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THE ACTION PATTERN OF *Penicillium lilacinum* DEXTRANASE

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ABSTRACT

The product distributions resulting from the action of *Penicillium lilacinum* dextranase on end-labelled oligosaccharides of the isomaltose series have been determined. The initial rates of formation of labelled products were measured for isomaltotriose up to isomalto-octaose, and the molar proportions and radioactivity of the final products from isomaltotriose up to isomaltohexaose were determined. D-Glucose was released only from isomaltotriose and isomaltotetraose, by hydrolysis of the first linkage from the reducing end (linkage 1); the terminal bonds of higher members of the series were not attacked. All oligosaccharides except isomaltotriose were hydrolyzed at more than one linkage. The main points of attack on isomaltotetraose up to isomalto-octaose were at linkage 2, and at the third linkage from the non-reducing end; these two positions coincide for isomaltopentaose. The degradation of isomaltotriose up to isomalto-octaose was entirely hydrolytic. The enzyme also catalyzed an extremely slow, concentration-dependent degradation of isomaltose, and this may have occurred *via* a condensation to isomaltotetraose, followed by hydrolysis of linkage 1 to give D-glucose and isomaltotriose.

INTRODUCTION

Little is known of the pattern of hydrolysis of dextran by endo-dextranases (E.C.3.2.1.11). Even the initial cleavage of the polymer cannot be random, because (1→6)-α-D-glucosidic linkages in the vicinity of the branch linkages possess a degree of resistance to attack[1]. The products of dextranase action will thus be branched oligosaccharides from the region of the branch points, and oligosaccharides of the isomaltose series from the linear portions of the polymer. The nature of the two types of product will depend on the specificity of the enzyme. The chemical structure of the branched oligosaccharides derived from dextran has been investigated only for the dextranases of *Penicillium funiculosum*[2], *P. lilacinum*[2], and *Lactobacillus bifidus*[3]; for other dextranases, the nature of the linear products alone has been reported. Isomaltose (IM_2) is the main product[4–7] released by many dextranases, while others[5–7] also produce smaller amounts of isomaltotriose (IM_3) and D-glucose.

Bacterial endo-dextranases may provide IM_3, IM_4, and IM_5[8]; IM_3 through to IM_6[9]; IM_2 to IM_6[10], or even series of oligosaccharides of higher molecular weight of which the lowest member is IM_4[11] or IM_5[10]. Such a variation in products indicates that dextranases from different sources exhibit as much diversity as alpha-amylases.

Our interest in dextranase stems from the fact that dextrans and related polysaccharides occur in dental plaque, and are implicated in dental caries. The extracellular dextranase of *P. lilacinum*, when administered to rats in relative gnotobiosis with cariogenic strains of *Streptococcus mutans*, caused the inhibition of both caries and plaque formation[12]. This enzyme also prevented the conversion of sucrose into polysaccharide *in vitro* by cariogenic streptococci[13].

We now present the results of our preliminary investigations on the hydrolysis of oligosaccharides of the isomaltose series by *P. lilacinum* dextranase, a study made possible by the availability of a homologous series of end-labelled saccharides[8] from isomaltotriose-^{14}C to isomalto-octaose-^{14}C.

MATERIALS AND METHODS

Enzyme preparation. — *P. lilacinum* NRRL 896 was grown on a simple salts medium[14], supplemented with a mixture of oral dextrans. The conditions for the growth of the organism, and for the subsequent purification of the extracellular dextranase, were those described previously[15] for *P. funiculosum* dextranase. The combined fractions, from the single peak for dextranase activity obtained from the isoelectric-focusing step, were applied to a column of Bio-Gel P-10 to remove ampholytes. The enzyme was then stored in 50mM sodium citrate buffer (pH 6). The specific activity of the final preparation was 600 (i.u./mg of protein), a value that is close to that obtained by Chaiet *et al.*[16] for a pure dextranase from *P. funiculosum*.

