The Structure of *Leuconostoc mesenteroides* Strain C Dextran. I. Methylation Analysis[1a]

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The extracellular dextran elaborated by *Leuconostoc mesenteroides* strain C (NRRL B-1298), which gives a strong precipitin reaction with type-12 pneumococcus antiserum and with concanavalin A, has been shown by methylation analysis to be composed of D-glucopyranose units linked by 1 → 6 glycosidic bonds with branches at C-2 and at C-3. Methylation and subsequent hydrolysis of the dextran afforded 2,3,4,6-tetra- (20.2 mol %), 2,3,4-tri- (53.3 mol %), 2,4,6-tri- (1.0 mol %), 3,4-di- (12.0 mol %), 2,4-di- (7,7 mol %), and 4-*O*-methyl-D-glucose (5.7 mol %); the molar proportions of the methylated sugars correspond to $\overline{CL}$ 4–5.

The dextrans constitute a heterogeneous class of extracellular D-glucans elaborated by bacteria primarily of the genus *Leuconostoc*.[2,3] These polymers apparently possess a common structural feature of chains of α-D-glucopyranose residues consecutively linked by 1 → 6 glycosidic bonds.[2] Most dextrans are somewhat branched at C-2, C-3, or C-4. They vary in the degree of branching from the *L. mesenteroides* NRRL B-512 dextran with 4% branch points at C-3[4] to the highly branched dextrans from *L. mesenteroides*, Birmingham strain, which has 15% branch points at C-3,[5] and B-1416 strain with 17% branch points at C-3 and C-4.[6] Preliminary studies have indicated that certain dextrans are still more highly branched with as many as 30 and 40% branched units.[7]

Substitution at C-3 now appears to be common in dextrans and indeed may be a structural feature typical of all dextrans.[8] Evidence has been presented that in one dextran the 1 → 3 linkages occur in the main chain as well as at branch points.[7] The occurrence of 1 → 2 linkages in certain dextrans was suggested first by the optical rotational shifts associated with cuprammonium dextran complex formation[9] and was confirmed later by the isolation of kojibiose from acetolysates of several dextrans.[8,10–12] Serological data[13] provided additional evidence for the presence of 1 → 2 linkages when kojibiose was found to be an inhibitor of the precipitin reaction of certain dextrans with antisera.

There is a marked similarity[8,14] in the capacity of the dextrans to precipitate concanavalin A,[15,16] a globulin in Jack bean meal, and their activity toward type-12 pneumococcus antiserum;[14] both of these precipitin reactions correlate with the content of 1 → 2 linkages (as revealed by the amount of kojibiose liberated during acetolysis).[8]

Relatively few dextrans have been submitted to a detailed structural examination and those dextrans containing 1 → 2 linkages have been examined by acetolysis fragmentation or by cuprammonium complex formation only.[17] Neither technique provides quantitative data for the proportion of the individual linkages and the degree of branching in the polymers. This paper is concerned with the constitution of the dextran elaborated by *Leuconostoc mesenteroides* strain C[18,19] which reacts strongly with concanavalin A and type-12 pneumococcus antiserum and has been shown[8] to contain the relatively rare 1 → 2 linkage.

L. mesenteroides strain C when grown on a medium containing sucrose as a carbon source produces two extracellular polysaccharides: the dextran, described herein, and a fructan.[20] The two polymers were separated readily by fractional precipitation with ethanol since the dextran was insoluble in 45% aqueous ethanol while the fructan ($[\alpha]^{25}D$ −40° in water) remained in solution. The crude dextran ($[\alpha]D$ +175° in water), representing 68% of the polysaccharide mixture, was purified further by fractional precipitation of the dextran acetate. The major fraction of the dextran acetate ($[\alpha]^{27}D$ +187° in chloroform) was recovered in 61% yield while a minor fraction ($[\alpha]^{27}D$ +190° in chloroform) was isolated in 11% yield. All structural studies were conducted on the major acetate fraction which on deacetylation gave the purified dextran having $[\alpha]^{27}D$ +182° in water.

