The Structure of the *Aerobacter aerogenes* A3(S1) Polysaccharide. II. Sequence Analysis and Hydrolysis Studies*

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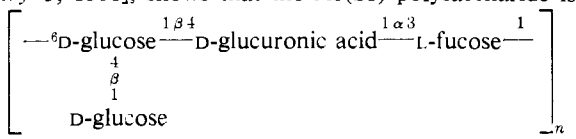
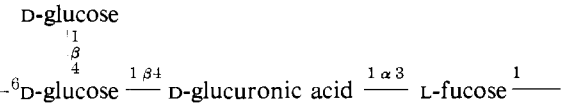
ABSTRACT: Quantitative radiochemical methods for determination of reducing equivalents and for radiochromatographic analysis of oligosaccharide mixtures have been developed for use in structural studies of the slime polysaccharide from *Aerobacter aerogenes*, A 3(S1). Partial acid hydrolysis of the polysaccharide yields a single neutral disaccharide, identified as cellobiose, and three glucuronic acid containing oligosaccharides—a di-, a tri-, and a tetrasaccharide—each of which has L-fucose at the reducing terminal. Sequence analysis, taken with the previous methylation data [Sandford, P. A., and Conrad, H. E. (1966), *Biochemistry 5*, 1508], shows that the A3(S1) polysaccharide is made up of repeating sequences of the tetrasaccharide shown. The acidic tri- and disaccharides are derived from this tetrasaccharide by the hydrolytic removal of one and two D-glucose residues, respectively. Assignments of the anomeric configurations are based on susceptibility of the oligosaccharides to cleavage by specific enzymes and on proton magnetic resonance data. On periodate oxidation the polysaccharide consumes 4 moles of periodate with the formation of 1 mole of formic acid for each anhydrotetrasaccharide unit, as expected from the proposed structure.

The first-order rate constant for hydrolysis of the L-fucosyl bond of the polysaccharide in 1 N sulfuric acid at 100° is 300-fold greater than that for the D-glucuronosyl bond and 10- to 20-fold greater than those for the D-glucosyl bonds. The relationship between the complexity of polysaccharide structures and the possible mechanisms for their biosynthesis is discussed.



The concept that the complex heteropolysaccharides are made up of repeating oligosaccharide sequences is widely accepted (Barker *et al.*, 1958a,b; How *et al.*, 1964) but has received experimental documentation in only a few instances (Staub and Tinelli, 1960; Robbins and Uchida, 1962; Osborn *et al.*, 1964; Jann *et al.*, 1965; Lüderitz and Westphal, 1966). For a large number of plant gums and mucilages (Smith and Montgomery, 1959; Whistler, 1959) and microbial polysaccharides (Stacey and Barker, 1960; How *et al.*, 1964) the available data contradict the repeating unit concept. An earlier report by Aspinall *et al.* (1956) indicated that the acidic slime polysaccharide from *Aerobacter aerogenes*, A3(S1), which is composed of D-glucose, D-glucuronic acid, and L-fucose, is a very complex nonrepeating structure. A reexamination of this polysaccharide using improved methylation methodology (Sandford and Conrad, 1966) showed that the D-glucose, D-glucuronic acid, and L-fucose are present in a simple molar ratio of 2:1:1, respectively, and that all of the D-glucuronic acid is linked through C-4, all of the L-fucose is linked through C-3, one-half of the D-glucose is at branch points linked through both C-4 and C-6, and the remaining D-glucose is at nonreducing ends of the polysaccharide. This paper describes a sequence analysis of the A3(S1) polysaccharide based on a study of the oligosaccharides obtained on partial hydrolysis. The data show that the polysaccharide has the following simple tetrasaccharide repeating unit.



Periodate oxidation of the polysaccharide gives data which are consistent with this structural assignment.

