THE STRUCTURAL ANALYSIS AND ENZYMIC SYNTHESIS OF A PENTASACCHARIDE ALPHA-LIMIT DEXTRIN FORMED FROM AMYLOPECTIN BY *Bacillus subtilis* ALPHA-AMYLASE*

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ABSTRACT

Crystalline *Bacillus subtilis* alpha-amylase hydrolyses amylopectin to a mixture of D-glucose, maltose, and branched oligosaccharides (alpha-limit dextrins). The smallest such dextrin formed under the conditions of the experiment is a pentasaccharide. A combination of methylation analysis, periodate oxidation, and fragmentation analysis with acid narrowed the pentasaccharide structure to two possibilities, but failed to distinguish between them. A rigid proof that the dextrin has the structure 6^2-α-maltosylmaltotriose was obtained by application of enzymic degradation. Finally, the structure of the pentasaccharide was confirmed by enzymic synthesis. It is shown that the structural analysis of such oligosaccharides, derived from amylopectin, can be made by the use of enzymes alone, without resort to the more time-consuming, less-specific, and less-sensitive methods of chemical analysis. Conclusions are drawn regarding the action pattern of the *B. subtilis* amylase.

INTRODUCTION

The hydrolysis of amylopectin by salivary alpha-amylase proceeds in at least two well-defined stages[1]. A relatively short exposure to a low concentration of enzyme results in the formation of maltose, maltotriose, and a series of dextrins containing the original (1→6)-branch points of the amylopectin, as well as (1→4)-bonds. The smallest of these dextrins is a pentasaccharide, 6^3-α-maltosylmaltotriose[2]. On longer exposure, with higher concentrations of enzyme, the maltotriose is observed to disappear, being converted into maltose and D-glucose. At the same time, the dextrins are further attacked and the smallest is now 6^3-α-D-glucosylmaltotriose[1,3]. This is the second stage of the alpha-amylolysis. It may also represent the final limit of degradation. It is concluded that D-glucose residues not in the immediate vicinity of the branch points will ultimately appear as D-glucose or maltose, by hydrolysis of (1→4)-bonds. The enzyme cannot split the (1→6)-branch point and the appearance of (1→4)-bonds

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday.

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in the resistant alpha-limit dextrins indicates that some such bonds are sterically protected from hydrolysis by virtue of their proximity to the branch point[1–4].

We have extended our studies of the action pattern of alpha-amylase to enzymes from other sources. It is already known that there is a variety of action patterns and products, but very few of the alpha-limit dextrins have been isolated and well characterized. A survey conducted in our laboratory by Hughes[5] indicated that all the alpha-amylases tested appeared capable of hydrolysing maltotriose and higher homologues, so that the same second stage of hydrolysis could be achieved as for salivary alpha-amylase. A first stage was not always easy to discern, and was, in any case, of minor interest since it represents only a pseudo end-point in the reaction.

This paper describes the full structural characterization of one of the alpha-limit dextrins formed when *Bacillus subtilis* alpha-amylase is allowed to act on amylopectin to the second stage of hydrolysis, that is when the initially formed maltotriose has been converted into maltose and D-glucose. Under these conditions, the limit dextrin in question, a pentasaccharide, is the smallest such dextrin observed. Hughes[5] had tentatively identified the pentasaccharide as one not previously observed among the salivary alpha-amylase limit-dextrins, *i.e.*, 6^2-α-maltosylmaltotriose (structure **2**, Fig. 1). The full characterization, described here, was accomplished by chemical and enzymic methods and establishes the superiority of enzymic methods for this type of structural analysis.

By analogy with classical chemical principles, we have confirmed the structure by enzymic synthesis of the pentasaccharide.

Some of these results have already been reported briefly[6].

MATERIALS AND METHODS

Waxy-maize starch (amylopectin) was prepared from hand-sorted, single-cross Tapicorn seeds (Bear Hybrid Corn Co., Decatur, Ill.) as described by Schoch[7]. Cyclomaltohexaose (Schardinger α-dextrin) was prepared as described by French *et al.*[8], and panose as by Smith and Whelan[9].

