase in light absorption in the region 400—500 m μ . The Figure also shows that the increase in absorption at the B.V. wave-length (680 m μ) is so small as to be within the limits of experimental error, in agreement with our earlier statement.

The fact remains that R-enzyme affects the structure of glycogen in such a way as slightly to enhance the light absorption of its iodine complex, although it has no detectable effect on the reducing power of glycogen or its susceptibility to β -amylase. An explanation might be found in the postulate that debranching by R-enzyme occurs to a very limited extent, which might well escape observation in the measurement of reducing power or degree of β -amylolysis. With regard to the latter criterion of activity it should be pointed out that, if the structure of glycogen is that envisaged in the Meyer-Bernfeld hypothesis (*Helv. Chim. Acta*, 1940, **23**, 875) and supported by the experiments of Larner *et al.* (*loc. cit.*), then about half of the branch linkages are so situated that their preferential scission would not lead to an enhancement of the extent of β -amylolysis.

We conclude that while most of the branch links of glycogen are inaccessible to R-enzyme, there occur a limited number of regions in which the spacing of the branches is not so close as to prevent R-enzyme action.

EXPERIMENTAL

Glycogen Samples.—The oyster glycogen was a commercial sample from British Drug Houses Ltd. The dried material yielded 96.4% of glucose after acidic hydrolysis by Pirt and Whelan's method (*J. Sci. Food Agric.*, 1951, **2**, 224). The rabbit-liver glycogen was prepared as follows. A rabbit was killed by decapitation and the liver removed and immediately plunged into boiling water (150 ml.) containing 2 drops of glacial acetic acid. After 10 min. the liver was removed, ground with sand, and returned to the original liquid for further boiling (10 min.). The liver debris and sand were filtered from the cooled liquid, and the glycogen was precipitated by the addition of 2 volumes of ethanol. It was then dried by treatment with ethanol and ether. It was purified by adding 4 volumes of glacial acetic acid to its 3% aqueous solution. The precipitate was redissolved in water and the acetic acid precipitation repeated twice more. Finally, the precipitate was dissolved in water, precipitated with 1.5 volumes of ethanol, and dried by washing on the centrifuge with alcohol and ether. Samples of glycogen prepared in this way contain at least 95% of carbohydrate (as glucose) as measured by Pirt and Whelan's method (*loc. cit.*).

Amylopectin β -Dextrin.—Waxy maize starch (10 g., dry wt.) was dispersed in warm 0.1N-sodium hydroxide (250 ml.), cooled, and neutralised (sulphuric acid). 0.2M-Acetate buffer (pH 4.8; 120 ml.), crystalline sweet potato β -amylase solution (27,000 units), and water (to 1 l.) were then added. The digest was incubated for 3 days under toluene at 35°. After this time a portion of the digest was removed, fresh enzyme was added, and quantitative measurements of intensity of iodine staining were made at intervals. Since no fall in the intensity of the iodine stain occurred the large digest was heated to 100° for 3 min., and then dialysed for 4 days against running distilled water. The impermeate was then concentrated to 100 ml. under reduced pressure and the β -dextrin was isolated by freeze-drying.

β -Amylase.—This was the crystalline sweet potato enzyme, kindly provided by Dr. A. K. Balls. Its activity was determined as in Part X (*J.*, 1950, 3566).

α -Amylase.—This was obtained from saliva and was purified by Whelan and Roberts's method (*J.*, 1953, 1298), which is essentially that of Meyer, Fischer, Staub, and Bernfeld (*Helv. Chim. Acta*, 1948, **31**, 2158).

R-Enzyme.—This was prepared as in Part XIV (*J.*, 1951, 1451). In some cases the time of preparation was shortened by using the following method as an alternative to the process of stirring the enzyme solution with starch for 24 hr. at 0°. The enzyme solution and starch were cooled to -5° , shaken for 15 min. at room temperature, then cooled to -5° for 15 min., and the shaking and cooling were each repeated twice more before removal of the starch and precipitation of the enzyme as in the original method.

Reducing Power Determinations.—These were made with the Somogyi reagent (*J. Biol. Chem.*, 1954, **160**, 61). Portions of digests removed for determination of reducing power were first deproteinised (*idem, ibid.*, p. 69). All digests were incubated under toluene.

Iodine-staining.—The solution of iodine contained 0.2% of iodine and 2% of potassium iodide. The Spekker photometer equipped with 4-cm. cells and Ilford No. 608 filters (maximum transmission 680 m μ) was used for determination of absorption value (A.V.) except in the experiment depicted in the Figure, where the Unicam SP. 500 spectrophotometer equipped with 1-cm. cells was used. One drop of 6N-hydrochloric acid was always added to the

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iodine-stained solutions. Water was used as the reference solution for B.V. determination, and iodine for the measurements given in the Figure.

