The Mechanism of Carbohydrase Action

5. ACTION OF HUMAN SALIVARY α -AMYLASE ON AMYLOPECTIN AND GLYCOGEN*

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When human salivary α -amylase acts on whole starch (amylose + amylopectin) there is produced a mixture of fermentable and unfermentable sugars (Myrbäck, 1948). The fermentable sugars consist of maltose, maltotriose and/or glucose (see below). The non-fermentable sugars (α -limit dextrins) are a mixture of higher oligosaccharides. Since the amylose component of starch, which consists essentially only of 1:4-linked α -glucose units, can be converted by the amylase completely into maltose and maltotriose (Whelan & Roberts, 1953) the α -limit dextrins must arise from the amylopectin component and may be assumed to contain the α -1:6-bonds which constitute the points of branching in the dendritic structure of this polysaccharide. The object of this investigation was to characterize the α -limit dextrins and thereby to define the action of salivary α -amylase on amylopectin and the related polysaccharide glycogen. A preliminary account of this work has already appeared (Whelan & Roberts, 1952).

EXPERIMENTAL

Materials

Enzymes. α -Amylase was prepared from human saliva as by Whelan & Roberts (1953), by the method of Meyer, Fischer, Staub & Bernfeld (1948). R-Enzyme was prepared from broad beans ('Prolific Longpod', Sutton and Sons, Reading) as by Hobson, Whelan & Peat (1951).

Substrates. Glycogen was prepared from the livers of pregnant rabbits. The livers were boiled with water as soon as possible after removal from the animal. After cooling, the liver was ground with acid-washed sand and re-extracted with the same liquid by boiling for 20 min. Then the solution was cooled, centrifuged, and the supernatant solution filtered through paper. The glycogen was precipitated with ethanol (2 vol.) and washed with 66% (v/v) ethanol, and ether. Finally it was purified by precipitation with acetic acid (Bell & Young, 1934; Schlamovitz, 1951).

The amylopectin was a gift from Dr T. J. Schoch. This was waxy-maize starch, which is almost pure amylopectin in the sense of being free from amylose and contains little,

if any, esterified phosphorus (Schoch, 1953). It was prepared from hand-sorted single-cross Tapicorn seed, (Bear Hybrid Co., Decatur, Illinois, U.S.A.) as by Schoch (1957).

Buffers. These were 0.2M-sodium acetate-acetic acid (pH 7.0) and 0.2M-sodium citrate-citric acid (pH 7.0).

Methods

Digests. Waxy-maize starch was moistened with ethanol and dissolved in 0.6N-sodium hydroxide by heating in a boiling-water bath for 5 min. After cooling, the alkali was neutralized to phenolphthalein either with sulphuric acid [digests (1) and (2), see below] or hydrochloric acid [digest (3)]. Glycogen and the enzymes were dissolved in water. After the substrate had dissolved, the buffer was added and then the enzyme. The digest was covered with toluene or toluene-nitrobenzene (3:1, v/v) and incubated at 35°. After incubation the enzyme was inactivated by heating in a boiling-water bath for 3 min.

Reducing power, developed during the digestions, was measured with the Somogyi (1945*a*) reagent, the time of heating with the reagent being 60 min., in order to develop full reducing power (Whelan, Bailey & Roberts, 1953). When R-enzyme was present the digest portion was treated with Somogyi's (1945*b*) deproteinizing reagents before measuring reducing power. A digest containing buffer and enzyme but no substrate was used in each case to estimate the reducing power of the non-substrate components.

Chromatography. Sugars were examined by chromatography on Whatman no. 54 paper in one or more of the following solvents: water-saturated phenol (Partridge, 1948), pyridine-fusel oil-water (1:1:1, by vol.), propanol-ethyl acetate-water (6:1:3, by vol.) and butanol-acetic acid-water (4:1:5, by vol.). The sugar spots were revealed with aniline hydrogen phthalate (Partridge, 1949), benzidine-trichloroacetic acid (Bacon & Edelman, 1951) or silver nitrate-sodium hydroxide (Trevelyan, Procter & Harrison, 1950).

Charcoal-Celite columns were prepared and used as by Whelan *et al.* (1953). All column dimensions given are those of the adsorbent. Sugars were detected in the fractions by measurement of optical rotation in a 4 dm. tube. All weights of products quoted were estimated by reducing power, where the product was glucose, or by acid hydrolysis to glucose, as by Pirt & Whelan (1951). The weights are quoted in terms of combined glucose ($C_6H_{10}O_5$).