Complete hydrolysis of the dextran in refluxing 1 *N* sulfuric acid liberated D-glucose as the only sugar detectable by paper chromatographic analysis. Since these conditions promote extensive degradation of fructose, the dextran was also subjected to hydrolytic conditions designed to cleave fructosyl bonds with minimum degradation of fructose. This demonstrated the efficiency of the fractionation procedures for removing

(1) (a) This paper, No. 6261 Scientific Journal Series, Minnesota Agricultural Experiment Station, was presented at the National Meeting of the American Chemical Society, Phoenix, Ariz., 1966, and forms part of the thesis submitted by M. J. S. to the Graduate faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D., July 1966. (b) Deceased.

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the fructan since even these mild conditions failed to reveal fructose.

The dextran was methylated by the Haworth and Purdie procedures giving the methylated product in 62% yield (OCH_3 44.93%). Fractional precipitation of the methylated dextran gave a major fraction ($[\alpha]^{25}D$ +222° in chloroform) in 90% yield. A minor fraction, which was recovered from the mother liquor, appeared to be structurally different as revealed by the lower rotation ($[\alpha]^{25}D$ +101° in chloroform) and was not examined further. With the exception of this minor fraction, the dextran was predominantly homogeneous by these fractionation procedures.

Cleavage of the methylated dextran into the component methylated glucosides by methanolysis was only partially successful. Refluxing with 1% hydrogen chloride in methanol for 33 hr did not effect complete solution of the methylated dextran. The soluble portion of the methanolysate (fraction i) was separated from the insoluble portion (fraction ii) by centrifugation and hydrolyzed in refluxing 1 *N* sulfuric acid. The methylated sugars from fraction i (see Table I) were separated by hydrocellulose column chromatography and identified by paper chromatography, by paper electrophoresis, and by comparison of physical constants of the crystalline compounds or their derivatives.

TABLE I

CLEAVAGE FRAGMENTS OF THE METHYLATED DEXTRAN

O-Methyl-D-glucose	Fraction i, mol %[a]	Fraction ii, mol %[a]
2,3,4,6-Tetra-	25.5	5.1
2,3,4-Tri-	48.2	69.5
2,4,6-Tri-	1.2	...
2,4-Di-	6.1	12.6
3,4-Di-	13.1	7.6
4-	6.1	5.2

[a] Fractions i and ii refer to the methanol-soluble and -insoluble fractions, respectively, formed during methanolysis of the methylated dextran. Fraction ii represents 21% by weight of the original methylated dextran.

Fraction ii, which represented 21% of the methylated dextran, was resistant to both methanolysis and dilute acid hydrolysis and was therefore treated successively with cold 72% sulfuric acid and refluxing 1 *N* sulfuric acid. The methylated sugars obtained thus were separated by paper chromatography and shown to be identical with those obtained from fraction i although they were obtained in different proportions from the two fractions (see Table I).

In view of the fact that an unusually low proportion of tetra-*O*-methyl-D-glucose was obtained from fraction ii as shown in Table I, it is reasonable to assume that this insoluble fraction had arisen by fragmentation of the original methylated polymer through the loss of side chains (and hence tetra-*O*-methyl-D-glucosyl residues). The possibility that the low proportion of tetra-*O*-methyl-D-glucose resulted from partial demethylation during treatment of fraction ii with cold 72% sulfuric acid is unlikely since it was demonstrated in a control experiment that more 2,3,4,6-tetra-*O*-methyl-D-glucose is degraded during the methanolysis and subsequent hydrolysis with 1 *N* sulfuric acid (10% degradation) than with the 72% sulfuric acid–1 *N* sulfuric acid treatment (7% degradation). In both cases the predominant methylated sugar formed was tri-*O*-methyl-D-glucose. Similar observations have been reported by Croon and coworkers.[21]

Since there is no evidence to indicate that fractions i and ii reflect a heterogeneity in the original dextran, the proportions of the different linkages in the dextran are based on the combined yields of the methylated sugars from fractions i and ii. Cleavage of the methylated dextran therefore afforded the methylated sugars in the following mole proportions: 2,3,4,6-tetra- (20.2 mol %), 2,3,4-tri- (53.3), 2,4,6-tri- (1.0), 3,4-di- (12.0), 2,4-di- (7.7), and 4-*O*-methyl-D-glucose (5.7).

