The Structure of the *Aerobacter aerogenes* A3(Sl) Polysaccharide. I. A Reexamination Using Improved Procedures for Methylation Analysis*

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ABSTRACT: The acidic slime polysaccharide from *Aerobacter aerogenes*, A3(Sl), has been converted in three reaction steps to a fully methylated, neutral polysaccharide. The permethylation reaction of Hakamori (Hakamori, S., 1964, *J. Biochem. (Tokyo) 55*, 205) gives a stoichiometric conversion of the polysaccharide to a completely methylated and esterified polymer which is reduced with lithium aluminum hydride and remethylated.

The progress of methylation has been followed by using [^{14}C]methyl iodide of known specific activity as the alkylating reagent and determining per cent methoxyl by isotope dilution assay. Hydrolysis of the fully methylated, neutral polysaccharide yields equimolar amounts of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose (derived from 2,3-di-O-methyl-D-glucuronic acid), 2,3-di-O-methyl-D-glucose, and 2,4-di-O-methyl-L-fucose. Analyses of A3(Sl) polysaccharide preparations obtained when the organism is grown under a wide variety of growth conditions yield identical structural results. The data show that *A. aerogenes*, A3(Sl), by a highly specific biosynthetic mechanism, produces a single polysaccharide with a structure much less complex than suggested by an earlier study (Aspinall, G. O., Jamieson, R. S. P., and Wilkinson, J. F., 1956, *J. Chem. Soc.*, 3483). The limitations of methylation analysis in assessing polysaccharide structure are discussed in relation to present concepts of the complexity of structure and biosynthesis of heteropolysaccharides.

The biosynthesis of heteropolysaccharides is a subject of increasing interest (Elbein and Heath, 1965; Nikaido, 1965; Weiner *et al.*, 1965; Wright *et al.*, 1965). To date it is not possible to outline for any complex polysaccharide the sequence of enzymatic steps by which its uniquely linked, specifically ordered monose sequence is synthesized. The number and specificities of the enzymes involved in the biosynthetic sequence must be a function of the complexity of the structure for any given polysaccharide; thus, a knowledge of the monosaccharide sequences is of considerable importance in understanding the biosynthetic mechanism.

Repeating sequences of specifically linked monosaccharide residues have been demonstrated only for a few complex polysaccharides (Robbins and Uchida, 1962; How *et al.*, 1964). For the great majority of heteropolysaccharides, however, structural analyses have indicated that repeating units, if present at all, are extremely complex, being composed of as many as 40 monose residues (Barker *et al.*, 1958a,b; How *et al.*, 1964). In the latter group of polymers no single structure has been established in detail. There has been no examination of the possibilities that the seeming structural complexities may be due to mixtures of polysaccharides in the fractions analyzed or to enzymatic nonspecificity in the ordering and/or linking of the monose residues.

The capsular and slime polysaccharides produced by *Aerobacter aerogenes* strains appear to be highly complex structures (Barker *et al.*, 1958a,b; Aspinall *et al.*, 1956b). Glucose, galactose, mannose, fucose, and uronic acids are found in varying amounts in these polysaccharides (Dudman and Wilkinson, 1956) but are not present in simple molar ratios. The slime polysaccharide from *A. aerogenes*, strain A3(Sl) (*Klebsiella* Type 54), *e.g.*, was reported to be composed of 48% D-glucose, 10% L-fucose, and 29% of an unidentified uronic acid. The simplest possible repeating unit, based on these analyses, would contain five residues of glucose, three of uronic acid, and one of fucose. A more detailed study of the A3(S1) polysaccharide (Aspinall *et al.*, 1956b) showed that the uronic acid was glucuronic acid and that cellobiose was obtained from the polysaccharide as an intermediate in acid hydrolysis. Methylation analysis gave a mixture containing low yields of more than 10 neutral methyl sugars and a relatively large uronic acid containing fraction. Periodate oxidation of the polysaccharide yielded 1 mole of formic acid/3 sugar residues. The authors concluded that the A3(Sl) polysaccharide is a very complex, highly branched polymer in which the glucuronic acid residues are probably present as nonreducing ends.

The present manuscript is addressed to a critical reexamination of the structure of the *A. aerogenes* A3(Sl) polysaccharide and its constancy as a function

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TABLE I: Media Used for Chemostat Production of *A. aerogenes* A3(S1) Polysaccharide.

Medium	Limiting Nutrient	Component[a]										
		$NaH_2PO_4 \cdot 2H_2O$ (g)	$(NH_4)_2HPO_4$ (g)	K_2SO_4 (g)	K_2HPO_4 (g)	NH_4Cl (g)	Tris (g)	0.1 M H_2SO_4 (ml)	MgO (g)	Concn, HCl (ml)	0.155 M EDTA (ml)	Glucose (g)
1	Glucose	31	238	70	...	...	..	...	2.0	10.3	200	400
2	Nitrogen	31	...	70	314	53.5	..	...	2.0	10.3	200	1600
3	Phosphorus	..	6.6	70	...	216	61	...	2.0	10.3	200	1200
4	Sulfur	31	238	..	...	...	..	100	2.0	10.3	200	1200
5	Magnesium	31	238	70	...	...	..	...	0.5	3.6	150	1200

[a] Amounts given are for 40 l. of medium. All media contained in addition the following salts, in g/40 l.: $CaCO_3$, 0.2; ZnO, 0.04; $FeCl_3 \cdot 6H_2O$, 0.54; $MnCl_2 \cdot 4H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.017; $CoCl_2 \cdot 6H_2O$, 0.024; $NaMoO_4 \cdot 2H_2O$, 0.024, and H_3BO_3, 0.006.