Characterization of products. The products of enzyme action were characterized by one or more of the following methods: measurement of $[\alpha]_D$ (in water) (4 dm. tube), concentration being estimated by acid hydrolysis to glucose; R_p and co-chromatography with authentic material; formation of the β -acetate (Whelan & Roberts, 1953), measuring the $[\alpha]_D$ (in chloroform) and m.p. (un-

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corrected); degree of β -amyolysis (Whelan & Roberts, 1954); measurement of degree of polymerization (DP), based on the reducing power, relative to that of maltose (Whelan *et al.* 1953).

α -Amyolysis of amylopectin (1)

Waxy-maize starch (8.063 g., moisture content 13.1%, starch content 6.77 g. by acid hydrolysis) was incubated at 35° in 20 mm-sodium citrate buffer (pH 7.0, 1 l.) with salivary α -amylase (1 g.). After 48 hr. the apparent percentage conversion into maltose was 71.6%. The enzyme was inactivated and a portion of the digest (982 ml., 6.65 g. of starch) was fractionated on a charcoal-Celite column (4.2 cm. $\times$ 95 cm.). No monosaccharide was eluted with water (3.3 l.) but di- (2.42 g.), tri- (1.74 g.) and higher oligo-saccharide fractions (1.97 g.) were obtained, a recovery of 92.4%. The di- and tri-saccharide fractions corresponded in R_F values to maltose and maltotriose respectively. The disaccharide had DP 2.02, $[\alpha]_D +134^\circ$, and its β -acetate m.p. 159°, $[\alpha]_D +63^\circ$. The trisaccharide had DP 3.00, $[\alpha]_D +169^\circ$, and its β -acetate m.p. 136°, $[\alpha]_D +87^\circ$. These are the properties of maltose, maltotriose and their derivatives (Whelan & Roberts, 1953).

The higher oligosaccharides had DP 8.89. A small-scale digestion with α -amylase showed an increase in reducing power. Accordingly, the dextrins (1.88 g.) were incubated at 35° in 8 mm-sodium citrate buffer (pH 7.0, 250 ml.) with salivary α -amylase (0.881 g.). The reducing power of a 0.5 ml. portion of digest rose from 3.90 ml. of 5 mM-thio-sulphate (45 min. heating) to 6.00, 6.14, 5.99 and 6.12 ml. after 7, 9, 11 and 14 days respectively. The activity of the enzyme remaining after this time was such that soluble starch was rendered achromic after a few minutes incubation. The enzyme was inactivated and a portion of the digest (234 ml., 1.76 g.) was fractionated on charcoal-Celite (4.0 cm. $\times$ 72 cm.). No monosaccharide was eluted with water (3.2 l.). Ethanol (7.5%) eluted maltose (0.103 g.) but 15% ethanol (5.4 l.) did not elute any trisaccharide. Ethanol (50%) desorbed higher oligosaccharides (1.358 g.). The recovery of maltose and oligosaccharides was therefore 82.4%. When corrected for this degree of recovery, assumed to be 82.4% for maltose itself, the total yield of maltose in the two experiments was 2.56 g. The molar ratio maltose:maltotriose (1.74 g., see above) was 2.20:1.

When examined by paper chromatography the higher oligosaccharides showed substances migrating approximately in the positions of malto-pentaose, -hexaose and -heptaose and spots of even lower R_F . The average DP was 7.06. When the dextrins (81.5 mg.) were incubated at 35° with R-enzyme (70 mg.) in 12 mm-sodium acetate buffer (pH 7.0, 25 ml.) the reducing power of a 3 ml. portion rose from 5.33 ml. of 5 mM-thiosulphate to 11.47 ml. after 41 hr., remaining constant thereafter, at 2.15 times the initial value.

α -Amyolysis of amylopectin (2)

Waxy-maize starch (11.42 g., wet wt., starch content 9.99 g. by acid hydrolysis) was incubated at 35° in 20 mm-sodium citrate buffer (pH 7.0, 1 l.) with salivary α -amylase (1.51 g.) in 1 l. of solution. After 9 days incubation the apparent conversion into maltose was 60.6%. A further amount of enzyme (0.827 g.) was added and the reducing power increased to 71.6% conversion during the next

24 hr. Thereafter an increase of about 0.5% conversion/day took place over 27 days and the reaction was terminated by inactivating the enzyme at 85.6% conversion. The digest (960 ml., 9.56 g. of starch) was fractionated on charcoal-Celite (5 cm. $\times$ 118 cm.). Glucose (0.29 g., estimated by optical rotation), maltose (4.64 g.) and maltotriose (1.88 g.) were obtained and their identities checked by measurement of R_F . The α -dextrins (2.60 g.) were eluted with 50% ethanol. When maltotriose was incubated with the salivary α -amylase used in this experiment a slow increase in reducing power took place, amounting to 10% after 59 days.

