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# PREPARATION OF ISOMALTOSE OLIGOSACCHARIDES LABELLED WITH $^{14}C$ IN THE NON-REDUCING TERMINAL UNIT, AND THEIR USE IN STUDIES OF DEXTRANASE ACTIVITY

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ABSTRACT

A series of end-labelled isomaltose oligosaccharides was prepared by the reaction of dextran-sucrase with sucrose-$^{14}C$ in the presence of excess of unlabelled isomaltose saccharides as alternative acceptor. The main product of each reaction contained one more D-glucose residue than the acceptor substrate, and the label was located at the non-reducing end. The end-labelled saccharides were used to determine the specificity of a bacterial dextranase that required five or more consecutive α-(1→6)-D-glucosidic linkages in the substrate. The third linkage from the reducing end of isomaltohexaose ($IM_6$) and of other substrates with longer chains ($IM_7$ and $IM_8$) was the most susceptible to attack, and the products from higher oligosaccharides were $IM_3$, $IM_4$, and $IM_5$. Isomaltopentaose ($IM_5$) was further hydrolysed to $IM_3$ and $IM_2$ when a 35-fold excess of enzyme was added, but there was no action on $IM_4$, $IM_3$, or $IM_2$ under these conditions. It was concluded that the dextranase hydrolysed linkages penultimate to either end of the chain only with difficulty, and that end linkages were completely resistant to attack.

## INTRODUCTION

The action pattern of dextran-sucrase (α-1,6-glucan: D-fructose 2-glucosyltransferase, E.C. 2.4.1.5) on sucrose is altered[1–4] by the addition of isomaltose. The rate of the reaction is increased, polysaccharide synthesis is decreased, and oligosaccharides become the main products. Other di- and tri-saccharides that are good acceptors for dextran-sucrase can also exert this influence[1–4], so that the synthesis of polysaccharide is largely replaced by oligosaccharide synthesis.

We now show that isomaltose saccharides containing up to seven D-glucose residues can act as alternative acceptors to sucrose, and use has been made of this fact to synthesize a homologous series of end-labelled saccharides from isomaltotriose-$^{14}C$ to isomalto-octaose-$^{14}C$. These were prepared by the action of dextran-sucrase on sucrose-$^{14}C$ in the presence of excess of unlabelled isomaltose saccharide as added acceptor.

End-labelled oligosaccharides have been used for many years to determine the action patterns of enzymes (*e.g.* alpha-amylase[5–7], potato D-enzyme[8]). End-labelled

isomaltose saccharides have not previously been available, and an example is given of their use as substrates for a bacterial dextranase (E.C. 3.2.1.11).

## MATERIALS AND METHODS

*Carbohydrates.* — Isomaltose saccharides were isolated from a partial, acid hydrolysate of dextran (Pharmachem. Corp., Bethlehem, Pa.) obtained by heating in 0.165M sulphuric acid for 6 h at 100°. The oligosaccharides were separated by chromatography on charcoal[9], and each was further purified by chromatography on paper. Sucrose-*U*-$^{14}C$ was purchased from International Chemical and Nuclear Corporation, Burbank, Calif.

*Enzymes.* — Dextran-sucrase was prepared from *Streptococcus mutans* OMZ 176 that was grown anaerobically ($N_2+CO_2$, 95:5) in a Microferm fermentor (New Brunswick Scientific Co.) for 24 h in a medium[10] containing 1% of D-glucose. The pH was automatically controlled at 6.0. The bacteria were removed by centrifuging at 2°, for 10 min at 4000 *g*, and the cell-free filtrate (2 l) was diluted with water (800 ml) and stirred for 5 h with hydroxyapatite, Bio-Gel HT (120 ml). The procedure described by Guggenheim and Newbrun[11] was followed thereafter. Dextran-sucrase, eluted from hydroxyapatite with 0.5M potassium phosphate buffer (pH 6.0), was dialysed against 50mM sodium citrate buffer (pH 6.0) for 18 h. The enzyme solution contained 0.235 mg of protein per ml, and had no hydrolytic action on sucrose, isomaltose saccharides, or dextran.

Dextran-glucosidase (α-1,6 glucan glucohydrolase) was prepared from *Streptococcus mitis* 439 by the method of Walker and Pulkownik[12].