of growth conditions of the cell. This paper describes the adaptation of the permethylation method described by Hakamori (1964) to yield the completely methylated polymer in a single reaction step. [^{14}C]-Methyl iodide has been used as the methylating reagent in a portion of this study to quantitate per cent methoxyl by isotope dilution assay and to examine some of the difficulties encountered in methylation studies. When the polysaccharide is completely methylated, reduced, and remethylated, it is quantitatively converted by acid hydrolysis into a mixture of methyl sugars containing equimolar amounts of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose (from 2,3-di-O-methyl-D-glucuronic acid), 2,3-di-O-methyl-D-glucose, and 2,4-di-O-methyl-L-fucose. Thus, the ratio of glucose–glucuronic acid–fucose is 2:1:1. The unique hydrolysis properties of the polysaccharide make its complete hydrolysis to monoses without concurrent degradation virtually impossible, thus complicating the analysis of the polysaccharide for monosaccharides (T. J. Kindt and H. E. Conrad, unpublished results). The data show that strain A3(Sl) of *A. aerogenes* synthesizes a single, structurally identical polysaccharide under a variety of growth conditions and that the polysaccharide structure is considerably less complex than previously indicated.

Methods

Preparation of Polysaccharide. A. aerogenes, strain A3(Sl) (ATCC 12658), was grown at 30° in an 8-l. chemostat with automatic pH and temperature controls restricting the concentration of a single nutrient to growth-limiting levels. The growth media are given in Table I. The organism was grown under conditions of glucose, nitrogen, phosphorus, sulfur, or magnesium limitation. The growth chamber was filled with 8 l. of sterile medium and inoculated to an OD_{660} of 0.05 (1-cm light path) from an 8-hr shake culture grown in the same medium. When the growth approached the maximum OD_{660} (*ca.* 5), fresh medium was introduced at a dilution rate of 0.1 fermentor volume/hr. The culture in the spent medium was withdrawn at the same rate and cells were removed by centrifugation in an air-driven Sharples supercentrifuge. The pH of the supernatant was adjusted to 2 with sulfuric acid and the polysaccharide was precipitated by slowly adding 1.25 volumes of acetone while stirring the solution vigorously. After the precipitated polysaccharide had settled, the supernatant was decanted, and the precipitate was filtered, washed twice each with 99% ethanol and ether, and air dried. The crude polysaccharide thus obtained was purified by stirring a 2% aqueous solution for 2 hr and removing any insoluble material by a 30-min centrifugation at 35,000*g*. The supernatant was dialyzed *vs.* two changes of deionized water for 20 hr and freeze dried. This procedure reduced the ash content to <4%. The final yield of purified polysaccharide was *ca.* 1 g/l. of growth medium.

Methylation of the Polysaccharide. The methylation procedure was essentially that described by Hakamori (1964) wherein the methylsulfinyl anion (Corey and Chaykovsky, 1962; Chaykovsky and Corey, 1962) was used to generate the polysaccharide alkoxide prior to addition of methyl iodide. Routinely 1-g samples of polysaccharide were methylated. The procedure described is for this amount but the size of the reaction may be altered readily.

The methylsulfinyl anion was prepared as follows. Into a dry, 300-ml three-necked round-bottom flask fitted at one neck with a rubber serum cap and containing a magnetic stirring bar was weighed 1.5 g of sodium hydride (55%, coated with mineral oil). The sodium hydride was washed three times by stirring with 30-ml portions of *n*-pentane and decanting the wash. After the third wash the flask was fitted with a thermometer and a stoppered condenser and residual *n*-pentane was removed by successive evacuations with a vacuum pump through an 18-gauge needle inserted into the serum cap. After each evacuation, the flask was

regassed with nitrogen. The stopper was then removed from the condenser and nitrogen was passed continuously through the flask *via* the needle. Using a hypodermic syringe 15 ml of dimethyl sulfoxide, distilled from calcium hydride under reduced pressure and stored over dried molecular sieves (Linde, type 4A), were transferred into the flask. The flask was placed in a Glasco heating mantle and stirred with a magnetic stirrer at 50° until the solution became clear and green and evolution of hydrogen gas ceased (*ca.* 45 min). The concentration of anion in the dimethyl sulfoxide solution was determined by withdrawing a 1-ml aliquot for titration with 0.1 N hydrochloric acid in aqueous solution.

For generation of the polysaccharide alkoxide, polysaccharide was first passed through a 200-mesh sieve and dried overnight at 60° *in vacuo*. Dried material (1 g) was added to 50 ml of dry dimethyl sulfoxide in a 300-ml three-necked round-bottom flask containing a magnetic stirring bar and fitted with a thermometer, a condenser, and a serum cap through which reagents were introduced and nitrogen gas was passed continuously. The suspension was heated at 60° and stirred with a magnetic stirrer until all of the polysaccharide dissolved (*ca.* 1 hr) and, after cooling to room temperature, 0.023 equiv of methylsulfinyl anion (*ca.* 10 ml) was added. The amount of base was a 35% excess over the number of equivalents of hydroxyl plus carboxyl present, calculated on the basis of a polysaccharide composed of 50% glucose and 25% each of fucose and glucuronic acid. Upon addition of the anion a gel formed immediately but gradually liquefied and, after stirring at room temperature for 30 min, the reaction mixture appeared homogeneous. The minimum time for complete alkoxide formation after addition of base was 4 hr.

For the methylation reaction the polysaccharide alkoxide solution was cooled to 20° in an ice-water bath and 3 ml of methyl iodide was added to the stirred solution at a rate such that the temperature did not rise above 25° (6–8 min). The amount of methyl iodide added was not critical as long as it was in molar excess of the base. Identical degrees of methylation were obtained with dimethyl sulfate but methyl iodide was used routinely because of its more desirable handling properties (more volatile, less toxic). Within a few minutes after addition of methyl iodide heat evolution ceased, the solution became clear, and the viscosity was markedly reduced. At this stage the reaction was complete. The reaction mixture was dialyzed overnight *vs.* running tap water and extracted continuously with chloroform. The chloroform extract was evaporated to dryness at 40° *in vacuo*. The dried product was dissolved in ether and filtered to remove a small amount of insoluble material. The ether-soluble material was dried *in vacuo* and analyzed.

