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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO A STUDY OF BRANCHING IN DEXTRANS

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ABSTRACT

The length of the side chains in dextrans has been examined by enzymic hydrolysis and l.c. The linear (IM_n) and branched (B_n) oligosaccharide-products of endodextranase activity were separated by l.c. in water. The branched fractions B_5–B_8, obtained from *Leuconostoc mesenteroides* NRRL B-512(F) dextran were resolved into two components by the same system. Treatment of the isolated components of B_5 with (1→6)-α-D-glucan glucohydrolase, showed that B_5-1, the oligosaccharide eluted in the first peak, was completely hydrolysed to D-glucose and B_4, whereas B_5-2 was not a substrate. The two components of B_6–B_8 were all hydrolysed to D-glucose and B_5-2. From a knowledge of the specificity of the two dextranases, together with the results of methylation analysis, it was concluded that the B_n-1 series were 3^3-α-isomaltosylisomaltosaccharides, and that the B_n-2 series were 3^3-α-D-glucosylisomaltosaccharides. The products are consistent with the structures previously proposed for B_5–B_8, and confirm directly that side chains containing two or more glucose residues occur in B-512(F) dextran. The B_n-1 series was obtained neither from *Streptococcus viridans* NRRL B-1351 dextran nor from a chemically synthesized, branched dextran in which the (1→3) branch linkages attached D-glucosyl side-chains exclusively. A determination of B_n-2 oligosaccharides and B_4 (3^3-α-D-glucosylisomaltotriose) in the final products indicates the proportion of glucosyl side-chains in dextrans.

INTRODUCTION

The dextran produced by *Leuconostoc mesenteroides* NRRL B-512(F) is a (1→6)-linked α-D-glucan, with some 5% of branch linkages attached to the 3-positions of the backbone[1–4]. The length of the side chains in many dextrans has been assumed to be one residue, but often without direct proof. Chemical studies on B-512(F) dextran indicated that ~40% of the side chains were single D-glucosyl groups, and some 45% were isomaltosyl groups[5]

Several (1→6)-glucosidic linkages in the vicinity of the branch point in

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dextrans are resistant to hydrolysis by endodextranases. These enzymes are therefore suitable for the partial degradation of dextrans into fragments that can more easily be characterized. Separation and identification of the products can provide insights into specific structural features of each dextran.

Previous studies on dextrans generally relied on the separation of enzymic degradation products by paper chromatography (p.c.)[6–8]. This technique permitted the separation of two homologous series consisting of linear (IM_n) and branched (B_n) oligosaccharides, respectively. However, analysis of the B_n series from B-512(F) dextran[8] indicated that oligosaccharides having $n \geqslant 5$ most probably contained two isomers that were not resolved by p.c. The suggestion was made that the two components would be expected from regions in the dextran backbone bearing 1-unit and 2-unit side-chains, respectively.

The present work has confirmed this proposal. The improved resolution of high performance liquid chromatography (l.c.) as compared to p.c. permitted quantitative separation of *two* homologous series of branched oligosaccharides from B-512(F) dextran, whereas a single series of branched oligosaccharides was obtained from a synthetic, branched dextran containing 1-unit side chains exclusively[9].

MATERIALS AND METHODS

Carbohydrates. — A synthetic, branched dextran was a generous gift from Professor C. Schuerch, and dextrans from *Leuconostoc mesenteroides* NRRL B-512(F) and *Streptococcus viridans* NRRL B-1351 were kindly provided by Dr. Allene Jeanes. Isomaltose (IM_2) and isomalto-oligosaccharides (IM_n) were separated from a partial, acid hydrolysate of B-512(F) dextran by column chromatography on charcoal[10], followed by chromatography on paper.