The α -dextrins had DP 7.13 and were not further attacked by α -amylase, when treated under the same conditions as the original amylopectin. They were identical in composition with the first preparation, as revealed by paper chromatography, and the two preparations were combined.

Enzymic debranching of amylopectin α -limit dextrins. The combined α -limit dextrins (see above, 3.47 g.) were incubated at 35° in 20 mm-sodium acetate buffer (pH 7.0, 500 ml.) with R-enzyme (0.924 g.). After 140 and 168 hr. the apparent conversion into maltose was constant at 62.0%. The initial value was 28.3% and the ratio final: initial reducing power was therefore 2.19:1. A portion of the digest (8 ml.) was then incubated for 24 hr. with R-enzyme (50 mg.), when no further increase in reducing power took place. Accordingly, the enzyme in the main digest was inactivated and a portion (465 ml., 3.22 g.) fractionated on charcoal-Celite. No glucose was found. Maltose and maltotriose were obtained in pure form but the last fractions containing the triose also contained maltotetraose and similarly some of the tetraose was contaminated with pentaose. The pentaose was followed by higher oligosaccharides, mainly hexaose and heptaose, which were combined together. To estimate the yields of individual components of mixed fractions the mixtures were treated with β -amylase under conditions which would hydrolyse maltotetraose and higher maltodextrins but not maltotriose (Whelan *et al.* 1953). The products were quantitatively analysed on charcoal-Celite (Whelan & Roberts, 1953; Whelan & Roberts, 1954). In the case of the maltotetraose-maltopentaose mixture, for example, the latter yields maltotriose on β -amyolysis, the former does not, but both produce maltose. Accordingly, from the amounts of maltose and maltotriose produced on β -amyolysis the composition of the original mixture could be assessed. The total yields of the saccharides produced from the α -limit dextrins and their properties are reported in Table 1. These properties, together with the correspondence of the R_F values of these substances with authentic maltodextrins, serve to justify their description as maltodextrins.

α -Amyolysis of amylopectin (3)

Waxy-maize starch (13.03 g., moisture content 7.15%, starch content 11.13 g. by acid hydrolysis) was incubated at 35° in 20 mm-sodium citrate (pH 7.0, 1 l.) with salivary α -amylase (1 g.). After dissolving the starch in alkali the solution had been neutralized with hydrochloric acid, not, as in Expts. (1) and (2), with sulphuric acid (see Methods). After 19 days the apparent conversion into maltose was 73.6%. More enzyme (0.510 g.) was added, and after 33, 43 and 48 days the conversion was constant at 76.8%. After inactivating the enzymes the digest (985 ml.; 10.99 g. of starch) was fractionated on charcoal-Celite (4.0 cm. $\times$

120 cm.) as usual. Glucose (81 mg.), maltose (4.73 g.) and maltotriose (3.00 g.) were obtained. The column was then eluted with 23% ethanol (4.5 l.), 25% ethanol (3 l.), 27.5% ethanol (4 l.), 40% ethanol (4 l.) and 50% ethanol (4 l.), fractions of 100 ml. or 200 ml. being collected. The fractions reserved for examination were *A* (eluted between 1.3 l. and 1.6 l. by 23% ethanol), *B* (3.0–3.8 l., 23% ethanol) and *C* (13.4–14.4 l. 40% ethanol).

Fraction A. This was a single substance migrating slightly more slowly on paper than maltopentaose. It had *DP* 4.85. A portion (10 mg.) was treated with R-enzyme (1 ml., 10 mg.) for 48 hr. at 35°. When fractionated on paper, substances migrating with maltose and maltotriose were seen.