Reduction and Remethylation of the Methylated Polysaccharide. The uronic acid residues in the polysaccharide, esterified in the methylation reaction, were reduced with lithium aluminum hydride. To 110 ml of a 1.35% ethereal suspension of lithium aluminum hydride 50 ml of a 2% solution of the methylated polysaccharide in anhydrous ether was added dropwise. The reaction mixture was stirred during the addition and for 18 hr afterwards. Excess hydride was destroyed by cautious dropwise addition of water to the vigorously stirred suspension. The ether solution was filtered and the insoluble residue extracted continuously with chloroform. The filtrate and extract were combined and evaporated to dryness at 40° *in vacuo*.

The primary hydroxyl groups formed from the uronic acid residues in the reduction reaction were methylated as described above using a 35% excess of methylsulfinyl anion, calculated on the basis of 25% uronic acid in the polysaccharide. The fully methylated, neutral polysaccharide was isolated by dialysis and extraction with chloroform and was purified by dissolution in ether, filtration, and removal of ether *in vacuo* at 40°.

Hydrolysis and Chromatography of the Methyl Sugars. The methylated polysaccharide was hydrolyzed as described by Garegg and Lindberg (1960). To 10 ml of 72% sulfuric acid at 0–5° was added 1.3 g of methylated polysaccharide. The suspension was stirred at room temperature for 1 hr to solubilize the polysaccharide. Water was then added to bring the acid concentration to 8% and the hydrolysis mixture was heated at 100° for 4 hr, then cooled to room temperature, and extracted continuously with chloroform for 24 hr. The aqueous phase was neutralized with barium carbonate and the resulting barium sulfate extracted exhaustively with ethanol to recover any mono or dimethyl sugars. The extracts were combined and evaporated to a syrup at 35° *in vacuo*.

The mixture of methyl sugars was separated by partition chromatography on a column of powdered cellulose (Hough *et al.*, 1949). This method was chosen because α- and β-forms of each sugar migrate as a single peak, thus facilitating identification and quantitation of the methyl sugar in each peak. Up to 1 g of syrup was applied to a column 3.5 cm in diameter packed to a height of 50 cm in water-saturated redistilled methyl ethyl ketone. The column was developed with this solvent and 10–15-ml fractions were collected. Fraction weights were obtained by drying each fraction at room temperature in a tared aluminum pan. The weight of material in each peak was determined by summing the weight of all fractions in the peak. Analysis of the dried fractions by paper chromatograms run in water-saturated methyl ethyl ketone and sprayed with aniline acid phthalate (Partridge, 1949) showed that separation was essentially complete.

Methoxyl Determination. The progress of methylation was followed by the ^{14}C isotope dilution assay described below or by the iodometric Zeisel micromethoxyl method which gave a precision of $\pm 0.6\%$ when 5-mg samples were analyzed. Infrared spectra run on 10% solutions (w/v) in carbon tetrachloride using a Perkin-Elmer Model 521 spectrophotometer were used to confirm the complete substitution of all free hydroxyls in the polysaccharide.

For the ^{14}C method polysaccharide was methylated using [^{14}C]methyl iodide of known specific activity.

TABLE II: Analytical Characterization of the A3(S1) Polysaccharide at Various Stages of Methylation.[a]

Methylation Stage	Yield[b,c] (%)	Methoxyl (%) Found Calcd[c]	Methoxyl (%) Found Micro-anal	Methoxyl (%) Found ^{14}C Method	Carbon (%) Calcd[c]	Carbon (%) Found	Hydrogen (%) Calcd[c]	Hydrogen (%) Found
1. Original polysaccharide[d]	100	0	0	...	44.5	44.8	5.9	7.0
2. Initially methylated	99	42.6	41.1	41.6	52.5	52.2	7.5	7.7
3. Methylated and reduced	87	40.0	40.0	39.9	52.7	52.5	7.8	8.2
4. Methylated, reduced, and remethylated	82	43.4	42.6	43.3	53.5	53.7	7.9	8.2

[a] Typical data taken from a reaction sequence in which polysaccharide produced by phosphate limited cells is methylated, reduced, and remethylated. [b] Calculated from weight of original polysaccharide. [c] Calculation based on polysaccharide composed of 50% glucose, and 25% each of glucuronic acid and fucose. Uronic acid residues are esterified in the initial methylation. [d] The starting material contained <0.5% nitrogen or sulfur.

[^{14}C]Methyl iodide (New England Nuclear Corp., 5.7 mc/mmole) was diluted with unlabeled methyl iodide to an approximate specific activity of 0.1 μc/mmole. The exact specific activity of the diluted [^{14}C]methyl iodide was calculated from the specific activity of cholesterol methyl ether prepared by the methylation procedure described above using the diluted [^{14}C]-methyl iodide. The cholesterol methyl ether was extracted from the reaction mixture with *n*-hexane, purified by adsorption on alumina, and recrystallized to constant specific activity.