The methylation data show that the dextran has a framework of (1 → 6)-linked D-glucopyranose residues with branches joined to some of these residues through C-2 and to a lesser extent through C-3. The ratio of 3,4-di- to 2,4-di-*O*-methyl-D-glucose indicates 50% more branches at C-2. A few of these residues may be branched at both C-2 and C-3 since the proportion of 4-*O*-methyl-D-glucose obtained from the methylated dextran is greater than can be attributed to incomplete methylation of the dextran considering the methoxyl content. The proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose is somewhat lower than that required by the di-*O*-methyl and mono-*O*-methyl sugars present and corresponds to an average chain length ($\overline{CL}$) of 5, whereas the $\overline{CL}$ calculated from the proportion of branch points is about 4. This is apparently one of the most highly branched dextrans that have been characterized although preliminary studies[7] indicate that a dextran with $\overline{CL}$ 2.5 occurs.

The identification of 3,4-di-*O*-methyl-D-glucose establishes that the 1 → 2 linkages previously demonstrated by acetolysis[8] represent branch points rather than linear residues. The structural significance of the 2,4,6-tri-*O*-methyl-D-glucose is questionable; this small amount could have arisen by demethylation of the tetra-*O*-methyl-D-glucose or by incomplete methylation of the dextran since C-3 is the most difficult to alkylate.

Experimental Section

All concentrations were effected *in vacuo* at 35–45° (bath temperature). Paper chromatography was performed on Whatman No. 1 paper, unless stated otherwise, by the descending method using the following solvent systems: (A) pyridine–ethyl acetate–water (2:5:7, upper phase), (B) 1-butanol–ethanol–water (3:2:1), (C) 2-butanone–water azeotrope, and (D) benzene–ethanol–water–ammonium hydroxide (200:47:15:1, upper phase). Paper electrophoresis was carried out on Whatman No. 1 paper using 0.1 *M* sodium tetraborate at 600 V for 1–2 hr.[22]

Chromatograms were sprayed with ammoniacal silver nitrate (for detection of sugars and polyols),[23] *p*-anisidine hydrochloride (reducing sugars),[24] or *p*-anisidine trichloroacetate (reducing sugars). Components were detected on electropherograms by spraying with *p*-anisidine trichloroacetate containing 2% phosphoric acid or by the Trevelyan method[25] after removal of borates with 10% hydrogen fluoride in acetone.[26]

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Thin layer chromatography (tlc) was performed on layers of silica gel G by the ascending method. Glass plates (20 × 20 cm) were coated mechanically and activated for 1 hr at 100°. Microscope slides (5 × 7.5 cm) were coated manually with a slurry of silica gel G in chloroform–methanol (1:1) and air dried for 30–60 min. Compounds were detected on air-dried plates with iodine vapor or with sulfuric acid and subsequent charring.

Optical rotations were obtained with a Rudolph Model 80 or a Bendix ETL-NPL automatic polarimeter, type 143A. Melting points were determined on a Fisher-Johns apparatus and are uncorrected.

The precipitin reaction with concanavalin A was performed as described previously[27] and the reactivity of the polysaccharide is expressed as the glycogen value (GV) with calf liver glycogen assigned a GV of 1.00.

Fractionation of the Extracellular Polysaccharides. Isolation of the Dextran.—The extracellular polysaccharides elaborated by *Leuconostoc mesenteroides* strain C (NRRL B-1298)[28] when grown on a medium containing sucrose were fractionated by alcohol precipitation. The crude polysaccharide mixture (25 g) was dissolved in water (1 l.), and the dextran was precipitated as a gel with ethanol (825 ml). The gel was collected by centrifugation, dissolved in water (180 ml), and poured into ethanol (2 l.). The fibrous dextran was washed with ethanol and petroleum ether (bp 30–60°) and dried *in vacuo:* yield 16.5 g (66% of the crude mixture); $[\alpha]^{25}D$ +171° (*c* 0.1 in water); GV 7.3. In two additional experiments the fractionation performed in the same manner afforded the dextran in yields of 68% with $[\alpha]^{21}D$ +175 and +181°.