Fraction B. As judged by *R_F*, this contained mainly heptasaccharide(s) with a small amount of hexasaccharide(s). It had *DP* 6.82. A portion (20 ml., 100 mg.) was reduced with sodium borohydride (1.5 ml., 22 mg.) for 3 hr. at room temperature. Unchanged borohydride was decomposed with acetic acid and the solution was neutralized and diluted to 25 ml. Of this, 20 ml. were incubated with R-enzyme (65 mg., 1 ml.) and acetate buffer (pH 7.0, 3 ml.). The reducing power became constant between 46 and 69 hr. Assuming the dextrin to be singly branched the reducing power at this time was 85.5% of that expected if complete debranching had occurred. The digest was examined by paper chromatography in comparison with that of an unreduced sample, also treated with R-enzyme. The observations are described in the Results section.

Fraction C. This had *DP* 14.1 and when treated with R-enzyme as above the reducing power of portions of the digest after 16.5 and 40 hr. were 8.98 and 9.15 ml. of 5 mM-thiosulphate, compared with an initial value of 2.63 ml. The mean final reducing power of 9.07 ml. therefore represented an increase of 3.44 times.

α-Amylolysis of glycogen

Rabbit-liver glycogen (7.75 g., glycogen content 7.26 g. by acid hydrolysis) was incubated at 35° in 20 mM-sodium citrate (pH 7.0, 1 l.) with salivary *α*-amylase (0.950 g.) for 9 days. The apparent conversion into maltose was then 42.0%. After adding more enzyme (0.750 g.) the conversion increased by about 2% per day until a value of 56.4% was reached, remaining constant for a further 8 days. After inactivating the enzyme the digest (7.53 g. of glycogen) was fractionated on charcoal-Celite (4.0 cm. × 120 cm.). No glucose was obtained. Maltose (and maltulose) (1.82 g.) were eluted with 7.5% ethanol and the higher dextrins (4.50 g.) were desorbed with 55% ethanol. These dextrins (4.39 g.) were incubated at 35° in 1 l. of 20 mM-acetate buffer (pH 7.0) with R-enzyme (2.88 g.) for 39 hr. The reducing power rose from 40.7 to 58.4%, as maltose. The addition of more enzyme (1.44 g.) did not change the reducing power appreciably, the values after a further 48 and 72 hr. being 59.1 and 59.2% respectively. The enzyme was inactivated and a portion of the digest, equivalent to 4.25 g. of the original glycogen, was fractionated on charcoal-Celite (4.0 cm. × 80 cm.) as in Expt. 2 for waxy-maize starch (see above). No glucose was obtained and the disaccharide fraction contained only maltose. The yields and properties of the products are in Table 2.

A second *α*-amyinolysis of glycogen was performed to determine the amounts of maltose and maltotriose obtainable by *α*-amyinolysis. (In the first experiment the maltotriose was not separated from the *α*-limit dextrins before they were debranched.) From 6.16 g. of *α*-amyolysed glycogen at an apparent conversion into maltose of 60.3% there were obtained 2.05 g. of maltose (+ maltulose) and 1.51 g. of maltotriose, a molar ratio of 2.01:1.

Table 1. *Properties of the maltodextrins obtained on debranching the amylopectin α-limit dextrins*

DP represents degree of polymerization, based on reducing power relative to that of maltose. Figures in parenthesis are theoretical degrees of amyinolysis (Whelan, Bailey & Roberts, 1953; Whelan & Roberts, 1953, 1954).

Dextrin	Yield (mg.)	<i>DP</i>	[α] _D in water	Degree of amyinolysis (%)	
				<i>α</i> -Amylase	<i>β</i> -Amylase
Maltose	526	2.00	135°	—	—
Maltotriose	785	2.94	159	—	98 (100)
Maltotetraose	1028	3.84	173	95 (100)	91 (100)
Maltopentaose	211	4.92	179	81 (80)	78 (80)
Higher dextrins	295	6.68	183	—	85

Total yield 2.845 g., from 3.22 g. of *α*-dextrins.

Table 2. *Properties of the maltodextrins obtained on debranching the glycogen α-limit dextrins*

Dextrin	Yield (mg.)	<i>DP</i>	[α] _D in water	Degree of amyinolysis (%)*	
				<i>α</i> -Amylase	<i>β</i> -Amylase
Maltose	561	2.00	135°	—	—
Maltotriose†	1930	3.00	154	—	94
Maltotetraose	852	4.00	164	92	85
Maltopentaose	215	4.92	174	81	74
Higher dextrins	247	6.11	—	—	83

Total 3805 g., from 4.25 g. of *α*-dextrins.

* See Table 1.

† This figure includes the maltotriose liberated in the initial *α*-amyinolysis of the glycogen and not separated from the *α*-limit dextrins before debranching (see Experimental section).