Samples were counted in a scintillation fluid containing 4 g of 2,5-diphenyloxazole/l. of reagent grade toluene using a Packard Model 314EX liquid scintillation spectrometer. The specific activity of [^{14}C]methyl ethers was determined by dissolving 100 mg of sample in 1.0 ml of absolute ethanol and counting an aliquot or by drying *ca.* 10 mg of sample in a tared counting vial prior to addition of scintillation fluid to dissolve the sample. Counting efficiencies were determined by the channels ratio method of Bush (1963) and all counting rates were converted to disintegrations per minute for use in calculations. The per cent methoxyl in the methylated polysaccharide and the derived methyl sugars was calculated using the relationship

$$\% \ OCH_3 = \frac{31 \times \text{dpm/mg of sample}}{\text{dpm/mmole of } CH_3I} \times 100$$

Rearrangement of this equation and substitution of the per cent methoxyl and the disintegrations per minute per milligram for cholesterol methyl ether permits the calculation of the specific activity of the methyl iodide used to methylate both cholesterol and the polysaccharide.

Results

Preparation of Fully Methylated Polysaccharide. The methylation procedure was studied using polysaccharide produced by phosphate limited cells. Data presented in Table II and Figure 1 show typical analyses of products at the various stages of a reaction sequence in which [^{14}C]methyl iodide with a specific activity of 0.067 μc/mmole was used for both methylation steps. Each step represents a single reaction. The data illustrate several points: (1) the initial methylation yields a product with 98% of the theoretical methoxyl content; (2) the uronic acid residues are esterified in the

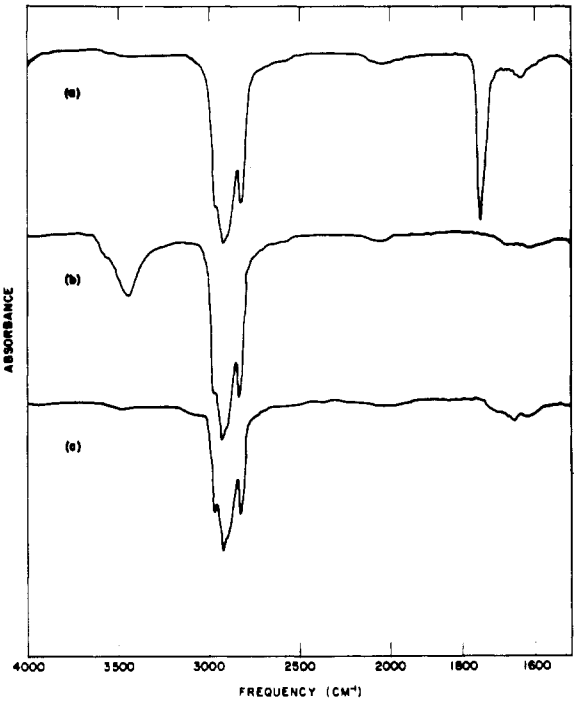

FIGURE 1: Infrared spectra of methylated polysaccharide at successive stages of the reaction sequence. (a) After initial methylation, (b) after reduction of the methylated polysaccharide, (c) after remethylation of the reduced product.

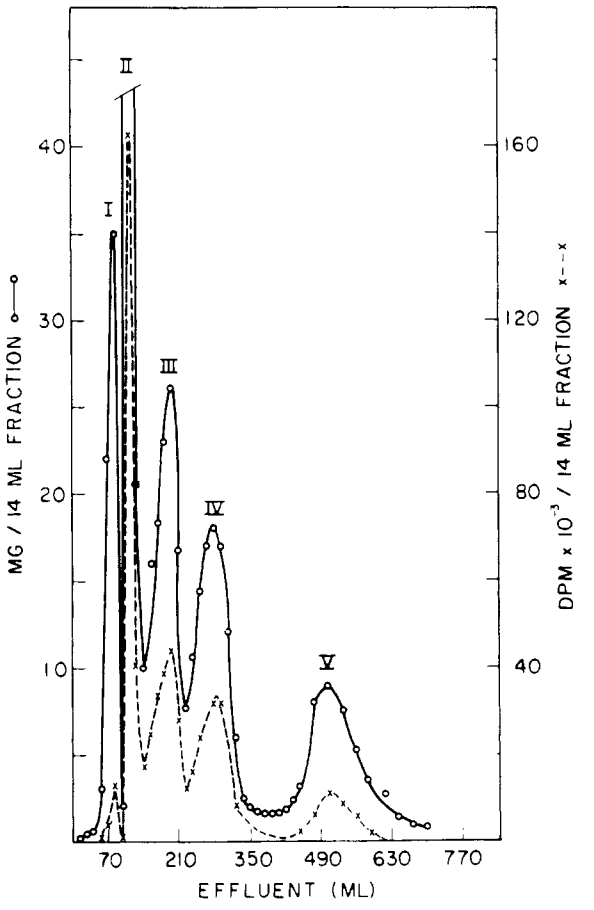

FIGURE 2: Cellulose column chromatography of the mixture of methyl sugars obtained on hydrolysis of ^{14}C-labeled fully methylated neutral polysaccharide. The column was developed with water-saturated methyl ethyl ketone. Peaks were identified as follows: I, degradation products; II, 2,3,4,6-tetra-O-methyl-D-glucose; III, 2,3,6-tri-O-methyl-D-glucose; IV, 2,4-di-O-methyl-L-fucose; and V, 2,3-di-O-methyl-D-glucose.

reaction as indicated by the infrared spectrum and the correspondence between the observed analytical data and those calculated for a methylated and esterified product; (3) reduction of the uronic acid esters is complete in one step as shown by disappearance of the ester carbonyl band in the infrared spectrum, the appearance of a corresponding hydroxyl band, and the agreement between theoretical and observed analytical values; (4) remethylation yields fully methylated, neutral polysaccharide; and (5) the recovery in each reaction is 90% or greater. Thus, the acidic polysaccharide is converted in better than 80% yield to a completely methylated, neutral product in three reaction steps.

Chromatographic Analysis. Chromatography of a mixture of methyl sugars from ^{14}C-labeled methylated, reduced, and remethylated polysaccharide is illustrated in Figure 2. Peak I contained degradation products formed during hydrolysis and is discussed in detail below. Peaks II–V were identified as 2,3,4,6-tetra-O-

TABLE III: Identification of Methyl Sugars from Fully Methylated Neutral Polysaccharide.

