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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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and purified human salivary α -amylase prepared by Dr P. J. P. Roberts and described by Roberts & Whelan (1960). Fractions from several such digests were combined and refracted on charcoal-Celite to yield about 1 g. of chromatographically and ionophoretically pure material having $[\alpha]_D^{20} + 181^\circ$ in water (c, 0.08). Maltopentaose has $[\alpha]_D + 180^\circ$ (Whelan *et al.* 1953). The dextrin was resistant to salivary α -amylase when tested as for maltodextrins as by Whelan & Roberts (1953).

Methylation experiments

Methylation of the pentasaccharide. The pentasaccharide (720 mg.) was first methylated as by Albon, Bell, Blanchard, Gross & Rundell (1953), being dissolved in 50% (v/v) aqueous dioxan and treated successively with dimethyl sulphate (3.65 ml.) and 30% (w/v) aqueous sodium hydroxide (7.3 ml.) at room temperature. The mixture was stirred for 15 hr., when all reducing power had disappeared. Sodium hydroxide (8 ml.) and dimethyl sulphate (4 ml.) were then added alternately at 10 min. intervals until totals of 80 and 40 ml. respectively had been added, when, after a further 20 min., the solution was heated on a boiling-water bath for 30 min. to decompose methyl sulphates. After cooling, the solution was neutralized with sulphuric acid. Most of the sodium sulphate was precipitated by adding ethanol (1 vol.) and storing at 0° overnight. The filtrate was evaporated to dryness and the residue again treated with the methylating agents. In all, three such methylations were performed and after the third methylation the neutralized reaction mixture was extracted with chloroform (3 $\times$ 50 ml.) to yield 855 mg. of soluble methylated sugar. This was methylated 10 times as by Purdie & Irvine (1903). In each treatment the dextrin was refluxed with methyl iodide (1.54 g., 10.8 m-moles) and silver oxide (1.20 g., 4.5 m-moles) for 4 hr. The iodide was distilled away and the residue extracted with chloroform, filtered, and the filtrate evaporated, the syrupy residue then being remethylated. Finally the syrup was dissolved in ethanol, filtered and evaporated, then in water, and filtered and evaporated. The residue weighed 520 mg. (56%) (Found: OMe, 49.3%. The fully methylated pentasaccharide, $C_{47}H_{86}O_{26}$, requires OMe, 49.4%).

Hydrolysis and fractionation of the methylated pentasaccharide. The methylated pentasaccharide (500 mg.) was heated in 0.33N-sulphuric acid (500 ml.) for 10 hr. at 100°

and then, because tests showed hydrolysis to be incomplete, for a further 12 hr. in N-sulphuric acid. After this time the reducing power had attained a constant value. The solution was neutralized with sodium hydroxide and evaporated to 100 ml. The solution (α_D 1.462°) was adsorbed on a charcoal-Celite column and subjected to gradient elution. A reservoir of water (4 l.) was connected to the top of the column and kept at constant level by the automatic addition of 30% aqueous ethanol, with stirring. Fractions (100 ml.) were collected under gravity. Fig. 1 shows the progress of the elution. After removal of fraction D the eluting solvent was changed to 25% aqueous ethyl methyl ketone, when material having a total rotation of $+0.088^\circ$ in 100 ml. was collected. This was not further examined. The total α_D of all fractions was $+1.301^\circ$, a recovery, in terms of rotation, of 91.3%. The fractions corresponding to each peak (A, B, C and D) were combined and evaporated to dryness.

Identification of fraction A. This fraction behaved as a homogeneous substance during paper-chromatographic and -electrophoretic analysis and had R_F 0.51 and M_G 0.00. The R_F of 2:3-di-O-methylglucose was 0.57 and the M_G of the 3:6-isomer was 0.82. The sugar was thought likely to be the 2:6-isomer, in which case treatment with phenylhydrazine would yield 6-O-methylglucosazone. The sugar (30 mg.) was heated at 100° with phenylhydrazine (70 mg.), sodium bisulphite (10 mg.) and 0.2M-sodium acetate-acetic acid buffer (pH 4.8, 5.0 ml.) for 24 hr. (Bell & Manners, 1954). The solution was evaporated and applied to 40 cm. lines drawn on six filter papers (Whatman no. 54, 18 $\frac{1}{2}$ in. $\times$ 22 $\frac{1}{2}$ in.). The papers were subjected to electrophoresis at 400 v for 6 hr. in 0.2M-sodium acetate-acetic acid buffer (pH 4.8) when the phenylhydrazine salts moved away from the origin, leaving a yellow band of osazone, which was cut out and extracted with ethanol. This solution was concentrated and applied to six filter papers (as above) which were irrigated with butanol-acetic acid-water (4:1:5, by vol.). The osazone moved with the solvent front, and was completely separated from the salts. The coloured bands were eluted from the air-dried papers with ethanol and the extract evaporated to a crystalline residue (11.4 mg., 21%) having m.p. $182-183^\circ$, $[\alpha]_D^{20} - 74.0^\circ \rightarrow -45.0^\circ$ (90 hr., const.) in ethanol (Found: N, 15.0. Calc. for $C_{18}H_{24}O_4N_4$: N, 15.0%). 6-O-Methylglucosazone prepared as by Freudenberg & Hüll (1941) had m.p. $185-186^\circ$, $[\alpha]_D^{20} - 74.2^\circ \rightarrow -45.8^\circ$ in ethanol. A mixture of the two specimens melted at 185° .