Isomaltose oligosaccharides. — The oligosaccharides were prepared by partial acid hydrolysis of dextran, as previously described[8]. Isomaltose oligosaccharides labelled with ^{14}C in the non-reducing terminal unit[8] were isolated as major products of the action of dextran-sucrase on sucrose-^{14}C in the presence of excess of unlabelled isomaltose saccharide as added acceptor. Isomalto-octaose (IM_8), in which the D-glucose residue adjacent to the non-reducing terminal unit was also labelled with ^{14}C, was isolated as a minor product of a similar reaction with IM_6 as acceptor. Evidence for the position of the label in both major and minor products of dextran-sucrase action was obtained by partial hydrolysis with (1→6)-α-D-glucan gluco-hydrolase by the method previously described[8].

Determination of dextranase activity. — The digest (1 ml), containing dextran from *Leuconostoc mesenteroides* NRRL B-512 (10 mg), 50mM sodium citrate buffer (pH 6.0), and enzyme, was incubated at 35°. Portions were withdrawn after 30 min for the determination of reducing power. One unit was defined as the amount of enzyme that released 1 μmole of apparent isomaltose per min.

Paper chromatography. — The separation of the products of dextranase activity was made on Whatman No. 3MM paper with ethyl acetate–pyridine–water (10:4:3).

Strips dipped in silver nitrate–sodium hydroxide[17] were used to locate unlabelled saccharides, and the position of ^{14}C-labelled saccharides was revealed by radioautography with X-ray film. D-Glucose was separated from D-glucitol by chromatography in butanone–acetic acid–saturated, aqueous boric acid[18] (9:1:1), and the dipping reagents contained pentaerythritol[19].

Analytical methods. — Isomaltose saccharides were determined with cysteine–sulphuric acid[20], and reducing power was measured by the method of Nelson[21]. D-Glucose was assayed with D-glucose oxidase[22] reagent, as modified by Dahlqvist[23]. Protein was determined by a modification[24] of the Folin–Ciocalteu procedure. Radioactivity of sugars in solution was measured with a gas-flow detector (Nuclear-Chicago Model D47), used with an automatic scaler (Ekco Electronics Model N530G). Radioactivity of sugars on portions of paper chromatograms was measured with a liquid scintillation counter (Packard Tri-Carb Model 3003). The paper strips (4 × 7.4 cm) lined the walls of glass vials, and a toluene scintillation solution (20 ml) containing *p*-terphenyl (0.3%) and dimethyl POPOP (0.01%) was added. The sugars remained on the paper in this solvent, and when required, they were eluted with water, after first removing the scintillators by extraction with toluene.

RESULTS

Initial radioactive products of the action of P. lilacinum *dextranase on end-labelled oligosaccharides.* — Digests (0.5 ml) each containing an isomaltose oligosaccharide (0.75 μmole, 0.125 μCi) labelled with ^{14}C in the non-reducing terminal unit, and a similar digest containing isomalto-octaose having two labelled D-glucose residues at the non-reducing end, were incubated with enzyme in 12.5mM sodium citrate (pH 6) at 35°. Isomaltotetraose and higher oligosaccharides (IM_4–IM_8) were hydrolysed with 0.02 unit of dextranase, and portions (0.1 ml) of the solution were withdrawn at intervals during 15–30 min. The lowest member of the series, isomaltotriose, was a poor substrate, and 100-fold more enzyme (2 units) and a longer incubation period (2 h) were required to achieve an equivalent extent of reaction. The portions were boiled, and then applied directly to paper for chromatography. After separation and counting of the radioactivity on areas of the paper corresponding to the position of the labelled products, bond-cleavage frequencies were calculated by plotting (counts corresponding to products/total counts applied to chromatogram) against (counts corresponding to *i*-mer/total counts applied to chromatogram). The slope of each line represented the bond-cleavage frequency for the bond giving rise to the product of chain length *i*. This treatment, devised by Mr. J.D. Allen, corrected for background counts due to trace impurities and also for variation in the size of the portions being analysed (see Appendix). The example given in Fig. 1 shows the hydrolysis data for isomalto-octaose (Table I). The data for the other oligosaccharides in the series were treated by the same method, and the results are shown in Fig. 2.