Enzymes. — (1→6)-α-D-Glucan glucohydrolase (EC 3.2.1.70) was prepared from *Streptococcus mitis* as described by Walker and Pulkownik[11]. Endodextranase (EC 3.2.1.11)[12] from *Penicillium funiculosum* QM 474 and from[13] *Bacillus coagulans* NRRL B-3949 were prepared as described previously. The unit of activity of the dextranases is defined as the amount of enzyme that liberates 1 μmol of reducing sugar per min.

Enzymic hydrolyses. — Dextrans (50 mg) were incubated at 35° with endodextranase (1.32 U) in digests (5 mL) buffered with sodium citrate (1.25mM) at pH 6.0. At intervals, from 2 h to 6 days, the digests were boiled for 15 min to inactivate the enzyme and samples were taken for the determination of reducing power[14] and carbohydrate[15]. The remainder was deionized with Amberlite MB-3 resin when necessary, and then filtered in preparation for chromatography. The samples were evaporated and redissolved in water (0.2 mL).

When *P. funiculosum* dextranase was incubated with B-512(F) dextran under these conditions, the apparent conversion into IM_2 was 78% at 2 h, 100% at 7 h, 118% at 24 h; thereafter the hydrolysis rose slowly to a maximum of 124% over 6 days. After all the IM_3 had been hydrolysed to D-glucose and IM_2 (3 days), the

percentage of D-glucose released remained steady at 14%. The dextranase retained 81% of its original activity after incubation for 9 days.

Branched oligosaccharides (0.75 mg) were incubated at 35° with (1→6)-α-D-glucan glucohydrolase (1.44 mU) in digests (0.3 mL) containing sodium citrate buffer (25mM), pH 6.0. After 24 h, the digests were boiled, and samples were taken for the determination of glucose[16] and carbohydrate[15]. The remainder of each digest was then prepared for l.c. by deionization, filtration, and concentration.

Chromatography. — Separation of the products of dextranase activity was made on Whatman No. 3MM paper in 10:4:3 ethyl acetate–pyridine–water by descending p.c. for 70 h. The oligosaccharides were revealed by the silver nitrate–sodium hydroxide dip procedure.

For l.c., a Varian model 5000 liquid chromatograph equipped with a manual-loop injector was used in conjunction with a Dextropak cartridge (0.8 × 10 cm) fitted with a precolumn insert and held in a radial-compression separating system (Z-module and RCSS C_{18} Guard PAK, Waters Associates). The solvent, water, was vacuum filtered through a Norganic purification-system (Millipore Corporation) comprising a scavenger resin and a membrane filter (HA, pore size 0.45 μm). The samples were prepared for l.c. by centrifuging through a microfilter (MF-1, Bioanalytical Systems Inc.) fitted with a regenerated-cellulose membrane (RC 58, pore size 0.2 μm). Separation of the oligosaccharides was monitored with a differential refractometer (R 401, Waters Associates) and peak areas were measured with a chromatography data system (CDS111, Varian).

Branched oligosaccharides were separated at a constant flow-rate of 2 mL/min. For a complete separation of glucose, IM_2, and IM_3 from higher oligosaccharides, the flow rate was linearly increased from 0.5 to 3.0 mL per min during the first 10 min of the operating programme.

RESULTS AND DISCUSSION

L.c. and p.c. of linear and branched oligosaccharides derived from dextrans. — It has been shown[17] that isomaltose oligosaccharides having d.p. 2–7 may be separated by the l.c. system used in this investigation. The ability of the system to separate linear α-D-(1→6)-linked oligosaccharides from those containing a single (1→3)-branch linkage has now been compared with that of paper chromatography (p.c.). Linear and branched oligosaccharides of the same d.p. were obtained from dextran by hydrolysis with a bacterial endodextranase. *Bacillus coagulans* dextranase has no action on isomaltotriose (IM_3) or IM_4, and the hydrolysis of IM_5 occurs only very slowly[13]. Hence these oligosaccharides accumulate in the digests, together with branched oligosaccharides (B_n) derived from regions in the vicinity of branch points in the dextran. Separation by p.c. (Fig. 1) of the products from B512(F) dextran showed that the main oligosaccharides were IM_3, IM_4, and IM_5, with traces of B_4 and B_5. The main products from the more highly branched B-1351 dextran were IM_3, B_4, IM_4, and B_5. The spots were of similar intensity, and were