Calculation of the degree of branching of amylopectin and glycogen

Method 1. When the α -limit dextrins of amylopectin were debranched with R-enzyme the reducing power rose to 215% of the original value (see Expt. 1). Therefore the dextrins (*DP* 7.06) contained 1.15 branch links per molecule. The yield of dextrins from 6.65 g. of amylopectin was 1.99 g., allowance being made for incomplete recoveries of material (92.4%, 82.4%) in the two fractionations required for the isolation of the dextrins. The only other products of the α -amyolysis were maltose and maltotriose. All the branch links of the amylopectin were therefore 'concentrated' in the α -limit dextrins. The number of glucose units for each enzymically hydrolysable branch point in the original amylopectin is therefore given by $(6.65/1.99) \times (7.06/1.15) = 20.4$.

In a branched molecule of high molecular weight such as amylopectin the number of branch points should equal the number of non-reducing-end groups. The number of such end groups determined by periodate oxidation of the same sample of amylopectin was 1 per 24.4 glucose residues (Peat, Whelan & Thomas, 1956).

Method 2. The number of molecules of α -limit dextrins can be calculated from the yields of maltodextrins obtained after debranching (Tables 1 and 2). If all the α -limit dextrins contained only 1 branch point per molecule this molar quantity would be half that of the maltodextrins. Limit dextrins with higher degrees of branching were, however, also present. To make an approximate calculation of the amount of these dextrins two assumptions were made, first that there were no α -limit dextrins containing more than two branch points, and secondly that the maltodextrins greater in size than maltopentaose ('higher dextrins', Tables 1 and 2) represented the base chains of the doubly branched dextrins. (The base chain is the sequence of 1:4-linked α -glucose units carrying the reducing group.) If the molar quantity of maltose-maltopentaose is M_1 and of higher maltodextrins is M_2 , then the molar quantity of α -limit dextrins is $M_2 + (M_1 - 2M_2)/2 = M_1/2$, and the 'molar' quantity of branch points is

$$2M_2 + (M_1 - 2M_2)/2 = M_2 + M_1/2.$$

The values in Table 1 correspond to 2.88 'm-moles' of branch points. These are associated with a total quantity of glucose residues in the maltodextrins of $2845/162 = 17.5$ m-moles (Table 1). The number of glucose residues per branch point is therefore $17.5/2.88 = 6.08$. This is to be compared with the value of $7.06/1.15 = 6.13$, determined by method 1. By further calculations made as in method 1 the number of glucose residues per branch point in the original amylopectin is 20.3. In view of the agreement of this value with that derived by method 1 the error introduced by the assumptions made in this second method of calculation does not seem to be of any consequence.

The same calculations can be made for glycogen. The results in Table 2 have to be adjusted to allow for the fact that the maltotriose produced by α -amylase was not separated from the α -limit dextrins before they were debranched with R-enzyme. The amount of maltotriose formed by α -amylase was determined in a separate experiment, described above.

The number of glucose residues per branch point of the α -limit dextrins, calculated from the quantities of malto-

dextrins formed from the limit dextrins by R-enzyme, is 6.07, the same as for amylopectin. The calculated proportion of branch links in the whole glycogen molecule is 1 per 12.0 glucose residues. By periodate oxidation [semi-micro method of Peat, Whelan & Turvey (1956)] Dr J. R. Turvey found the non-reducing end-group content of this glycogen to be 1 per 12.8 glucose residues.

RESULTS AND DISCUSSION

α -Amyolysis of waxy-maize starch

Amylopectin was treated with α -amylase and when the rate of reaction had become very low the products were examined by paper chromatography in propanol-ethyl acetate-water. Substances were seen which migrated with maltose and maltotriose but a spot corresponding to maltotetraose was absent. After maltotriose, in order of descending R_F , was a substance migrating slightly more slowly than maltopentaose, followed by a series of spots extending to the origin. Large-scale separation of the digest components enabled the maltose and maltotriose to be characterized and identified while the larger molecules were collected as a single fraction, having an average *DP* 8.89. These were found still to be susceptible to α -amyolysis, the reducing power being raised by about 70% on further treatment with enzyme. When this digestion mixture was fractionated maltose was obtained, but not maltotriose. The higher oligosaccharides were no longer susceptible to α -amyolysis, and had *DP* 7.06. Glucose was not detected in the first or second digestions, either on paper chromatography of the whole digest or during the large-scale fractionation. The molar ratio total maltose:maltotriose was 2.20:1. This is different from the ratio (2.39:1) of the two sugars produced from amylose (Whelan & Roberts, 1953). Taken with the fact that maltotriose was not formed at all in the second digestion the results indicate that the 1:6-branch points of amylopectin, not present in amylose, exert a steric effect on the course of the enzyme action on those parts of the molecule giving rise to these two sugars.