Dextranase-CB was a product of Calbiochem, Los Angeles. A portion (8 mg) was dissolved in 5mM sodium citrate buffer (pH 6.0) and incubated at 35° for 1 h to degrade the dextran in the preparation. The products of the hydrolysis were removed by dialysis against 10mM sodium citrate buffer (pH 6.0), then the enzyme was dialysed against 2M urea–1% glycine prior to isoelectric focusing in an LKB column of 110-ml capacity. A pH gradient of 3–7 (Ampholine) was employed, and the density gradient was prepared with glycerol. After focusing for 72 h at 600 volts, fractions (2 ml) were withdrawn from the bottom of the column, and the fraction with maximum dextranase activity was dialysed against 50mM sodium citrate buffer (pH 6). This procedure removed all the invertase activity from the commercial preparation and gave a preparation that showed a single band on disc gel-electrophoresis[13].

*Analytical methods.* — Sucrose was determined by a modification of the anthrone method[14]. Isomaltose saccharides were estimated with cysteine–sulphuric acid[15], and reducing power was measured by the method of Nelson[16]. Protein was determined by a modification[17] of the Folin–Ciocalteu procedure. Radioactivity was measured with a gas-flow detector (Nuclear-Chicago Model D47) used with an automatic scaler (Ekco Electronics Model N530G). D-Glucose was assayed with D-glucose oxidase[18] reagent, as modified by Dahlqvist[19].

*Paper chromatography.* — The separation of homologous isomaltose saccharides

was made on Whatman No. 3MM paper in ethyl acetate–pyridine–water (10:4:3). Strips dipped in silver nitrate–sodium hydroxide[20] were used to locate unlabelled saccharides, and the position of $^{14}C$-labelled saccharides was revealed by preparing radioautograms using X-ray film. D-Glucose was separated from D-glucitol by chromatography in butanone–acetic acid–saturated aqueous boric acid[21] (9:1:1), and the dipping reagents contained pentaerythritol[22].

## RESULTS

*Preparation of end-labelled isomaltose saccharides.* — In previous experiments with dextran-sucrase, where isomaltose or isomaltotriose were added as alternative acceptors to sucrose, the products were a series of isomaltose saccharides[1–4]. In the present work, the aim was to synthesize one main product, containing only one more D-glucose residue than the acceptor. Conditions were therefore chosen so that, throughout the reaction of dextran-sucrase with sucrose-$^{14}C$, the added, unlabelled acceptor remained in excess over the end-labelled product.

The labelled saccharides were prepared by incubating sucrose-$^{14}C$ (1.47 mg, 7.7 $\mu$Ci), isomaltose saccharide (18 $\mu$moles), and dextran-sucrase (47 $\mu$g, 0.02 unit) in 0.5 ml of solution at 35° and pH 6. After all the sucrose had reacted, the digests were heated to inactivate the enzyme, cooled, and treated with Biodeminrolit mixed-bed resin (The Permutit Co. Ltd.) in the carbonate form, to remove buffer salts. The solution was concentrated, and the products of the reaction were separated from the unlabelled substrate by paper chromatography. Examination of the radioautograms revealed that each reaction had yielded two labelled isomaltose saccharides. The main product contained one more D-glucose residue, and the minor product contained two more D-glucose residues than the acceptor substrate, respectively. The labelled saccharides were eluted with water into volumetric flasks (1 ml), and the specific radioactivities are recorded in Table I. In each reaction, the combined weight of the two products accounted for all the sucrose that had reacted, and 80% of the transferred D-glucosyl residues appeared in the main product. No transfer of D-glucosyl residues to water had occurred, and D-fructose was the only monosaccharide product. The

TABLE I

SPECIFIC ACTIVITIES OF END-LABELLED ISOMALTOSE SACCHARIDES (IMS)

| *Unlabelled IMS acceptor* | *Major product* | | | *Minor product* | | |
|---|---|---|---|---|---|---|
| *D.p.* (n) | *D.p.* (n+1) | *Weight* (mg) | *Specific activity* (counts/sec/mg) | *D.p.* (n+2) | *Weight* (mg) | *Specific activity* (counts/sec/mg) |
| 2 | 3 | 1.34 | 24102 | 4 | 0.29 | 43040 |
| 3 | 4 | 2.21 | 19140 | 5 | 0.32 | 32900 |
| 4 | 5 | 2.68 | 14560 | 6 | 0.36 | 23100 |
| 5 | 6 | 3.49 | 11320 | 7 | 0.47 | 20200 |
| 6 | 7 | 3.68 | 10320 | 8 | 0.56 | 18530 |

specific activities of the end-labelled saccharides were in accord with the presence of one D-glucosyl-$^{14}C$ residue in the main product, and of two labelled D-glucose residues in the minor product.