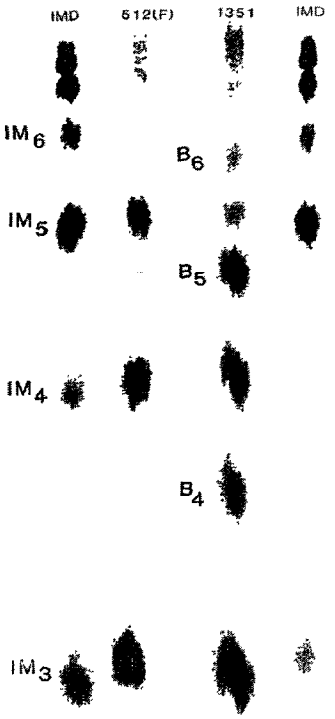


Fig. 1. Separation by paper chromatography of the main products of *B. coagulans* endo-(1→6)-α-D-glucanase action on *L. mesenteroides* NRRL B-512(F) dextran and *S. viridans* NRRL B-1351 dextran. The standard (IMD) contains oligosaccharides of the isomaltose series The B_n oligosaccharides also contain a (1→3)-glucosidic linkage.

far stronger than that of IM_5. Separation of the enzymic-digestion products by l.c. confirmed and augmented the p.c. results, and provided a quantitative result for the widely different ratio of IM_5 to B_5 obtained from the two dextrans (Fig. 2). The separation of IM_n from B_n improved with increasing values of n. IM_4 and B_4 were not well separated by l.c. in aqueous solution, although the leading edge of the tetrasaccharide peak was mainly B_4, with the trailing edge being mainly IM_4. Addition of ammonium sulphate (M) to the eluant[18] permitted the complete separation of B_4 from IM_4 (not shown).

The products of hydrolysis of dextrans with Penicillium funiculosum *dextranase.* — Fungal dextranases, unlike many bacterial dextranases, hydrolyse IM_4 and higher isomalto-oligosaccharides as rapidly as dextran, and IM_3 is also slowly hydrolysed to IM_2 and D-glucose. The main products of dextran hydrolysis by *P. funiculosum* dextranase, separated by p.c., were glucose, isomaltose, and branched oligosaccharides. We have now examined the products from three different dextrans by l.c. The dextrans were (*1*) a chemically synthesized dextran[9], V39,

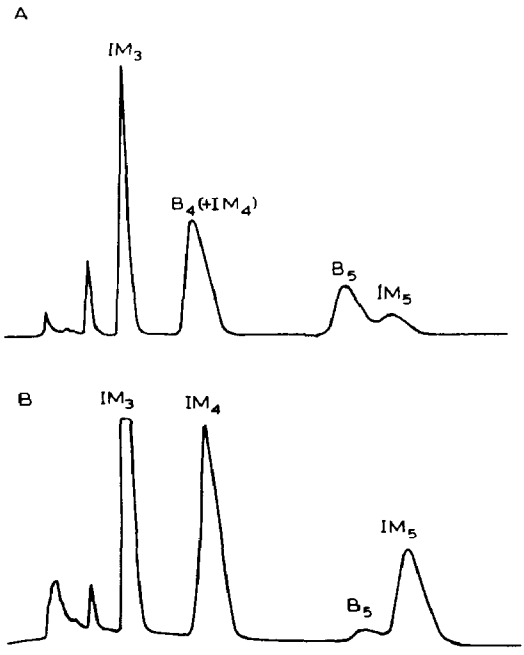


Fig. 2. Separation by l.c. of low-mol.-wt. products of *B. coagulans* endodextranase on A, B-1351 dextran, B, B-512(F) dextran. Symbols are as in Fig. 1.

containing 10% of (1→3) branch linkages, and with side chains consisting entirely of single D-glucosyl groups, (*2*) dextran from *S. viridans* NRRL B-1351 having 10.5% of (1→3) branch linkages[19], and (*3*) dextran from *L. mesenteroides* NRRL B-512(F) containing 4.6% of (1→3) branch-linkages. Separation of the products from each dextran by p.c. revealed single spots in positions corresponding to glucose, isomaltose, B_4, and B_5–B_8.