Although there was a 300-fold difference between the rate of hydrolysis of isomaltotriose and isomaltotetraose, the difference between the extent of reaction of

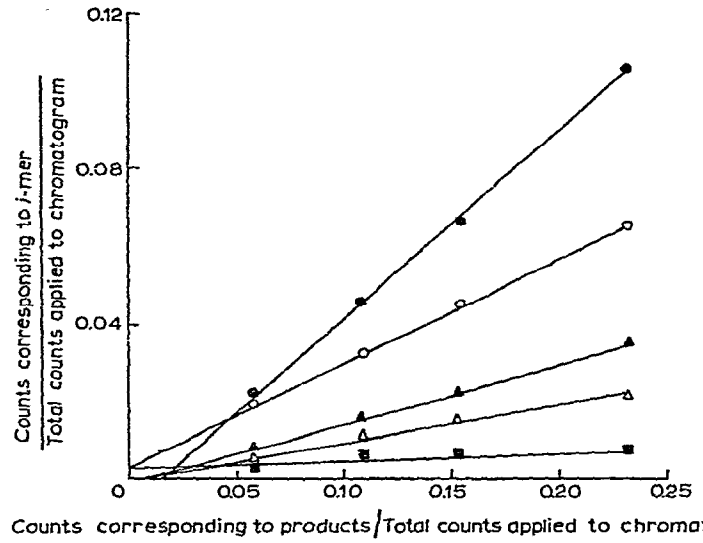


Fig. 1. Hydrolysis data for end-labelled isomalto-octaose. The slope of the lines gives the bond-cleavage frequency for the bond giving rise to the following labelled products: IM_3 (●), IM_6 (○), IM_4 (▲), IM_5 (△), and IM_2 (■). The radioactive saccharides were released from the non-reducing end of the substrate.

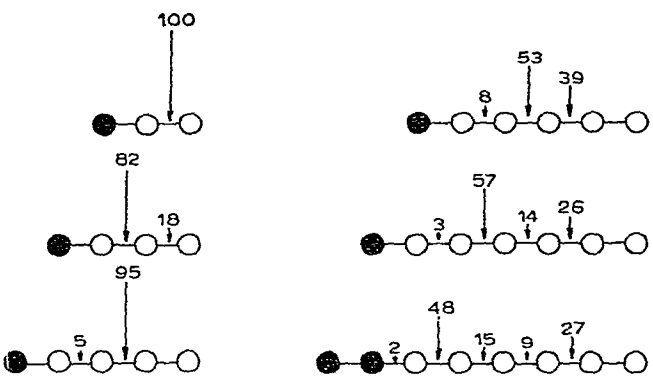


Fig. 2. Distribution of linkage hydrolysis of end-labelled isomaltose saccharides. ○, D-Glucose residue; ●, D-glucose-^{14}C residue; ———, (1→6)-α-D-glucosidic linkage. The reducing end is to the right. Arrows indicate the point and extent of hydrolysis (%). Linkages that are not arrowed are resistant to dextranase.

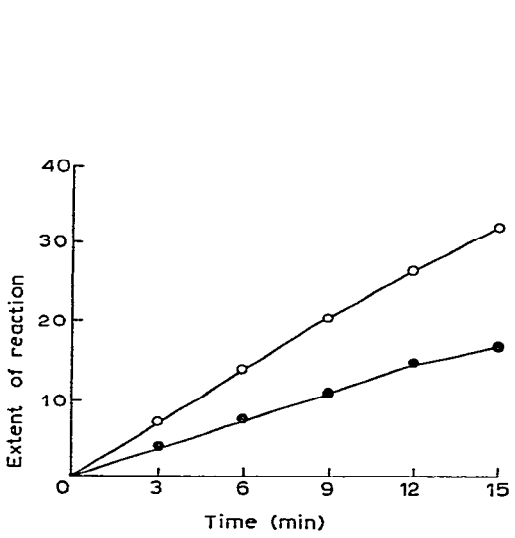
isomaltotetraose and isomaltopentaose with dextranase, under identical conditions, was only 2-fold (Fig. 3).