Sugars were examined by separation on Whatman No. 3MM paper previously washed in 1% acetic acid and distilled water. Descending chromatograms were irrigated with ethyl acetate–pyridine–water (10:4:3, by vol.) or, in the case of methyl sugars, with 1-butanol–ethanol–water–ammonia (d, 0.88) (40:10:49:1, by vol.). The spray reagents were silver nitrate–sodium hydroxide[10] and aniline–diphenylamine–phosphoric acid[11]. Charcoal–Celite columns were prepared and used as described by Whelan *et al.*[12], and sugars in the eluates were detected by measuring their optical rotation in a 4-dm tube.

Solutions of the separated sugars were concentrated under diminished pressure at 40°, keeping the pH between 5 and 6. Electrolytes were removed with Biodeminrolit mixed-bed ion-exchange resin (Permutit Co. Ltd., London, England) which had been converted into the carbonate form[13].

The reducing powers in digests of alpha-amylase and amylopectin were measured

with a copper reagent[14] to which D-glucose (6 mg/100 ml) had been added to compensate for loss of cuprous oxide by re-oxidation. The concentrations of oligo- and poly-saccharide solutions were estimated by acid hydrolysis to D-glucose[15], and the concentrations of solutions of methyl ethers of D-glucose were measured by hypoiodite oxidation[16].

alpha-Amylase was isolated from *Bacillus subtilis* as described by Fischer and Stein[17] and crystallized five times. The same authors' method was used for assay of the enzyme. Twice-crystallized, sweet-potato beta-amylase was purchased from the Worthington Biochemical Corp., Freehold, N. J., and activities were determined by the method of Hobson *et al.*[18]. In all beta-amylase digests, other than the activity-assay digests, glutathione (0.05%) and serum albumin (0.05%) were included as stabilizing agents[19]. A purified preparation of amyloglucosidase from *Aspergillus niger* was kindly given by Dr. I. D. Fleming. *Bacillus macerans* transglycosylase was obtained and assayed as described by French *et al.*[8].

EXPERIMENTAL AND RESULTS

Isolation of alpha-limit pentasaccharide from amylopectin. — Amylopectin (15 g) was wetted with methanol and suspended in water (500 ml) made alkaline with 3M sodium hydroxide (20 ml). The suspension was heated with stirring in a boiling water-bath until the starch had dissolved, and then cooled and neutralized to phenolphthalein by addition of 1.5M sulphuric acid. The amylopectin was incubated under toluene at 37° with *B. subtilis* alpha-amylase (4.5 units/ml) in a digest (1 litre) containing 15mM sodium glycerophosphate (pH 5.7). Samples were withdrawn at intervals for measurement of reducing power. After 66 h, it was apparent that hydrolysis had fallen to a low rate, and the digest was therefore heated to 90° for 5 min in a boiling water-bath. The digest was cooled and filtered, and the filtrate evaporated at 40° under diminished pressure to a volume of 250 ml. The concentrate was absorbed at the head of a charcoal–Celite column and fractionated by successive elution of the column with water, followed by 7.5, 15, and finally 30% aqueous ethanol. The fraction eluted with 30% ethanol was concentrated under diminished pressure at 40° and examined by paper chromatography. It contained only dextrins possessing R_F values less than that of maltotriose. This fraction was evaporated to dryness under diminished pressure and dissolved in water (50 ml). The solution was treated with *B. subtilis* alpha-amylase under the conditions described above. When there was no further hydrolysis (67 h), the enzyme was inactivated and the digest was applied to a charcoal–Celite column which was eluted as above, except that 20% aqueous ethanol was used as the final eluant. The desired pentasaccharide, eluted with 20% ethanol, was contaminated by incompletely hydrolysed maltosaccharides, *i.e.* maltotetraose and maltopentaose. This fraction was therefore incubated for 4 h with beta-amylase under conditions defined by Walker and Whelan[19] for the complete hydrolysis of maltotetraose without hydrolysis of maltotriose. The enzyme was inactivated by heating on a boiling water-bath for 3 min, and chromatographic examination of the deionized

solution revealed that the maltosaccharides higher than maltotriose had been completely hydrolysed. The mixture was separated on sheets of Whatman No. 3MM paper and the pentasaccharide located on guide strips cut from the chromatograms. The areas of paper containing pentasaccharide were cut out and eluted with water. Three such experiments resulted in the isolation of 1.85 g of chromatographically pure pentasaccharide alpha-limit dextrin, $[\alpha]_D$ +197° (*c* 1.5, water), from a total of 145 g of waxy-maize starch.