Acetylation of the Dextran.—Dextran (10 g) was dissolved in formamide (200 ml) and treated with pyridine (110 ml) and acetic anhydride (74 ml) at room temperature for 24 hr.[29] The acetate, which precipitated as the reaction mixture was poured into water, was collected (centrifuge), washed with water, ethanol, and petroleum ether, and air dried. The product was reacetylated in the same manner except that the reaction mixture was held at 50–55° for the first 2 hr. The acetylated dextran was isolated as before and dried *in vacuo*. Fractional precipitation of the dextran acetate from chloroform (800 ml) with petroleum ether (550 ml) gave fraction A which was collected (centrifuge), washed with petroleum ether, and dried *in vacuo:* yield 11 g (61%); $[\alpha]^{27}D$ +187° (*c* 0.8 in chloroform). Addition of petroleum ether (550 ml) to the supernatant solution from the precipitation of fraction A afforded fraction B: yield 2 g (11%); $[\alpha]^{27}D$ +190° (*c* 0.5 in chloroform). Fraction B was not investigated further.

Dextran acetate A (12.5 g) was deacetylated in chloroform (600 ml) with 1 *N* methanolic potassium hydroxide (220 ml). The precipitate which separated after 10 min was washed with ethanol and dissolved in 0.3 *N* sodium hydroxide to ensure saponification of all acetyl groups. After 45 min the solution was neutralized with acetic acid and poured into ethanol (2 l.). The precipitate was removed, dissolved in water (150 ml), and again precipitated in ethanol (2 l.). The white fibrous dextran was washed with ethanol and petroleum ether and dried *in vacuo:* yield 7.8 g; $[\alpha]^{27}D$ +182° (*c* 0.7 in water); GV 8.2.

The dextran was treated with 0.1 *N* sulfuric acid at 65° for 6 hr, and the solution was deionized. No monosaccharides or oligosaccharides which moved from the origin were detected by paper chromatographic examination (solvent A). Hydrolysis of the dextran with refluxing 1 *N* sulfuric acid for 7 hr afforded glucose only which was characterized by conversion into *N*-*p*-nitrophenyl-D-glucopyranosylamine: mp 180–182° and $[\alpha]^{29}D$ −194° (*c* 0.7 in pyridine) (lit.[30] mp 180–182° and $[\alpha]D$ −191°).

Methylation.—Dextran acetate A (4.35 g) was dissolved in acetone (200 ml) and methylated with 30% sodium hydroxide (360 ml) and dimethyl sulfate (110 ml) over a period of 3 hr. The bath temperature was kept at 25° for the first 2 hr and at 55° for 1 hr; it was then raised to 100° for 0.5 hr. The excess alkali was neutralized and the reaction mixture was dialyzed. The dialyzed material was concentrated to a small volume and the methylation was repeated four times in the same way with the bath temperature maintained at 55°; acetone was added into keep the product in solution.

After the fifth methylation the product was extracted with chloroform, and the extract was washed with dilute acetic acid and water and concentrated to a syrup (3.37 g). The syrupy product was methylated with methyl iodide (50 ml) and silver oxide (6 g, added in 12 portions) with continual stirring and refluxing for 22 hr. The product was extracted with chloroform and after removal of solvent it was remethylated four times in the same manner (yield 1.96 g; OCH_3, 44.93%). On fractionation of the methylated dextran from chloroform with petroleum ether, 90% of the product precipitated at 90–92% petroleum ether: $[\alpha]^{25}D$ +222° (*c* 0.7 in chloroform). A second fraction ($[\alpha]^{25}D$ +101 in chloroform) recovered by concentration of the mother liquor was not investigated further.

Identification of the Hydrolysis Products of the Methylated Dextran.—The methylated dextran (1.28 g) was heated in refluxing 1% methanolic hydrogen chloride (100 ml) for 33 hr. A small amount of insoluble methylated dextran (fraction ii, 0.27 g) was removed by centrifugation and the supernatant solution (fraction i) was neutralized with lead carbonate, filtered, and concentrated. The methyl glycosides (fraction i) were hydrolyzed in refluxing 1 *N* sulfuric acid (100 ml) for 20 hr. The hydrolysate was neutralized with barium carbonate, filtered, and concentrated, and the mixture (1.04 g) was separated on a hydrocellulose column[31] with solvent C.

The components were identified by direct comparison with authentic compounds by paper electrophoresis and paper chromatography using solvents B, C, and D. The identification was confirmed by crystallization of the component or a derivative.

A. 2,3,4,6-Tetra-*O*-methyl-D-glucose (0.237 g) had mp 86–88° and $[\alpha]^{26}D$ +82.7° (*c* 0.2 in water) after recrystallization from petroleum ether (lit.[32] mp 95–96° and $[\alpha]D$ +83.8° in water).