B-512(F) dextran. Previous results[8] had indicated that the B_5 fraction, and possibly the higher branched oligosaccharides from B-512(F) dextran, consisted of more than one component. The l.c. separation of products from B-512(F) dextran confirmed this hypothesis, and two well resolved peaks were found for B_5–B_8 (Fig. 3, Table I). Because of the ability of the l.c. system to resolve anomers of some oligosaccharides, it was necessary to prove that the double peaks were not anomers. Several injections of B_5 and B_6 material eluted from p.c. were made, and each peak was collected, concentrated, and reinjected. Single peaks corresponding to each of the expected peaks (Fig. 3) were obtained. The samples were kept overnight at room temperature to permit mutarotation. Reinjection the next day still revealed single peaks, confirming that the B_5 and B_6 fractions obtained from p.c. each consisted of two different branched fractions, corresponding to peak 1 and peak 2.

Plots of log(retention times) relative to that of B_4 against d.p. gave two parallel lines for the peak 1 and peak 2 series, showing that they were homologous series

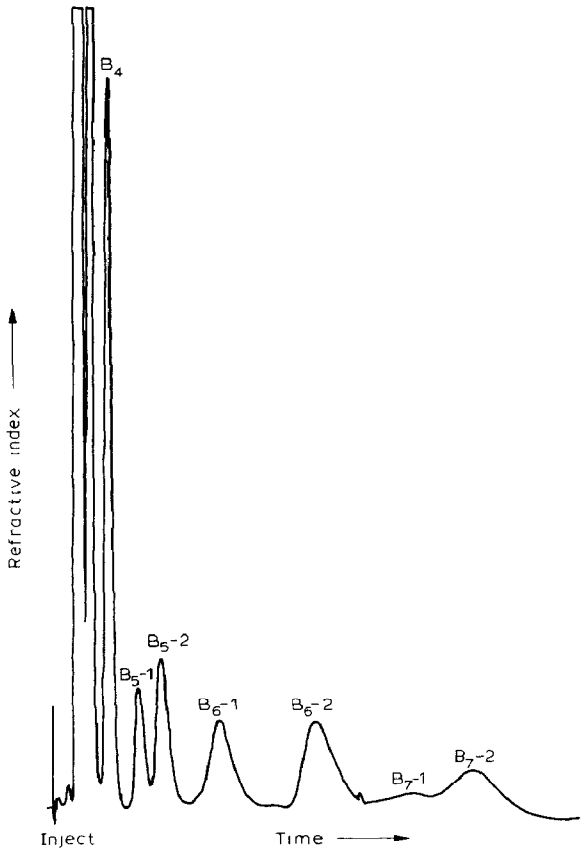


Fig. 3. Separation by l.c. of the branched oligosaccharide-products of the action of *P. funiculosum* endodextranase on *L. mesenteroides* NRRL B-512(F) dextran. Incubation time was 24 h.