Peak[a]	Identity[b]	Melting Point (°C)		$[\alpha]_D^{20}$ (deg)		Methoxy (%)			Carbon (%)		Hydrogen (%)	
		Lit. Value	Found	Lit. Value	Found	Calcd	Found: Micro-anal	Found: ^{14}C Method	Calcd	Found	Calcd	Found[d]
II	2,3,4,6-Tetra-O-methyl-D-glucose	96	96	+92 → +84	+94 → +84 (*c* 1.8, H_2O)	52.5	52.1	52.0	51.0	51.1	8.5	8.7
III	2,3,6-Tri-O-methyl-D-glucose	121–23	120–21	+70	+64 (*c* 2.1, H_2O)	41.6	42.4	41.6	48.5	48.6	8.0	8.1
IV	2,4-Di-O-methyl-L-fucose	131–32	131–32	−85	−105 → −86 (*c* 1.0, H_2O)	32.3	31.8	31.6	50.0	49.1	8.3	8.3
V	2,3-Di-O-methyl-D-glucose	85–87	79–82	+32 → +48	+50 (*c* 2.6, acetone)	29.8	29.8	30.2	46.2	46.4	7.7	7.9

[a] From Figure 2. [b] Identities further established by comparison of R_G values with standards and by demethylation with BCl_3 (Bonner *et al.*, 1960) to identify the parent monosaccharides.

methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose, 2,4-di-O-methyl-L-fucose, and 2,3-di-O-methyl-D-glucose, respectively, by their characteristic physical properties given in Table III. The methoxyl values determined by the microanalytical procedure were obtained on recrystallized samples, while those calculated by the ^{14}C method were obtained using the dried syrup from those aluminum pans containing the peak fractions without recrystallization to constant specific activity. It is seen that the ^{14}C method not only gives accurate values for methoxyl content but also permits a simple estimate of the degree of methyl substitution in an unknown methyl sugar.

The quantitative recoveries of the four methyl sugars from polysaccharide samples synthesized by the organism under a variety of growth conditions are given in Table IV. Molar ratios of recovered sugars are calculated relative to the amounts of tetramethylglucose in each analysis. From all polysaccharide samples which were methylated, reduced, and remethylated (samples 1,3–6) the four sugars were recovered in equimolar amounts. When the polysaccharide was methylated and reduced but not remethylated (sample 2), 2,3,6-tri-O-methyl-D-glucose, found in the remethylated polysaccharide, was absent and was replaced by an equimolar amount of 2,3-di-O-methyl-D-glucose. This result indicates that 2,3,6-tri-O-methyl-D-glucose is formed by methylation of 2,3-di-O-methyl-D-glucose which in turn is derived from the uronic acid residues in the polysaccharide. Therefore, all of the uronic acid in the polysaccharide is linked through C-4. The constancy of the ratio of recovered methyl sugars indicates that the production of slime polysaccharide by this organism is under strict genetic control and that only one extracellular polysaccharide is formed. If more than one polysaccharide were produced, or if a single polysaccharide were synthesized by a random selection of activated sugars from the metabolic pool, the different growth conditions chosen would be expected to yield varying ratios of methyl sugars from the polysaccharide samples analyzed.

Degradation of Methyl Sugars during Hydrolysis. When the methylated polysaccharide was added to 72% sulfuric acid at 0° and then stirred at room temperature for 1 hr to solubilize the polysaccharide, a marked darkening of the solution occurred. Under the same conditions polysaccharide substituted with [^{14}C]-methyl groups lost 20% of the label after the 1-hr treatment. When the hydrolysis mixture was maintained at 0–5° during this initial stage, the polysaccharide was solubilized so that the second stage of hydrolysis (dilution to 8% sulfuric acid and heating at 100° for 4 hr) could be carried out in a homogeneous reaction mixture, but only 7% of the label was lost. No further darkening or loss of label took place in the second stage of hydrolysis in either case.

The degradation products were recovered in peak I (Figure 2 and Table IV) and in the water wash (Table IV) from the cellulose column. Neither of these fractions contained carbohydrate as judged by negative phenol–sulfuric acid reactions (Dubois *et al.*, 1956)

TABLE IV: Chromatographic Analysis of Methyl Sugar Mixtures Obtained on Hydrolysis of Methylated Polysaccharides

Sample[a]	Limitation during Biosynthesis	Applied to Column (mg)	Recovery (mg)							Molar Ratios of Recovered Sugars (moles/mole of Me_4Glu)		
			Peak I Degrad Products[b] (mg)	Peak II Me_4Glu	Peak III Me_3Glu	Peak IV Me_2Fuc	Peak V Me_2Glu	Water Wash[b] (mg)	Total (mg)	Total (%)	Me_3Glu	Me_2Fuc	Me_2Glu
1[c]	Phosphorus	450	57	102	102	89	88	2	449	100	1.05	1.05	0.97
2	Phosphorus	550	170	84	0	68	160	35	517	94	0	1.00	2.16
3	Glucose	800	78	190	178	128	143	52	749	94	1.00	0.83	0.86
4	Nitrogen	670	33	176	158	136	160	10	673	100	0.96	0.95	1.03
5	Magnesium	340	118	58	45	39	51	3	314	92	0.83	0.86	0.99
6	Sulfur	580	41	132	125	99	125	30	522	90	1.05	0.91	1.07

[a] All samples were methylated, reduced, and remethylated except sample 2 which was methylated and reduced but not remethylated. For sample 1 the first stage of hydrolysis was carried out at 0–5°; for other samples the first stage was carried out at room temperature. [b] Products resulting from complete degradation of a fraction of each methyl sugar, see Results. [c] The chromatographic separation of sample 1 is illustrated in Figure 2.

