The Structure of the Serotype 2 Capsular Polysaccharide of *Aerobacter aerogenes**

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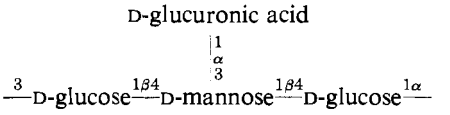
ABSTRACT: The four serologically identical capsular polysaccharides produced by the NCTC 243, 418, 5055, and 9504 strains of *Aerobacter aerogenes* are shown to be structurally identical and to be made up of repeating sequences of the tetrasaccharide, *O*-β-D-glucopyranosyl-(1→4)-[*O*-α-D-glucopyranosyluronic acid-(1→3)]-*O*-β-D-mannopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-. The identity of the four polysaccharides is established by methylation analysis and by paper chromatographic fingerprinting of the oligosaccharides formed on partial acid hydrolysis. A detailed quantitative examination of the NCTC 243 polysaccharide by sequence analysis, periodate oxidation, and proton magnetic resonance spectroscopy establishes unequivocably the repeating nature of the polysaccharide structure. Radioisotopic methods are described for determination of (1) per cent uronic acid in a polysaccharide, (2) identity and relative amounts of unknown uronic acids, and (3) degree of polymerization of an unknown oligosaccharide.

Complex polysaccharides occurring as surface layers of microbial cells have sufficiently variable, yet specific, biological properties that they are widely used in the immunological classification of the organisms which produce them. The pioneering work of Heidelberger, Goebel, and Avery (Heidelberger and Avery, 1923; Heidelberger *et al.*, 1925) has established the direct correlation of polysaccharide structural features with their antigenic specificity (Heidelberger, 1964; How *et al.*, 1964). However, lack of detailed structural information has prevented determination of the degree of structural similarity required in polysaccharides to yield serotypically identical antigens. The possibility has remained that identical antigens merely have identical immunodominant mono- or oligosaccharide portions occurring regularly along the polymer rather than totally identical structures (Beale and Wilkinson, 1961; Lüderitz *et al.*, 1966). Experimental evaluation of immunodominance *vs.* total identity has appeared particularly difficult because of the apparent structural complexity of antigenic heteropolysaccharides.

The data presented in this paper show that four *Aerobacter aerogenes* capsular polysaccharides which have been classified as serotype 2 are identical in all details of their structure. One of these, the polysaccharide from the NCTC 418 strain, has been studied previously by Barker *et al.* (1958) who, on the basis of methylation, periodate oxidation, and oligosaccharide sequence data, concluded that the polysaccharide has a branched structure in which the "average repeating unit" is a sequence containing about 40 monosaccharides. The data presented here indicate that the polysaccharides from *A. aerogenes* NCTC strains 243, 418, 5055, and 9504 all have the following simple tetrasaccharide repeating unit.



Materials

NCTC 243, 418, 5055, and 9504 strains of *Klebsiella aerogenes* were gifts from the National Collection of Type Cultures. All produced mucoid colonies on microassay agar and were classified on the basis of their capsular antigens as serotype 2 strains (Kauffmann, 1951; Edwards and Fife, 1952). Polysaccharides were purified from phosphorus-limited chemostat cultures as described earlier (Sandford and Conrad, 1966; Tempest, 1965). Under these growth conditions the capsules apparently were sheared from the cells and were recovered as slimes from the spent medium in yields of approximately 1 g/l. of medium. The starting materials for structural analysis contained less than 5% ash and 0.4% nitrogen or sulfur.

Dimethyl sulfoxide was redistilled from calcium hydride under reduced pressure and stored over dried Molecular Sieves (Linde, type 4A). Methylal (dimethoxymethane) was dried over metallic sodium and redistilled before use. [^{14}C]Methyl iodide, uniformly labeled [^{14}C]glucose, [^{3}H]lithium aluminum hydride, and [^{3}H]-

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sodium borohydride were obtained from New England Nuclear Corp. as chemically and radiochemically pure reagents. The latter was obtained at a specific activity of 13.7 mc/mmole and was used without dilution; the others were diluted with unlabeled reagents to obtain the desired specific activities indicated below.

Methods

Total carbohydrate was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and expressed in terms of equivalents of appropriate standards, as described in Results. D-Glucose was assayed by the glucostat method (Worthington Biochemical Corp., Freehold, N. J.). Descending paper chromatography was run on a Whatman No. 1 paper in the following solvents: (A) ethyl acetate–glacial acetic acid–formic acid–water (18:3:1:4), (B) ethyl acetate–pyridine–water (8:2:1), (C) 1-butanol–glacial acetic acid–water (2:1:1), and (D) methyl ethyl ketone, saturated with 1% aqueous ammonia. Reducing substances were detected with aniline acid phthalate spray reagent (Partridge, 1949). Nonreducing sugars were located with periodate–benzidine spray reagent (Cifonelli and Smith, 1954). Proton magnetic resonance spectra were run at 60 Mcycles on 20% solutions (w/v) in deuterium oxide after replacing exchangeable hydrogens by evaporating samples to dryness several times from deuterium oxide. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (Tiers and Coon, 1961), a gift from Dr. H. Foster, E. I. du Pont de Nemours and Co. (obtainable from Brinkmann Instruments, Inc.), was used as internal standard (τ 10) at a concentration of 0.5%.

Methylation Analysis. Polysaccharides were methylated, reduced, and remethylated using [^{14}C]methyl iodide (0.087 μc/mmole) as described previously (Sandford and Conrad, 1966), with the following modification of the lithium aluminum hydride reduction procedure. In the initial methylation the uronic acid residues were completely esterified as before but, in contrast to the earlier work, the hydroxyls were incompletely substituted and the polysaccharides therefore could not be dissolved in diethyl ether for the reduction reaction. Consequently, after the initial methylation, each lyophilized product was extracted exhaustively with methylal using a Soxhlet extractor and the resulting solutions were adjusted to a concentration of 20 mg of polysaccharide/ml of methylal prior to the reduction. Reductions were carried out using a solution of [^{3}H]-lithium aluminum hydride (52.4 μc/mmole) prepared by stirring 15 g of the unlabeled hydride in 750 ml of methylal for 5 hr. Insoluble hydroxides were removed by an initial centrifugation followed by filtration under nitrogen to obtain a clear solution. The concentration of the solution was determined by measuring manometrically the volume of hydrogen evolved when an aliquot was treated with water. The solution was then diluted with methylal to a concentration of approximately 1.5% (w/v) and 2 mg of [^{3}H]lithium aluminum hydride (100 mc/mmole) was dissolved in the solution with stirring. The resulting solution was used in the reduction of all four polysaccharides and for the reduction of benzophenone to determine the specific activity of the lithium aluminum hydride. For the reduction of methylated polysaccharide 1 g was dissolved in 50 ml of methylal and added dropwise to 33 ml of the [^{3}H]lithium aluminum hydride solution. The products were worked up and remethylated with [^{14}C]methyl iodide as described previously. The resulting fully methylated, doubly labeled, neutral products were hydrolyzed and analyzed by cellulose column chromatography (Sandford and Conrad, 1966) to determine molar ratios of the monomers.

