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,

stated that 'no glucose was ever detected in actions of salivary amylase on amylose' but that maltotriose was slowly hydrolysed by large amounts of the enzyme. Myrbäck and coworkers (Myrbäck & Leissner, 1943; Svanborg & Myrbäck, 1953) also concluded that salivary amylase does not attack maltotriose. On the other hand, Pazur (1953) found the sugar was attacked by crude saliva. Jansen & Wydeveld (1958) reported the hydrolysis of *p*-nitrophenyl α -maltoside by saliva and concluded that maltotriose would also be hydrolysed. We have examined two possible explanations of these results: either an enzyme distinct from the starch-splitting amylase hydrolyses the maltotriose, or the failure to detect such hydrolysis results from using too low a concentration of enzyme. It is clear that if the amylase does hydrolyse maltotriose there must be an enormous disparity in the rate of this action as compared with that on the next higher maltodextrin homologue, maltotetraose. Whelan & Roberts (1953) reported the complete hydrolysis of maltotetraose under conditions in which there was no detectable breakdown of maltotriose. It is already known, however, that phosphorylase (Whelan & Bailey, 1954) and β -amylase (Whelan, Bailey & Roberts, 1953) each attack these two sugars at greatly different rates, so that hydrolysis of maltotriose by pure α -amylase might occur when sufficient enzyme is used. With the same specimen of crystalline enzyme used in the present work Pazur & Budovich (1955) reported such hydrolysis. The present work confirms and amplifies this finding and deals with a similar question about products of the α -amylolysis of amylopectin and glycogen, from which, in addition to glucose, maltose and maltotriose, salivary α -amylase liberates molecules containing the α -1:6-branch linkages of the polysaccharide (α -limit dextrins) (Roberts & Whelan, 1960). The reported formation of different α -limit dextrins can be attributed to the use of different amounts of enzyme.

MATERIALS AND METHODS

Enzymes. Except in one instance (Table 3) saliva was collected from the same subject (W.J.W.), diluted with an equal volume of water, covered with toluene and centrifuged after 1 hr. to remove mucins. The diluted saliva was dialysed in cellophane against running tap water and then running distilled water at room temperature, each dialysis lasting at least 24 hr. Human salivary α -amylase, crystallized four times, was a gift from Dr Jytte Muus (Muus, 1953). All digests were incubated at 30° under a layer of toluene.

Substrates. Maltose hydrate was supplied by Thomas Kerfoot Ltd., Vale of Bardsey, Lancs. Maltotriose and maltotetraose were prepared as by Peat, Whelan & Kroll (1956). The starch used in determining α -amylase activity

was prepared from AnalaR soluble starch as by Hanes & Cattle (1938). The unmodified starch was used in other experiments requiring whole starch. The amylopectin was a gift from Dr T. J. Schoch. Rabbit-liver glycogen was prepared as by Schlamovitz (1951). Oyster glycogen was a commercial sample (British Drug Houses Ltd.). Some properties of these three polysaccharides are given by Walker & Whelan (1960), who used the same specimens. Amylose dextrin was prepared from potato amylose (Hobson, Pirt, Whelan & Peat, 1951). The amylose (2 g.) was dissolved in alkali (Peat, Pirt & Whelan, 1952), neutralized and diluted to 500 ml. Dialysed saliva (5 ml.) was added and the digest incubated at 30° for 11 min. The enzyme was inactivated by heating for 3 min. in a boiling-water bath. Precipitated amylose was filtered away and amylose in solution precipitated with acetone (2 vol.), recovered on the centrifuge, dissolved in the minimum of water and dialysed as for saliva.

Except for maltose, which was assumed to be the pure hydrate, all substrate concentrations were determined by hydrolysis to glucose (Pirt & Whelan, 1951).

Buffers. Phosphate-citrate buffers (McIlvaine, 1921) were used in the determination of optimum pH of enzyme activity; otherwise the buffers were made from citric acid-sodium hydroxide, sodium acetate-acetic acid, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-hydrochloric acid or triethanolamine-hydrochloric acid.

Reducing-power determinations. The copper reagent of Shaffer & Hartmann (1921) was used.

Amylase activity. The method of Schwimmer (1947) and Schwimmer & Balls (1949) was used, except that the pH of the digest was 6.3, not 4.7, and sodium chloride was used in place of calcium chloride.

Paper chromatography. The methods were as given by Walker & Whelan (1960). Before being applied to the chromatograms the digests were heated at 100° for 3 min. to inactivate the enzyme and then freed from inorganic matter with Bio-Deminrolit mixed-bed resin (The Permutit Co. Ltd.) in the carbonate form (Woolf, 1953).