Identification of fraction B. This fraction crystallized on being evaporated to dryness and was further crystallized twice from isopropyl ether-chloroform (9:1, v/v). It had m.p. $121-122^\circ$, $[\alpha]_D^{20} + 68.7^\circ$ in ethanol (c, 0.16). Authentic 2:3:6-tri-O-methylglucose had m.p. 121° , $[\alpha]_D^{20} + 69.5^\circ$, the m.p. being unchanged when mixed with fraction B.

Identification of fraction C. A portion of the syrup (68 mg.) was kept at 30° for 45 hr. with aniline-ethanol (1:1 ml.; 2:3, v/v). Water (10 ml.) was added and distilled away. The syrup crystallized on standing and was recrystallized four times from light petroleum (b.p. $40-60^\circ$)-ether (1:1, v/v). The crystals had m.p. $145-145.5^\circ$, $[\alpha]_D^{19} - 102^\circ$ in ethanol (c, 0.045) (Found: C, 61.0; H, 7.93; N, 4.74. Calc. for $C_{18}H_{23}O_5N$: C, 60.6; H, 7.74; N, 4.72%). Authentic 2:3:4-tri-O-methylglucose anilide had m.p. 146° , $[\alpha]_D - 103^\circ$ and was unchanged in m.p. on being mixed with the anilide of fraction C.

Fig. 1. Fractionation of the hydrolysate of the methylated pentasaccharide on charcoal-Celite. Details are given in the Experimental section. A, 2:6-Di-O-methyl-D-glucose; B, 2:3:6-tri-O-methyl-D-glucose; C, 2:3:4-tri-O-methyl-D-glucose; D, 2:3:4:6-tetra-O-methyl-D-glucose.

Identification of fraction D. The syrupy residue crystallized on standing and was twice recrystallized from light petroleum (b.p. 40–60°)–ether (9:1, v/v). A portion (47.6 mg.) was converted into the anilide by heating at 100° with aniline (0.30 ml.) and ethanol (0.45 ml.) for 3 hr. Crystals were formed on cooling and were twice recrystallized from ethanol. The compound had m.p. 136–137°, $[\alpha]_D^{20} + 233^\circ$ in ethanol (c, 0.07) (Found: N, 4.72. Calc. for $C_{16}H_{25}O_5N$: N, 4.50%). 2:3:4:6-Tetra-*O*-methylglucose anilide had m.p. 136–137°, $[\alpha]_D^{20} + 235^\circ$ and was unchanged in m.p. when mixed with the anilide of fraction D.

Acid-catalysed demethylation of methylglucoses. 0.1% Solutions of 2:3-di-*O*-methylglucose (325 mg.), 2:3:6-tri-*O*-methylglucose (262 mg.) and 2:3:4:6-tetra-*O*-methylglucose (413 mg.) in 0.33*N*-sulphuric acid were heated at 100° for 10 hr., and then in *N*-acid for a further 12 hr., as in the hydrolysis of the methylated pentasaccharide (see above). The solutions were neutralized and then adsorbed on and eluted from charcoal–Celite as for the pentasaccharide. In terms of optical rotation the recoveries of di-, tri- and tetra-*O*-methylglucoses were 91.3, 95.5 and 93.7% respectively. All fractions collected before the emergence of the main sugar peak were combined, evaporated and dissolved in 100 ml. of water for determination of optical rotation. These rotations correspond to 2:1, 2:4 and 1:2% respectively of the rotations of the di-, tri- and tetra-*O*-methylglucoses before treatment with acid. Paper-chromatographic examination of these fractions showed the probable presence of mono-*O*-methylglucose in the product from di-*O*-methylglucose, mono- and di-, and mono-, di- and tri-*O*-methylglucoses in the other two products respectively.