*Position of $^{14}C$-labelling in isomaltose saccharides.* — The position of the labelled D-glucose residue in the saccharides depends on the direction of chain elongation. Recent experiments to determine the direction of propagation of polysaccharide synthesis from sucrose by amylosucrase showed that D-glucosyl additions were made at the non-reducing end of the growing polymer[23]. The claim that dextransucrase and levan-sucrase elaborate their polysaccharides towards the reducing end by means of an insertion-type mechanism[24] has not found general acceptance.

It was shown, as follows, that the reaction of dextran-sucrase with sucrose-$^{14}C$ and isomaltose produced isomaltotriose-$^{14}C$ that was not labelled at the reducing end. Sodium borohydride (7.5 mg), dissolved in ice-cold water (0.2 ml), was added to isomaltotriose-$^{14}C$ (0.22 mg, 25 $\mu$l) at 4°. After standing at room temperature for 3 h, the solution was neutralized with 1.5M sulphuric acid. The isomaltotri-itol was hydrolysed with 0.25M sulphuric acid for 4 h at 100°, and the cooled and neutralised hydrolysate was then treated with Amberlite MB3 resin and concentrated to dryness. Methanol (3 × 5 ml) was distilled from the residue to remove boric acid, and then the products were separated by paper chromatography. D-Glucose and D-glucitol were eluted from appropriate sections of the paper, and portions were dried on aluminium planchets. The radioactivity of the D-glucitol, expressed as counts/100 sec, was 30, while that of the D-glucose fraction was 21,000. Thus, less than 0.15% of the label was at the reducing end of isomaltotriose-$^{14}C$.

Evidence that the saccharides were labelled exclusively at the non-reducing end was obtained by degradation with dextran-glucosidase. This exo-dextranase removes D-glucosyl residues one at a time from the non-reducing end of isomaltose saccharides and dextran[12], and a partial, enzymic hydrolysis of an isomaltose saccharide with chain length $n$ gives all possible intermediates $n$, $n-1$, $n-2$, down to 1 (D-glucose). Dextran-glucosidase (1.25 i.u.) was incubated with each $^{14}C$-labelled saccharide (275 $\mu$g) at pH 6 and 35°, until the hydrolysis to D-glucose, as measured with the D-glucose oxidase reagent, was ~30%. The products were separated by paper chromatography, and after a radioautogram had been prepared, the position of the saccharides on the paper was revealed with silver nitrate. Large spots of each possible intermediate product from each substrate were seen on the paper, but the corresponding radioautogram showed only two spots in each case. One of these corresponded to undegraded substrate, the other was glucose. The absence of radioactivity in the intermediate products proved conclusively that all the label was situated at the non-reducing end.

*Action of dextranase-CB on dextran.* — Dextranase-CB was supplied as a high-purity bacterial enzyme that hydrolyzes α-(1→6)-D-glucosidic linkages in dextran. The main product was claimed to be isomaltotriose. The action of the purified preparation of dextranase (0.03 unit) was tested on dextran B512-F (2 mg) in a digest (1 ml) containing 6mM sodium citrate buffer (pH 6.0). After incubation at

35° for 3 h, the development of reducing power corresponded to an apparent 85% conversion into isomaltotriose, (Fig. 1). A portion of the digest was examined by paper chromatography, and the main products of low molecular weight were isomaltotriose and isomaltotetraose, with a smaller amount of isomaltopentaose. Traces of isomaltose were seen, but D-glucose was completely absent. After a further incubation for 24 h, the reducing power had not increased, and the same products $IM_3$, $IM_4$, and $IM_5$ were observed on a paper chromatogram. These were therefore stable products under the conditions of this experiment.