A detailed study of the hydrolysis of the polysaccharide has been carried out using a newly developed reducing value method based on incorporation of tritium when aliquots taken during hydrolysis are reduced with sodium [³H]borohydride. This method gives identical molar responses with all reducing groups regardless of their mode of combination. Rate constants for hydrolysis of the linkages in the polysaccharide vary over a 300-fold range under the hydrolysis conditions used. Radiochromatography of [³H]-borohydride-reduced aliquots taken at intervals during

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hydrolysis of the polysaccharide permits quantitative determination of the amount of each oligosaccharide present as hydrolysis progresses and shows a precursor–product relationship among the oligosaccharides which is consistent with the assigned structure.

Methods

Polysaccharide from *A. aerogenes* A3(S1) (ATCC 12658) was isolated and purified as described previously (Sandford and Conrad, 1966; Tempest, 1965). Hydrolyses were carried out at 100° with 2% solutions of polysaccharide in 1 N sulfuric acid. Hydrolysates were examined by descending paper chromatography on Whatman No. 1 paper using the following solvents: (A) ethyl acetate–glacial acetic acid–formic acid–water (18:3:1:4), and (B) ethyl acetate–pyridine–water (8:2:1). Reducing substances on chromatograms were detected with aniline acid phthalate spray reagent (Partridge, 1949). Total carbohydrate was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956) and expressed as glucose equivalents. D-Glucose was assayed by the glucostat method (Worthington Biochemical Corp., Freehold, N. J). Proton magnetic resonance spectra were run on 30% solutions (w/v) in deuterium oxide after replacing exchangeable hydrogens by evaporating samples to dryness several times from deuterium oxide to eliminate the HDO resonance line. Tetramethylsilane was used as the external standard (τ 10). Spectra were run at 60 Mc.

Fucose Determination. Fucose was measured by the piperazine–nitroprusside reaction with the acetaldehyde released by periodate oxidation of fucose-containing samples (Desnuelle and Naudet, 1945; Edward and Waldron, 1952). Aliquots containing 80–800 μg of fucose (yielding 0.5–5 μmoles of acetaldehyde) in 0.1 ml of 1 N sulfuric acid were diluted to 2.5 ml with water and oxidized at room temperature in parafilm-sealed tubes with 0.5 ml of 0.2 N periodic acid for 16 hr. Free L-fucose gave quantitative yields of acetaldehyde by the end of the first hour of oxidation, but the oligosaccharides with fucose-reducing terminals required a much longer period of oxidation for quantitative release of acetaldehyde. For the 16-hr oxidation it was essential to have the tubes tightly sealed with parafilm to prevent loss of acetaldehyde by volatilization. The acetaldehyde in the oxidized samples was determined by measuring the maximum absorbancy attained at 570 mμ in the reaction tube upon rapid and simultaneous addition of 0.75 ml of piperazine reagent (25 g of piperazine hexahydrate plus 1.3 ml of 87% formic acid made up 100 ml in water) and 0.25 ml of freshly prepared 4% aqueous sodium nitroprusside. The blue color develops rapidly, reaching a maximum at approximately 1 min, then fades equally rapidly. The maximum absorbancy reached under these conditions is a linear function of the amount of acetaldehyde present in the sample. Values obtained for unknowns were converted to fucose equivalents using a standard curve obtained by treating L-fucose in an identical manner. Since all fucose in the A3(S1) polysaccharide is linked through C-3, all oligosaccharides derived from the polysaccharide which have fucose at the reducing terminal will yield acetaldehyde on periodate oxidation.

Reducing Value Measurements. The desirability of a reducing value method which gives an identical molar response with all reducing carbohydrates led to our development of a procedure which utilizes low specific activity sodium [^{3}H]borohydride (576 μc/mmole, New England Nuclear Corp.; specific activity determined by counting an aliquot of pentan-3-ol formed by [^{3}H]borohydride reduction of pentan-3-one and purified by preparative vapor phase chromatography). The method depends on the stoichiometric reduction of all reducing groups to the corresponding alcohol with the incorporation of one atom of hydrogen from the labeled borohydride into the product. After destruction of the excess borohydride, the tritium which remains in the reduced sample is a measure of reducing equivalents originally present.

