# DETERMINATION OF THE STRUCTURE OF A BROTH DEXTRAN PRODUCED BY A CARIOGENIC STREPTOCOCCUS

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ABSTRACT

The broth polysaccharide from the cariogenic bacterium *Streptococcus mutans* E49 has been shown by periodate oxidation and methylation studies to be a highly branched dextran. The dextran is produced by a dextransucrase when the organism is cultured in sucrose. Periodate oxidation of the dextran indicated that 69% of the D-glucose was in a (1→6)-like linkage, 13% in either a (1→2)- or (1→4)-like linkage, and 18% in a (1→3)-like linkage. Methanolysis of the methylated dextran gave methyl 2,3,4,6-tetra-, 2,3,4-tri-, 2,4-di-, and 2,3,6-tri-*O*-methyl-D-glucopyranoside (minor proportion). The degree of branching (18%) indicated by periodate oxidation is substantiated by the presence of equimolar proportions of 2,4-di- and 2,3,4,6-tetra-*O*-methyl-D-glucose (20 mole percent). A modification of the sodium hydride–methyl iodide method of methylation is presented.

## INTRODUCTION

The structure of the dextrans produced by the cariogenic streptococci is not fully known. Dextrans are produced by a dextransucrase when the organisms are cultured on sucrose[1]. Most of the structural information is based on qualitative Smith degradations of periodate-oxidized dextran and on serological crossreactions with sera directed against the *Leuconostoc mesenteroides* B512 dextran[2,3]. The general resistance of the dextrans to dextranase produced by *Penicillium lilacinum* has been suggested as being due to a highly branched structure[4,5].

*Streptococcus mutans* strain E49 produced caries in hamsters, and this cariogenic ability seems to be related to its production of dextran[6]. Study of the structure of the dextran precipitated by ethanol from the culture medium is described in this paper. The dextran was characterized by periodate oxidations coupled with Smith degradations, and by methylation followed by methanolysis and g.l.c. analysis of the

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methylated methyl D-glucopyranosides. The structure is characteristic of dextran, *viz.*, (1→6)-linkages with a high proportion of (1→3)-branches.

## EXPERIMENTAL

*General.* — Methylated methyl D-glucopyranosides used as reference compounds were prepared according to published procedures cited elsewhere[7]. Thin-layer chromatography (t.l.c.) of the methylated derivatives was performed on plates of Silica Gel G with 1:1 (v/v) benzene–acetone as the developer, and the sugars were detected by spraying with 10% sulfuric acid and heating at 105°. Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard Model 5750B instrument equipped with a column of Carbowax 20M pre-esterified with 2-nitroterephthalic acid as the liquid, on Silica Gel G pretreated with dimethylsilane[7]. Methoxyl determinations were made by Galbraith Laboratories, Knoxville, Tennessee. The anomers of methyl 2,4-di-*O*-methyl-D-glucopyranoside were gifts from Dr. H. W. Kircher, University of Arizona, Tucson, Arizona. Infrared spectra were recorded with a Perkin–Elmer Model 137 or 457 spectrophotometer for pellets in potassium bromide or for oils on plates of potassium bromide. Unless otherwise stated, all evaporations were performed at 40°/50 torr. Analyses for carbohydrate were conducted by the cysteine–sulfuric acid method[8], by the phenol–sulfuric acid method[9], and, after hydrolysis, by the D-glucose oxidase–peroxidase method[10].

*Production of the dextran.* — The bacteria, *Streptococcus mutans* strain E49, were obtained from Dr. R. J. Fitzgerald, National Institutes of Health, Bethesda, Maryland. The organism was cultured in Trypticase Soy Broth (Baltimore Biological Laboratory) supplemented with 5% of sucrose (which was autoclaved separately). The dextran was precipitated from the cell-free broth with 95% ethanol (2 ml/ml), and purified by two reprecipitations from water with ethanol. The analysis of the dextran for total carbohydrate showed that 95% of it was carbohydrate. D-Glucose oxidase–peroxidase analysis following acid hydrolysis (18 h at 100°, 1N) demonstrated that the dextran contained 95% of D-glucose. Paper chromatography of the hydrolyzate revealed that glucose was the only reducing sugar present. From these results, the material was considered to be a homopolymer of D-glucose.