Complete hydrolysis of end-labelled isomaltotriose, isomaltotetraose, isomaltopentaose, and isomaltohexaose. — The linearity of the Allen plots (Fig. 1) proved that there was no secondary attack on the oligosaccharides, therefore hydrolysis of the products had not occurred during the incubation period. Consideration was then given

TABLE I

RADIOACTIVITY OF THE PRODUCTS OF DEXTRANASE ACTION ON END-LABELLED ISOMALTO-OCTAOSE

Time (*min*)	*Counts per min*						*Extent of reaction* (%)
	IM_2	IM_3	IM_4	IM_5	IM_6	IM_8	
3	96	685	263	190	613	29843	5.8
6	170	1404	460	339	980	27469	10.9
9	204	2114	718	481	1430	27176	15.4
15	183	2981	996	596	1859	21874	23.2



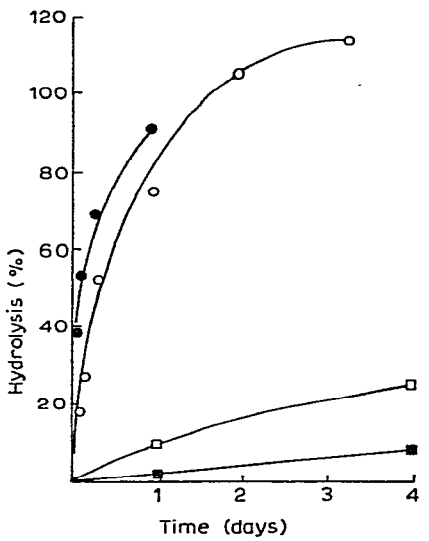


Fig. 3. Relative rate of hydrolysis of isomaltotetraose (●) and isomaltopentaose (○). The digests (0.5 ml) contained substrate (0.75 μmole), dextranase (0.02 unit), and sodium citrate buffer (12.5mM, pH 6.0).

Fig. 4. Hydrolysis of isomaltose (□, ■) and isomaltotriose (○, ●). The open and filled-in symbols refer to substrate concentrations of 10 and 1.5mM, respectively. The conditions are described in the text. Hydrolysis (100%) of isomaltotriose is defined as the release of one mole of D-glucose from one mole of isomaltotriose.

to the possibility that condensation or transfer reactions had contributed to the results in Fig. 2. This was explored by determining the molar proportion as well as the radioactivity of each product obtained from IM_3, IM_4, IM_5, and IM_6. Condensation reactions are more likely to occur with substrates, such as IM_3, that are not rapidly hydrolyzed. Digests identical to those described above were incubated with dextranase until the reducing power of controls containing unlabelled substrate had doubled. A portion was withdrawn from the control isomaltotetraose digest for the determination of D-glucose with the D-glucose oxidase reagent. Each digest was then boiled,

deionized with mixed-bed resin (Bio-Rad AG501-x8), and subjected to paper chromatography. The separated products were eluted from the paper into volumetric flasks (1 ml), and portions were withdrawn for the determination of carbohydrate and of radioactivity. The distribution of radioactivity among the labelled products from IM_3–IM_5 was close to that obtained for the early stages of the reactions, and the molar proportions of the products from all the oligosaccharides were those expected for true hydrolysis (Table II).

TABLE II

DISTRIBUTION AND RADIOACTIVITY OF THE END PRODUCTS OF DEXTRANASE ACTION ON END-LABELLED ISOMALTOSE OLIGOSACCHARIDES

Products	*Substrates*							
	IM_3		IM_4		IM_5		IM_6	
	Counts/ sec	*Mol. prop.*	*Counts/ sec*	*Mol. prop.*	*Counts/ sec*	*Mol. prop.*	*Counts/ sec*	*Mol. prop.*
Glc	30	1.0	0	0.10	0	0	0	0
IM_2	2394	1	1944	1	133	1.0	916	1.1
IM_3			397	0.11	2197	1	1362	1