Since the desired measurements were to be made on 0.1-ml aliquots from hydrolysates of 2% polysaccharide solutions in 1 N sulfuric acid, all standards were prepared as 2% solutions in 1 N sulfuric acid. For assay 0.1-ml aliquots containing from 1.0 to 10 μequiv of reducing groups were placed in 1.0-ml volumetric tubes. To the aliquots were added in succession 0.12 ml of 1 N sodium hydroxide, 0.10 ml of 0.5 M sodium [^{3}H]borohydride (19 mg/ml in 0.1 N sodium hydroxide), and 0.10 ml of 0.1 M boric acid (618 mg/100 ml of water). The reduction was carried out at 50° in a water bath for 1.5 hr. Samples were then removed from the path and excess borohydride was destroyed by cautious addition of 0.1 ml of 1 N sulfuric acid. Vigorous evolution of hydrogen gas at this stage assured that the borohydride was in excess during the entire reduction period. After effervescence ceased (5–10 min) the flasks were made to volume with water and stoppered, and the contents was mixed by inversion. Using disposable pipets (Falcon Plastics, Los Angeles, Calif.), 0.5 ml of each solution was transferred to a porcelain spot plate, 1 drop of 1% phenolphthalein in ethanol was added, and the aliquots were neutralized to the acid side of phenolphthalein with 0.1 N solutions of sodium hydroxide and sulfuric acid. The samples were then evaporated to dryness in a forced draft oven at 110° for 60 min. This step was necessary to remove the last traces of dissolved hydrogen gas which otherwise give high counting rates even in the absence of reducing substances. The dried samples were redissolved on the cooled spot plate in 1.0 ml of water and 0.5 ml of this solution was transferred to a scintillation bottle prepared for suspension counting (Blanchard and Takahashi, 1961) as follows. The counting bottle was filled to three-fourths its volume with Cab-O-Sil (Cabot Corp.). Then 15 ml of a scintillation fluid prepared by mixing 5.6 g of 2,5-diphenyloxazole, 70 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene, 300 ml of absolute ethanol, and 400 ml of toluene was added and the sample was introduced. Samples were counted in a Packard