*Periodate oxidation.* — Periodate oxidations of the dextran were performed as described by Rankin and Jeanes[11]. The oxidation of the dextran (1 mg/ml) with sodium periodate (5 mg/ml) was conducted in the dark at room temperature. Glucose and glycerol were qualitatively determined by paper chromatography [4:1:5 (v/v) butyl alcohol–ethanol–water] of the product obtained after oxidation with periodate, reduction with sodium borohydride, and acid hydrolysis[12]. Glycerol was the only alditol that could be detected. At the end of the oxidation, glucose was detected on the chromatogram; the quantity in the Smith-degradation products was determined by means of D-glucose oxidase–peroxidase[10].

The consumption of periodate, production of formic acid, and the proportion of unoxidized D-glucose (by D-glucose oxidase assay) of the broth dextran were

measured. The consumption of periodate and production of formic acid were constant after 24 h, and there was no detectable overoxidation of the dextran during periods of up to 72 h. The periodate consumption (1.51 moles), formic acid production (0.69 mole), and unoxidized D-glucose (0.18 mole) are relative to one mole of anhydrous D-glucose[11].

The production of formic acid is a measure of the (1→6)-linkages and terminal D-glucosyl groups, and accounts for 69% of the dextran linked in this manner. As two moles of periodate are consumed for each mole of formic acid produced, the total number of moles of periodate consumed minus twice the number of moles of formic acid produced is a measure of (1→2)- and (1→4)-linkages[11]. This difference amounts to 13% of the dextran linked either (1→2), or (1→4), or both. The rest of the dextran contains (1→3)-linkages, which are resistant to periodate oxidation; this linkage amounts to 18% of the dextran and is in agreement with the D-glucose remaining after the oxidation (as measured, after acid hydrolysis, by use of D-glucose oxidase).

*Methylation.* — The initial methylation was performed by a modification[13] of the Haworth method. The dextran (1.5 g) was methylated six times by this procedure, with a purification after the third methylation. The methylated dextran (1.7 g; methoxyl 33.6%) was precipitated from solution in chloroform by the addition of petroleum ether. The partially methylated dextran (1.65 g) was dissolved in *N,N*-dimethylformamide, and remethylated by a modification[14] of the Purdie method, for a 48-h period, during which two additions of methyl iodide and silver oxide were made. The salts were removed by filtration, and the methylated dextran (1.7 g; methoxyl 39.5%) was isolated in the usual way and precipitated from chloroform by means of petroleum ether.

The partially methylated dextran (250 mg) was dissolved in carbon tetrachloride, and remethylated with 30% sodium hydroxide (5.5 ml) and methyl sulfate (2.5 ml). The reagents were added slowly to the stirred solution, so that a one-phase system was maintained. After the reagents had been added, the temperature was raised from 45 to 100° in 1 h, and kept for 1 h at 100°. The mixture was cooled, and made neutral, and the product was extracted into carbon tetrachloride, and remethylated. The resulting, methylated dextran (200 mg) had a methoxyl content of 42.06%.

The methylated dextran was remethylated with sodium hydride–methyl iodide by the general methods of Anderson and Cree[15] and Sanford and Conrad[16]. The foregoing product (120 mg) was dried over phosphorus pentaoxide under vacuum for 18 h at 78°, and then dissolved in methyl sulfoxide (freshly distilled from calcium hydride under vacuum). The reaction flask was dried by flaming under vacuum, and then flushed with dry nitrogen; it was kept connected to a nitrogen line and a pressure of nitrogen was maintained throughout the reaction. The flow of nitrogen was increased when subsequent additions were made, in order to ensure a flow of nitrogen out of the flask. The dispersion of sodium hydride (35 mg of a 57% dispersion in oil) was washed three times with hexane predried over sodium; it was added in one portion, and the mixture was stirred magnetically for 30 min at 25°. The temperature was slowly raised to 60°, and the dextran dissolved completely. The color of the