The aldobiouronic acid was methylated, reduced, and remethylated in the same manner using [^{14}C]methyl iodide and unlabeled lithium aluminum hydride. Products from the methylation reactions were recovered from dimethyl sulfoxide by adding four volumes of water and extraction three times with one-half the final volume of chloroform. The chloroform extracts were combined, back-extracted with water to remove residual dimethyl sulfoxide, and evaporated to dryness at 40° *in vacuo* for analysis.

Characterization of Methyl Sugars by Periodate Oxidation. To determine the positions of methyl substitution, methyl sugars were analyzed by periodate oxidation. For measurement of periodate consumption and formic acid release 10–20 mg of methyl sugar was oxidized with 6 ml of 0.15 M sodium metaperiodate in the dark at room temperature. Periodate uptake was determined by titrating a 0.5-ml aliquot of the oxidation mixture in a solution containing 2.5 ml of 1 N sulfuric acid and 2.5 ml of 20% potassium iodide to a starch–iodine end point with 0.05 N sodium thiosulfate. Formic acid was determined by titration of a 0.5-ml aliquot of the oxidation mixture to a phenolphthalein end point with 0.05 N sodium hydroxide 5 min after mixing the aliquot with 0.6 ml of ethylene glycol in 10 ml of water. To determine formaldehyde release a second sample (10–20 mg) was oxidized in the dark at room temperature with 0.15 M periodic acid. Formaldehyde was determined colorimetrically on 0.05-ml aliquots using chromatropic acid (Speck and Forist, 1954). All values are reported for aliquots taken at 30 min, at which time oxidation was complete.

Isolation of Oligosaccharides. Oligosaccharides were isolated after hydrolysis of 2% solutions of polysaccharide in 1 N sulfuric acid at 100° for periods of time chosen for maximum accumulation of the desired oligosaccharides. Hydrolysates were neutralized with barium hydroxide and examined by descending paper chromatography after removal of precipitated barium sulfate. Gross separations of oligosaccharides were achieved by Dowex 1 (OAc) chromatography as before (Conrad *et al.*, 1966). After removing the neutral fraction in the water wash, acidic oligosaccharides were removed sequentially in order of their decreasing degree of polymerization using as eluent 4 N acetic acid in 20% aqueous ethanol. Further purification of the acids was achieved by preparative paper chromatography on Whatman 3MM paper sheets using solvents A and C. The neutral fraction did not contain significant amounts

of oligosaccharides and was discarded.

Degree of Polymerization of Oligosaccharides. The degree of polymerization of the various oligosaccharides formed on partial hydrolysis of the polysaccharide was determined by radiochromatographic analysis of hydrolysates of uniformly labeled ^{14}C polysaccharide. The labeled polysaccharide was isolated from a shake culture grown on a defined medium using uniformly labeled [^{14}C]glucose as the sole carbon source. The growth medium, containing 8 mg of $(NH_4)_2SO_4$, 4 mg of K_2SO_4, 4 mg of NaCl, 0.44 mmole of Tris·HCl (pH 8.5), 7.6 mg of $Na_2HPO_4 \cdot 7H_2O$, 130 μg of $CaCl_2 \cdot 2H_2O$, and 18 μg of $FeSO_4 \cdot 7H_2O$ in 44 ml, was inoculated from a fresh slant and growth was allowed to proceed for 21 hr at 30° on a gyrorotatory shaker. The culture was homogenized in a Waring Blendor for 0.75 min to decapsulate the cells. After sedimentation of the cells at 35,000*g* for 30 min the supernatant was acidified to pH 2 and ^{14}C polysaccharide was precipitated with an equal volume of acetone. The precipitate was collected by centrifugation, redissolved in H_2O, dialyzed against deionized water for 20 hr, and lyophilized. Partial hydrolysates of the ^{14}C polysaccharide were analyzed by radiochromatography in solvent C after [^3H]borohydride reduction and the ratios of ^{14}C to ^3H along the chromatographic strip were determined. These ratios indicate the number of carbons per reducing equivalent and thus the degree of polymerization. Assuming uniform counting efficiencies throughout the paper strip for both isotopes, division of the ^{14}C:^3H ratio obtained for any separated oligosaccharide on the chromatogram by the ratio obtained for the glucitol (DP = 1) segment of the chromatogram gives the degree of polymerization of the oligosaccharide.

The oligosaccharides from the ^{14}C polysaccharide were identified with the oligosaccharides isolated on a larger scale by chromatographic comparison of the reduced samples using solvents A and C.

Isolation of Glucosylmannose from Periodate-Oxidized Polysaccharide. Polysaccharide from the NCTC 243 strain was oxidized with periodate in acetate buffer as described earlier (Conrad *et al.*, 1966) for 18 days at which time periodate uptake had reached 0.84 mole of IO_4/162 g of polysaccharide. Excess periodate was destroyed with ethylene glycol and the oxidized polysaccharide solution was dialyzed overnight against running water. The solution was adjusted to pH 8 with 1 N sodium hydroxide to redissolve the partially precipitated polyaldehyde and was stirred with 2.1 g of sodium borohydride at room temperature for 12 hr. Excess borohydride was destroyed by cautious addition of 6 N sulfuric acid to pH 1. After stirring for 30 min the resulting polyalcohol solution was readjusted to pH 7 with 1 N sodium hydroxide and concentrated *in vacuo* at 40°. Total carbohydrate was determined and the polyalcohol was then hydrolyzed at 100° in 1 N sulfuric acid for 1 hr. The hydrolysate was neutralized with barium hydroxide and the barium sulfate was centrifuged, washed with water, and discarded. The water-soluble product was deionized with Amberlite MB3 resin and concentrated to a syrup and the saccharide components were isolated by preparative paper chromatography using solvent B.

Radiochromatography. Radiochromatographic analyses were performed using the [^3H]sodium borohydride method described previously (Conrad *et al.*, 1966) with the following modification of the reduction procedure. To 0.1-ml aliquots of hydrolysates containing 1–10 μequiv of reducing groups in 1 N sulfuric acid were added, in succession, 0.1 ml of 2 N sodium carbonate and 0.2 ml of 0.5 M [^3H]sodium borohydride (19 mg/ml of 0.1 N sodium hydroxide). The reaction mixture was heated at 50° in a 1.0-ml volumetric tube for 40 min. Excess borohydride was destroyed by cautious addition of 0.5 ml of 1 N sulfuric acid to the cooled reaction mixture. The volume was brought to 1.0 ml and analyses were carried out as described earlier. Counts were converted to reducing equivalents using standard curves obtained by carrying aliquots of a D-glucose standard solution through the identical procedure.