and by their failure to yield colored zones on heavily spotted paper chromatograms sprayed with aniline acid phthalate (Partridge, 1949). Therefore, simple demethylation products were not formed in the hydrolysis. Peak I, which represented the major fraction of the degradation products, contained only 7.9% methoxyl as calculated by the ^{14}C method. Thin layer chromatography of peak I showed a mixture of more than five noncarbohydrate components, all less polar than tetramethylglucose. Of the six samples described in Table IV, only sample 1 was maintained at 0–5° during the entire first stage of hydrolysis. As indicated above and borne out in Table IV, this modification of the hydrolysis procedure significantly reduced the amount of degradation. The reason for the variation in the extent of degradation in samples 2–6 has not been investigated further.

These degradation products must be formed from the four methyl sugars in the methylated polysaccharide. This is a necessary conclusion since the correspondence between the theoretical and observed analytical values for the polysaccharide at all stages of methylation (Table II) indicates that the polymer contains no noncarbohydrate moieties. To assess the relative contribution of each of the isolated methyl sugars to the degradation products, the purified methyl sugars were subjected to the same hydrolysis conditions used for the polysaccharide and the recovery of total carbohydrate was measured using the phenol–sulfuric acid method. The data are given in Table V. All four methyl sugars are partially destroyed but the extent of destruction does not vary markedly from one sugar to the next. Thus, it appears that all sugars are recovered in sufficiently high yields after acid hydrolysis so that their ratios accurately reflect their relative proportions in the intact methylated polysaccharide.

Volatility of Tetramethylglucose. The possibility of preferential loss of the more highly methylated sugars due to volatilization during work-up of the hydrolysates was examined by measuring the recovery of pure [^{14}C]-tetramethylglucose when concentrated under conditions similar to those used in this work. When labeled tetramethylglucose was dissolved in ethanol and evaporated *in vacuo* (using a water aspirator) at 50° on a rotatory evaporator, 1.8 and 3.0% of the label was lost after 3 and 4 hr, respectively. At 40°, 100% of the label was recovered after 4 hr. Since in this work all samples were evaporated at 35° for periods no longer than 1 hr, it was concluded that neither tetramethylglucose nor any of the less volatile sugars in these mixtures was lost by volatilization.

TABLE V: Effect of Conditions Used for Hydrolysis of Fully Methylated Polysaccharide on Recovery of Methyl Sugars.[a]

Methyl Sugar	Glucose Equivalents[b] (mg/ml) Initial	After Hydrolysis	Recovery (%)
Fully methylated neutral polysaccharide	13.0	11.6	90
2,3,4,6-Tetra-O-methyl-D-glucose	21.0	18.9	90
2,3,6-Tri-O-methyl-D-glucose	24.4	22.4	92
2,4-Di-O-methyl-L-fucose	17.1	16.6	97
2,3-Di-O-methyl-D-glucose	12.4	12.0	97

[a] Methyl sugars were dissolved in 72% H_2SO_4 at 0° and stirred at room temperature for 1 hr followed by dilution to 8.0% H_2SO_4 and heating for 4 hr at 100°. [b] Measured by the phenol–sulfuric acid method using D-glucose as standard. Initial and final values both calculated on basis of final volume. Methyl sugars give same color yield on a molar basis as their parent sugar.

Discussion

Methylation analysis has been widely applied in the elucidation of the types of linkages present in complex polysaccharides. For determination of the complete structure of a heteropolysaccharide this method is a necessary supplement to those which establish the monosaccharide sequence. The value of methylation analysis has been limited by a number of experimental difficulties (Bouveng and Lindberg, 1960; Wallenfels *et al.*, 1963) which may be summarized as follows: (1) complete methylation of all uncombined hydroxyls requires multiple reaction steps, often by more than one method; (2) destruction and demethylation of the different methyl sugars during hydrolysis of the methylated polysaccharide occurs at rates which may not be identical and which are difficult to quantitate; (3) the more highly methylated fragments may not be recovered quantitatively due to their loss by volatilization during work-up and analysis of the hydrolysate; and (4) precise determination (±0.1%) of per cent methoxyl is difficult and consumes *ca.* 20 mg of sample. With acidic polysaccharides the problem is further complicated by an unusual difficulty in obtaining complete methylation and by the resistance to hydrolysis of uronic acid glycosidic bonds. Thus, hydrolysis requires more drastic conditions with increased degradation and demethylation of the monose fragments. Reduction of the uronic acid moieties prior to hydrolysis has been used to circumvent the latter difficulty (Abdel-Akher and Smith, 1950; Aspinall *et al.*, 1956a) but leaves unanswered the question of which methyl sugars are derived from glucuronic acid (or galacturonic acid) when both glucose and glucuronic acid (or galactose and galacturonic acid) are present in the original polysaccharide.

Classical methylation methods have relied upon the initial conversion of the hydroxyl groups to alkoxides

by reacting the polysaccharide with base in aqueous solution. The added methylating reagent then reacts with the alkoxides to yield methyl ethers. Any free base in the reaction mixture competes with the alkoxide for the alkylating reagent. In the initial reaction, an

$$ROH + B^- \rightleftharpoons RO^- + BH$$

equilibrium is established at a point dependent on the strength and concentration of the base, B^-. Complete conversion to the alkoxide requires a base stronger than OH^-. However, the strongest base that can exist in aqueous solution is the OH^- ion since stronger bases will react with water to form this ion. Thus, in the standard methylation procedures the extent of ether formation is influenced by the point of equilibrium in the base-catalyzed reaction which in turn is limited by the base strength of OH^-. In the method applied here these limitations are overcome by use of the strongly basic methylsulfinyl carbanion (Corey and Chaykovsky, 1962) in non aqueous medium to generate the polysaccharide alkoxide. It is apparent that the equilibrium in this reaction lies almost completely in the direction of alkoxide formation since, upon addition of the alkylating reagent, formation of the methyl ether is complete within a few minutes. The competing reaction between the excess methylsulfinyl anion and methyl iodide does not interfere as long as methyl iodide is added in excess of total base. Generation of the alkoxide requires *ca.* 4 hr under our conditions.