Periodate-oxidation experiments

Periodate oxidations and measurements of formaldehyde were carried out as by O'Dea & Gibbons (1953) and O'Dea (1953) except that the ratio sugar solution:*p*-hydroxybenzaldehyde:periodate–bicarbonate was 9:1:10 (by vol.). AnalaR *D*-glucose was used as the standard source of formaldehyde. The results are shown in Table 1.

RESULTS AND DISCUSSION

Acid and enzymic hydrolysis of the pentasaccharide

When a partial acid hydrolysate of the pentasaccharide was fractionated by paper chromatography two disaccharides were found, having the R_f values of maltose and isomaltose. The only monosaccharide present was glucose. Roberts & Whelan (1960) had previously found that R_{enzyme} believed to be specific for the hydrolysis of α -1:6-glucosidic linkages (Hobson *et al.* 1951), split the pentasaccharide into two sugars, having the R_f values of maltose and maltotriose. The tentative conclusion that the pentasaccharide was one of the five structures pictured in Fig. 2 enabled experiments to be designed to decide between the various alternatives.

Methylation analysis of the pentasaccharide

The pentasaccharides in Fig. 2 can be divided into two groups. Methylation and hydrolysis of one

group (*Q*, *R*, *T*) should yield 2:3:4:6-tetra-, 2:3:6-tri- and 2:3-di-*O*-methylglucose in the molar ratio 2:2:1. The second group (*P*, *S*) should yield the first two of these sugars with 2:3:4-tri-*O*-methylglucose, in the molar ratio 1:3:1. The pentasaccharide was exhaustively methylated, apparently to the extent calculated for complete etherification. On hydrolysis and fractionation (Fig. 1) four methyl-sugars were isolated and identified. These were 2:3:4:6-tetra-, 2:3:6-tri-, 2:3:4-tri- and 2:6-di-*O*-methylglucose. The unexpected feature of the experiment was the isolation of the dimethylglucose, since its formation could be interpreted as meaning the presence in the pentasaccharide of 1:3-links. Wolfson & Thompson (1956) have indeed isolated nigerose (3-*O*- α -glucosylglucose) from partly hydrolysed waxy-maize amylopectin and Hamilton & Smith (1956) have found that the same amylopectin contains 0.2–0.5% of glucose units resistant to periodate oxidation. These could be 1:3-linked units. An alternative explanation of the origin of the dimethyl-sugar was the de-etherification of the tri- and tetra-methylglucose during acid hydrolysis of the methylated pentasaccharide. Control experiments showed, however, that the amount of de-etherification occurring could not account for the 14% (molar basis) yield of the 2:6-isomer. A third possibility was incomplete methylation of the pentasaccharide and we are inclined to this as the correct explanation. (In this case the apparently full degree of methylation of the pentasaccharide, as shown by the methoxyl content, could have been due to adhering traces of ethanol.) It is known that the C-3 hydroxyl group of glucose is much less easily substituted than are the other hydroxyl groups. Full methylation of phenyl α -glucoside should give the 2:3:4:6-tetra-*O*-methyl derivative, but the reaction at the C-2, C-4 and C-6 hydroxyl groups proceeds so much more

Fig. 2. Possible structures of the pentasaccharide α -limit dextrin. O, Glucose unit; O with a slash, reducing-end unit; —, α -1:4-link; ↓, α -1:6-link.

rapidly than at C-3 that the direct methylation of this glucoside is a practical method for preparing 2:4:6-tri-*O*-methylglucose (Richtmyer, 1939). 2:6-Di-*O*-methylglucose was obtained from methylated glycogen, and as a result 1:3-links were claimed to be present (Bell, 1948); this was later withdrawn (Bell & Manners, 1954), as also was the claim for 1:3-links in inulin based on the isolation of 2:4:6-tri-*O*-methylglucose from methylated inulin (Hirst, McGilvray & Percival, 1950; Aspinall & Telfer, 1953). The 2:6-di-*O*-methylglucose appeared to arise as a result of incomplete methylation of those glucose residues which should, on full methylation, have given the 2:3:6-tri-*O*-methyl-sugar. There was no sign in the hydrolysate of 2:4:6-tri- or 2:4-di-*O*-methylglucose, which have R_f values distinctly different from any of the four products *A-D*. These two sugars would have been formed if the glucose units giving 2:3:4:6-tetra- and 2:3:4-tri-*O*-methylglucose respectively had been incompletely methylated. On this assumption the experimental molar ratio 2:3:4:6-tetra-:2:3:6-tri- + 2:6-di-:2:3:4-tri-*O*-methylglucose of 1:2:8:1.1 indicates that the pentasaccharide molecule contains one non-reducing-end group and one 1:6-link and that it must have structure *P* or *S* (Fig. 2).