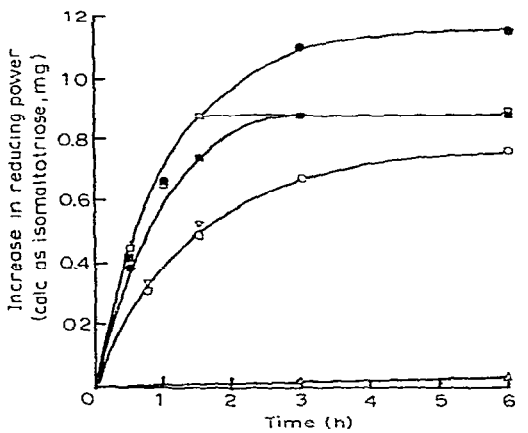


Fig. 1. Action of dextranase-CB on isomaltose saccharides and dextran. The enzyme (0.015 unit) was incubated with 1 mg of dextran (■), and with 0.75 μmole of $IM_5$ (△), $IM_6$ (○), $IM_7$ (▽), $IM_8$ (□), and $IM_9$ (●) at 35° and pH 6.0.

*Action of dextranase-CB on isomaltose saccharides.* — The relative rate of hydrolysis of isomaltopentaose through to isomaltononaose was studied at 35° in digests (0.5 ml) that contained substrate (1.5mM), sodium citrate buffer (15mM, pH 6.0), and dextranase (0.015 unit). Under these conditions, hydrolysis of $IM_5$ was very slow, and $IM_6$ was the smallest substrate to be rapidly hydrolysed. The hydrolysis of $IM_8$ and $IM_9$ proceeded at twice the rate of $IM_6$ and $IM_7$ (Fig. 1). When $IM_5$ and $IM_4$ were incubated in similar digests containing a higher concentration of enzyme (0.75 unit), isomaltopentaose was completely hydrolysed within 2 h, but there was no action on isomaltotetraose. No products of higher molecular weight were observed.

*Action of dextranase-CB on end-$^{14}$C-labelled isomaltose saccharides.* — The digests (0.5 ml) contained an amount of labelled substrate such that the radioactivity (0.125 μCi), expressed in counts/sec, was ~2600. Unlabelled isomaltose saccharide was added as necessary to raise the substrate concentration to 1.5mM. $^{14}$C-Labelled isomaltotetraose and isomaltopentaose were incubated at 35° for 6 h with dextranase (0.5 unit). The digests were heated to inactivate the enzyme, buffer salts were removed with Amberlite MB3, and the products were separated by paper chromatography.

Radioautograms revealed that there were no products from $IM_4$, and that the products from $IM_5$ were $IM_3$ and $IM_2$. Again, no products of higher molecular weight were seen.

$^{14}$C-Labelled isomalto-hexaose, -heptaose, and -octaose were incubated in similar digests for 6 h with a lower concentration (0.015 unit) of enzyme. After paper chromatography of the products, the strips were eluted with water into volumetric flasks (1 ml). Portions were then taken for the determination of carbohydrate and of radioactivity, and the results are shown in Table II. Yields of counts and weight were ~88%. The losses occurred by absorption of the sugars on resin during the deionization procedure.

TABLE II

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

| *Products* | *Substrates* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $IM_5$ *Counts/sec* | *Mol. prop.* | $IM_6$ *Counts/sec* | *Mol. prop.* | $IM_7$ *Counts/sec* | *Mol. prop.* | $IM_8$ *Counts/sec* | *Mol. prop.* |
| $IM_2$ | 318 | 1 | 5 | 0.02 | — | — | — | — |
| $IM_3$ | 1975 | 1.1 | 1870 | 1 | 407 | 1.02 | 404 | 1.1 |
| $IM_4$ | — | — | 90 | 0.02 | 751 | 1 | 706 | 1.0 |
| $IM_5$ | — | — | — | — | — | — | 1095 | 1 |

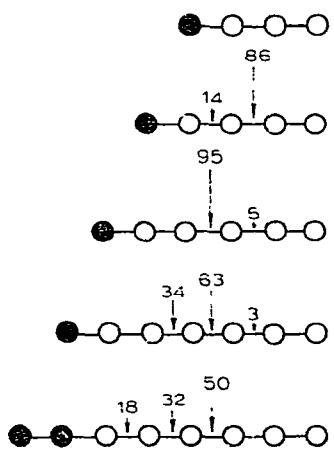


Fig. 2. Distribution of linkage hydrolysis of end-labelled isomaltose saccharides. ○, D-Glucose residue; ●, D-glucose-$^{14}C$ residue; —, α-(1→6)-D-glucosidic linkage. Arrows indicate the point and extent of hydrolysis (%). Linkages that are not arrowed are resistant to dextranase.