mixture varied from green-yellow to muddy yellow, depending on the proportion of sodium hydride present in excess. The mixture was cooled to 25°, freshly distilled methyl iodide (100 mg) was added, and the mixture was stirred for 12 h at 25°; methylation was then repeated without intermediate isolation of the product. An excess of methyl iodide was added at the end of the reaction, in order to ensure complete decomposition of the excess of sodium hydride. Chloroform and water were added to the mixture, which was then placed in a dialysis bag, and dialyzed exhaustively against running tap-water and then against several changes of distilled water. The two layers were added to one flask and evaporated to dryness under vacuum. The residue was dissolved in chloroform, the solution was dried (sodium sulfate), and the product was precipitated by the addition of petroleum ether (95 mg; methoxyl 44.09%). Two further methylations were conducted on the methylated dextran (80 mg), and the product was isolated as before (65 mg; methoxyl 45.46%). The i.r. spectra of E49 dextran, a partially methylated dextran, and the final methylated product are given in Fig. 1.

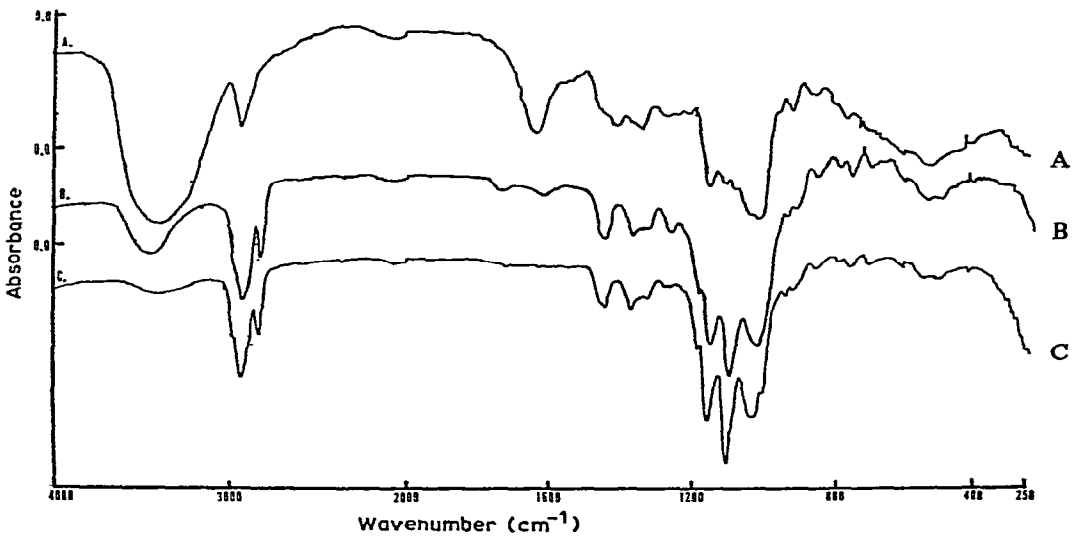


Fig. 1. I.r. spectra of (*A*) the broth dextran, (*B*) partially methylated dextran (methoxyl 39.46%), and (*C*) fully methylated dextran (methoxyl 45.46%).

*Methanolysis of the methylated dextran.* — In order to establish appropriate conditions of methanolysis for the methylated dextran, a suspension of 20 mg in 3% methanolic hydrogen chloride (3 ml) was boiled for 8 days under reflux. Samples (100 $\mu$l) were removed every 24 h, made neutral with silver carbonate, and analyzed by t.l.c. (Less severe treatment during 8 days did not cause complete hydrolysis.) Six compounds, corresponding to three pairs of anomers, were found on the t.l.c. plate; no material was present at the origin. Solutions of reference compounds for t.l.c. (and g.l.c.) were refluxed under the same conditions, in order to ensure the same ratio of the anomers as in the hydrolyzates. Qualitative examination of the plates revealed

appreciable amounts of methyl di- and tetra-*O*-methyl-D-glucosides, in addition to the methyl tri-*O*-methyl-D-glucosides (see Table I). It was not possible to identify which of the isomers were present (an identification necessary for structural analysis), but it was determined that the dextran was highly branched.