Methylation analysis. — The *B. subtilis* alpha-limit pentasaccharide (950 mg) was methylated[20,21] by being suspended in *N*,*N*-dimethylformamide (30 ml) and treated with methyl iodide (6 ml) followed by freshly prepared silver oxide (6 g), added in portions with shaking at room temperature. The mixture was shaken for 23 h, and further portions of methyl iodide (6 ml) and silver oxide (6 g) were then added, shaking being maintained for another 44 h. The solution was centrifuged, the residue was washed with *N*,*N*-dimethylformamide (2 × 15 ml), and the combined supernatant and washings were evaporated to dryness. The yellow residue was refluxed with chloroform (150 ml) for 1 h, and the extract was centrifuged and evaporated to dryness. The residue, dried over phosphoric oxide in *vacuo*, weighed 970 mg (Found: OMe, 41.0%; calc. for the fully methylated pentasaccharide: OMe, 49.5%).

The product was remethylated under similar conditions, but in the extraction procedure the product was treated with potassium cyanide[21]; yield, 810 mg (Found: OMe, 41.8%). A preliminary, small-scale hydrolysis of a portion of this product revealed the presence of only di-, tri-and tetra-*O*-methylglucoses. Hypoiodite estimation of these three methyl sugars gave molar ratios of 1:2.1:2.2, which are close to the theoretical values (1:2:2) expected for a pentasaccharide of the postulated structure[5]. This result indicated that the pentasaccharide was probably fully methylated and that the low value for the methoxyl content was due to a chloroform-soluble impurity. The methylated sugar was found to contain combined iodine, and subsequent experience with this method of methylation indicated that it is essential, at each stage of working up, to use cyanide to render any silver derivatives soluble in water and non-extractable by chloroform. If this step is omitted, subsequent treatment with the methylating agents causes the formation of the chloroform-soluble, iodine-containing impurity.

A portion of the methylated pentasaccharide (340 mg) was hydrolysed by the method of Croon *et al.*[22]. The material was shaken with 72% (v/v) sulphuric acid (2.5 ml) and allowed to stand at room temperature. After 30 min, water (20 ml) was added and the solution was heated in a boiling water-bath for 4 h. The cooled hydrolysate was deionized, evaporated to a small volume, and fractionated on Whatman No. 3MM paper. Three fractions (I–III), corresponding to di-, tri-, and tetra-*O*-methylglucoses, were isolated and their molar yields, as estimated by hypiodite oxidation, were found to be in the ratios of 1:1.95:1.98, respectively.

Identification of the methylglucose fractions. — Fraction I had $[\alpha]_D$ +52.5° (*c* 0.7, acetone) (*cf.* +50.9° for 2,3-di-*O*-methyl-D-glucose[23]). The acetone solution was

then evaporated to dryness and the residue dissolved in 1% methanolic hydrochloride. During 24 h at room temperature, α_D changed $+0.835° \rightarrow -0.650°$. The change to a negative rotation indicates the formation of a furanose ring which is only possible if HO-4 is not methylated. The possibility of a methoxyl group at either C-5 or C-6 was excluded by the release by periodate of one mol. of formaldehyde from an aqueous solution of the sugar[24]. The ability of hypoiodite to oxidise the sugar indicated that C-1 was unmethylated and the two methyl groups present, as indicated by the R_F value, must therefore have been at C-2 and C-3.

Fraction II was crystallized five times from chloroform–di-isopropyl ether (1:9, v/v) and had m.p. 120–121°, $[\alpha]_D$ +71.8° (*c* 0.3, water); *cf.* 2,3,6-tri-*O*-methyl-D-glucose, m.p. 121–122°, $[\alpha]_D$ +70.5°.