The stoichiometric methylation reaction requires complete solution of the polysaccharide prior to addition of the methylsulfinyl anion, a condition which is greatly facilitated by lyophilization and sieving of the polysaccharide before attempting to dissolve it in dimethyl sulfoxide. The uronic acid moieties, esterified in the methylation reaction, are readily reduced with lithium aluminum hydride. The methylated and reduced product may be hydrolyzed directly and analyzed for methyl sugars or remethylated prior to hydrolysis. Comparison of the mixtures of methyl sugars obtained before and after remethylation allows ready identification of the methyl sugars derived from glucuronic acid.

The development of a single step methylation reaction makes feasible the use of [^{14}C]methyl iodide as the alkylating reagent. The specific activity of the labeled methyl iodide has been used in calculating the per cent methoxyl in the intact methylated polysaccharide as well as in the methyl sugars obtained on hydrolysis. The accuracy of this method for methoxyl determination is limited only by the accuracy with which the sample weight can be determined since the sample counting rate as well as the specific activity of the [^{14}C]-methyl iodide can be readily measured to $\pm 1\%$. The method thus offers the advantage of high accuracy as well as speed and simplicity.

One of the problems usually associated with methylation analysis is the unknown extent and nature of the degradation of methyl sugars during hydrolysis of a fully methylated polysaccharide (Croon *et al.*, 1960). Obviously, if degradation involves the loss of methyl groups with the formation of the corresponding sugars with fewer substitutions, the ratio of methyl sugars recovered may be altered in an indeterminant way, especially if the rates of demethylation vary for the different sugars. Our results confirm that under hydrolysis conditions shown to minimize degradation (Whistler, 1965) there is a loss of methoxyl content during hydrolysis. There is, however, a concomitant decrease in total carbohydrate. The latter is not consistent with simple demethylation without destruction of the sugars since in the phenol–sulfuric acid test used here (Dubois *et al.*, 1956) methyl sugars give the same molar color yield as their parent sugar. The major fraction of the hydrolysis degradation products emerges from the cellulose column ahead of tetramethylglucose and, by the ^{14}C method, has a methoxyl content of 7.9%. The data show that this fraction is a mixture of non-carbohydrate components. That they are derived from the known carbohydrate components of the polysaccharide rather than from some unidentified moiety is established by the close agreement between the analytical values (C, H, OCH_3) observed and those calculated for a polysaccharide composed solely of glucose, glucuronic acid, and fucose in a ratio of 2:1:1 (Table II). Throughout this work there has been no indication of demethylation without further degradation of the sugars. Control experiments show that the rates of destruction of each of the methyl sugars are very similar. Since it has also been demonstrated that there was no loss of methyl sugars due to volatilization, the ratios of undegraded methyl sugars recovered from the hydrolysates accurately reflect the ratios in the intact polysaccharide. The question of whether there is degradation of the polysaccharide under the methylation conditions described here has not been examined.

In many methylation studies the errors due to experimental difficulties appear to have been compounded to such an extent that the amounts and kinds of methyl sugars recovered in an analysis deviate significantly from what would be present in the fully methylated polysaccharide. This point is amply demonstrated by comparison of our results with those of Aspinall *et al.* (1956b) in which the same polysaccharide was examined. In the latter work the polysaccharide was methylated four times by the method of Fear and Menzies (1926) to obtain a product containing 39.6% methoxyl in a 60% yield. Recovery on hydrolysis was 74% (on a weight basis) and on column chromatography, 67%. Thus, the over-all yield of methyl sugars was 30%. The sharpest contrast between the present results and those of Aspinall *et al.* is in the distribution of recovered methyl sugars. In the earlier work the total methylglucose fraction recovered represented only 17% of the hydrolyzed sample applied to the column and was composed of varying amounts of 10 different glucose derivatives. The fucose was recovered in three fractions [2,4-di-O-methyl-L-fucose (Gardiner and Percival, 1958), 2-O-methyl-L-fucose, and free fucose] which together represented 9% of the material applied to the column. The acid fraction (47% of the hydrolyzed polysaccharide) apparently contained methyl

derivatives of glucuronic acid and unidentified oligosaccharides, and was refractory to further analysis. There was no correspondence between the molar amounts of branching residues (methyl sugars containing two or more free hydroxyls) and nonreducing end residues (fully methylated sugars).

In the work reported here the total recovery of the four methyl sugars from the chromatographic column was *ca.* 80% of theoretical based on the weight of original polysaccharide before methylation. Recovery of equimolar amounts of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose (derived from 2,3-di-O-methyl-D-glucuronic acid), 2,4-di-O-methyl-L-fucose, and 2,3-di-O-methyl-D-glucose indicates that: (1) all of the glucuronic acid is linked through C-4; (2) all of the fucose is linked through C-3; (3) half of the glucose is at a branch point linked through both C-4 and C-6; and (4) the remaining glucose is at a nonreducing end of the polysaccharide. The high recoveries assure that the molar ratios of methyl sugars recovered accurately reflect their proportion in the fully methylated polymer. These results show that the *A. aerogenes* A3(Sl) polysaccharide has a much simpler structure than indicated by the earlier work and that its structure is inalterable with respect to the proportion of each type of linkage when the organism synthesizes it under different limiting growth conditions. The constancy of its structure also shows that only one extracellular polysaccharide is produced by the organism (although homodispersity has not been established).