TABLE I

$R_F$ VALUES OF METHYL ETHERS OF METHYL α,β-D-GLUCOPYRANOSIDE, AND OF THE PRODUCT FROM METHANOLYSIS OF THE METHYLATED DEXTRAN

| *Methyl ethers* | $R_F$ |
|---|---|
| Of methyl α,β-D-glucopyranoside | |
| 2,3-di- | 0.19, 0.23 |
| 2,4-di- | 0.18, 0.26 |
| 2,3,6-tri- | 0.37, 0.44 |
| 2,3,4-tri- | 0.35, 0.42 |
| 2,3,4,6-tetra- | 0.55, 0.62 |
| In methanolyzate of methylated dextran | 0.18, 0.26, 0.35, 0.43, 0.55, 0.62 |

*Gas–liquid chromatography of the methanolyzate.* — The methylated dextran was methanolyzed by boiling a suspension of it in 3% methanolic hydrogen chloride for 8 days under reflux. The hydrolyzate was made neutral with silver carbonate, and analyzed by g.l.c. The specific details of the separation with the 2-nitroterephthalic acid–Carbowax 20M column, together with the detector response, have been

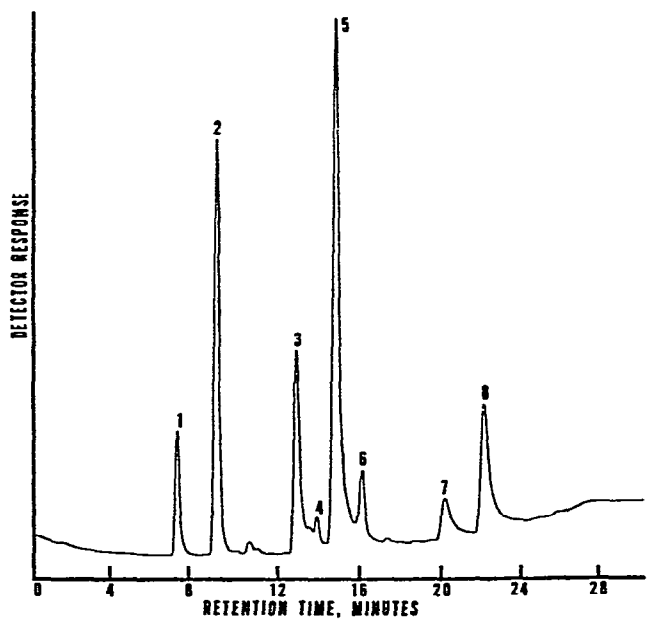


Fig. 2. Chromatogram of the products of methanolysis of the methylated dextran (methoxyl 45.46%). *1* and *2*, 2,3,4,6-tetramethyl ether; *3* and *5*, 2,3,4-trimethyl ether; *4* and *6*, 2,3,6-trimethyl ether; *7* and *8*, 2,4-dimethyl ether of methyl α,β-D-glucopyranoside.

reported[7]. A typical chromatogram of the methanolyzates of the fully methylated dextran is shown in Fig. 2. The number of micromoles in each peak was determined from the detector-response curve established from reference compounds analyzed under identical conditions. The number of micromoles in each peak for the methanolyzate of the various partially and totally methylated dextrans was calculated, and is expressed as mole percent (see Table II). The proportion of terminal D-glucose residues, as methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside, increases with increase in methylation, and the linear portion of the chain (as methyl 2,3,4-tri-*O*-methyl-D-glucoside) decreases. The proportion of branching points (as methyl 2,4-di-*O*-methyl-D-glucoside) decreases as the degree of methylation is increased and, probably, yields more methyl tri-*O*-methyl-D-glucosides. The completely methylated dextran shows equimolar proportions of branching points and terminal groups.

TABLE II

ANALYSIS OF THE METHYL *O*-METHYL-D-GLUCOPYRANOSIDES FROM THE DEXTRAN AT VARIOUS DEGREES OF METHYLATION

| *Methoxyl content* (%) | *Methyl O-methyl-D-glucopyranoside (mole %)* | | | |
|---|---|---|---|---|
| | *2,4-Di-* | *2,3,6-Tri-* | *2,3,4-Tri-* | *2,3,4,6-Tetra-* |
| 39.46 | 24.0 | 4.0 | 59.5 | 12.5 |
| 42.06 | 22.0 | 4.0 | 60.0 | 14.0 |
| 44.09 | 12.0 | 6.0 | 58.0 | 18.0 |
| 45.46 | 20.0 | 8.0 | 52.0 | 20.0 |