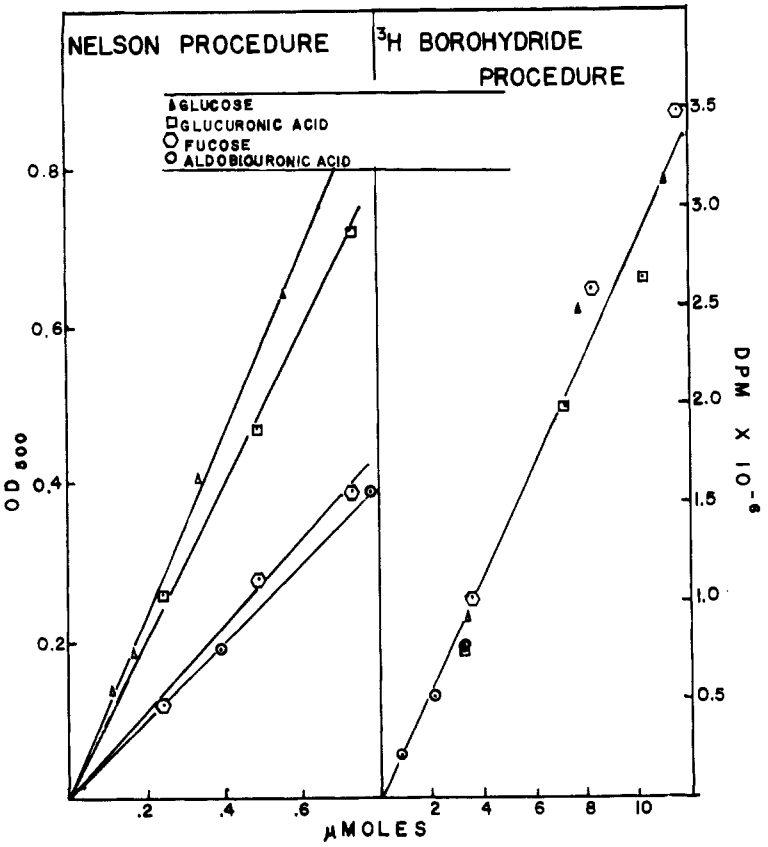

FIGURE 1: Comparison of Nelson (1944) and [^{3}H]borohydride procedures for determination of reducing groups.

Model 3000 liquid scintillation spectrometer equipped for external standardization. Counting efficiencies were determined from a standard curve obtained by calibrating the counting rate of the external standard against a series of quenched [^{3}H]toluene standards, and all counting rates were converted to disintegrations per minute for use in calculations. Blanks containing no reducing sugar were run simultaneously with the samples and sample counts were corrected for residual tritium in the blanks.

The linearity of molar response using this reducing value method is compared with that obtained using the Somogyi–Nelson method (Somogyi, 1952; Nelson, 1944) in Figure 1. This figure shows the wide variation in the amount of Cu^{2+} reduced by equimolar amounts of different sugars in the Somogyi–Nelson procedure. In the [^{3}H]borohydride method the molar response of these sugars is identical. The constancy of molar response for a number of standards analyzed by this method is shown in Table I.

Radiochromatographic Determination of Oligosaccharide Accumulation during Hydrolysis. Aliquots (0.1 ml) were taken at intervals during hydrolysis of the polysaccharide and reduced with high specific activity sodium [^{3}H]borohydride (11.8 mc/mmole, New England Nuclear Corp., Boston, Mass.) as described in the analytical procedure above. After destroying excess borohydride and making the samples to 1.0 ml, four 5-μl aliquots were spotted successively at the starting line of a 1-in. wide strip of Whatman No. 1 chromatographic paper. Chromatograms were developed in solvent A for 20 hr, dried, and cut into 0.5-in. segments which were counted in the Packard scintillation counter using a scintillation fluid containing 8 g of 2,5-di-

TABLE I: Molar Response in the [^{3}H]Borohydride Method[a] for Determination of Reducing Sugars.

Reducing Sugar	Dpm/μmole × 10^{-5}	Reducing Sugar	Dpm/μmole × 10^{-5}
Cellobiose	2.90	D-Glucuronic acid	2.92
2-Deoxy-D-ribose	2.95	Lactose	2.84
D-Fructose	3.36	D-Mannose	2.73
L-Fucose	2.92	Melibiose	2.60
D-Glucosamine	2.87	Glucuronosyl-fucose	2.73
D-Glucose	2.95		

[a] Analyses were carried out as described in Methods using sodium [^{3}H]borohydride having a specific activity of 576 μc/mmole.

phenyloxazole and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 l. of toluene. Counting efficiencies were determined by reducing, chromatographing, and counting a standard glucose sample in an identical manner. Counting rates for each segment were converted to micromoles of reducing compound by comparison with the counts per minute per micromole of glucose obtained from the standard glucose chromatogram. Alternatively, the value for micromoles of glucose per milliliter was determined independently for each aliquot using the glucostat procedure and all counting rates were converted to micromoles of reducing compound per milliliter by multiplying by the factor required for converting the glucitol counting rate to micromoles of glucose per milliliter. Both procedures are dependent upon the assumption that counting efficiencies for tritium are the same for all paper segments counted.