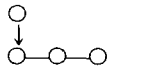
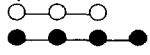
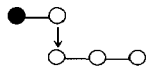
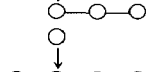
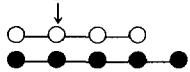
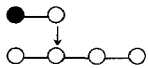
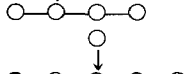
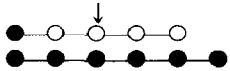
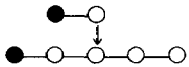
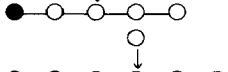
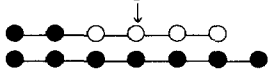
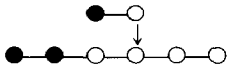
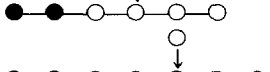
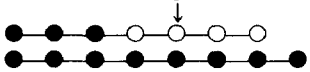
that differed by a constant factor (Fig. 4). A plot of log(retention times) for isomalto-oligosaccharides relative to IM_4 against d.p. gave a line that was identical to the peak 2 series (B_n-2). This result indicated that the peak 2 branched series were oligosaccharides containing increasing numbers of glucose residues joined by (1→6) linkages to the main chain of B_4.

V39 and B1351 dextrans. By contrast, l.c. separation of the products from V39 and B-1351 dextrans yielded single peaks for B_5–B_8 (Figs. 5 and 6). The retention times of the branched oligosaccharides corresponded to those of the B_n-2 series derived from B-512(F) dextran . As V39 contains 1-unit side chains exclusively, the B_n-2 series of oligosaccharides must also contain single D-glucose residues attached by a (1→3) branch linkage to the main chain.

Analysis of branched oligosaccharides isolated by l.c. — Confirmation of the structure of the two series of oligosaccharides B_n-1 and B_n-2 was achieved by enzymic degradation and methylation analysis. Enzymic analysis of the B_5 fraction obtained by p.c. from products of hydrolysis of several dextrans[8] had previously

TABLE I

RETENTION TIMES OF OLIGOSACCHARIDES DERIVED FROM *Leuconostoc mesenteroides* NRRL B-512(F) DEXTRAN

Fraction[a]	*Retention time (min)*[b]	*Proposed structure*[c]	*Oligosaccharide*
B_4	1.6		3^3-α-D-Glucosylisomaltotriose
IM_4	1.9		Isomaltotetraose
B_5-1	2.2		3^3-Isomaltosylisomaltotriose
B_5-2	2.7		3^3-α-D-Glucosylisomaltotetraose
IM_5	3.2		Isomaltopentaose
B_6-1	3.8		3^3-Isomaltosylisomaltotetraose
B_6-2	5.4		3^3-α-D-Glucosylisomaltopentaose
IM_6	5.8		Isomaltohexaose
B_7-1	6.7		3^3-Isomaltosylisomaltopentaose
B_7-2	8.4		3^3-α-D-Glucosylisomaltohexaose
IM_7	9.6		Isomaltoheptaose
B_8-1	10.9		3^3-Isomaltosylisomaltohexaose
B_8-2	13.4		3^3-α-D-Glucosylisomaltoheptaose
IM_8	17.6		Isomaltooctaose

[a]Each IM_n or B_n fraction had been separated by p.c. from a partial acid or enzymic (*P. funiculosum* dextranase) hydrolysate, respectively. [b]Column for l.c.: Dextropak. Flow rate 3 mL/min. [c]—, α-(1→6)-D-glucosidic linkage. ↓, α-(1→3)-D-glucosidic linkage. ○, D-glucose residue. ●, D-glucose residue that is released by α-(1→6)-α-D-glucan glucohydrolase.

indicated that two different branched pentasaccharides were present. When these were incubated with (1→6)-α-D-glucan glucohydrolase, the products were glucose, B_4, and a resistant B_5, now shown to be B_5-2. In the present work, we have found that other susceptible substrates, such as IM_4, when added to a mixture of B_5-2 and glucohydrolase, were completely hydrolysed to glucose, leaving B_5-2 intact (results not shown).