Condensation reactions followed by hydrolysis also lead to an altered location of the label in the products. The position of the label in isomaltose released by hydrolysis of end-labelled isomaltotriose was ascertained by reduction followed by hydrolysis. Sodium borohydride (4.5 mg), dissolved in ice-cold water (0.1 ml), was added to isomaltose-^{14}C (0.2 mg, 0.5 ml) at 4°. After standing at room temperature for 4 h, the solution was neutralized with 1.5M sulphuric acid. The isomaltitol was hydrolysed with 0.25M sulphuric acid for 4 h at 100°, and the cooled and neutralized hydrolysate was then treated with Amberlite MB3 resin and concentrated to dryness. Methanol (3 × 10 ml) was distilled from the residue to remove boric acid, and then the products were separated by paper chromatography. Sections of the paper containing D-glucose and D-glucitol were placed in vials for counting by liquid-scintillation spectrometry. The radioactivity of D-glucitol (expressed as counts/min) was 30, whereas that of D-glucose was 6,210. Thus, 99.5% of the label was at the non-reducing end of isomaltose-^{14}C.

Effect of substrate concentration on the action of dextranase on isomaltotriose and isomaltose. — In the absence of condensation or transfer reactions, product ratios are independent of substrate concentration. Accordingly, the action of dextranase on end-labelled isomaltotriose was examined at a higher substrate concentration (10mM). During the early stages of the reaction, 0.3% of the hydrolysis occurred at linkage 2, compared with 0.4% found with 1.5mM isomaltotriose. Towards the end of the reaction when 93% of IM_3 had reacted, a higher proportion of D-glucose-C^{14} (8.3%) appeared among the products from 10mM isomaltotriose than was found (1.4%) from

1.5mM isomaltotriose. Reduction and hydrolysis of the labelled isomaltose obtained from the reaction with 10mM isomaltotriose revealed that no shift in position of the label had occurred, showing that condensation of isomaltotriose was not the explanation for the altered hydrolysis pattern. The possibility was then considered that D-glucose-^{14}C arose from the degradation of isomaltose.

The rate of apparent hydrolysis of isomaltose relative to that of isomaltotriose was determined in digests (0.5 ml) that contained dextranase (2 units) and two concentrations of substrate (1.5 and 10mM). Portions were withdrawn at intervals for the determination of D-glucose release with the D-glucose oxidase reagent. Degradation of isomaltose, which was slow compared with that of isomaltotriose (Fig. 4), was more rapid at the higher concentration of substrate. This was in contrast to the effect of substrate concentration on the hydrolysis of isomaltotriose.

Action of P. funiculosum *dextranase on isomaltotetraose and isomaltose.* — Bond-cleavage frequencies were calculated for the hydrolysis of end-labelled isomaltotetraose by *P. funiculosum* dextranase. The major proportion of the hydrolysis (92%) occurred at linkage 2, and the remaining 8% hydrolysis occurred at linkage 1, releasing D-glucose and end-labelled isomaltotriose.

The release of D-glucose from isomaltose (10mM) by the dextranases of *P. funiculosum* and *P. lilacinum* was then compared. The results (Table III) showed that *P. lilacinum* dextranase, the enzyme that produced the more D-glucose from isomaltotetraose, also had the more rapid action on isomaltose.

TABLE III

RELATIVE RATE OF D-GLUCOSE RELEASE FROM ISOMALTOSE BY DEXTRANASE FROM TWO SPECIES OF *Penicillium*

Time (days)	*Dextranase*	
	P. lilacinum	P. funiculosum
1	10	6
4	27	17
7	41	23
13	58	31

DISCUSSION

The studies of Bourne *et al.*[25] demonstrated that the products of the action of the extracellular dextranases of *Penicillium funiculosum* and *P. lilacinum* on *Streptococcus bovis* dextran were isomaltose and isomaltotriose, together with small amounts of D-glucose. These products were also released from isomaltotetraose, isomaltopentaose, and isomaltohexaose. Isomaltose was reported to be the major product of dextranase action on all these substrates.