A second α -amyolysis of amylopectin was performed which was prolonged much beyond the time of the first experiment. In this case a small amount of glucose was obtained, with maltose, maltotriose and α -limit dextrins. It seemed that the glucose was formed from the maltotriose and not from the α -limit dextrins. There were three reasons for this conclusion. First, the α -limit dextrins had the same *DP* (7.13) as those in the first experiment, when glucose was not formed. Secondly, the paper-chromatographic pattern of the two limit-dextrin preparations was the same. Thirdly, assuming the glucose to have arisen from maltotriose, the molar ratio maltose:maltotriose

from this hydrolysis would have been 2:21:1, the same as in the first experiment.

When the two limit-dextrin preparations were combined and treated with R-enzyme to hydrolyse the 1:6-linkages, the products proved to be maltose, malto-triose, -tetraose, -pentaose and higher maltodextrins (Table 1).

Action pattern of salivary α -amylase on amylopectin

It is evident that the α -1:6-branch links of amylopectin confer resistance to α -amylolysis on α -1:4-bonds in their vicinity. If this were not so then isomaltose (6-O- α -D-glucopyranosyl-D-glucose) would be found as a product of the amylolysis. The fact that the smallest α -limit dextrin found was a pentasaccharide suggests that three α -1:4-bonds close to the branch link are rendered stable to α -amylase action. This pentasaccharide was split by R-enzyme into maltose and malto-triose. There are five ways in which these two sugars can be joined to each other through an α -1:6-link and in formulating a hypothesis of α -amylase action we assumed previously that the pentasaccharide had the most symmetrical of these structures, that in which maltose is joined to the central glucose unit of maltotriose, i.e. 6³- α -maltosylmaltotriose [Whelan & Roberts (1952); nomenclature as by Whelan (1960)]. Structural analysis of the pentasaccharide, however, proved the maltose to be joined to the non-reducing-end unit of maltotriose, i.e. the pentasaccharide is 6³-maltosylmaltotriose (Fig. 2a, Bines & Whelan, 1960). The amylase-resistant links are therefore not symmetrically disposed around the branch link but are situated as in Fig. 1; the hypothesis of α -amylase action therefore needs to be revised.

Under the conditions employed in these experiments salivary α -amylase rapidly hydrolyses non-terminal α -1:4-bonds of starch but does not attack the terminal bonds at the reducing or non-reducing chain ends (Whelan & Roberts, 1953). Maltotriose, containing only terminal bonds, is therefore resistant to α -amylolysis and only begins to break

down under conditions of prolonged enzyme action (digestion no. 2) or with relatively high concentrations of enzyme (Walker & Whelan, 1960). An α -limit dextrin formed under the chosen experimental conditions will therefore be one in which the α -1:4-bonds are resistant to amylolysis either by reason of their proximity to the 1:6-link or because they occupy terminal positions. There are eight structures which fit this requirement (Fig. 2). These are the pentasaccharide (Fig. 2a), of proven structure, and a range of hexa- to octa-saccharides, whose structures have been derived from that of the pentasaccharide by adding one or more terminal-linked glucose units. Each of these complies with the above definition of an α -limit dextrin. In these dextrans the base chain, i.e. that carrying the reducing group, varies in length from 3 to 5 glucose units, and the side chain from 2 to 3 glucose units. On debranching with R-enzyme the α -limit dextrans should therefore give rise to maltose, malto-triose, -tetraose and -pentaose, and these were in fact found. In addition, however, maltohexaose and higher maltodextrins were also found but their presence can be accounted for (see below). Further evidence in support of the hypothesis was obtained by subfractionating the α -limit dextrans to yield a heptasaccharide component. When this was debranched with R-enzyme the products were maltose, malto-triose, -tetraose and -pentaose. (The triose and tetraose were the major components of the mixture.) This agrees with the predicted products from dextrans e, f and g (Fig. 2). When the heptasaccharide was reduced with sodium borohydride and then debranched the only reducing sugars formed were maltose and maltotriose. The side chains of the dextrin were therefore maltose and maltotriose and the base chains malto-tetraose and -pentaose. This again agrees with prediction.