Isolation of Oligosaccharides. Oligosaccharides were formed by hydrolysis of 2% solutions of purified polysaccharide in 1 N sulfuric acid at 100°. Hydrolysis was stopped at different times according to the maximum accumulation of the desired oligosaccharide (see Figure 3). Hydrolysates were neutralized with barium hydroxide and filtered on a Büchner funnel coated with Celite 545 (Johns-Manville Co.). The filter cake was washed several times with water and then filtrate and washings were combined and concentrated *in vacuo* to a thin syrup. The mixture was introduced at pH 8 onto a column (3 × 450 cm) of Dowex 1 resin (X2, 200–400 mesh) prepared in the acetate form. The column was washed exhaustively with water to remove the neutral fraction and then the total acid fraction was eluted with 4 N acetic acid. Eluates were concentrated *in vacuo* at 40° and the components of each mixture were separated by cellulose column chromatography (Hough *et al.*, 1949). For separation of the acid fraction solvent A was used; for the neutrals, solvent B.

Periodate Oxidation. Polysaccharide was oxidized in 0.1 M sodium acetate buffer, pH 3.9, at 5° in the dark to minimize overoxidation (Bobbitt, 1956). The oxidation mixture contained 2 g of polysaccharide and 25 mmoles of sodium metaperiodate in 500 ml of buffer. A control periodate solution from which polysaccharide was omitted was run simultaneously under identical conditions. Appropriate aliquots were withdrawn at intervals and analyzed for periodate consumption and formic acid production. Periodate was determined by iodometric titration (Malaprade, 1928; Kolthoff and Sandell, 1943). Formic acid was quantitated by titration with 0.05 N sodium hydroxide to a phenolphthalein end point. The zero time titration was subtracted from all subsequent values to correct for the buffer and the uronic acid residues in the polysaccharide.

Results

Oligosaccharide Accumulation. The oligosaccharide intermediates formed from the A3(S1) polysaccharide after 2.5- and 10-hr hydrolysis periods are illustrated in Figure 2. The mixture of oligosaccharides observed in the 2.5-hr sample is completely hydrolyzed to glucose and aldobiouronic acid after 10 hr. Only small amounts of free fucose and glucuronic acid, each of which represents one-fourth of the total monosaccharide content of the polysaccharide, are obtained and more than 40% of the weight of the original polysaccharide can be recovered as aldobiouronic acid even after 10 hr of hydrolysis.

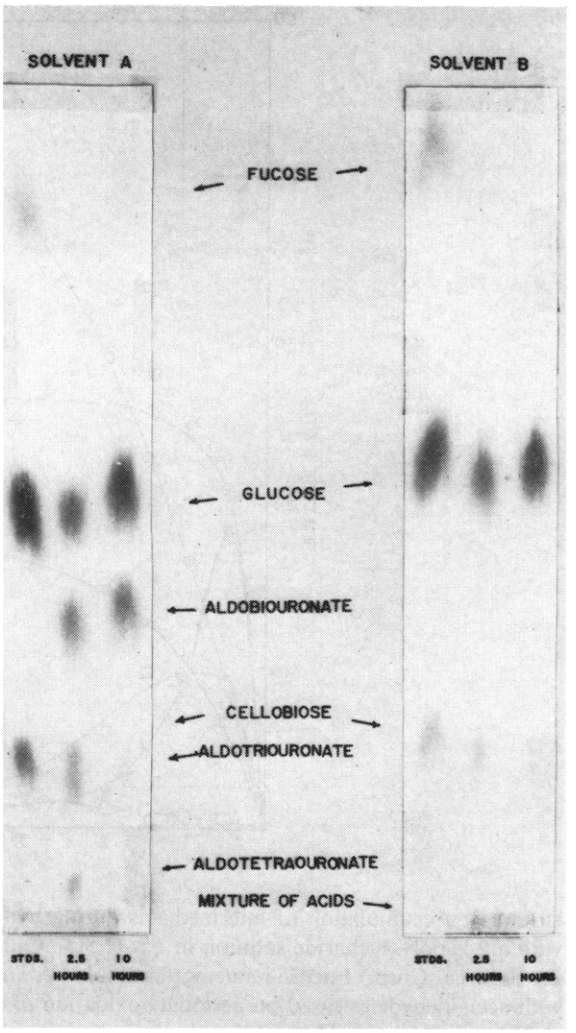

FIGURE 2: Paper chromatography of A3(S1) polysaccharide hydrolysates. Solvent A: ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Solvent B: ethyl acetate–pyridine–water (8:2:1).