RESULTS

Action of crude and crystalline salivary α -amylase on maltotriose and maltose

Dialysed saliva was allowed to act on maltotriose in the presence of chloride ion, which activates salivary α -amylase. The concentration of enzyme was about 100 times that used by Whelan & Roberts (1953) in the hydrolysis of amylose. A substantial hydrolysis of maltotriose was observed during 22 hr., the rate of reaction being the same in three different buffers (Table 1).

Dialysed saliva was then heated in the presence and absence of chloride to destroy some of the amylase. Digestions of maltotriose carried out in chloride with the same amount of amylase, measured by its action on starch, each resulted in about the same degree of hydrolysis of maltotriose (Table 2). Next, dialysed saliva from two subjects was incubated with maltotriose, with and without chloride ion, equipotent amounts of enzyme in

Table 1. *Action of dialysed saliva on maltotriose*

The digests contained 0.4% of maltotriose, dialysed saliva (0.27 unit/ml.), and 40 mM-buffer, pH 7.0. Sodium chloride, to 20 mM, was added to the citrate digest; the other buffers already contained chloride.

Buffer	Hydrolysis of maltotriose (%)	
	6 hr.	22 hr.
Citrate	10	33
Tris	12	31
Triethanolamine	12	30

Table 2. *Action of heat-inactivated saliva on maltotriose*

Dialysed saliva was heated at 56.5° for 15, 35 and 60 min., when the activities towards starch were 55, 34 and 18% respectively of the original value. Another sample of saliva, containing 10 mM-sodium chloride, was heated at 63.5° for 25 and 73 min., when the activities were 73 and 47% respectively of the original. Digests were prepared from these samples containing 0.08% of maltotriose, 40 mM-citrate (pH 6.5), 80 mM-sodium chloride and α -amylase (0.6 unit/ml.). Reducing powers were measured initially and after 4.7 hr. Figures in parentheses indicate the time of heating (min.) of the enzyme.

Hydrolysis of maltotriose (%)	
Enzyme heated without chloride	Enzyme heated with chloride
31 (0)	27 (0)
32 (15)	32 (25)
35 (35)	31 (73)
36 (60)	

Table 3. *Comparison of the actions of saliva and crystalline α -amylase on maltotriose*

(a) The digests contained 0.4% of maltotriose, dialysed saliva (0.075 unit/ml. with chloride present, 0.225 unit/ml. with chloride absent), and 50 mM-citrate buffer, pH 6.5. Sodium chloride, when present, was 0.1M. Dialysed saliva (G.J.W.) initially contained 1.5 units/ml. and dialysed saliva (W.J.W.) 2.7 units/ml. Incubation was for 48 hr.

Saliva	Hydrolysis of maltotriose (%)	
	Chloride present	Chloride absent
G.J.W.	40	10.1
W.J.W.	37	9.7

(b) The digests contained 0.28% of maltotriose, dialysed saliva or crystalline α -amylase (0.068 unit/ml.), buffer and chloride as in (a), and were incubated for 24 hr.

	Hydrolysis of maltotriose (%)
Dialysed saliva	21
Crystalline enzyme	22

terms of starch hydrolysis being used. Hydrolysis of maltotriose took place in all cases, but much more in the presence of chloride (Table 3a). Finally, equipotent amounts of dialysed saliva and crystalline α -amylase hydrolysed maltotriose in the presence of chloride to the same degree (Table 3b).

When dialysed saliva acted on maltose under the conditions described in Table 2, maltose being used at the same molarity as that specified for maltotriose, the degree of hydrolysis in 24 hr. was 16%, in both the presence and absence of chloride, each result being the mean of four experiments. Crystalline salivary α -amylase did not act on maltose under these conditions.

In these reactions there was no evidence of transglycosylation. Crude or crystalline α -amylase was incubated with maltotriose or maltose as in Table 2, with added [¹⁴C]glucose, and the digests were fractionated by paper chromatography. No radioactivity was detected in the oligosaccharides.

Effect of chloride ions on the α -amylolysis of maltotriose, maltotetraose and starch

The action of dialysed saliva on maltotriose, maltotetraose and starch in chloride at concentrations varying from μ M to M was investigated. The chloride concentration in the preparation of dialysed enzyme was not measured but must have been very low since starch and maltotetraose hydrolysis was activated by added chloride in the range 1–10 μ M (Fig. 1). The degree of amylolysis of these two substrates was affected in a similar manner by chloride, but no activation of the hydrolysis of maltotriose was noticed until the chloride concentration was raised to 10 mM.