It remains to account for the formation of malto-hexaose and larger maltodextrins on debranching the α -limit dextrans. The reason for this was suggested from the fact that during the debranching process the reducing power was more than doubled, increases of 2.15 and 2.19 times being noted in two experiments. This was suggestive of the presence of dextrans containing more than one branch point. Such dextrans were obtained by subfractionating the α -limit dextrans. The particular fraction examined had average DP 14.1 and on debranching the reducing power increased 3.44 times. Evidently there were dextrans with two, three and perhaps more branch points per molecule. These dextrans would be expected to arise from regions of the molecule in which the branch points are so close that all intervening links are resistant to α -amylase. In constructing possible formulae for such dextrans it became evident that to satisfy the

Fig. 1. A segment of the amylopectin or glycogen molecule in the vicinity of a branch point, showing the positions of α -1:4-links resistant to α -amylase. O, α -Glucose unit; —, hydrolysable 1:4-link; ~, resistant 1:4-link; ↓, 1:6-link.

requirement that they should contain maltohexaose and higher maltodextrins within the molecule they would have to exhibit multiple branching. By this is meant the occurrence of two or more C₆-substituted glucose residues in a maltodextrin chain and waxy-maize starch is known to contain multiple branches (Peat, Whelan &

Thomas, 1952, 1956). Fig. 3 shows the largest α -limit dextrins containing two and three branch links per molecule which the hypothesis permits. Both the singly- and multiply-branched molecules are shown. The singly-branched dextrins do not contain a maltodextrin chain longer than five units but the upper limit is nine for the multiply-

Fig. 2. Singly-branched α -limit dextrins of amylopectin and glycogen. $\circ$, α -Glucose unit; $\oslash$, reducing-end glucose unit; —, 1:4-link; $\downarrow$, 1:6-link.

Fig. 3. Structures of the largest doubly- and triply-branched α -limit dextrins exhibiting single (a, b) and multiple (c, d) branching. Symbols as in Fig. 2.

branched dextrans. The presence of the malto-dextrans of $DP > 5$ in the debranched α -limit dextrans is therefore consistent with our hypothesis of salivary α -amylase action.

α -Amylolysis of glycogen

The pattern of the α -amylolysis of glycogen seems to be the same as for amylopectin. Maltose, maltotriose (molar ratio 2.03:1) and α -limit dextrans of the same type as from amylopectin are formed and the products of debranching of the dextrans are the same (Table 2). Glucose was not found after α -amylolysis or after debranching with R-enzyme, but a disaccharide product was found which was not encountered, except in very small amount, with amylopectin. This was maltulose (4-O- α -D-glucopyranosyl-D-fructose) and this finding has already been reported (Peat, Whelan & Roberts, 1952). The yield of maltulose varied in different experiments according to the source of the rabbit-liver glycogen and its origin is the subject of continuing investigation.

Correlation of this work with other studies of α -amylolysis

The products we have characterized as resulting from the α -amylolysis of amylopectin and glycogen, i.e. maltose, maltotriose and α -limit dextrans are end products of α -amylolysis in the sense that when tested with a concentration of enzyme similar to that used in their preparation no further breakdown took place. By using far greater amounts of enzyme, with the incorporation of chloride ions to activate the enzyme, the maltotriose and α -limit dextrans can be caused to undergo further hydrolysis (Walker & Whelan, 1960). The products described here, except maltose, are not therefore true end products, but the attainment of the levels of α -amylolysis at which these substances persist in a metastable condition is readily reproducible, as evidenced by the number of such experiments reported here. The difficulty of defining the true limit of α -amylolysis of amylopectin and glycogen is discussed by Walker & Whelan (1960). The level of amylolysis achieved here seems to represent the lowest in what is possibly a series of discrete stages of amylolysis, readily reproducible and defined in terms of the conditions of the experiment, i.e. concentration of enzyme and time of incubation. A recognition of the existence of these stages in the enzyme action serves to explain why different workers report different 'end products' of α -amylolysis (Walker & Whelan, 1960).