Fraction III was twice crystallized from ether–light petroleum (b.p. 40–60°) (1:9, v/v) and had m.p. 73–75°, $[\alpha]_D$ +82.5° (*c* 0.16, water). The m.p. was unchanged in admixture with authentic 2,3,4,6-tetra-*O*-methyl-D-glucose, $[\alpha]_D$ +84°, m.p. 96°[23]; a m.p. of 78–84° has also been reported[25]. The "anilide" had m.p. 136–137°, $[\alpha]_D$ +230° (*c* 0.72, ethanol); lit.[23] m.p. 136–137°, $[\alpha]_D$ + 235°.

Periodate oxidation. — The D-glucose residue at the reducing end of the alpha-limit pentasaccharide was converted into D-glucitol by treatment with sodium borohydride[26]. Periodate over-oxidation of the sugar alcohol, as described by Parrish and Whelan[24], and measurement of the formaldehyde released, resulted in the rapid formation (0.25 h) of 1.96 mol. of formaldehyde. The amount of aldehyde remained almost unchanged thereafter, being 2.00 mol. at 24 h.

Partial, acid hydrolysis. — The sodium borohydride-reduced pentasaccharide was heated in 50mM sulphuric acid in a boiling water-bath for 8 h. The solution was cooled, deionized, and evaporated to dryness, and borate was removed as methyl borate by repeated evaporations of small volumes of methanol from the residue. Reducing and non-reducing sugars present in the residue were separated from each other by high-voltage paper electrophoresis (50 volts/cm) in molybdate buffer[27] at pH5.0. The component sugars of the separated, reducing and non-reducing fractions were then examined by paper chromatography. Substances having R_F values corresponding to isomaltose, maltotri-itol, and panitol were detected.

Action of Aspergillus niger *amyloglucosidase.* — The alpha-limit pentasaccharide and panose were each incubated with amyloglucosidase (10 mg) in 15mM sodium acetate buffer (pH 4.8, 2 ml) at 35°. The reducing powers were measured at intervals during 4 h, and the pentasaccharide digest was also examined by paper chromatography. The panose was hydrolysed slowly, the pentasaccharide rapidly. When the reducing power of the pentasaccharide digest had increased by an amount corresponding to the release of two mol. of D-glucose, the rate of hydrolysis decreased approximately to that of panose (Fig. 2). Chromatographic separation of the products of the hydrolysis of the pentasaccharide revealed that it was being degraded *via* a tetrasaccharide intermediate to panose and D-glucose. The change in rate of hydrolysis must therefore have reflected the slower action of the enzyme on the accumulated panose. An extended chromatographic separation revealed that the tetrasaccharide

intermediate was, in fact, a composite fraction containing two sugars having R_F values between those of panose and the pentasaccharide. A large-scale hydrolysis of the pentasaccharide (100 mg) with amyloglucosidase was stopped at a point (4 h) where the rate of formation of reducing sugars had decreased (Fig. 2), and the two main components of the hydrolysate were separated by paper chromatography. The molar ratio, calculated from the optical rotation of the separated sugar solutions, was found to be 2:0.85 (D-glucose:panose). The identity of the panose component was confirmed by determination of the physical constants of the dodeca-acetyl derivative of the reduced sugar; this had m.p. 151.5–152.5°, $[\alpha]_D$ +127° (*c* 0.21, chloroform). An authentic specimen had m.p. 149–150°.

Action of beta-amylase. — The alpha-limit pentasaccharide was treated with sweet-potato beta-amylase (2,500 units/ml) at pH 4.8 for 24 h at 35°. Examination of the digest by paper chromatography revealed only unchanged pentasaccharide.