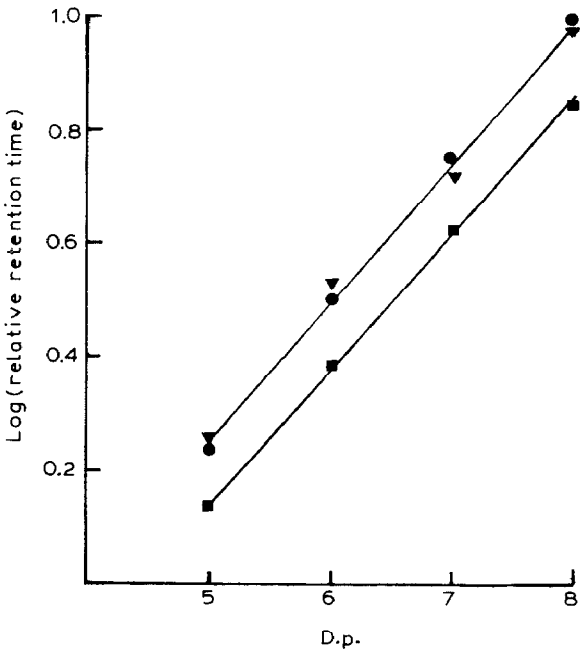


Fig. 4. Log(retention times) on l.c. of isomaltose saccharides (▼) relative to IM_4, and of branched oligosaccharide series B_n-1 (■) and B_n-2 (●) relative to B_4.

Methylation analysis of B_5-2 was consistent with several possible structures, for instance 3^2- or 3^3-α-D-glucosylisomaltotetraose or even 3^2-isomaltosylisomaltotriose. However, the specificity of action of *S. mitis* (1→6)-α-D-glucan glucohydrolase[11], towards (1→6)-linkages in the vicinity of branch points, ruled out isomers having the side chains attached to the second glucosyl residue from the reducing end. Oligosaccharide B_5-2, obtained from B-512(F) and B-1351 dextran, was not a substrate for the glucohydrolase, and was therefore 3^3-α-D-glucosylisomaltotetraose.

The next member of the B_n-2 homologous series, B_6-2, after incubation with the enzyme, yielded glucose and B_5-2 in equimolar proportions. This branched hexasaccharide was obtained from all of the dextrans studied, including V39, having 1-unit side-chains. B_6-2 was thus identified as 3^3-α-D-glucosylisomaltopentaose, and the B_n-2 series were 3^3-α-D-glucosylisomalto-oligosaccharides, B_4 being the lowest member.

Enzymic hydrolysis, together with methylation analysis, successfully identified B_5-1 as 3^3-α-isomaltosylisomaltotriose. This oligosaccharide contains a (1→6)-linkage at the nonreducing end that is adjacent to a (1→3)-linkage *not* involved in a branch point, hence one glucose residue was released by the glucohydrolase (see Table I). Equimolar proportions of glucose and B_4 were separated by l.c. The retention time of B_4, and its R_F value on p.c., identified it as the same B_4, 3^3-α-D-glucosylisomaltotriose, that was released from all of the dextrans by *P. funiculosum*