Fig. 1. Effect of chloride on the α -amylolysis of maltotriose (M3), maltotetraose (M4) and starch (S). The digests contained 0.4% of substrate, 40 mM-citrate buffer (pH 6.5), dialysed saliva (0.26 unit/ml.) and sodium chloride in concentrations from μ M to M. The starch and maltotetraose digests were incubated for 5 min. and maltotriose for 26.5 hr.

Amylolysis of starch and maltotetraose, but not the hydrolysis of maltotriose, was inhibited by 0.1–1.0M-chloride (Fig. 1). The small (5–8%) hydrolysis of maltotriose taking place over the chloride-concentration range μM – mM was confirmed by paper chromatography of the digests, which showed the formation of maltose and glucose from the maltotriose.

Optimum pH of α -amylase action

Starch and maltotetraose were incubated with dialysed saliva in the presence and absence of chloride at various hydrogen-ion concentrations. With chloride, the pH optimum for starch was 6.5–7.0 and for maltotetraose 6.5. Without chloride the optimum was at a rather more acid pH and was less clearly marked (Fig. 2). Experiments with maltotriose were performed with dialysed saliva and crystalline amylase, no great difference between the two preparations being noted. The results for the crystalline enzyme are given in Fig. 3. With chloride the optimum pH was at pH 5.5. In contrast with starch and maltotetraose hydrolysis the pH optimum without chloride was at a more alkaline pH.

Stages in the α -amylolysis of amylose, amylopectin and glycogen

Amylose was subjected to a slight degree of α -amylolysis and the amylose dextrin isolated (see

above). This was preferred to native amylose as a substrate for quantitative studies because of the retrogradation which takes place when undegraded amylose is treated with α -amylase in the absence of chloride (Whelan & Roberts, 1953). The amylose dextrin was treated, in parallel with maltotriose, with crystalline α -amylase in two concentrations, in the presence and absence of chloride. The ratio of the two concentrations of enzyme was 25:1. At the lower concentration, with chloride, the hydrolysate of amylose dextrin attained a constant reducing power, equivalent to an apparent conversion of 95% of the amylose dextrin into maltose (Fig. 4). In the corresponding maltotriose digest there was a negligible hydrolysis after 72 hr. The 95% conversion is close to that of 90.8% noted by Whelan & Roberts (1953) after the α -amylolysis of amylose, which yielded only maltose and maltotriose as end products. At the higher amylase concentration, with chloride the curve of the reaction with amylose dextrin was inflected at about 95% conversion, then rising slowly to a constant value of 115% after 45 hr. In the corresponding maltotriose digest the sugar was completely hydrolysed after 72 hr. Paper chromatograms were made of the four 72 hr. digests containing chloride. At the high enzyme concentration the amylose dextrin and maltotriose digests contained maltose and glucose with a very faint trace of maltotriose. At the low concentration the amylose dextrin digest contained maltose and maltotriose with a trace of

Fig. 2. Effect of hydrogen-ion concentration on the α -amylolysis of starch (S) and maltotetraose (M4) in the presence (—) and absence (---) of chloride ion. The starch digests contained 1.32% of starch, 40 mM-phosphate-citrate buffer, dialysed saliva (0.01 unit/ml.) and 0.1M-sodium chloride, when present. Incubation was for 1 hr. The maltotetraose digest contained 0.44% of maltotetraose, 50 mM-buffer, dialysed saliva (0.17 unit/ml.) and 0.1M-sodium chloride, when present. Incubation was for 5 min.

Fig. 3. Effect of hydrogen-ion concentration on the α -amylolysis of maltotriose. When chloride was absent (---) the digests contained 0.4% of maltotriose, 50 mM-phosphate-citrate buffer, crystalline α -amylase (0.41 unit/ml.), and with 0.1M-chloride present (—) the digests contained 0.24% of maltotriose, 23 mM-phosphate-citrate buffer and 0.13 unit of crystalline enzyme/ml. Incubation was for 43.5 hr. The activity of the enzyme was tested after 24 hr. and in each case was within 2% of the amount added.

Fig. 4. α -Amylolytic of amylose dextrin with high (*H*) and low (*L*) concentrations of enzyme in the presence (—) and absence (---) of chloride. The digests contained 0.36% of dextrin, 20 mM-citrate buffer (pH 6.5), dialysed saliva (0.25 or 0.01 unit/ml.) and 0.1M-sodium chloride, when present. Maltotriose was treated similarly and the results are reported in the text.

glucose, and the maltotriose digest contained the trisaccharide and barely detectable amounts of maltose and glucose. After 193 hr. incubation of the four amylose dextrin digests the enzyme was still active in each case.