Enzymic synthesis of 6²-α-maltosylmaltotriose. — French and co-workers[28,29] showed that chains of α-(1→4)-linked D-glucose residues are transferred by *Bacillus macerans* transglycosylase from cyclomaltohexaose to either or both of the free HO-4 groups of the trisaccharide, panose (*a*, Fig. 3). The enzyme also catalyses a reversible transfer of chain segments of varying size from one chain end to another, resulting in a disproportionation of the chain lengths. Therefore, under appropriate conditions, the equilibrium mixture will contain a series of molecules possessing an increasing number of D-glucose residues. Included in this mixture will be panose and the isomeric tetrasaccharides and pentasaccharides illustrated in Fig. 3. One of these (**2**, Fig. 3) is the desired pentasaccharide, 6²-α-maltosylmaltotriose. If sufficiently long, the side chains may be hydrolysed in a stepwise manner by beta-amylase, an enzyme that cleaves a β-maltose unit from the non-reducing chain ends. The limit of degradation of components of the mixture will not always be represented by panose. French and co-workers[28,29] demonstrated that two of the isomeric pentasaccharides (**8** and **4**, Fig. 3) are degraded by beta-amylase to panose and maltose; the desired pentasaccharide (**2**, Fig. 3), however, is entirely resistant to the action of the enzyme. This pentasaccharide was therefore prepared and isolated as follows. The cyclohexane complex of cyclomaltohexaose [4 g, equivalent to 3.4 g of uncomplexed dextrin] was suspended in water and boiled until the dextrin dissolved and cyclohexane had been driven off. The solution was evaporated to dryness and dissolved in water (10 ml) containing twice-crystallized panose [975 mg]. *B. macerans* transglycosylase [10 ml, 75 units] was added, together with thymol (100 mg) as an antiseptic, The digest was incubated at 30° for 5 days. The enzyme was inactivated by heating the digest to boiling over a flame. After cooling, 1,1,2,2-tetrachloroethane (2 ml) was added and emulsified with the digest. The mixture was stored overnight, the cyclomaltohexaose–tetrachloroethane complex was removed by filtration, and the solution was evaporated to dryness under diminished pressure. Examination by paper chromatography revealed panose to be the sugar of lowest molecular weight; two tetrasaccharides (see *b* and *c*, Fig. 3), at least two partially resolved pentasaccharides, and sugars of higher molecular weight, extending to the line of origin, were also detected.

The mixture was dissolved in water (7.5 ml) and there were added, in order, 0.2M sodium acetate–acetic acid buffer (3 ml, pH 4.8), 1% human-serum albumin (1.25 ml), 10mM reduced glutathione (1.25 ml), and beta-amylase (12.5 ml, 30,240 units). The mixture was incubated at 35°, and at intervals, portions (0.05 ml) were removed for determination of reducing power, as glucose .The initial value of reducing power was equivalent to 0.325 g of glucose and rose to a constant value of 1.37 g after 43 h. The approximate, average degree-of-polymerization of the oligosaccharide mixture decreased from 12.5 to 3.

The beta-amylase was heat-inactivated (3 min at 100°) and the digest deionized. After evaporation to dryness under diminished pressure, the entire mixture was fractionated on sheets (22.5 × 18.25 in.) of Whatman No. 17 paper, using the ethyl acetate–pyridine–water solvent. Sugars corresponding in R_F values from di- to octa-maltosaccharides were seen on guide strips cut from chromatograms. The areas of paper containing pentasaccharide were cut out, eluted with water, and evaporated to dryness to give a syrup (310 mg) behaving as a single substance on paper chromatography and having the same R_F value as the *B. subtilis* alpha-limit pentasaccharide. This product was dissolved in water (1 ml) and again treated with beta-amylase for 24 h at 35°. The products were worked up as before and fractionated on filter paper into pentasaccharide (200 mg), panose, and maltose. The pentasaccharide was again treated in the same way with beta-amylase, but no further hydrolysis took place during 24 h. The chromatographically homogeneous product finally isolated weighed 154 mg and had $[\alpha]_D$ +194° (water).

Identity of synthetic 6²-α-maltosylmaltotriose with the alpha-limit pentasaccharide. — The synthetic pentasaccharide was examined by methods similar to those used with the alpha-limit pentasaccharide. The following points of similarity were noted.