The identity of each compound was established by collecting it at the exit port of an Aerograph Autoprep gas–liquid chromatograph (8.5 ft × 0.25 in. o.d.). The same column packing as before was used, but it was not possible to reproduce the linear temperature-program used with the analytical chromatograph. The anomers of methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside were identified by their relative retention-times and by comparison of their i.r. spectra with those of reference compounds. Methyl 2,3,4-tri-*O*-methyl-β-D-glucopyranoside was identified by its retention time, m.p. of 92–94° (*cf.* Ref. 13), and i.r. spectrum. The α-D anomer was an oil, identified by its retention time and i.r. spectrum. The anomers of methyl 2,3,6-tri-*O*-methyl-D-glucopyranoside were identified by their retention times and i.r. spectra. The methyl 2,4-di-*O*-methyl-α- and -β-D-glucopyranosides were collected, and identified by their respective melting points (α, 80°; β, 122°; *cf.* Ref. 16), their retention times, and their i.r. spectra.

## DISCUSSION

The extracellular polysaccharide produced by *Streptococcus mutans* strain E49 is a homoglucan. Because of their resistance to dextranase[4,5], the general type of dextrans from cariogenic bacteria has been thought to be highly branched. This may

play a role in the difficulty in methylation of the dextran, which has been experienced by others for highly branched dextrans[19].

When once the necessary anhydrous conditions have been established, the sodium hydride–methyl iodide method described here seems to be the easiest method of methylation. The total volume of the reaction mixture does not increase appreciably with subsequent methylations, and good recovery of the product, and, eventually, total methylation, are possible. In several variations on this type of methylation, the anions have been generated either (a) in the presence of the polymer[15,20,21] or (b) separately and then added to the polysaccharide[16]. The method described here seems preferable, if only for the ease of maintaining anhydrous conditions during the reaction and during subsequent additions to the system.

The dextran is highly branched, containing 20% of the D-glucose residues as (1→3)-branched points (determined by methylation). This result is substantiated by the equimolar amount of terminal D-glucosyl groups. The linear portion of the polymer has (1→6)-linkages, as, after methylation and hydrolysis, 52% of the D-glucose is obtained as 2,3,4-tri-*O*-methyl-D-glucopyranose. These results are consistent with periodate-oxidation data which reveal that this dextran contains 69% of (1→6)-linkages (linear and terminal groups) and 18% of (1→3)-linkages. Antisera directed against the E49 dextran crossreact with the *Leuconostoc mesenteroides* B512F dextran, of which 95% is α-D-(1→6)-glucan[22]. The antisera also crossreact with several dextrans containing 50 to 95% of (1→6)-linkages (unpublished results). Based on the crossreaction and hapten-inhibition studies with the antisera, the E49 dextran has, most likely, the α-D configuration. The i.r. spectrum of the unmethylated dextran shows absorptions assigned to α-D-(1→6)-linkages (917 and 768 $cm^{-1}$), α-D anomer (841 $cm^{-1}$, weak), and α-D-(1→3)-linkages (794 $cm^{-1}$)[19,24].

The presence of (1→4)-linkages is possible as, after methylation and hydrolysis, 8% of the hydrolyzate consists of methyl 2,3,6-tri-*O*-methyl-D-glucopyranosides. Smith degradation of the periodate-oxidized dextran did not reveal the presence of erythritol [which would have shown a (1→4)-linkage]. It seems likely that the dextran contains (1→4)-linkages, or is contaminated by amylose, rather than containing (1→2)-linkages, as some cariogenic streptococci are capable of synthesizing amylose when cultured with D-glucose[24,25]. The possibility that amylose is a contaminant is now under investigation.

The work reported here, in conjunction with the serology, leads us to consider two types of branched structure for the broth dextran. One is a glycogen-like structure, with a linear (1→6)-backbone and (1→3)-branches. The other involves a (1→3)-D-glucose chain, with branches from O-6 of every D-glucose residue in the chain. The glycogen type of structure is much more reasonable, as Bowen[4] has shown that several dextrans from cariogenic streptococci are only partially hydrolyzed by dextranase. The degree of hydrolysis by dextranase varied from 1 to 35%, depending on the dextran tested[4]. If the second type of structure proposed is correct, almost complete hydrolysis of the branches, leaving only the (1→3)-backbone, might be expected.

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