The accumulation of oligosaccharide intermediates during hydrolysis is illustrated in Figure 3. The concentration of each fragment at the intervals analyzed was determined by radiochromatography (see Methods) of [^{3}H]borohydride-reduced aliquots using solvent A. The acidic trisaccharide and the neutral disaccharide are measured together since they are poorly separated in this solvent (Figure 2). The rate of cleavage of the

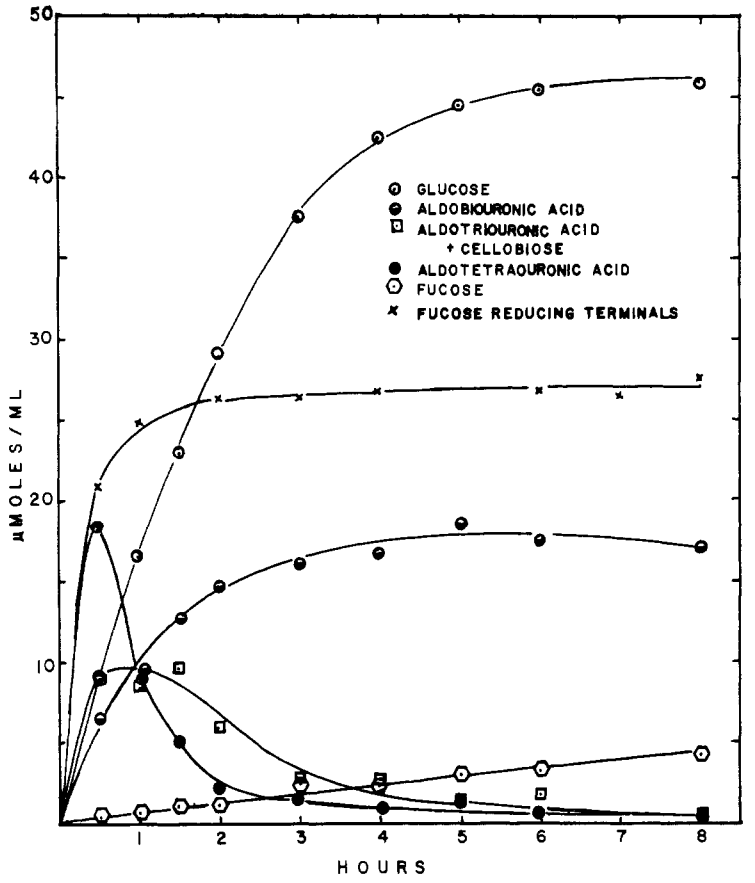

FIGURE 3: Accumulation of intermediates during hydrolysis of A3(S1) polysaccharide. Hydrolysis was carried out with a 2% polysaccharide solution in 1 N H_2SO_4. Glucose was determined using the glucostat reagent (Worthington Biochemical Corp.) Fucose-reducing terminals were measured by the colorimetric piperazine–nitroprusside reaction with acetaldehyde released on periodate oxidation of aliquots. All other values were obtained by quantitative radiochromatography (see Methods).

fucosyl bond of the polysaccharide, determined by measuring the total amount of acetaldehyde formed upon periodate oxidation of each aliquot, is also illustrated in Figure 3. These values are used in calculation of the rate constant for hydrolysis of the fucosyl bond as described below. By stopping large-scale hydrolyses at appropriate times it was possible to maximize yields and minimize difficulties involved in separation of the oligosaccharides desired for sequence studies.

Determination of Oligosaccharide Structures. Since the methylation analysis reported previously (Sandford and Conrad, 1966) showed clearly that D-glucose, D-glucuronic acid, and L-fucose are present in the polysaccharide in a ratio of 2:1:1, and that all of the L-fucose is linked through C-3, all of the D-glucuronic acid through C-4, one-half of the D-glucose through both C-4 and C-6, and the remaining D-glucose at nonreducing terminals, the complete structure of the A3(S1) polysaccharide requires only the establishment of the sequence of these sugars in the polymer and the anomeric configurations.