(1) The synthetic pentasaccharide and the alpha-limit dextrin behaved in identical fashion when examined by paper chromatography. They had similar values of specific optical rotation, $[\alpha]_D$ +194° and +197° (water), respectively, and both were resistant to the action of beta-amylase.

(2) Periodate over-oxidation of the synthetic pentasaccharide yielded the 1 mol. of formaldehyde expected for the reducing oligosaccharide.

(3) The synthetic pentasaccharide was degraded by *A. niger* amyloglucosidase in the same manner and at exactly the same rate as was the alpha-limit dextrin.

(4) As previously shown for the pentasaccharide limit-dextrin[5], the α-(1→6)-linkage in the synthetic pentasaccharide was split by broad-bean R-enzyme to yield maltose and maltotriose, identified by paper chromatography.

DISCUSSION

Structure of the pentasaccharide. — The pentasaccharide was derived from amylopectin and could therefore be assumed to contain only (1→4)- and/or (1→6)-linked α-D-glucopyranosyl residues. That the linkages are in the α-D configuration was confirmed by the high positive value of the specific optical rotation of the sugar, while

the products of hydrolysis of the methylated pentasaccharide indicated that the linkages were indeed (1→4)- and (1→6)- and in the ratio 3:1. [This was already evident from the hydrolysis of the pentasaccharide by R-enzyme, specific for α-(1→6)-linkages, to maltose and maltotriose[5].] Fig. 1 shows all ten possible structures for a D-glucose pentasaccharide containing one α-(1→6)- and three α-(1→4)-bonds. Although methylation analysis provides quantitative information about the pentasaccharide and identifies the types of linkage present, only two different mixtures of methylglucoses could be obtained from all ten sugars shown in Fig. 1. Methylation analysis by itself therefore eliminates only four of the possible structures (Table I).

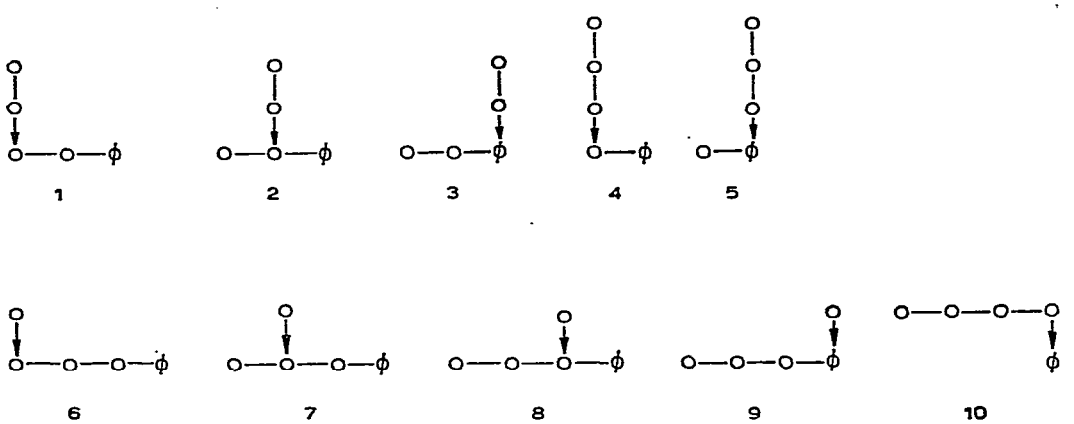

Fig. 1. Possible structures of the pentasaccharide alpha-limit dextrin formed from amylopectin by *B. subtilis* alpha-amylase Key: O = α-D-glucopyranosyl unit; ϕ = reducing-end unit; ↓ = (1→6)-bond; ——— = (1→4)-bond.