Most of the isomaltose saccharides were hydrolyzed to give more than one labelled product, showing that the enzyme could react with the substrate in more than

one way. The proportion of attack at each linkage was calculated from the distribution of radioactivity among the labelled products. The results are shown diagrammatically in Fig. 2.

## DISCUSSION

Dextran-sucrase is an extracellular enzyme produced by certain strains of *Streptococcus*[1,10,11], *Leuconostoc*[2,3,25], and *Lactobacillus spp.*[26]. The ability to transfer a D-glucosyl residue from sucrose to isomaltose would appear to be a general property of dextran-sucrases, for there are reports on the production of isomaltose saccharides by *Streptococcus bovis*[1] and by *Leuconostoc mesenteroides*[3] dextran-sucrases when isomaltose was added as an alternative acceptor. End-labelled isomaltose saccharides could therefore be prepared with enzyme from any of these sources. It should be emphasized, however, that some strains of oral streptococci (*e.g. S. mutans, S. mitis*) that produce dextran-sucrase may also contain enzymes that will hydrolyse the donor (sucrose) or the acceptor and product isomaltose saccharides ($IM_n$) of the reaction:

$$\text{Sucrose-}^{14}C + IM_n \rightarrow \text{D-Glucosyl-}^{14}C\text{-}IM_n + \text{D-Fructose-}^{14}C$$

Hence, to achieve a quantitative result, the dextran-sucrase must be free from invertase-type enzymes[27], and also from dextran-glucosidase[12], an enzyme that rapidly hydrolyses isomaltose and isomaltose saccharides.

The dextran-sucrase preparation from *S. mutans* OMZ 176 used in the present work contained no enzymic impurities that could influence the above reaction, and all the radioactivity from the utilization of sucrose-$^{14}C$ was recovered as D-fructose-$^{14}C$ and end-labelled saccharide. Nevertheless, the enzyme preparation consisted of several glucosyltransferases[11], some of which synthesized α-(1→3)-D-glucan under conditions different from those described here. It has previously been established that the oligosaccharides produced by the glucosyltransferases of *S. mutans* OMZ 176, in the presence of isomaltose, contained α-(1→6)-D-glucosidic linkages exclusively[4].

Experiments designed to locate the position of the $^{14}$C-label in isomaltose saccharides proved that a D-glucosyl-$^{14}C$ residue had been transferred from sucrose to the non-reducing end of the acceptor substrate. The provision of end-labelled isomaltose saccharides enabled the specificity of a bacterial dextranase to be investigated. Results with unlabelled substrates had indicated that two linkages at each end of the isomaltose saccharides might be resistant to attack, for D-glucose and isomaltose were normally absent from the reaction products. Also, the dextranase had no action on isomaltose or isomaltotriose, which contain only end-linkages, and D-glucose was not released from higher oligosaccharides or from dextran. Isomaltotetraose contains one penultimate linkage, and a high concentration of enzyme failed to hydrolyze this substrate. Isomaltopentaose was unique in being the only substrate that yielded isomaltose as a major product of hydrolysis. With end-labelled isomaltopentaose, it was shown that both the inner linkages could be hydrolyzed with a high

concentration of enzyme, and that linkage 2 (counting from the reducing end) was the most susceptible to attack (Fig. 2).

A far lower concentration of enzyme achieved a rapid hydrolysis of isomaltohexaose and higher saccharides. This indicated that dextranase-CB required the presence of at least five consecutive α-(1→6)-D-glucosidic linkages in the substrate before rapid attack could occur. It was not possible to decide whether the hydrolysis of linkage 3 in isomaltohexaose occurred because of its distance from the reducing end, or because it was the third linkage from the non-reducing end. This point was elucidated with isomaltoheptaose-$^{14}C$, in which linkages 3 and 4 were both hydrolysed, but where most of the hydrolysis (63%) occurred at linkage 3. The results with isomalto-octaose supported this conclusion. The attack was again mainly at linkage 3 (50%), with a lesser amount of hydrolysis (32% and 18%) occurring at linkages 4 and 5, respectively. The release of equimolar proportions of isomaltotriose and isomaltopentaose from isomalto-octaose, and the absence of isomaltose from the reaction products, indicated that the enzyme concentration was too low in this experiment to allow any hydrolysis of isomaltopentaose. Since the rate of hydrolysis increased with chain length up to the octasaccharide, it is possible that the hydrolysis of linkage 3 proceeds most rapidly if it is five linkages from the non-reducing end. The initial rates of hydrolysis of isomalto-octaose and isomaltononaose were identical (Fig. 1), and this could indicate that linkage 3 was hydrolysed with equal ease, whether it was five, six, or more linkages away from the non-reducing end. The fact that reducing groups were released at equivalent rates from isomalto-octaose, isomaltononaose, and dextran gives support to this suggestion.