B. 2,3,4-Tri-*O*-methyl-D-glucose (0.442 g) had $[\alpha]^{27}D$ +73.2° (*c* 3.0 in methanol) (lit.[32] $[\alpha]D$ +69.1° in methanol). Treatment of component b with aniline afforded *N*-phenyl-2,3,4-tri-*O*-methyl-D-glucosylamine which was recrystallized from ethyl ether: mp 142–143°, and $[\alpha]^{23}D$ −88° (*c* 0.3 in ethanol) (lit.[33] mp 150° and $[\alpha]^{23}D$ −103° in ethanol). Acylation of component b with *p*-nitrobenzoyl chloride in pyridine at 80° for 2 hr afforded a mixture of the anomeric di-*p*-nitrobenzoates as revealed by tlc with benzene–ethyl acetate (95:5) as solvent (α anomer, R_f 0.14, and β anomer, R_f 0.35). The mixture was separated by chromatography on a column of silica gel using the same solvent to give the following esters of 2,3,4-tri-*O*-methyl-D-glucose: β-1, 6-di-*p*-nitrobenzoate, mp and mmp 135–136° and $[\alpha]^{29}D$ −13.6° (*c* 1.0 in chloroform), after recrystallization from methanol (lit.[34] mp 138–139° and $[\alpha]^{22}D$ −12° in chloroform); α-1, 6-di-*p*-nitrobenzoate, mp 151–152° (recrystallized from methanol) and $[\alpha]^{29}D$ +22.6° (*c* 1.1 in chloroform).

Anal. Calcd for $C_{23}H_{24}O_{12}N_2$: C, 53.0; H, 4.7; N, 5.4. Found: C, 53.0; H, 4.7; N, 5.4.

Reduction with sodium borohydride and subsequent oxidation with periodic acid of the 2,3,4-tri-*O*-methyl-D-glucose afforded one component identical with 2,3,4-tri-*O*-methyl-L-xylose as revealed by paper chromatography using solvents B and C.

C. 2,4,6-Tri-*O*-methyl-D-glucose (0.012 g) had $[\alpha]^{22}D$ +72.5° (*c* 0.4 in methanol) (lit.[35] $[\alpha]D$ +70° in methanol).

D. 3,4-Di-*O*-methyl-α-D-glucose (0.112 g) had mp 118–120° and $[\alpha]^{22}D$ +139° → +100° (*c* 0.3 in ethanol) (recrystallized from ethyl acetate) (lit.[36] mp 114–118.5° and $[\alpha]D$ +80 → +76° in water for the α anomer; lit.[37] mp 113° and $[\alpha]^{16}D$ +94.5 → +99.5° in ethanol for the β anomer).

E. 2,4-*O*-methyl-D-glucose (0.052 g) had $[\alpha]D$ +63.8° (*c* 0.9 in water) (lit.[38] $[\alpha]D$ +76.5° in water). Component e was converted into the crystalline *N*-*p*-nitrophenyl-2,4-di-*O*-methyl-D-glucosylamine, mp and mmp 250–251° and $[\alpha]^{26}D$ −250 → −262° (*c* 0.2 in pyridine) (after recrystallization from ethanol) (lit.[38] mp 250–251° and $[\alpha]D$ −252 → −268° in pyridine).

F. 4-*O*-Methyl-D-glucose (0.049 g) had $[\alpha]^{31}D$ +63° (*c* 0.4 in methanol) (lit.[39] $[\alpha]^{20}D$ +53° in water). This fraction afforded

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4-O-methyl-D-glucosazone, mp 154–155°, after recrystallization from 30% aqueous acetone (lit.[39] mp 158°).

A portion of the insoluble methylated dextran (0.157 g) which resisted methanolysis (fraction ii) was solubilized in 72% sulfuric acid (2 ml) at 5° for 3 days. The solution was diluted with water to give 1 *N* sulfuric acid and refluxed for 11 hr. The hydrolysate was neutralized with barium carbonate, filtered, and concentrated. The methylated sugars were separated by chromatography (Whatman 3 MM paper, solvent C) giving 2,3,4,6-tetra- (0.006 g), 2,3,4-tri- (0.077 g), 2,4-di- (0.013 g), 3,4-di- (0.008 g), and 4-O-methyl-D-glucose (0.004 g). Each sugar was identified by paper electrophoresis and paper chromatography as described previously.