Isotope Dilution Analyses. Per cent methoxyl in methylated polysaccharides and in methyl sugars obtained on hydrolysis was measured by the ^{14}C method described earlier (Sandford and Conrad, 1966). The molar per cent uronic acid in the fully methylated, neutral polysaccharides which had been reduced with [^3H]lithium aluminum hydride was calculated using the relationship, % uronic acid = [(2 × 204 × ^3H dpm/mg of sample)/(dpm/mmole of $LiAlH_4$)]100. The derivation of this relationship is based upon the fact that each uronic acid residue incorporates two nonexchangeable hydrogens from lithium aluminum hydride upon reduction to the neutral sugar; thus, a reduced uronic acid has one-half the molar specific activity of the [^3H]lithium aluminum hydride. The average equivalent weight of the anhydrohexose residues in the neutral methylated polysaccharide is 204 (trimethylanhydrohexose). The specific activity of the [^3H]lithium aluminum hydride was calculated from the specific activity of recrystallized [^3H]benzhydrol formed by reduction of 1 g of pure benzophenone in 50 ml of methylal with 20 ml of the same 1.5% lithium aluminum hydride solution used in the polysaccharide reductions (see above).

Scintillation Counting. The doubly labeled methylated polysaccharides and methyl sugars derived from them were counted in a Packard Model 3000 liquid scintillation spectrometer equipped for external standardization. Counting efficiencies were determined from standard curves obtained by calibrating the counting rate of the external standard against two series of ethanol-quenched standards (one with [^3H]toluene and one with [^{14}C]-toluene) using counter settings chosen for optimal dual label counting.

Similarly, segments from paper chromatograms were counted without prior elution using either the Packard counter or a Beckman liquid scintillation spectrometer, again under conditions chosen for optimum single or dual label counting. Here counting efficiencies were obtained by counting ^{14}C- or ^3H-labeled glucose standards spotted on paper segments.

The scintillation fluid used for samples counted in the Packard counter contained 8 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in

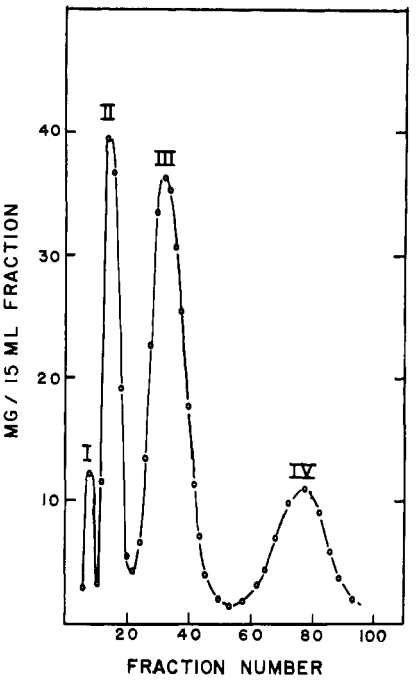

FIGURE 1: Cellulose column chromatography of the mixture of methyl sugars obtained on hydrolysis of the fully methylated neutral 243 polysaccharide. The column was developed with water-saturated methyl ethyl ketone. Peaks were identified as follows: I, degradation products; II, 2,3,4,6-tetra-*O*-methyl-D-glucose; III, an equimolar mixture of 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucoses; and IV, 2,6-di-*O*-methyl-D-mannose.

1 l. of toluene. That for samples counted in the Beckman counter contained 5 g of 2,5-diphenyloxazole in 1 l. of toluene.

Results

Methylation Analysis. The properties of serotype 2 polysaccharides at various stages (I–V) of the methylation sequence are compared in Table I. Although the initial methylation yields products (stage II) in which the hydroxyls are not fully substituted, infrared spectra indicate that the uronic acid moieties are completely esterified. The relatively poor yields in the extraction into methylal (stage III) are due to the incomplete methylation since, in a separate experiment, the NCTC 5055 polysaccharide was methylated twice prior to extraction bringing the per cent methoxyl to 42.7 and increasing the extraction yield at stage III to 94%. When the latter sample was carried through the complete methylation analysis, results identical with those described below were obtained.

Yields in the subsequent steps (reduction and remethylation) are comparable to those obtained previously with the A3 (S1) polysaccharide (Sandford and Conrad, 1966). The final products are completely methylated, neutral polysaccharides which are labeled

TABLE I: Analytical Characterization of Serotype 2 Polysaccharides at Various Stages of Methylation.[a]

	Methylation Stage									
	I	II		III	IV	V				
	Initial	Methylation		Extraction	Reduction	Remethylation				
NCTC Strain	$[\alpha]_D^{25}$ (deg)	Yield (%)[b]	Methoxyl (%)[b]	Yield (%)[b,c]	Yield (%)[b,c]	Yield (%)[b,c]	Methoxy (%)[b]	Uronic Acid (%)[d]	$[\alpha]_D^{25}$ (deg)
Calculated			43.6				45.6		
243	+74 (1.0, H_2O)	86	39.6	58	119	98	45.2	25.8	+93 (0.5, EtOH)
418	+77 (1.0, H_2O)	91	40.7	47	92	88	44.6	25.8	+82 (0.5, EtOH)
5055	+79 (1.0, H_2O)	92	39.5	57	112	86	44.3	25.1	+80 (0.7, EtOH)
9504	+79 (1.0, H_2O)	92	37.7	25	100	87	44.0	24.7	+90 (0.1, EtOH)

[a] Typical data taken from a reaction sequence in which polysaccharide (stage I) is methylated (stage II), extracted into methylal (stage III), reduced (stage IV), and remethylated to form a fully methylated neutral polysaccharide (stage V). [b] Based on polysaccharide composed of 50% D-glucose and 25% each of D-glucuronic acid and D-mannose. Uronic acid residues are esterified in the initial methylation. [c] Yields calculated on basis of starting weight for each step. [d] Calculated from the ^3H specific activity of stage V products as described in Methods.

TABLE II: Identification of 2,3,4,6-Tetra-*O*-methyl-D-[6-^{3}H]glucose.

NCTC Strain	Methoxyl (%)	Mp (°C)	$[\alpha]_D^{25}$ (deg)	^{3}H Sp Act. (μc/mmole)	% of Total Fraction[a]	
					Glucose	Mannose
Calculated	52.5	96	+92→+84	26.2[b]		
243	52.4	96	+83 (1.0, H_2O)	26.2	99.7	<0.25
418	51.6	96	+83 (0.5, H_2O)	25.1	99.6	<0.35
5055	53.0	96	+84 (0.6, H_2O)	25.2	99.6	<0.35
9504	52.7	96	+83 (1.3, H_2O)	26.9	99.5	<0.50

[a] Calculated from the ^{3}H counts in the glucose and mannose regions of paper chromatograms of the demethylated tetramethylhexose fractions run in solvent B. [b] Calculated value is one-half the specific activity of the [^{3}H]lithium aluminum hydride used in the reduction of the polysaccharide, assuming that all tetramethylhexose is derived from uronic acid moieties.