The specificity of dextranase-CB for the hydrolysis of linkage 3 in isomaltohexaose was almost absolute, whereas all the other substrates were hydrolysed at two or more positions (Fig. 2). This could mean that isomaltohexaose was bound to the enzyme in a special way, indicating the capacity of the active site to bind six D-glucose residues.

Some depolymerizing enzymes can transfer glycosyl residues to acceptors other than water, or catalyze condensation reactions. It was necessary, therefore, to ensure that the product distributions observed with dextranase-CB were entirely due to hydrolysis. If transferase or condensation reactions occurred, hydrolysis of the product of higher molecular weight would ensue. For example, condensation or transfer reactions with isomaltopentaose would provide products that would subsequently be hydrolysed to give a proportion of isomaltotetraose. But the weights and molar proportions of the products, isomaltose and isomaltotriose, obtained from isomaltopentaose (Table II) indicated that hydrolysis was the only method of degradation. Similarly, transferase action on isomaltohexaose, followed by hydrolysis, would have led to such alternative products as isomaltotetraose and isomaltopentaose in addition to the main product, isomaltotriose. It is concluded that, under the conditions of these experiments, reactions other than hydrolysis did not occur. It is possible, however, that, with higher concentrations of saccharides, the action pattern of the enzyme might be different. Pig pancreatic alpha-amylase[7] and *Penicillium*

*lilacinum* dextranase[28] showed changes in the pattern of degradation of trisaccharides as the concentration of these substrates, which were not readily hydrolysed, was increased. The possibilities that dextranase-CB might act on isomaltotetraose, and degrade isomaltopentaose by a different mechanism, at substrate concentrations higher than 1.5mM, have not been examined.

Dextranase-CB may be compared with the extracellular dextranase[29] produced by a rumen strain of *Lactobacillus bifidus*. The end products of the action of *L.bifidus* dextranase on dextran were isomaltotriose, isomaltotetraose, and isomaltopentaose, with a smaller amount of isomaltohexaose and a trace of isomaltoheptaose, but no D-glucose or isomaltose. The inability of *L. bifidus* dextranase to hydrolyse isomaltopentaose, and the slow rate of attack on isomaltohexaose, were the main differences between this enzyme and dextranase-CB. Both enzymes hydrolysed isomaltoheptaose and isomalto-octaose rapidly. From a consideration of the action on reduced saccharides[30], it was deduced that linkages 3 and 4 in reduced isomaltoheptaose, and linkages 3, 4, and 5 in reduced isomalto-octaose were hydrolysed by *L. bifidus* dextranase. This specificity is closely similar to that of dextranase-CB. These two bacterial dextranases are markedly different from the other known endo-dextranases, most of which produce isomaltose[31–34] as a main product from dextran, while some[32–34] also produce smaller amounts of D-glucose or isomaltotriose.

More information on the action pattern of dextranase-CB could be obtained with isomaltose saccharides of higher d.p. The nonasaccharide is the smallest substrate that could be used to determine whether multiple attack can occur. Because there are indications that the enzyme may attack from the reducing end, the label in higher isomaltose saccharides should preferably be located at the reducing D-glucose residue, so that the labelled products released in the early stages of the reaction would indicate the point of attack. This kind of labelled substrate could not be synthesized with dextran-sucrase, because glucose is not an efficient acceptor. Reduction with sodium borohydride-*t* is a favoured method for labelling the reducing end of oligosaccharides, but this changes the nature and conformation of the reducing unit, thus producing substrates that are unacceptable for specificity studies.

Since dextranase-CB has no action on end linkages, isomaltose saccharides having two labelled D-glucose residues at the reducing end would be suitable substrates for further studies of specificity. These could easily be prepared by modifying the procedure described in this paper so that dextran-sucrase transferred D-glucosyl residues from unlabelled sucrose to isomaltose-$^{14}C$, producing a series of isomaltose saccharides labelled at the reducing end.

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