Periodate oxidation of the pentasaccharide

A method of distinguishing between structures *P* and *S* was devised, based on the over-oxidation reaction of periodate. The same method was developed independently at the same time by Hough & Perry (1956) and since these authors have explained the basis of the method this will not be elaborated here. Structure *P* would be expected to be eroded by periodate from the reducing end of the molecule, the reaction progressing towards the

non-reducing end until arrested at the 1:6-linkage. Two glucose units, the reducing-end unit and that next to it, would be completely converted into formic acid, formaldehyde and carbon dioxide before the reaction ceased, the yield of formaldehyde amounting to 2 mol.prop. Structure *S*, on the other hand, would yield only 1 mol.prop. of formaldehyde. Model substances, maltose, panose, melibiose and isomaltotriose, were tested under oxidation conditions described by O'Dea & Gibbons (1953) and O'Dea (1953), namely in periodate-bicarbonate at 50°. The calculated yields of formaldehyde were 2, 1, 0 and 0 mol.prop. respectively. The experimental results showed, however, that at 50° the reactions with panose, melibiose and isomaltotriose progressed beyond their expected limits (Table 1). When the temperature was lowered to 20° the abnormal reaction did not take place (Table 1) and the pentasaccharide was accordingly oxidized under these conditions. The yield of formaldehyde was 1.86 mol.prop. and from maltose was 1.80 mol.prop. (calc. 2 mol.prop.). It was clear that structure *P* and not *S* represented the constitution of the pentasaccharide.

The splitting of the pentasaccharide by R-enzyme into maltose and maltotriose shows the structure to be restricted to one of the five substances *P-T* (Fig. 2). Only one of these, substance *P*, would yield more than 1 mol.prop. of formaldehyde. The conclusion that the pentasaccharide is substance *P* can therefore be reached independently of the methylation analysis.

The same conclusion can be formed independently of the R-enzyme experiment, provided that the explanation of the origin of the 2:6-di-*O*-methylglucose in the methylation experiment is correct. The results of methylation analysis show that the

Table 1. *Yields of formaldehyde on periodate oxidation of oligosaccharides*

The methods are given in the Experimental section.

Time (min.)	Formaldehyde (mol.prop.) formed at 50°			
	Maltose	Panose	Melibiose	Isomaltotriose
10	1.82	0.74	1.02 (100 min.)	0.88 (100 min.)
30	1.98	1.14	—	—
50	2.00	1.70	—	—
70	2.02	1.70	—	—
90	2.00	—	—	—
Time (hr.)	Formaldehyde (mol.prop.) formed at 20°			
	Maltose	Panose	Melibiose	Pentasaccharide
4	—	0.91	—	—
6	—	1.08	—	—
12	1.47	1.06	—	—
16	1.80	1.06	—	—
18	1.80	1.06	0.09	1.85
41	—	—	—	1.87
45	—	—	—	1.86

molecule contains one non-reducing end group, one 1:6-linked glucose unit and three 1:4-linked glucose units. The high positive $[\alpha]_D$ of the pentasaccharide shows all the links to have the α -configuration. There are four molecules which can be constructed from this evidence. These are structures *P* and *S* (Fig. 2), 6⁴- α -glucosylmaltotetraose and 6- α -maltotetraosylglucose [nomenclature as defined by Whelan (1960)]. The respective yields of formaldehyde from these sugars would be 2, 1, 3 and 0 mol.prop. Structure *P* is therefore singled out as the only one consistent with all the facts, and the pentasaccharide is therefore *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose (6³- α -maltosylmaltotriose).

SUMMARY

1. A pentasaccharide α -limit dextrin formed from amylopectin by human salivary α -amylase has been subjected to structural analysis.

2. Methylation analysis shows the pentasaccharide to contain one non-reducing-end group and one 1:6-link.

3. The results of periodate oxidation indicate that the 1:6-linkage is the antepenultimate bond, relative to the reducing end of the molecule.

4. The foregoing evidence, taken with the results of acid and enzymic hydrolysis and the high positive rotation of the pentasaccharide, shows the molecule to be 6³- α -maltosylmaltotriose.

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The Mechanism of Carbohydrase Action

7. STAGES IN THE SALIVARY α -AMYLOLYSIS OF AMYLOSE, AMYLOPECTIN AND GLYCOGEN

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Conflicting views have been expressed on the nature of the end products of the action of human salivary α -amylase on amylose (see review by Whelan, 1958). Whelan & Roberts (1953) recovered maltose and maltotriose in high yield (92.4%) as the only detectable products of the action of a

purified enzyme preparation on amylose. There is general agreement that maltose is entirely resistant to the enzyme, but it has been disputed whether maltotriose is a stable end product; it could be further hydrolysed to maltose and glucose. Hopkins & Bird (1954), using unpurified saliva,