TABLE III: Identification of 2,3,6- and 2,4,6-Tri-*O*-methyl-D-glucoses in Mixtures.[a]

NCTC Strain	Methoxyl (%)	$[\alpha]_D^{25}$[b] (deg)	Periodate Oxidation[d]		
			IO_4 Uptake	HCOOH Formed	HCHO Formed
Calculated[c]	41.6	+70	0.50	0.00	0.00
243	41.6	+70	0.57	0.00	0.07
418	42.2	+71	0.49	0.00	0.09
5055	41.6	+70	0.59	0.00	0.07
9504	41.9	+70	0.49	0.00	0.08

[a] Only glucose was found upon demethylation. [b] *c* 1.0, H_2O. [c] Calculated for an equimolar mixture of 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucoses. The calculated periodate oxidation values uniquely characterize this mixture of trimethylglucoses. [d] Moles per mole of sugar.

with ^{3}H on C-6 of the residues derived from uronic acids and which show no hydroxyl or carbonyl bands in their infrared spectra. Each polysaccharide contains one uronic acid moiety per four anhydrohexose residues. Initial and final optical rotations of the four polysaccharides are consistent with the immunochemical implication that they are structurally similar.

Chromatographic separation of the methyl sugars derived from the NCTC 243 polysaccharide is shown in Figure 1. Identical elution patterns were obtained for hydrolysates of the other three polysaccharides. Peak I contained degradation products formed during hydrolysis. The identity of peak II as 2,3,4,6-tetra-*O*-methyl-D-glucose is established by the properties given in Table II. Molar specific tritium activities of the tetramethyl fractions are exactly one-half of that of the [^{3}H]lithium aluminum hydride used in the reductions, indicating that the tetramethylglucose is derived solely from the D-glucuronic acid residues in the polysaccharides. Any tetramethylglucose arising from the D-glucose residues in the original polymer would be unlabeled and would dilute these specific activities. Upon paper chromatography of the BCl_3 demethlyation products (Bonner *et al.*, 1960), only glucose was detectable with aniline acid–phthalate spray reagent. Furthermore, when the unsprayed chromatograms were counted, the number of ^{3}H counts in the mannose segments were insignificant relative to those recovered in the glucose segments. Thus, in contrast to the results of Barker *et al.* (1958), these polysaccharides do not contain mannuronic acid.

Although peak III emerges as a symmetrical peak, paper chromatography in solvent D shows it to contain two components which are partially separated on the column and which have R_G values identical with 2,3,6- and 2,4,6-tri-*O*-methyl-D-glucoses. For characterization the entire peak was combined and the mixture was analyzed as shown in Table III. Demethylation yielded only glucose. Methoxyl values show that these are trimethylglucoses. Optical rotations, although very nearly identical for all tri-*O*-methyl-D-glucoses, are consistent with the assigned structures and show the D configuration of the glucose. The experimentally determined

TABLE IV: Identification of 2,6-Di-*O*-methyl-D-mannose.[a]

NCTC Strain	$[\alpha]_D^{25}$ (deg)	Periodate Oxidation: Before BH_4 Reduction[c] IO_4 Uptake	HCOOH Formed	HCHO Formed	Periodate Oxidation: After BH_4 Reduction[c] HCHO Formed
Calculated[b]	+9.1	2.00	1.00	0.00	0.00
243	+9.3 (2.4, H_2O)	1.99	0.96	0.00	0.18
418	+8.1 (2.1, H_2O)	2.14	1.01	0.00	0.14
5055	+9.8 (5.3, H_2O)	2.16	0.99	0.00	0.11
9504	+12.5 (8.2, H_2O)	2.19	0.95	0.00	0.04

[a] Only mannose was found upon demethylation. [b] Calculated for 2,6-di-*O*-methyl-D-mannose. The calculated periodate oxidation values uniquely characterize this derivative. [c] Moles per mole of sugar.

periodate oxidation values uniquely describe an equimolar mixture of 2,3,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose.

Peak IV is identified as 2,6-di-*O*-methyl-D-mannose (Table IV) on the basis of demethylation, optical rotation, and periodate oxidation before and after sodium borohydride reduction. The physical constants and analytical values uniquely define the structure of this component.

The molar ratios of recovered methyl sugars are shown in Table V. All of the polysaccharides yield 2 moles of trimethylglucose and 1 mole of dimethylmannose per mole of tetramethylglucose. The tritium in the uronic acid residues is recovered quantitatively in the tetramethylglucose fraction proving that *all* of the D-glucuronic acid residues occur as nonreducing terminals in these polysaccharides. As observed earlier (Conrad *et al.*, 1966) hydrolysis of the fully methylated polysaccharides causes formation of significant amounts of degradation products which emerge from the cellulose column in peak I and in the water wash. The conclusion that these are degradation products of the methyl sugars rather than noncarbohydrate moieties in the polysaccharide is based on the fact that analytical values for C, H, and OCH_3 in the fully methylated, neutral polysaccharides (Table I) are consistent with the values calculated assuming that no noncarbohydrate constituents are present.

The data in Tables II–IV show that the same methyl sugars are derived from all of the serotype 2 polysaccharides. As shown in Tables III and V these methyl sugars are recovered from all of the polysaccharides in the same molar ratios. The four polysaccharides are therefore identical on the basis of methylation analysis. The data indicate that D-glucose, D-glucuronic acid, and D-mannose are present in a molar ratio of 2:1:1 and that all of the D-glucuronic acid residues are at nonreducing terminals, all of the D-mannose residues are at branch points linked through both C-3 and C-4, half of the D-glucose residues are linked through C-3, and the remaining D-glucoses are linked through C-4.

Sequence Analysis. Chromatographic comparison of 0.5-hr hydrolysates of the four polysaccharides is shown in Figure 2. The qualitative identity of these "fingerprints," when considered with the results of methylation analysis, indicate that the four polysaccharides are structurally identical. Therefore, sequence analysis was performed only on the NCTC 243 polysaccharide.

Partial hydrolysis gives a series of acidic but no neutral oligosaccharides. The monosaccharide sequences in the purified oligosaccharides have been deduced from the data presented in Table VI. The aldobiouronic acid (IIA) is the only acidic disaccharide found in hydrolysates and can be recovered from short hydrolysates (3–4 hr) in yields of almost 50% of the weight of the polysaccharide. Since this disaccharide is composed of D-glucuronic acid and D-mannose, it is apparent that these two sugars are present in the polysaccharide entirely in this aldobiouronic acid combination. Thus, this disaccharide must be present in all of the higher acidic oligosaccharides. Furthermore, since the D-glucuronic acid residues are nonreducing terminals and the D-mannose residues are branch points in the polysaccharide, the branches in these polymers contain only one monosaccharide unit.