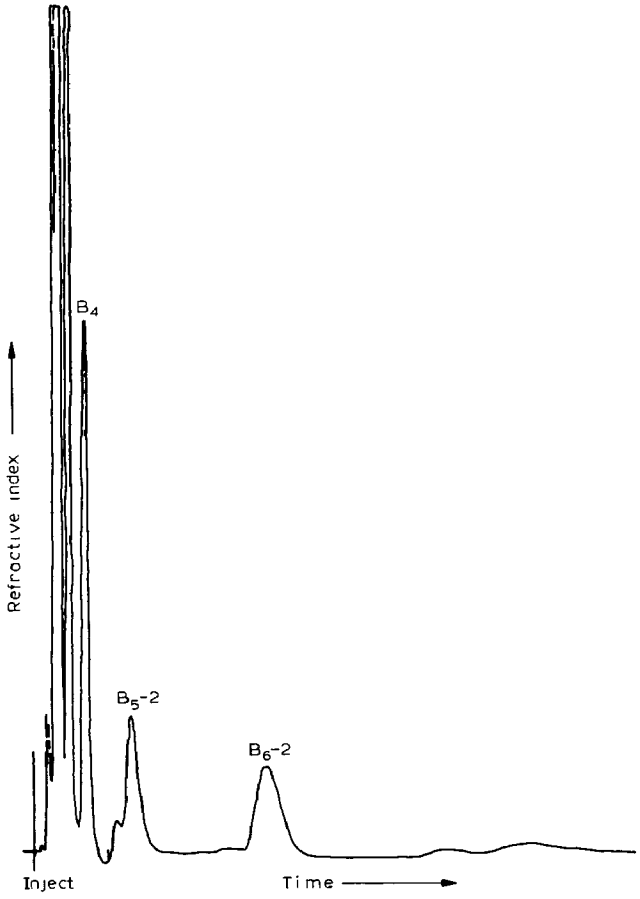


Fig. 5. Separation by l.c. of the branched oligosaccharide-products of the action of *P. funiculosum* endodextranase on V 39 dextran. Incubation time was 24 h.

dextranase. The structure proposed for B_5-1 is also consistent with the specificity of *P. funiculosum* dextranase, namely, the α-(1→6) linkage at the nonreducing side of the branch point may be hydrolyzed, but two α-(1→6) linkages on the reducing side are resistant[6]. The absence of the B_n-1 series from hydrolysates of V39 dextran is in accord with this series' consisting of 3-isomaltosylisomalto-oligosaccharides.

Higher branched-oligosaccharides, B_n-1 and B_n-2, where $n = 6$ or 7, could not be distinguished from the extent of their hydrolysis with glucohydrolase. Members of both series from B-512(F) dextran yielded equal numbers of D-glucose residues, leaving the same resistant B_5-2 (Table I). Likewise, methylation analysis would give identical results for the two series. However, there were significant differences between B_n-1 and B_n-2 regarding their rate of hydrolysis by the two dextranases. First, the strong preference of (1→6)-α-D-glucan glucohydrolase to release glucose residues from the main chain rather than from side chains in dextran[11] has now

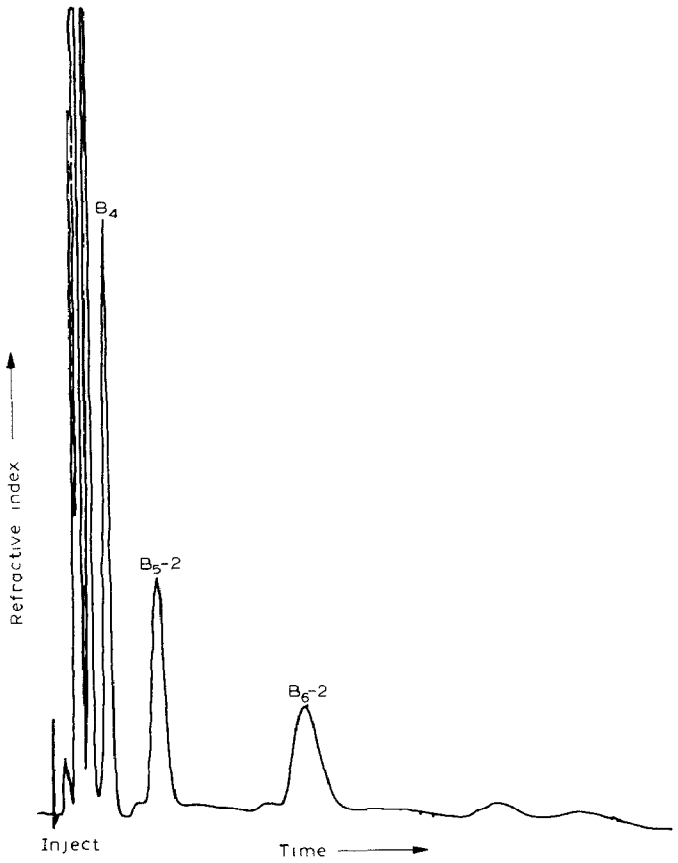


Fig. 6. Separation by l.c. of the branched oligosaccharide-products of the action of *P. funiculosum* endodextranase on B-1351 dextran. Incubation time was 24 h.

been noted with branched oligosaccharides. For instance, the intermediate product from B_7-1 was B_6-1, which was then hydrolysed to B_5-2. Because glucose residues are released only from the main chain of B_n-2 oligosaccharides, the intermediate products from this series are also D-glucosylisomalto-oligosaccharides.