The present study of the action pattern of a purified preparation of *P. lilacinum* dextranase was facilitated by the availability of a homologous series of isomaltose

oligosaccharides labelled with ^{14}C at the non-reducing D-glucose terminal[8]. Analysis of the initial products of hydrolysis of these oligosaccharides revealed that dextranase released D-glucose from isomaltotriose and isomaltotetraose by the hydrolysis of linkage 1; no attack on the terminal linkages of all the higher oligosaccharides could be detected. The hydrolysis of isomaltotriose to give isomaltose and D-glucose was extremely slow, and D-glucose was produced rapidly only from isomaltotetraose. However, the main cleavage of isomaltotetraose occurred at the inner linkage.

It was further shown that isomaltose (IM_2) and isomaltotriose (IM_3) were the major products from the higher isomaltose oligosaccharides, and that isomaltose resulted mainly from the hydrolysis of linkage 2, while most of the isomaltotriose was released by hydrolysis of the third linkage from the non-reducing end. Isomaltotriose was the major product from isomaltopentaose, isomaltohexaose, up to isomaltooctaose. The fact that IM_3 was not reported to be a major product by Bourne *et al.*[25] can be explained by the high concentration of enzyme used. Under such conditions, IM_3, being subject to further degradation, could not be determined accurately. In our experiments, secondary attack of isomaltose and isomaltotriose did not occur, and the frequency distribution of bond cleavage shown for IM_4 and IM_5 (Fig. 2) did not alter throughout the reaction. Thus, by choosing conditions whereby IM_3 could not be further hydrolysed, the product specificity of dextranase towards IM_3 was revealed.

Hydrolysis of all the oligosaccharides except isomaltotriose occurred at more than one position. The coincidence in IM_5 of linkage 2 with the third linkage from the non-reducing end led to 95% of hydrolysis taking place at linkage 2. It has been suggested[26] that the sum of the chain lengths of the major products of hydrolysis of a polymer is equal to the span of the active site of the enzyme. This would indicate the capacity of the active site of *P. lilacinum* dextranase to bind five D-glucose residues, for IM_2 and IM_3 are the main products of the hydrolysis of dextran[25]. However, when subsite binding energies were systematically varied to secure the best fit between experimental and computed product ratios[29], it was found that a five subsite model did not adequately account for the action pattern of the enzyme. A larger number of subsites may be necessary to fit the data, and a subsite histogram can be furnished only when the dependence of Michaelis parameters on the chain length of the oligosaccharides has been ascertained.

Care was taken to ensure that the product distributions were due to hydrolysis, for the alternatives of condensation or transfer reactions would result in an altered pattern of products. In a similar system, the action of pig pancreatic alpha-amylase[27] on maltotriose and maltotetraose, which were poor hydrolytic substrates, was found to be strongly concentration-dependent. With *P. lilacinum* dextranase, however, the product distribution at early stages in the reaction with isomaltotriose did not alter when the substrate concentration was raised from 1.5 to 10mM. Towards the end of the reaction, the proportion of labelled D-glucose in the total labelled products increased and the effect was more marked in the digest that initially contained the higher concentration of isomaltotriose.

Fig. 5 shows the probable sequence of events if condensation of two molecules

of IM_3 occurred to give IM_6. Since 39% of the hydrolysis of IM_6 occurs at linkage 2 to give IM_4, which is then hydrolysed at linkage 1 to give D-glucose, this sequence would explain the release of labelled D-glucose from isomaltotriose. The other consequences of such a pathway would be (*1*) the release of isomaltose labelled at the reducing unit, and (*2*) that isomaltose and D-glucose would not be produced in molar proportions. Analysis of isomaltose released from IM_3 proved that all the label was at the non-reducing D-glucose residue. Furthermore, no evidence was found for any change in the molar proportions of D-glucose and isomaltose from that expected for the hydrolysis of one mole of isomaltotriose, to give one mole of D-glucose and one mole of isomaltose.