The degree of polymerization of each oligosaccharide described in Table VI is determined by the ^{14}C:^{3}H ratio procedure described in Methods and is found to vary from 2 to 5. The monosaccharide content of each oligosaccharide was determined by radiochromatography (solvent A) of the products obtained on hydrolysis for 9 hr in 1 N sulfuric acid at 100°. Under these hydrolysis conditions D-glucose is released quantitatively but the aldobiouronic acid linkage is incompletely hydrolyzed. Although solvent A separates the reduced forms of aldobiouronic and D-glucuronic acids, the reduced D-mannose, D-glucose, and D-glucuronic acid migrate at similar rates and must be determined to-

TABLE V: Chromatographic Analysis of Methyl Sugar Mixtures Obtained on Hydrolysis of Methylated Polysaccharides.[a]

NCTC Strain	Applied to Column (mg)	Recovery (mg)					Recovered Sugars			
		Peak I	Peak II	Peak III	Peak IV	Water	Molar Ratios[c]		^{3}H Recovery (%)	
		Degradation Products[b]	Me_4Glc	Me_5Glc	Me_2Man	Wash[b]	Me_5Glc	Me_2Man	Me_4 Fraction	All Others
243[e]	1000	22	215	443	210	108	2.2	1.1	100	0
418	515	21	140	260	124	54	2.0	1.0	96	0
5055	743	39	151	325	153	56	2.3[d]	1.2[d]	96	0
9504	854	15	194	399	183	49	2.2	1.0	102	0

[a] All samples were methylated, reduced, and remethylated (Figure 1). [b] Products resulting from complete degradation of a fraction of each methyl sugar. [c] The chromatographic separation of the NCTC 243 monosaccharides is illustrated in Figure 1. [d] Confirmed by radiochromatography after reduction of hydrolysis mixture with [^{3}H]sodium borohydride. [e] Moles per mole of Me_4Glc.

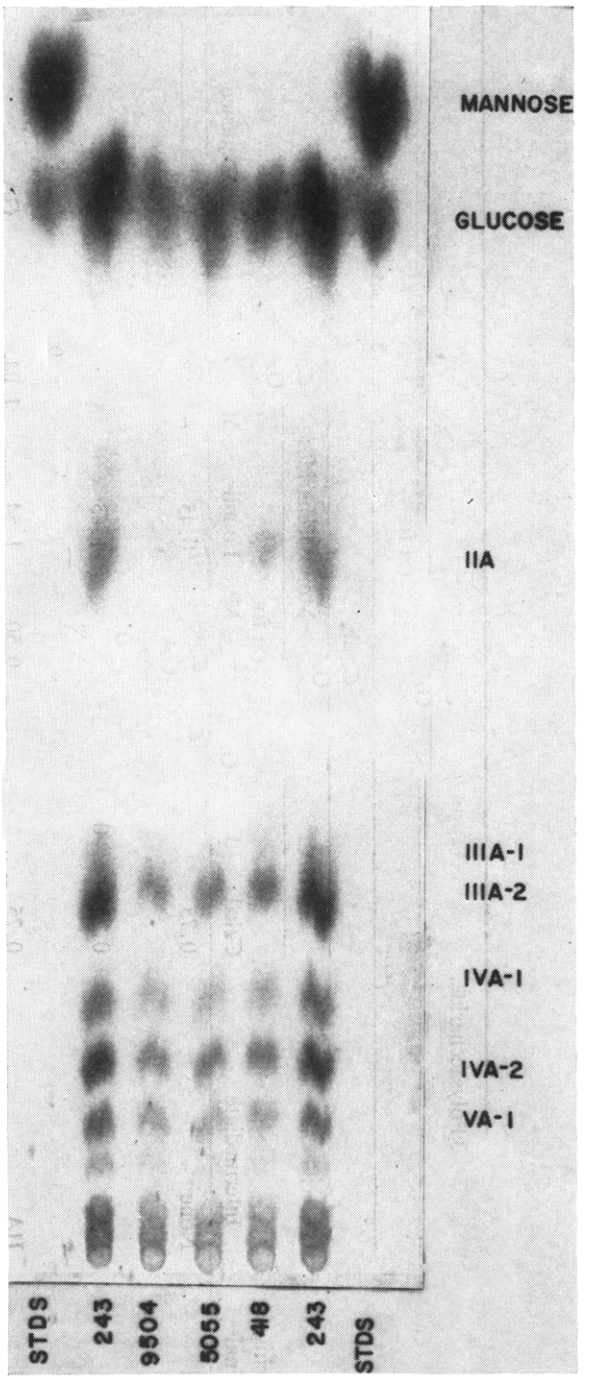

FIGURE 2: Paper chromatographic comparison of partial hydrolysates of the four serotype 2 capsular polysaccharides. Hydrolysis was carried out for 0.5 hr with 2% solutions of polysaccharide in 1 N sulfuric acid at 100°. The chromatogram was run in solvent C (1-butanol–acetic acid–water (2:1:1)).

gether. By use of the rate constant for aldobiouronic acid hydrolysis (Table X) and the time period of hydrolysis, the amount of D-glucuronic acid and D-mannose in the monosaccharide fraction on the chromatogram can

TABLE VI: Characterization of Oligosaccharides from NCTC 243 Polysaccharide.

Oligosaccharide[a]	DP[b] Hexose/ Reducing Group	Reducing Terminal[c]	Hydrolysis Products[d] Intermediate	Final[e] IIA Calcd	IIA Found	Monosaccharides Calcd for GA + M[e]	Monosaccharides Found	Monosaccharides Δ(=G)	Sequence
IIA	2.1	M	None	0.75	0.66	0.50	0.45	−0.05	GA→M—OH
IIIA-1	3.0	G	IIA	0.75	0.69	0.50	1.43	0.93	GA→M→G—OH
IIIA-2	3.0	M	IIA	0.75	0.75	0.50	1.54	1.04	GA→G→M—OH
IVA-1	3.8	G	IIA, IIIA-1	0.75	0.75	0.50	2.53	2.03	GA→M→G→G—OH
IVA-2	3.8	G	IIA, IIIA-1, IIIA-2	0.75	0.74	0.50	2.56	2.03	GA→G→M→G—OH
VA-1	5.1	G	IIA, IIIA-1, IIIA-2, IVA-1, IVA-2	0.75	0.75	0.50	3.46	2.96	GA→G→M→G→G—OH

[a] See Figure 2. [b] Degree of polymerization, determined by the radiochromatographic isotope ratio procedure (Methods). The ratio of ^{14}C to ^{3}H counts per minute in the monosaccharide portion of the chromatogram was multiplied by an appropriate factor to give 1.0 (one hexose per reducing group) and the corresponding ratio for the segment containing each oligosaccharide was then multiplied by the same factor to calculate the degree of polymerization of the oligosaccharide. [c] Determined by comparison of hydrolysates of [^{3}H]sodium borohydride reduced oligosaccharides with standards (glucose and IIA) reduced in the same manner. M = D-mannose; G = D-glucose; GA = D-glucuronic acid. [d] Hydrolysis in 1 N sulfuric acid at 100°. Intermediate oligosaccharides observed by paper chromatography after 0.5 hr. Final products were determined by radiochromatography after 9 hr. [e] Moles per mole of oligosaccharide.