TABLE I

PROOF OF THE STRUCTURE OF PENTASACCHARIDE LIMIT-DEXTRIN

Method of examination	*Structures permitted by method of examination (Fig. 1)*
A. Methylation analysis	2 3 5 7 8 9
B. Periodate over-oxidation	2 4 8
C. Partial, acid hydrolysis	2 8
D. Action of *A. niger* amyloglucosidase	2 4 8
E. Action of beta-amylase	1 2 3 5 6 7
F. Action of R-enzyme	1 2 3 4 5

Periodate oxidation of the reduced pentasaccharide resulted in the rapid release of 2 mol. of formaldehyde and no further amount was released during the next 24 h. Under the conditions of the oxidation, a 4-substituted glucose or glucitol is rapidly oxidized, yielding one or two mol. of formaldehyde, respectively, and a further slow

release of formaldehyde will occur by over-oxidative erosion from the reducing chain-end. Formaldehyde is released from the next and successive D-glucopyranose residues until a D-glucose residue substituted at C-6 is encountered, when the release of formaldehyde ceases[2,24,30]. Therefore the yield of only 2 mol. of aldehyde from the reduced pentasaccharide indicates that the D-glucose residue next to the reducing terminus was 6-substituted. This experiment alone eliminates seven of the structures in Fig. 1 from consideration. For example, reduction and oxidation of structure **6** would have yielded 4 mol., and of structure **3**, only 1 mol. of formaldehyde.

When the same reduced pentasaccharide was partly hydrolysed with acid and the reducing and non-reducing (D-glucitol-terminated) fragments were separated and chromatographed, there were detected substances migrating with isomaltose, maltotriitol, and panitol. Maltotriose and isomaltitol could not be found. Only structures **2** and **8** could have yielded the oligosaccharides detected. Taken together, these three chemical methods of examination proved that the pentasaccharide has structure **2** or **8**, but there was no straightforward chemical method that could be used to decide between these alternatives. The only such chemical technique that could have been used is fragmentation by acid, followed by separation and identification of the oligosaccharides. However, in order for this method alone to have given an unequivocal proof of structure, it would have been necessary to identify, or prove the absence of, tetrasaccharide fragments that have themselves not yet been isolated and characterized.

Recourse was therefore had to enzymic degradation. The action of amyloglucosidase in forming 2 mol. of D-glucose and one of panose (4-α-isomaltosyl-D-glucose) was consistent only with structures **2**, **4**, and **8**. The failure of beta-amylase to attack the pentasaccharide eliminated structures **4** and **8**, leaving **2** as the only possibility. Therefore, the use of these two enzymes alone permitted the choice of **2** as the actual structure, independent of the use of any chemical evidence. A third line of

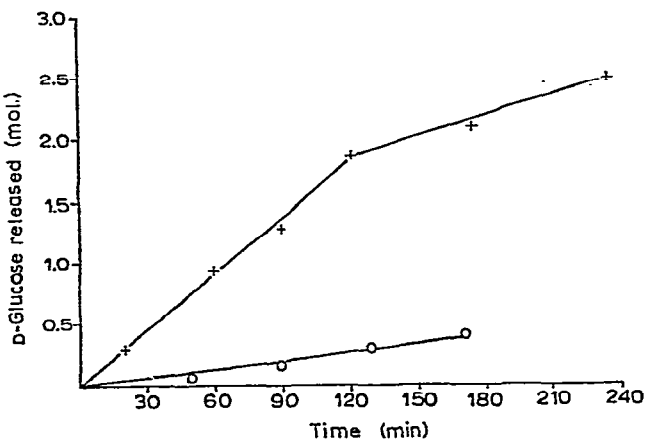

Fig. 2. Hydrolysis of the pentasaccharide alpha-limit dextrin (+) and panose (0) by *A. niger* amyloglucosidase. Oligosaccharide (35 μmoles) and amyloglucosidase (10 mg, freeze-dried powder) in 15mM sodium acetate buffer (pH 4.8) were incubated at 35° in 2-ml digests.

enzymic information was available, the fact that R-enzyme split the pentasaccharide to maltose and maltotriose[5]. This, with the results of partial, acid hydrolysis (Table I), also picks out structure **2** as the only possibility.