Methyl 2,3,4,6-tetra-O-methyl-α-D-glucoside was subjected to the two procedures that were used for cleavage of the methylated dextran, namely, (A) methanolysis followed by 1 *N* sulfuric acid hydrolysis (for fraction i) and (B) 72% sulfuric acid followed by 1 *N* sulfuric acid hydrolysis (for fraction ii). The extent of demethylation and degradation of the methylated sugar was ascertained by quantitative analysis (phenol–sulfuric acid method[40]) of each component of the hydrolysates after separation by paper chromatography (solvent C). Methanolysis (procedure A) resulted in a loss of 10% of the tetra-O-methyl-D-glucose, whereas 7% was lost in the 72% sulfuric acid method (procedure B); in both procedures tri-O-methyl-D-glucose was the major methylated sugar formed by demethylation.

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Registry No.—2,3,4-Tri-O-methyl-D-glucose α-1,6-di-*p*-nitrobenzoate, 16780-52-2.

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The Structure of *Leuconostoc mesenteroides* Strain C Dextran. II. Fragmentation Analysis[1a]

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Partial acid hydrolysis of the dextran produced by *Leuconostoc mesenteroides* strain C (NRRL B-1298) has afforded the homologous series of isomaltodextrins, whereas acetolysis gave kojibiose, nigerose, isomaltose, and, in addition, trisaccharides derived from the branch points. Further evidence for the detailed structure of the dextran was obtained from periodate oxidation studies.

Methylation studies (part I of this series[2]) have shown that the dextran synthesized by *Leuconostoc mesenteroides* strain C (NRRL B-1298) is a highly branched D-glucan (CL 4-5) linked predominantly by 1 → 6 glycosidic bonds. Twelve per cent of the D-glucose units have branches at C-2 and 7.7% at C-3, while a few units appear to be doubly branched at these positions. To obtain further information on the sequential arrangement of the linkages and to confirm the α-anomeric configuration suggested by the high positive rotation of the dextran and its derivatives, the dextran was fragmented by partial acid hydrolysis, by partial acetolysis, and by Smith degradation.

Since the order of stability of glycosidic linkages involving primary and secondary hydroxyls is reversed in acid hydrolysis compared with acetolysis, these two reactions are complimentary methods for fragmentation of glycans containing mixed linkages. Thus (1 → 6)-linked oligosaccharides are obtained from such glycans by partial acid hydrolysis, whereas glycosidic linkages involving secondary hydroxyls tend to be preserved in the oligosaccharides resulting from partial acetolysis.[3,4]

When the dextran was heated in 0.1 *N* oxalic acid, the homologous series of isomaltodextrins from isomaltose to isomaltooctaose was obtained in addition to D-glucose. Each oligosaccharide was identified by paper chromatography and by partial acid hydrolysis to give the lower homologs of the series. Isolation of this homologous series of oligosaccharides establishes the presence in this dextran of sequences of consecutive (1 → 6)-linked D-glucopyranose units.

Acetolysis of the dextran afforded D-glucose pentaacetate and a mixture of oligosaccharide acetates. After deacetylation the oligosaccharides were separated by gradient elution from a charcoal column and purified further by paper chromatography. The disaccharides were obtained in good yield and were characterized by conversion into crystalline derivatives. In two separate experiments kojibiose and nigerose were isolated in yields of 7–13 and 2%, respectively, whereas isomaltose was obtained in only 2–3% yield. The yield of kojibiose in particular (35% of the maximum yield theoretically possible on the basis of the methylation data) confirms previous observations[5,6] on the value of this procedure for obtaining non-(1 → 6)-linked oligosaccharides. These results are in accordance with those of Suzuki and Hehre[5] who isolated kojibiose and nigerose from this dextran in yields of 11.7 and 2.5%, respectively, under similar acetolysis conditions.

The trisaccharide fraction obtained from the ace-

(1) (a) This paper, was presented in part at the National Meeting of the American Chemical Society, Phoenix, Ariz., 1966, and forms part of the thesis submitted by M. J. S. to the Graduate faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D., 1966. (b) Deceased.

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