TABLE VII: Characterization of Products from Periodate-Oxidized NCTC 243 Polysaccharide.[a]

Sample	Total Carbohydrate[b] mg	Total Carbohydrate[b] % of Original	D-Glucose[c] (% of Carbohydrate)	Hydrolysis[c] Products
243 polysaccharide	1970	100	50	G, M, GA, IIA
Polyalcohol	1020	49.5		G, M
Disaccharide (II N)	400	19.8	51	G, M
Reduced disaccharide (II N_{red})			92[d]	G, mannitol

[a] Polysaccharide (0.4%, w/v) oxidized with 0.05 M sodium periodate in 0.04 M acetate buffer (pH 4.0) for 18 days at 0° in the dark. Final IO_4 uptake: 0.84 mole/162 g of polysaccharide. [b] Expressed as equivalents of an equimolar mixture of D-glucose and D-mannose. [c] Determined after hydrolysis for 9 hr at 100° in 1 N sulfuric acid. D-Glucose was determined by the Glucostat procedure. Abbreviations are listed in footnote *c* of Table VI. [d] Sample analyzed contained 43 μg of total carbohydrate, as glucose. After the 9-hr hydrolysis period 26% of the glycosidic bonds had been cleaved and 10.2 μg of D-glucose was released (Table X).

be calculated from the amount of unhydrolyzed aldobiouronic acid found on the same chromatogram. The amount of total monosaccharide in excess of that calculated for D-glucuronic acid plus D-mannose is the amount of D-glucose.

The number of moles of oligosaccharide originally present in the aliquot analyzed is determined by radiochromatographic analysis of the hydrolysis mixture at zero time. Thus, for the final hydrolysis mixture the moles of unhydrolyzed aldobiouronic acid and of total monosaccharide per mole of original oligosaccharide can be determined. The concentration of original oligosaccharide can also be used to calculate the expected amount of unhydrolyzed aldobiouronic acid in the final hydrolysate. For all of the oligosaccharides described in Table VI the number of moles of aldobiouronic acid remaining in the final hydrolysate is consistent with the calculation which assumes only a single aldobiouronic acid residue per oligosaccharide. Thus, the data in Table VI show that these oligosaccharides all contain 1 mole of the glucuronosylmannose moiety plus 0 to 3 D-glucose residues. For these calculations it is assumed that the rate of cleavage of the aldobiouronic acid linkage is the same in all of its oligosaccharide combinations.

The reducing terminal of each oligosaccharide is established by [^{3}H]borohydride reduction of the oligosaccharide followed by hydrolysis to IIA plus monosaccharides. Hydrolysates are chromatographed in solvent A and analyzed for ^{3}H. Since the D-glucuronic acid is combined in the aldobiouronic acid moiety of each oligosaccharide and is found only at nonreducing terminals in the polysaccharide, the reducing terminals of all of the oligosaccharides must be either D-mannose or D-glucose residues. Recovery of the bulk of the tritium in the reduced aldobiouronic acid area of the chromatogram establishes D-mannose as the reducing terminal of the oligosaccharide, whereas recovery of the tritium in the reduced monosaccharide area indicates that D-glucose is at the reducing end. Results were confirmed by spraying chromatograms of these hydrolysates of the [^{3}H]-borohydride-reduced oligosaccharides with aniline–acid phthalate and noting a corresponding absence of reducing sugar spot in the same area of the chromatogram which contained the tritium.

Final evidence required to establish the sequence of each oligosaccharide was obtained by examining chromatographically the oligosaccharides occurring at intermediate stages of hydrolysis. The sequence assignments shown in Table VI are the only ones consistent with the methylation data above and sequence analysis described here. The tetrasaccharide repeating sequence suggested by these data is confirmed by the periodate oxidation studies below.

Periodate Oxidation of the Polysaccharide. The methylation data show that one of the D-glucose residues is linked through C-3 and the other through C-4, while D-mannose is linked through both C-3 and C-4. Thus, upon periodate oxidation of the polysaccharide, all of the D-mannose and one of the D-glucoses will remain unoxidized. The sequence analysis indicates that these two residues will be present in the oxidized polysaccharide as an intact disaccharide. The data presented in Table VII show that 50% of the original polysaccharide is recovered after an extended period of periodate oxidation and that the unoxidized portion of the polysaccharide is present exclusively in a single glucosylmannose disaccharide (II N). These data establish unequivocally the repeating nature of the polysaccharide structure and prove that the glucosyl–glucose linkage in the repeating unit is 1→3 while the mannosyl–glucose linkage is 1→4. Proof of the repeating nature of the structure is based upon the demonstration that *all* of the D-glucuronic acid residues and *all* of the 3-linked D-glucose residues in the polysaccharide are linked to mannose. The resulting trisaccharide residues, which account quantitatively for three-fourths of the total polysaccharide, must be uniformly separated from each other and joined through the remaining 4-linked D-glucose residue since, if sequences of the trisaccharides were joined directly, one

TABLE VIII: Determination of the Aldobiouronic Acid Linkage by Methylation Analysis.

Sample	Recov (μmoles)	Methoxyl (%) Calcd	Methoxyl (%) Found	Effect of IO_4
1. IIA	840	0	0	Destroyed
2. IIA, methylated	740[a]			
3. Sample 2, reduced	620[a]	49.2	50.4	
4. Sample 3, remethylated	550[a]	54.5	55.5	
5. Hydrolysate of sample 4				
Me_4 glucose	432[b]			None
Me_3 mannose	431[b]			None
6. Control for IO_4 effect, 2,3,6-Me_3 glucose				Destroyed

[a] Calculated from recovered sample weight. [b] Determined by radiochromatography of the hydrolysate.

TABLE IX: The Anomeric Configurations in the NCTC 243 Polysaccharide.