Action of R-enzyme on Oyster Glycogen.—Four digests were prepared as detailed below and diluted to 27 ml.:

Digest no.	1	2	3	4
Glycogen (mg.)	20	20	0	0
R-Enzyme (mg.)	40	0	40	0
0.2M-Sodium acetate buffer, pH 7.0 (ml.)	3.4	3.4	3.4	3.4

The digests were incubated at 35.5° for 16.25 hr. Then portions (5 ml. each) were removed for determination of reducing power. No reducing power was detected in any digest. Portions (equiv. to 1 mg. of glycogen) were also removed from digest 1 and stained with iodine (0.25 ml.) in 25 ml. for determination of A.V. (680 mμ). The values recorded at 0, 3.25, and 16 hr. were, respectively, 0.021, 0.019, and 0.019. At 16 hr. the A.V. (680 mμ) of digest 2 was 0.019. At 17 hr. portions (15 ml. each) of each digest were removed, heated in stoppered flasks on a boiling-water bath for 10 min., cooled, and treated with N-acetic acid (0.35 ml.) to adjust the pH to 4.8. β-Amylase (800 units) and water to 25 ml. were then added. The digests were incubated at 21°, and the reducing powers of each digest determined (5-ml. portions) after 5 and 23 hr. Digests 3 and 4 provided the means of determining the reducing powers of the enzymes. After these times the glycogen in digest 1 had yielded 22.6 and 22.6% of maltose, and in digest 2, 22.6 and 23.7% maltose.

After 17.7 hours' incubation of the original digest 1 a portion (1.6 ml.) was mixed with amylopectin β-dextrin (13 mg.; 3.75 ml.) and 0.2M-citrate buffer (pH 7.0; 1.5 ml.) and incubated at 35.5°. The values of A.V. (680 mμ) of portions (1 ml. each) stained with iodine (0.5 ml.) in a final volume of 50 ml. were 0.253, 0.255, 0.344, 0.350, and 0.379 after 0, 5, 29, 33, and 48 hr. respectively.

Action of R-Enzyme on Rabbit-liver Glycogen.—This was carried out as for oyster glycogen above. The R-enzyme caused no increase in reducing power or in intensity of iodine staining. The percentage conversions of R-treated and untreated glycogen by β-amylase after 5 hours' incubation were, respectively, 47.0 and 46.5%.

Actions of R-Enzyme and β-Amylase on Rabbit-liver Glycogen and Amylopectin β-Dextrin after Treatment with α-Amylase.—Glycogen (40 mg.; 20 ml.) and β-dextrin (40 mg.; 20 ml.) were incubated separately at 35° with 0.2M-acetate buffer (pH 7.0; 3 ml.) and α-amylase (4 mg.; 2 ml.). The progress of each reaction was followed by measurement of reducing power and after 7 hr. a portion (24 ml.) of each digest was heated at 100° for 10 min., then divided into three parts. Digests were prepared as follows: (1) α-treated polysaccharide (5 ml.), R-enzyme (25 mg.; 1 ml.), and β-amylase (100 units; 0.2 ml.); (2) as (1), water replacing R-enzyme; (3) as (1), water replacing β-amylase. Reducing powers were measured after incubation for 13.25 hr. at 35° and the results are given in the Table.

Action of R-Enzyme on Rabbit-liver Glycogen β-Dextrin.—Two digests were prepared containing, in (1) glycogen (20 mg.; 20 ml.), 0.2M-acetate buffer (pH 4.8; 3 ml.), and β-amylase (800 units; 2 ml.), and in (2) buffer, enzyme, and water (20 ml.). After incubation for 14.5 hr. at 35.5° the reducing power (as maltose) was measured on a 5-ml. portion of each digest. At the same time, portions (10 ml. each) were heated at 100° in closed flasks for 10 min., cooled, and treated with N-sodium hydroxide (0.15 ml.) to adjust the pH to 6.3, and R-enzyme (40 mg.; 2 ml.) was added. After incubation for 5.5 hr. at 35.5° the enzyme was inactivated as above, and N-acetic acid (0.15 ml.) was added to each digest to adjust the pH to 4.8, followed by β-amylase (800 units; 2 ml.) and water to 25 ml. The reducing powers of each digest (5 ml. portions) were determined after 3.5 and 16.5 hr. of incubation at 35.5°. The experiment was repeated exactly except that at the second stage water (2 ml.) was added to the digest instead of R-enzyme. Digest (2) in each case provided the means of determining the reducing power of the enzyme. In the first experiment the initial conversion into maltose by β-amylase was 50.3%; after further treatment with R-enzyme and β-amylase the values at the stated times were 49.2 and 53.2%. The three corresponding values in the second series (absence of R-enzyme) were 49.0, 50.0, and 51.6%.

Simultaneous Actions of R-Enzyme and β-Amylase on Rabbit-liver Glycogen.—Two digests were prepared containing (1) glycogen (24.3 mg.; 12 ml.), 0.2M-acetate buffer (pH 7.0; 3 ml.), R-enzyme (30 mg.; 1.5 ml.), β-amylase (300 units; 1.5 ml.), and water to 25 ml.; R-enzyme was omitted from digest (2). Reducing powers (2-ml. portions) were measured at intervals during incubation at 35°. After 22.3, 46.8, and 71.3 hr. the respective conversions into maltose were

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(1) 39·3, 38·1, and 40·1%, (2) 36·4, 39·7, and 41·2%. After 74·3 hr. β -amylase (750 units; 1 drop) was added to each digest. After 94·3 hr. the respective conversions were 40·1 and 40·1%.

Action of R-Enzyme on Rabbit-liver Glycogen. Detection of Increase in Intensity of Iodine Staining.—Glycogen (50 mg.; 25 ml.) was incubated at 35° with 0·2M-acetate buffer (pH 7·0; 3·5 ml.) and R-enzyme (100 mg.; 2·5 ml.). Portions (1·55 ml. each; equiv. to 2·5 mg. of glycogen) were removed at intervals during incubation at 35° and stained with iodine (2·5 ml.) in a total volume of 25 ml. for determination of light absorption in the range 400—700 m μ with the Unicam SP. 500 spectrophotometer and 1-cm. cells. The results are plotted in the Figure.

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