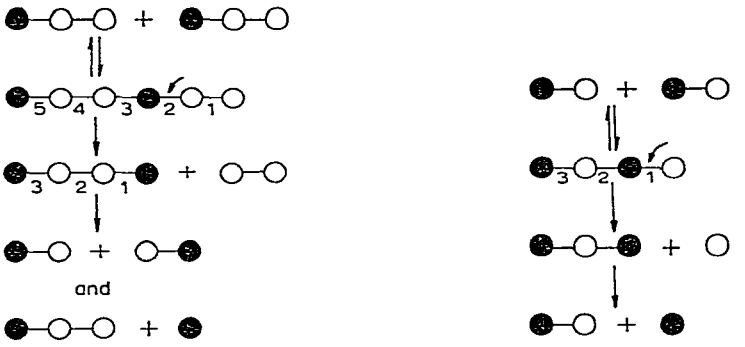


Fig. 5. Condensation of 2 molecules of isomaltotriose to give isomaltohexaose (IM_6). Cleavage at linkage 3 of IM_6 returns the original substrate, and cleavage at linkage 2 (arrowed) gives IM_4, which is labelled at both ends, and is hydrolysed at linkage 1 and linkage 2. The symbols are the same as in Fig. 2.

Fig. 6. Condensation of 2 molecules of isomaltose to give isomaltotetraose (IM_4). Cleavage of linkage 2 of IM_4 returns the original substrate, and cleavage of linkage 1 (arrowed) gives IM_3 which is labelled at both ends, and is rapidly hydrolysed to give labelled products. The symbols are the same as in Fig. 2.

The possibility that the product IM_2, released during the hydrolysis of IM_3, might condense to give IM_4 (Fig. 6) was then examined. Hydrolysis of IM_4 at linkage 1 would give isomaltotriose labelled at both end-residues, and subsequent hydrolysis of this IM_3 would inevitably release labelled D-glucose. Direct evidence for this type of degradation of IM_2 has not been obtained. It was established that *P. lilacinum* dextranase could slowly release D-glucose from IM_2 (Fig. 4), and the following considerations indicate that the degradation may proceed *via* a bimolecular mechanism. (*1*) The degradation of IM_2 was strongly dependent on concentration (Fig. 4), and an increase in substrate concentration from 1.5 to 10mM raised the initial rate of apparent hydrolysis by 5-fold. This effect was not observed with isomaltotriose, where the degradation was proved to be hydrolytic. (*2*) A condensation reaction with IM_2 would produce no net reaction if the subsequent hydrolysis of IM_4 occurred only

at linkage 2. Detection of condensation thus relies on the ability of the enzyme to hydrolyse IM_4 at either linkage 1 or 3. D-Glucose is then both an initial product, and also a secondary product from the hydrolysis of isomaltotriose. The dextranase of *P. funiculosum* differed from that of *P. lilacinum* with respect to the pattern of hydrolysis of isomaltotetraose. With *P. lilacinum* dextranase, 18% of the hydrolysis occurred at linkage 1, while the corresponding figure for the *P. funiculosum* enzyme was 8%. If these two enzymes degraded isomaltose by a condensation mechanism, it would be expected that the action of *P. lilacinum* dextranase would be the more easily detected. When isomaltose (10mM) was incubated with dextranase (2 units) from *P. lilacinum* and *P. funiculosum*, the relative rate of D-glucose release in the two digests was 1.8:1 (Table III). Thus, the ability of the two enzymes to act on isomaltose was related to the pattern of their action on isomaltotetraose. (*3*) Condensation and subsequent hydrolysis of isomaltose would explain why the product ratio for D-glucose, $R_1 = {}^{14}C$ in D-glucose/${}^{14}C$ in products, increased towards the end of the reaction of *P. lilacinum* dextranase with isomaltotriose. Since condensation of isomaltotriose does not occur, the increase in R_1 can only arise from the further degradation of isomaltose, and this could occur either by hydrolysis or by the pathway shown in Fig. 6. With 1.5mM isomaltotriose, the value of R_1 increased from 0.004 for the initial velocity measurements to 0.014 when 94% of the isomaltotriose had reacted. With 10mM isomaltotriose, the values rose from an initial 0.003 up to 0.083 when the extent of reaction of IM_3 was 93%. The concentration-dependence of this increase in R_1 strongly indicated that the slow degradation of isomaltose proceeded *via* a condensation mechanism.