Oligosaccharide			No. of Protons/ Total Ring Protons	
Designation[a]	Structure	τ Value (ppm)		Proton Assignment[b]
II N	$G\overset{1\ 4}{—}M\text{–}OH$	4.81	0.51	α-M-OH
		5.07	0.33	β-M-OH
		5.50	0.92	β-G-M
IIA	$GA\overset{1\ 3}{—}M\text{–}OH$	4.70	1.0	α-GA-M
		4.83	0.58	α-M-OH
		5.07	0.42	β-M-OH
IIIA-1	$GA\overset{1\ 3}{—}M\overset{1\ 4}{—}G\text{–}OH$	4.75	1.54	α-GA-M + α-G-OH
		5.19	1.0	β-M-G
		5.39	0.51	β-G-OH
IIIA-2	$GA\overset{1\ 3}{—}M\text{–}OH$ with $G\overset{4}{\underset{1}{\vert}}$	4.83	1.53	α-GA-M + α-M-OH
		5.40	0.53	β-M-OH
		5.52	1.0	β-G-M
IVA-1	$GA\overset{1\ 3}{—}M\overset{1\ 4}{—}G\overset{1\ 3}{—}G\text{–}OH$	4.68 + 4.73	2.43	α-GA-M + α-G-G + α-G-OH
		5.15	1.28	β-M-G + HDO
		5.39	0.52	β-G-OH

[a] See Figure 2. [b] Signals for the α-OH and β-OH assignments are those obtained for the anomeric protons when the OH's on the reducing group are in the α and β positions, respectively. Abbreviations are given in Table VI, footnote *c*.

would expect to recover from the periodate-oxidized polysaccharide higher oligosaccharides composed of multiples of glucosylmannose disaccharide. No such oligosaccharides were observed. Furthermore, the latter alternative to the tetrasaccharide repeating structure would require that these polysaccharides contain regions of linear 1,4-linked D-glucose residues, a requirement which is inconsistent with the amounts and types of oligosaccharides recovered from partial hydrolysates of the polysaccharide.

Linkages to Mannose. D-Mannose is a branch point in the polysaccharide to which D-glucuronic acid is linked as a one unit branch through *either* C-3 or C-4 of the D-mannose. To establish the point of the linkage of D-glucuronic acid to D-mannose, 300 mg of IIA was methylated, then reduced with lithium aluminum hydride, and remethylated as shown in Table VIII. The resulting fully methylated neutral disaccharide was hydrolyzed to yield 2,3,4,6-tetra-*O*-methyl-D-glucose (from D-glucuronic acid, identified by R_F only) and 2,4,6-tri-*O*-methyl-D-mannose. Since the physical properties of 2,3,6- and 2,4,6-tri-*O*-methyl-D-mannoses are

TABLE X: Rate Constants for Hydrolysis of the Glycosidic Bonds in the NCTC 243 Polysaccharides.

Bond Cleaved[c]	Oligosaccharide Hydrolyzed for Rate Measurement[a]	Rate Constant (sec^{-1}),[b] $\times 10^4$
$GA\overset{1\alpha 3}{—}M$	IIA	0.11
$M\overset{1\beta 4}{—}G$	IIIA-1	1.19
$G\overset{1\alpha 3}{—}G$	IVA-1	2.85
$G\overset{1\beta 4}{—}M$	IIIA-2	3.62
	II N	3.58
$G\overset{1\beta 4}{—}M_{red}$	II N_{red}	0.37

[a] See Figure 2 and Tables VI and IX. [b] Hydrolysis in 1 N sulfuric acid at 100°. [c] See Table VI, footnote *c*, for abbreviations.

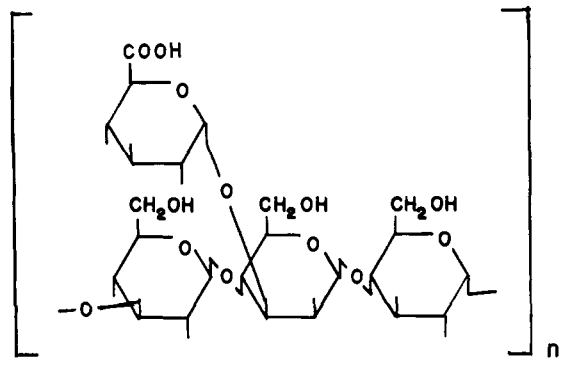

FIGURE 3: The repeating sequence of the *Aerobacter aerogenes* serotype 2 polysaccharide.

quite similar, the positions of methoxyl substitution on the tri-*O*-methyl-D-mannose from the aldobiouronic acid were established by observation of the effect of periodate spray reagent on the position of paper chromatographic migration of the reduced trimethylmannose. The mixture of sugars in the hydrolysate was reduced with [^{3}H]borohydride and separated using solvent D. The section of the strip containing the reduced tri-*O*-methyl-D-mannose was located using a radiochromatographic strip scanner and sprayed with 0.02 M sodium metaperiodate. This sprayed section was cut out and the radioactivity was washed to the center with ethanol. The section was then sewn into a second strip for rechromatography in solvent D. The tritium was recovered quantitatively in the region of the chromatogram where the original trimethylmannose migrated. It is concluded therefore that the trimethylmannose is substituted in positions 2, 4, and 6. In a control experiment 2,3,6-tri-*O*-methyl-D-[^{3}H]glucitol was oxidized by periodate to a product which migrated faster than the unoxidized sugar and which was presumed to be 2,3,di-*O*-methyl-L-threose. Thus, D-glucuronic acid is linked to the 3 position of the D-mannose. It follows that the 3-linked D-glucose in the polymer is linked through C-4 of the D-mannose.

Configurations of the Anomeric Linkages. The nuclear magnetic resonance data presented in Table IX establish the anomeric configurations of all of the linkages in the polysaccharide. Assignments of chemical shifts are based on previous literature reports (Lee and Ballou, 1965; van der Veen, 1963; Lenz and Heeschen, 1961). Oligosaccharides were examined in the order of increasing degree of polymerization. After assignment of the anomeric configurations in the lower oligosaccharides each additional anomeric configuration in the higher oligosaccharides is assigned on the basis of the increment in number of α or β protons over the number found in that smaller oligosaccharide which contains identical linkages except for the one under consideration. It is seen that identical chemical shifts are found for a given linkage in all of the oligosaccharides in which the linkage occurs. The data therefore are internally consistent for the assignments given. The complete structure of the tetrasaccharide repeating unit of the polysaccharide is shown in Figure 3.

Hydrolysis Rates of the Individual Bonds in the Polysaccharide. Rate constants for hydrolysis of individual glycosidic bonds in 1 N sulfuric acid at 100° are given in Table X. For hydrolysis of the disaccharides the rate of release of reducing groups during hydrolysis was determined by radiochromatographic measurement of the decrease in disaccharide concentration during hydrolysis. To determine the rates of cleavage of the glucosyl–mannose and mannosyl–glucose bonds the rate of release of glucose from IIIA-2 and IIIA-1, respectively, was measured by radiochromatography with appropriate corrections for aldobiouronic acid cleavage as described for the data in Table VI. For obtaining the rate constant for hydrolysis of the glucosylglucose bond the rate of glucose release from IVA-1 was compared with that for IIIA-1 and the difference was taken as the rate of cleavage of the glucosylglucose bond. Assumptions concerning the effect of multiple linkages in an oligosaccharide on the rate of cleavage of any particular linkage in the oligosaccharide were essentially those discussed earlier (Conrad *et al.*, 1966).