The data of Aspinall *et al.* (1956b) were obtained using standard techniques of polysaccharide chemistry and are typical of many literature reports on polysaccharide structure. The general conclusions drawn from such studies have been that heteropolysaccharide structures are extremely complex. Our data suggest that a more valid conclusion is that standard techniques are subject to errors of considerable magnitude and that structures of heteropolysaccharides may be much simpler than heretofore realized.

The constant physical and chemical properties of a polysaccharide produced by a given species indicate that the structures are genetically regulated and that there is a high degree of specificity in their biosynthesis. Such specificity, on the basis of current information (Weiner *et al.*, 1965; Wright *et al.*, 1965), would appear most reasonably to arise through a group of enzymes, each one specific for the formation of a definite linkage in the polymer. Thus, the greater the number of unique linkages, the larger the proportion of its enzymatic machinery the cell must devote to the synthesis of its heteropolysaccharide. At the present time there is no evidence for the alternate possibility that the specificity arises through a template mechanism. Therefore, studies which indicate that a given heteropolysaccharide has a very complex but genetically controlled monosaccharide sequence suggest as a corollary that a large number of enzymes are involved in the biosynthesis. While this corollary may turn out to be correct, it is difficult to understand the survival of such an extensive biosynthetic apparatus when, as in the case of many heteropolysaccharides, there is no apparent advantage accruing to the organism from the presence of the final product (Wilkinson, 1958). Thus, the demonstration of the relative simplicity of the A3(Sl) polysaccharide has important implications with reference to the biosynthesis of heteropolysaccharides. The data suggest that these polymers may in fact have considerably less complex structures than many reports have indicated and that, as improved methodology permits a more accurate aparaisal of polysaccharide structure, the need for extensive or novel mechanisms to describe heteropolysaccharide biosynthesis may be lessened.

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Nucleotides in the Encysted Embryos of *Daphnia magna**

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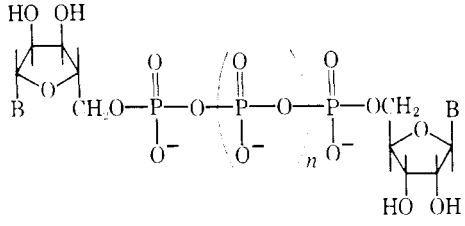
ABSTRACT: The encysted embryos of *Daphnia magna* contain guanosine-5′ phosphate, guanosine-5′ diphosphate, P^1,P^3-diguanosine-5′ triphosphate, and P^1,P^4-diguanosine-5′ tetraphosphate. Ion-exchange chromatography on diethylaminoethylcellulose provides a very satisfactory method for separation of these nucleotides.

The α,ω-dinucleoside-5′ polyphosphates (I) are a class of compounds which have recently attracted attention. P^1,P^2-Adenosine-5′ tetraphosphate (I, B =



I

adenine, $n = 2$) was suggested as an intermediate in the reaction of adenosine-5′ diphosphate with dicyclohexylcarbodiimide (Smith and Khorana, 1958), and this nucleotide together with its homologs are produced in dismutation reactions of adenosine-5′ polyphosphates (Verheyden *et al.*, 1965; Wehrli and Moffatt, 1965). General procedures for synthesis of α,ω-nucleoside-5′ polyphosphates have been devised (Reiss and Moffatt, 1965). The accolade of natural occurrence followed from the demonstration of P^1,P^3-diguanosine-5′ triphosphate (I, B = guanine, $n = 1$) and P^1,P^4-diguanosine-5′ tetraphosphate (I, B = guanine, $n = 2$) as major constituents of the encysted embryos of the brine shrimp, *Artemia salina* (Finamore and Warner, 1963; Warner and Finamore, 1965a).

The present study was directed at the nucleotides of the encysted embryos of *Daphnia magna*. Both P^1,P^2-diguanosine-5′ triphosphate and P^1,P^4-diguanosine-5′ tetraphosphate were present but in amounts differing from those found in *A. salina*. Ion-exchange chromatography on diethylaminoethylcellulose, using ammonium bicarbonate as eluting agent, provided a convenient method for isolation of α,ω-diguanosine-5′ polyphosphates. This procedure was also applicable to the isolation of these nucleotides from encysted embryos of *D. magna* and *A. salina*.

Experimental Methods and Results

Analytical Methods. Paper chromatography was carried out on Whatman 40 paper, using descending technique, in solvent 1, isobutyric acid–concentrated ammonia–water (66:1:33); solvent 2, isobutyric acid–1 M ammonia–0.1 M sodium ethylenediaminetetraacetate (100:60:1.6); solvent 3, 1 M ammonium acetate, pH 3.8–ethanol (7:3). Chromatography using ascending technique was carried out in solvent 4, 0.1 M sodium phosphate, pH 6.8–solid ammonium sulfate–1-propanol (100:60:2). Authentic nucleotides were always chromatographed alongside unknowns. Nucleotides were detected by viewing under an ultraviolet light, and mobilities are recorded in Table I. Ribose was determined by the orcinol method (Mejbaum, 1939). Total phosphate was determined after digestion in 70% perchloric acid and labile phosphate after hydrolysis in 1 M hydrochloric acid (7 min at 100°) using King's procedure (1932). Guanosine was determined from its ultraviolet absorption using a Beckman DU spectrophotometer assuming a molar extinction coefficient of 13,700 at 252 mμ at pH 7.0.

The hydrolysis of nucleotides by snake venom diesterase (Worthington) was carried out using published procedures (Razzell, 1963; Finamore and Warner, 1963), the products being examined chromatographically after removal of magnesium by treatment with a cation exchanger (Dowex 50) in the ammonium form.

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