ACKNOWLEDGMENTS

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APPENDIX

*Bond-cleavage frequency analysis**. — The analysis of bond-cleavage frequency of a radiolabelled oligosaccharide is complicated by both natural and sample background radiation. The natural background, which should be constant for any given counting system, is easily corrected for by determining the counting rate in the absence of a sample. This value is then subtracted from all subsequent measurements.

The sample background is usually larger and more difficult to determine accurately. This background arises primarily from contamination of the substrate

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sample by traces of radiolabelled impurities. The usual procedure for correcting for sample background is to spot an aliquot at zero time and, after making a correction for different aliquot size, subtract the activity on the chromatogram from the activity corresponding to the hydrolysis products[27,28]. The problem with this method is that any error in the zero-time determination is then introduced into all subsequent determinations. This error becomes more significant when the activity of the oligosaccharide is low in comparison with the sample background or when there is a large error in zero-time determination.

The procedure of subtracting a zero-time determination is based on the tacit assumption that the relative background for each product is constant throughout the hydrolysis, that is, that the sample background for each product is proportional to the total radioactivity of the aliquot applied to the chromatogram. This assumption is valid if the background due to streaking is insignificant. This can be shown to be true by reference to a specific case. The action of porcine, pancreatic alpha-amylase on maltopentaose having the reducing D-glucose residue labelled yields exclusively labelled maltose[27]. If streaking were significant, the apparent amount of maltotriose and maltotetraose would be expected to increase, since maltose has a larger R_F and the total radioactivity of maltose is increasing. As can be seen from Table I of Ref. 27, the radioactivity corresponding to maltotriose and maltotetraose remains constant within experimental error. Therefore, we conclude that it is reasonable to expect the sample background to be proportional to the size of the aliquot applied to the chromatogram.

The method described here for determining bond-cleavage frequencies does not depend on a single measurement of sample background by a zero-time determination. It, therefore, avoids the unnecessary propagation of error that results from subtracting the sample background. This method automatically corrects for different aliquot size and is easily adaptable to a least-squares analysis.

In the hydrolysis of an end-labelled oligosaccharide of chain length n, radioactive products may be produced of chain length $i = 1, 2, 3, \ldots, n-1$. When this mixture is chromatographed and counted, the activity of chain length i may be represented as G_i, and the sample background as B_i, so that radiation due only to products of hydrolysis is $G_i - B_i$. The fraction of this product to all products, F_i, is expressed as

$$F_i = (G_i - B_i) / \sum_{j=1}^{n-1} (G_j - B_j) \qquad (1)$$

The sample background is proportional to the total radioactivity applied to the chromatogram, thus

$$B_i = K_i \sum_{j=1}^{n} G_j, \qquad (2)$$

where K_i is the proportionality constant for chain length i. Therefore, equation *1* may

be written as

$$F_i = \frac{G_i - K_i \sum_{j=1}^{n} G_j}{\sum_{j=1}^{n-1} \left(G_j - K_j \sum_{j=1}^{n} G_j \right)} \tag{3}$$

Rearranging and dividing through by $\sum_{j=1}^{n} G_j$, gives

$$\frac{G_i}{\sum_{j=1}^{n} G_j} = F_i \frac{\sum_{j=1}^{n-1} G_j}{\sum_{j=1}^{n} G_j} - F_i \sum_{j=1}^{n-1} K_j + K_i \tag{4}$$

By plotting $\dfrac{\sum_{j=1}^{n-1} G_j}{\sum_{j=1}^{n} G_j}$ against $\dfrac{G_i}{\sum_{j=1}^{n} G_j}$

as the hydrolysis progresses, a line of slope F_i will be obtained. If this is done for every product, the fraction of each product, which is the frequency of bond cleavage to give that product, is obtained.

Any secondary attack on the products in the later stage of the reaction will be seen as deviation from a straight line, since the bond-cleavage frequency will no longer be independent of the degree of hydrolysis. To determine cleavage frequency, a least-squares fit is made to the points, omitting any points in the later stage of the hydrolysis that obviously manifest secondary attack. The lack of deviation of the points from a straight line attests to the correctness of the assumption that background is proportional to the total radioactivity of the aliquot applied to the chromatogram.

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