The curves for digests without chloride had similar trends to those with chloride, but the rates of reaction were much lower (Fig. 4). After 72 hr. only 12% of maltotriose was converted at the higher enzyme concentration and none at the lower.

Two samples of glycogen and one of amylopectin were subjected to α -amylolysis by high and low concentrations of enzyme in the presence of chloride, as in Fig. 4. After 48 hr. incubation the digests were applied to paper. The results of the chromatography of amylolyzed rabbit-liver glycogen are shown in Fig. 5, and were the same with oyster glycogen and waxy-maize starch as substrates. At high enzyme concentration the products, identified by R_f , were glucose, maltose and a series of sugars, the fastest moving of which had approximately the R_f of maltotetraose. At the low enzyme concentration the products were maltose, maltotriose and a sugar series with a substance having approximately the R_f of maltopentaose as its fastest-moving member.

Fig. 5. Paper-chromatographic fractionation of the products of α -amylolysis of glycogen. Rabbit-liver glycogen was treated for 48 hr. with high (*H*) and low (*L*) concentrations of crystalline α -amylase in the presence of chloride as in Fig. 4. The percentage conversions into maltose were 88 and 60% respectively. The reference substances on the chromatogram are glucose (*G*), maltose (*M2*), maltotriose (*M3*) etc.

DISCUSSION

Action of α -amylase on maltotriose and maltose

High concentrations of crude and crystalline salivary α -amylase hydrolysed maltotriose (Tables 1-3), confirming the results of Pazur (1953) and Pazur & Budovich (1955). The rate of hydrolysis relative to that of starch was, however, so low (see below) that four experiments were performed to find whether the maltotriose-splitting activity was due to an enzymic impurity. The results were as follows: (i) Like the hydrolysis of starch, that of maltotriose is activated by chloride ion (Table 3a). (ii) Two subjects had the same amount of maltotriose-splitting activity in their saliva, relative to starch-splitting activity (Table 3a). This is unlikely if the two activities were those of different enzymes. (iii) No significant change in maltotriose-splitting activity relative to starch-splitting activity occurred when the amylase was partly inactivated by heat (Table 2). (iv) Crude saliva and four-times-crystallized α -amylase had the same ratio of hydrolytic activities with both starch and maltotriose (Table 3b). It is reasonable to conclude that maltotriose is hydrolysed by salivary α -amylase.

It has been suggested that since there is only one starch-splitting enzyme in saliva (Meyer, Duckert & Fischer, 1950) it is justifiable to ascribe products arising from the action of saliva on starch to the action of α -amylase (Pazur, 1953). This, however, is

not valid as far as the production of glucose is concerned because saliva contains a second enzyme, forming glucose from maltose. The action seems to be one of hydrolysis rather than of transglycosylation, since no transfer to [^{14}C]glucose was observed, and formation of glucose was accompanied by an increase in reducing power. Chloride did not activate the reaction and crystalline amylase did not hydrolyse maltose. The hydrolysis was therefore due to an enzyme other than α -amylase.

The actions of salivary α -amylase on starch and maltotetraose are very similar, both in respect of optimum pH and the effect of chloride on the rate of reaction (Figs. 1, 2). With maltotriose, however, the pH optimum in the presence of chloride is distinctly different from that for starch and maltotetraose hydrolysis (Figs. 2, 3), and the relative rates of hydrolysis of these two substrates as compared with maltotriose vary in different ways as the chloride concentration is changed (Fig. 1). Although it seems proven that the amylase does hydrolyse maltotriose, the rate of its action on this molecule is of an entirely different order from that on larger molecules. Starch and maltotetraose, representing the two extremes of molecular size on which the enzyme acts rapidly, are not hydrolysed at greatly different rates (Fig. 1). However, whereas 63% of maltotetraose was hydrolysed in 5 min. in 0.1M-sodium chloride, only 33% of maltotriose was hydrolysed by the same amount of enzyme in 26.5 hr. (Fig. 1). There is a close parallel here with phosphorylase and β -amylase. These enzymes also act on maltotetraose and starch at rather similar rates, but act on maltotriose at very much lower rates (Whelan, 1958).

Stages in the α -amylolysis of amylose, amylopectin and glycogen

The vast difference in the rate of α -amylolysis of maltotriose, as compared with larger substrate molecules, makes it clear that the α -amylolysis of amylose proceeds in two stages. The first stage is the conversion of the amylose into maltose and maltotriose. The second stage involves the hydrolysis of the maltotriose into maltose and glucose. By the use of the appropriate amount of enzyme the reaction can be stopped, or nearly so, at the first stage (Fig. 4). This was the reaction described by Whelan & Roberts (1953) and Hopkins & Bird (1954). The second stage, where all the amylose has become maltose and glucose, is apparently the final arrest point in the reaction.