Proportions of 1:6-links in amylopectin and glycogen

The isolation and analysis of the products of α -amylolysis and of enzymic debranching of the α -limit dextrans were performed as far as possible on

a quantitative basis. It thus became possible to calculate the proportions of 1:6-branch links in amylopectin and glycogen. Two methods were used and are described in the Experimental section. The values so obtained were in reasonable agreement with the estimates of non-reducing end-group content made by periodate oxidation. This measure of agreement permits three conclusions. First, R-enzyme splits all the branch points in the α -limit dextrans. This is in contrast with its incomplete action on amylopectin and its almost complete lack of action on intact glycogen (Peat, Whelan & Thomas, 1956; Peat, Whelan, Hobson & Thomas, 1954). Secondly, α -amylase does not split any of the branch points susceptible to R-enzyme action. If it did then the determined proportion of branch points would be lower than of non-reducing end groups. Thirdly, if, as is believed (Hobson *et al.* 1951), R-enzyme is specific for the hydrolysis of α -1:6-links, then most, if not all, of the branch links of amylopectin and glycogen are of this type.

SUMMARY

1. Amylopectin (waxy-maize starch) is degraded by low concentrations of human salivary α -amylase to yield maltose (42%), maltotriose (28%) and α -limit dextrans containing the α -1:6-branch links of the amylopectin. Glycogen gives the same products.
2. The smallest α -limit dextrin is a pentasaccharide, the α -1:6-link of which is split by R-enzyme to yield maltose and maltotriose. α -Limit dextrans containing two and three branch links per molecule are also present.
3. The maltodextrans formed by R-enzyme action on the mixed α -limit dextrans have been separated, identified and analysed. A hypothesis to explain the formation of the limit dextrans has been formulated. It is suggested that under the chosen experimental conditions three α -1:4-bonds in defined positions adjacent to the branch link are resistant to α -amylase action. The hypothesis accounts for the presence of the α -limit dextrans containing more than one branch link only if the parent polysaccharides exhibit multiple branching.
4. By quantitatively analysing the products of α -amylolysis and those formed on subsequently debranching the α -limit dextrans, the proportions of α -1:6-links in amylopectin and glycogen have been calculated. The results agree with the measured proportions of non-reducing-end groups and it is concluded that salivary α -amylase does not split the α -1:6-links in these polysaccharides.

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The Mechanism of Carbohydrase Action

6. STRUCTURE OF A SALIVARY α -AMYLASE LIMIT DEXTRIN FROM AMYLOPECTIN

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Roberts & Whelan (1960) studied the action of a low concentration of human salivary α -amylase on amylopectin and at the limit of the reaction found the products to be maltose, maltotriose and a series of larger molecules, termed α -limit dextrins. These were split by R-enzyme, believed to be specific for the hydrolysis of α -1:6-glucosidic linkages (Hobson, Whelan & Peat, 1951), into maltodextrins. The α -limit dextrins therefore contained the amylase-resistant branch linkages of the original polysaccharide. The smallest of these dextrins is a pentasaccharide (Peat, Whelan & Roberts, 1956) and this communication describes its structural analysis. A preliminary report has already been made (Whelan & Bines, 1955).

EXPERIMENTAL

Methods. All evaporations were carried out under reduced pressure at about 40°. Measurements of optical rotation were made in a 4 dm. tube. R_F values of sugars

were measured in butanol-acetic acid-water (4:1:5, by vol.) or propanol-ethyl acetate-water (6:1:3, by vol.), and of methylated sugars in butanol-ethanol-water-ammonia (sp.gr. 0.88) (40:10:19:1, by vol.). Sugar spots were revealed with benzidine-trichloroacetic acid (Bacon & Edelman, 1951) or silver nitrate-sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Ionophoresis was carried out in 0.2M-boric acid-0.05M-borax (2:3, v/v; pH 8.7) and M_g values calculated as by Foster (1953). Charcoal-Celite columns were prepared as by Whelan, Bailey & Roberts (1953). Solutions of the pentasaccharide used for measurement of optical rotation and of pentasaccharide, panose and isomaltotriose (Turvey & Whelan, 1957) for periodate oxidation were treated with Somogyi's (1945) deproteinizing reagents before use. This was because these sugars had been isolated from charcoal-Celite columns (see Whelan *et al.* 1953). The concentrations of these solutions were determined by acid hydrolysis to glucose (Pirt & Whelan, 1951).

Preparation of methyl sugars. 2:3-Di-O-methyl-D-glucose was prepared as by McCloskey & Coleman (1945) and 2:3:6-tri-O-methyl-D-glucose as by Haworth, Hirst & Webb (1928). Other methyl sugars used were kindly given by Drs D. J. Bell, E. J. Bourne and A. G. Ross.

Preparation of the pentasaccharide. The pentasaccharide was isolated from digestion mixtures of waxy-maize starch

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