Table I lists the six methods used to obtain structural information. As already noted, no combination of purely chemical evidence yields a unique answer, whereas enzymic evidence alone does do so. There are other combinations of chemical and enzymic evidence that also pinpoint structure **2**. One additional piece of information gained during the degradation with amyloglucosidase (Fig. 2) could be used to derive structure **2** entirely from this single experiment. The intermediate tetrasaccharide component was resolved into two components by extended paper chromatography; neither of these was maltotetraose, and the R_F values were those expected of the tetrasaccharides (*b*) and (*c*) shown in Fig. 3[31]. Of structures **2**, **4**, and **8**, which had been selected by the formation of D-glucose and panose by amyloglucosidase, only structure **2** would yield two such tetrasaccharides (Fig. 3).

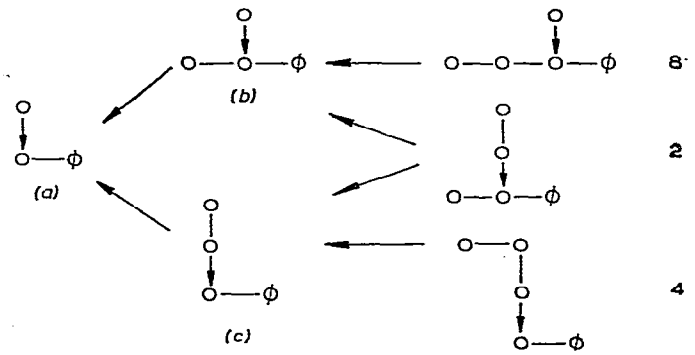

Fig. 3. The tetra- (*b*, *c*) and penta-saccharides (**2**, **4**, **8**) are members of a family of oligosaccharides that can be formed by the interaction of cyclomaltohexaose and panose (*a*), catalysed by *B. macerans* transglycosylase. The arrows indicate the pathways that each pentasaccharide would follow in being re-converted into panose by the action of amyloglucosidase. For key, see Fig. 1.

Finaliy, the pentasaccharide was synthesized from panose by an enzymic route described above. The identity of the synthetic and amylopectin-derived pentasaccharides was established by several criteria.

The total amount of analytical information brought to bear on this structural problem is much more than would normally be necessary to provide acceptable evidence of structure. The reason for introducing this degree of thoroughness into the analysis was to establish that the enzymic methods, which are far more rapid and require much less material than methylation or acid-fragmentation analysis, are in themselves entirely reliable. The use of amyloglucosidase, beta-amylase, and debranching enzymes therefore enable a complete structural analysis to be made on any oligosaccharide of the same type as the pentasaccharide, that could be derived from amylaceous polysaccharides such as starch, glycogen, or pullulan. The increasing availability of enzymes of known specificity should ensure that enzymic methods

will find increasing applications in complementing or replacing the classical chemical methods used in structural analysis.

Action pattern of B. subtilis *α-amylase.* — Based on the structure tentatively assigned to the pentasaccharide, Hughes[5] and Whelan[4] have already drawn conclusions as to the action pattern of the enzyme in the vicinity of the amylopectin branch-points. The confirmation of the correctness of the assigned structure reinforces the conclusions regarding the enzyme action. It is of fundamental interest that two species of alpha-amylase, from saliva and from *B. subtilis*, give different alpha-limit dextrins when permitted to hydrolyse amylopectin to the same degree. The smallest of these dextrins are 6^3-α-D-glucosylmaltotriose for the salivary enzyme, and 6^2-α-maltosyl-maltotriose for the *B. subtilis* enzyme. The question that may be posed is whether either of these are true end-products, or whether they, or some of the larger dextrins formed at the same time, could be broken to smaller products. This question cannot presently be answered. We can only define the products in terms of the experimental conditions under which they were produced. These are conditions under which maltotriose is degraded, essentially completely, to maltose and D-glucose, maltose itself being stable to further degradation.

It could be that if the concentration of enzyme and time of exposure of amylopectin to the enzyme were substantially increased, still further degradation, even of maltose, would be noted. The problem here would be one of distinguishing between genuine alpha-amylase action, and action of a trace impurity such as an α-D-glucosidase. The concentration of enzyme used in the present experiments is already rather high and such degradation as is observed probably represents the limit that is achieved under physiological conditions, as opposed to what might be seen *in vitro* if the enzyme concentration were to be raised to a level equimolar to that of substrate.

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