Secondly, B_6-2 and B_7-2 are not stable products of endodextranase action on dextran. Although they are released early in the reaction (Fig. 7), both are susceptible to further hydrolysis. By contrast, B_6-2 is a true end-product, and the isomaltosyl side-chain in B_7-1 also obstructs further degradation to give isomaltose and B_5-1. The final products having d.p. >4 from B-512(F) dextran were thus B_5-1, B_5-2, B_6-1, and B_7-1. These were different from the major products found at intermediate stages (Fig. 7), when B_5-2, B_6-2, and B_7-2 predominated. The present work also demonstrates that B_5-2 would become the major branched pentasaccharide product if the endodextranase chosen for the hydrolysis were contaminated with (1→6)-α-D-glucan glucohydrolase.

The proportion of the final products having 1-unit and 2-unit side-chains indi-

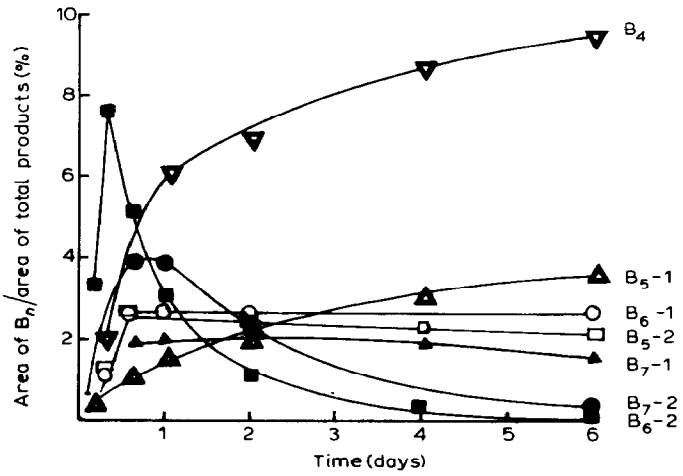


Fig. 7. Distribution of branched oligosaccharide-products during prolonged hydrolysis of B-512(F) dextran with *P. funiculosum* endodextranase. After separation by l.c., the proportion of each oligosaccharide in the total products was determined by measurement of peak areas; B_4 (▽), B_5-1 (△), B_5-2 (□), B_6-1 (○), B_6-2 (■), B_7-1 (▲), and B_7-2 (●).

cated that at least half of the (1→3)-branch linkages in B-512(F) dextran attached D-glucosyl residues to the main chain. This method would readily permit the detection of only 1% of branch linkages joining longer side-chains, but B-1351 dextran is apparently free from 2-unit side-chains. However, side chains that are longer than one glucosyl group may occur in several dextrans[11].

Information on the structure and relative amounts of branched oligosaccharide products is of key importance in improving knowledge of the structure of branched polysaccharides. The l.c. system described in this paper provides a greatly improved and faster separation of oligosaccharides than p.c., and the pressurized Dextropak cartridge has proved superior to the μBondapak carbohydrate column used by Smiley *et al.*[20] for the separation of oligosaccharides derived from B-512(F) dextran. We expect that quantitation and analysis of the products derived from the enzymic degradation of various dextrans will prove as rewarding as similar studies have been for investigations into amylopectin and glycogen[21]. Moreover, branched products from (1→6)-glucans and (1→4)-glucans[22,23] provide model oligosaccharides for further defining the specificity of the respective endo- and exo-glucanases.

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