A recognition of these two clearly separated stages in the α -amylolysis of amylose helps in an understanding of the α -amylolysis of the branched polysaccharides amylopectin and glycogen. As with amylose, there are reports of different end products. Roberts & Whelan (1960) found that the

products of the α -amylolysis of amylopectin and glycogen are maltose, maltotriose and a series of α -limit dextrins. These dextrins consist of maltodextrins joined to each other through the α -1:6-branch links of the parent polysaccharide, the smallest being a pentasaccharide. This is 6 3 - α -maltosylmaltotriose (Fig. 6b) [Bines & Whelan (1960), nomenclature as defined by Whelan (1960)]. Nordin & French (1958), however, reported the products to be glucose, maltose and α -limit dextrins, of which the smallest is a tetrasaccharide (6 3 - α -glucosylmaltotriose, Fig. 6a). It is likely that these differences in the α -limit dextrins are related to the concentration of enzyme used in their formation. Conditions conducive to the hydrolysis of maltotriose would seem also to facilitate a further hydrolysis of the α -limit dextrins that accumulate at the end of the first stage of the amylolysis. Amylolysates of amylopectin and glycogen at the limits of the first and second stages analysed by paper chromatography revealed patterns of sugar spots in agreement with the results of Roberts & Whelan (1960) and Nordin & French (1958) (Fig. 5).

It will be seen from Fig. 6 that the penta- and tetra-saccharide limit dextrins carry the same maltotriose base chain (the three glucose units terminating in the reducing-end group), but differ in the nature of the side chain. It may be that in the second stage of α -amylolysis the tetrasaccharide is formed from the pentasaccharide by a slow splitting of glucose from the maltose side chain. More likely it is formed from a hexasaccharide (6 3 - α -maltotriosylmaltotriose), believed by Roberts & Whelan (1960) to be one of the first-stage α -limit dextrins and shown in Fig. 6c. The maltotriose side chain of this hexasaccharide, like free maltotriose, could perhaps be split to yield the tetrasaccharide and maltose.

The question may be posed whether the second stage of salivary α -amylolysis represents the final limit of its action. Glucose and maltose seem to be true end products, but the α -limit dextrins, with the tetrasaccharide (Fig. 6a) as the smallest of them, might still be degraded further. Meyer & Gonon (1951) contended that isomaltose was the

Fig. 6. α -Limit dextrins formed in the salivary α -amylolysis of amylopectin and glycogen. $\bigcirc$, Glucose unit; $\bigcirc$ with slash, reducing-end glucose unit; —, α -1:4-link; $\downarrow$, α -1:6-link.

end product of pig-pancreatic α -amylase action. Ahnström (1958) studied α -limit dextrins formed from Lintner starch by saliva and found the trisaccharide panose (4- α -isomaltosylglucose) as the smallest. The tetrasaccharide fraction was a mixture of possibly three substances. The conditions of the experiment were, however, such that it is uncertain whether the formation of panose represented a third stage of α -amylolysis. The digest contained a high concentration of enzyme [5% (v/v) of filtered saliva] and was incubated for 3 months. Analysis of glucose and maltose after this time indicated that 'a rather strong hydrolysis of maltose to glucose must have taken place in the reaction mixture, i.e. the sample of saliva used contained maltase' (Ahnström, 1958). The same maltase, acting on α -limit dextrins, might have produced the panose.

SUMMARY

1. The actions of crude saliva and crystalline salivary α -amylase on starch and maltodextrins were compared, with particular reference to the question whether the α -amylase hydrolyses maltotriose. It is concluded that salivary α -amylase hydrolyses maltotriose to maltose and glucose. The hydrolysis is activated by chloride ion and is far slower than that of maltotetraose.

2. By varying the enzyme concentration the salivary α -amylolysis of amylose can be made to proceed in two stages. The first stage, at a low enzyme concentration, is a hydrolysis to maltose and maltotriose. The second stage, with enzyme at 25 times the concentration of that in the first stage, involves the hydrolysis of the maltotriose to glucose and maltose.

3. The α -amylolysis of amylopectin and glycogen also proceeds in two stages. The first-stage products are maltose, maltotriose and branched α -limit dextrins, ranging from a pentasaccharide upwards. The second-stage products are glucose, maltose and α -limit dextrins, of which the smallest is a tetrasaccharide.

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