Several points are of interest. First, the rate constant for cleavage of the 4-*O*-β-D-glucopyranosyl-D-mannose linkage is identical when determined using either the disaccharide or IIIA-2 to calculate the rate. Thus, the substitution of the D-glucuronic acid on C-3 of the mannose has no apparent effect on the rate. Second, when the reducing group of the 4-*O*-β-D-glucopyranosyl-D-mannose is reduced with borohydride, the rate constant for hydrolysis of the glucosyl bond is decreased by an order of magnitude. Finally, the relative rates for the glycosidic linkages in the main chain of the polysaccharide are consistent with our failure to recover significant amounts of either the glucosylglucose disaccharide or the third possible tetrasaccharide that one might expect from the repeating unit.

Discussion

The four serotype 2 capsular polysaccharides described here were isolated in several widely separated parts of the world (England, U. S., and Java) and submitted to the National Collection of Type Cultures over the years beginning with the 418 strain in 1911.[1] The complete structural identity of the four antigenic capsules, although not unexpected, affirms the remarkable specificity of the serological methods which have led to the suggestion of their intimate structural relationship (Heidelberger, 1964). In an examination of 256 strains of *Aerobacter*, Edwards and Fife (1952) found that among the 57 serotypes represented, 13% of the strains had serotype 2 capsular antigens. This suggests a basic biological selection for the tetrasaccharide sequence described here and an identity in those portions of the genome controlling capsular polysaccharide biosynthesis. This is especially notable when one considers the number of structures possible for a repeating tetrasaccharide composed of these same monosaccharides in the ratios found here.

Our data differ from those of Barker *et al.* (1958) at several significant points. Most important, the serotype 2 polysaccharide has a repeating sequence of 4 monosaccharide units in contrast to 40 as suggested earlier. The structure does not contain mannuronic acid nor does it appear to vary in uronic acid content when growth conditions are varied (Sandford and Conrad, 1966). Finally, the glucuronosylmannose linkage is 1→3 instead of 1→4 as reported by Barker *et al.* The present results reinforce our previous suggestion (Conrad *et al.*, 1966) that classical methods for determining polysaccharide structures are subject to errors of considerable magnitude. Once more reduction of the polysaccharide structure to a relatively simple repeating unit allows the consideration of nontemplate mechanisms for biosynthesis of these organism-specific complex polysaccharides.

The monose content of the serotype 2 polysaccharide differs from that of the serotype 54 polysaccharide (Conrad, *et al.*, 1966) simply in the substitution of a D-mannose residue in type 2 for the L-fucose residue in the type 54 polymer. This observation, at the outset of this work, suggested that the structures might be identical except for a D-mannose for L-fucose replacement. Since guanosine diphosphate D-mannose is enzymatically converted to guanosine diphosphate L-fucose (Ginsburg, 1960), such structural similarities in these polysaccharides would imply a role for the guanosine portion of the activated monosaccharide in the specific positioning of the monosaccharide in the polymer. However, marked differences have been found in the sequences and linkages of the serotypes 2 and 54 polymers, the only apparent similarities being (1) the positioning of the guanosine diphosphate activated sugar (D-mannose or L-fucose) between D-glucuronic acid and D-glucose in both polymers and (2) the identical α-1,3-D-glucuronosyl linkages to the guanosine diphosphate activated sugars. Results of enzymatic studies in the two organisms must be obtained before one may attribute any significance to these correlations.

[1] Personal communication from W. M. Whipp, National Collection of Type Cultures.

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Addendum

S. A. Barker and I. R. Siddiqui

We (Barker and Siddiqui, 1958) distinguish two polysaccharide preparations. One of these was prepared from an authentic 418 strain obtained directly from the National Collection of Type Cultures and we do not report this as containing mannuronic acid. Furthermore, methylation data showed the presence of glucuronic acid end groups, glucose end groups, 1→3-linked glucose units, 1→4-linked glucose units, and hexose units involved in branch points. Except for the percentage of end groups these results are in agreement with those cited above.

The other batch of polysaccharide was prepared from what we were careful to distinguish as the Oxford University Physical Chemistry Laboratory strain of *A. aerogenes* although we were informed by Professor Hinshelwood that parent strains of this organism were deposited with the National Collection of Type Cultures as 418 strain. The properties of this polysaccharide differed in several ways from those cited above (*e.g.*, optical rotation, proportion of glucose to mannose) and when examined contained mannuronic acid.

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The Archibald Molecular Weight Determination. A General Theory for Data Extrapolation and Its Application*

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ABSTRACT: Archibald's method of determining molecular weights by ultracentrifugation has several advantages over others. Its over-all effectiveness is impaired, however, by the necessity of extrapolation to obtain certain critical data. This necessity is created by optical artifacts occurring near the meniscus. Although various empirical extrapolation methods are known, no theoretically valid, completely general method has yet been described. Such a method, based on the Fujita–MacCosham solution to Lamm's differential equation [Fujita, H., and MacCosham, V. J. (1959), *J. Chem. Phys. 30*, 291], is described in this paper. Its application is effected by an iterative, self-correcting computer program. Data generated from the Fujita–MacCosham equation are used to test the influences of various parameters on the efficacy of the method, and β-lactoglobulin B is used as a "real" test system. A separate determination of the optical constant is made optional by a technique based on the principle described by R. Trautman and C. F. Crampton [(1959), *J. Am. Chem. Soc. 81*, 4036]. The molecular weight obtained is almost unaffected by another practical difficulty of the Archibald method, namely, the inability to measure the true meniscus position. The output data of the computer program appear to provide a very sensitive criterion for nonideal behavior.

Archibald's method of determining molecular weights in the ultracentrifuge has a unique complement of advantages: (i) it is faster than conventional equilibrium methods; (ii) it is based on equilibrium theory, making the interpretation of results more straightforward than in sedimentation–diffusion methods; and (iii) the fractionation occurring during a run facilitates a study of the composition of a paucidisperse system. Unfortunately, artifacts arising from optical phenomena invalidate data obtained at the ends of the liquid column, where the equilibrium condition exists. Thus it is difficult to obtain either the exact position of, or the concentration gradient at, the meniscus, using the schlieren optical system. This is the major drawback of the Archibald method. A technique for obtaining the meniscus position by using data obtained at different times of centrifugation was described some years ago (Trautman, 1958). There are several empirical methods for extrapolating the concentration gradient to the meniscus. Recently, conditions leading to linearity in $(\partial c/\partial r)$ *vs.* r have been derived (LaBar, 1966a,b) on the basis of the Fujita–MacCosham (1959) equation. These conditions are somewhat restrictive, especially in experiments designed to deplete markedly the solute concentration at the inner meniscus.

The major purpose of this study was to derive a generally valid procedure for extrapolating data to the menisci, based on the Fujita–MacCosham equation. By evaluating some of the terms in this equation numerically, it is possible to derive a simple function, which I call "recip" because it is directly related to $1/M$, correlating the measured variables. Recip is proportional to r^2, where r is the distance to the center of rotation. The numerical evaluations occurring in recip explicitly depend upon a value of the molecular weight, which is the

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