The aldobiouronic acid was shown to be 3-*O*-α-D-glucopyranosyluronic acid-L-fucose by the following observations. The pure material was esterified with methanolic hydrogen chloride and then reduced with sodium borohydride (Whistler and Wolfrom, 1963) to the methyl glycoside of a neutral disaccharide. On hydrolysis the neutral disaccharide yielded only glucose and fucose, identified by chromatography in solvents A and B. Analysis of the aldobiouronic acid for fucose by the periodate–piperazine–nitroprusside method gave 1 mole of acetaldehyde/mole of aldobiouronic acid, indicating that fucose is at the reducing end of the disaccharide and that the linkage of D-glucuronic acid to L-fucose is not through C-4 or C-5 of the fucose. Since methylation analysis showed that all of the L-fucose in the polysaccharide is linked through C-3, it is concluded that the aldobiouronic acid is a 3-*O*-D-glucopyranosyluronic acid-L-fucose. This was confirmed by methylation with dimethyl sulfate and sodium hydroxide followed by reduction with lithium aluminum hydride. Hydrolysis of the reduced material yielded 2,3,4-tri-*O*-methyl-D-glucose, identified by paper chromatography and by preparation of the anilide, mp 135°, and 2,4-di-*O*-methyl-L-fucose,

mp 131°. The anomeric configuration was shown to be α as follows. (1) The aldobiouronic acid was not cleaved by a preparation of β-glucuronidase which was active against phenolphthalein-β-glucuronidate; and (2) the proton magnetic resonance spectrum of the aldobiouronic acid showed 1.5 anomeric protons at τ = 4.17 ppm and 0.5 anomeric proton at τ = 4.9 ppm. After reduction of the terminal L-fucose with sodium borohydride, the 4.9 signal disappeared and a single proton remained at τ = 4.17 ppm. The latter signal is assigned to an anomeric proton at an α-glycosidic linkage by comparison with the spectra of borohydride-reduced cellobiose and maltose (van der Veen, 1963).

The molar content of D-glucose in each of the other oligosaccharides was determined by the glucostat procedure following acid or β-glucosidase cleavage. These data are recorded in Table II. The *neutral di-*

TABLE II: Glucose Content of A3(S1) Oligosaccharides.

Oligosaccharide	Moles of D-Glucose[a] Released/Mole of Oligosaccharide: β-Glucosidase	H_2SO_4 (N)
Aldotriouronic acid	0	1.0
Aldotetraouronic acid	0.8	2.1
Reduced aldotetraouronic acid	0.8	1.9
Cellobiose	1.8	—

[a] Determined using glucostat reagent (Worthington Chemical Corp., Freehold, N. J.)

saccharide was converted quantitatively to D-glucose by β-glucosidase at a rate equal to that obtained with authentic cellobiose. On paper chromatograms the disaccharide migrated at the same rate as cellobiose under conditions which separate cellobiose from gentiobiose (the only other β-linked glucose–glucose disaccharide consistent with the methylation data). The identity of the crystalline product as cellobiose was confirmed on the basis of mp 223° (lit. 225°) and $[\alpha]_D^{20}$ +34° (*c* 2, water) (lit. +35°). These data are in agreement with those presented previously by Aspinall *et al.* (1956).

The *aldotriouronic* and *aldotetraouronic acids* both yield the glucuronosylfucose on acid hydrolysis. In addition they yield 1 and 2 moles of D-glucose, respectively (see Table II). Partial acid hydrolysis of the aldotetraouronic acid gives cellobiose as an intermediate, indicating that its two D-glucose residues are joined through a β-1,4 linkage. On treatment with β-glucosidase the aldotetraouronic acid is converted

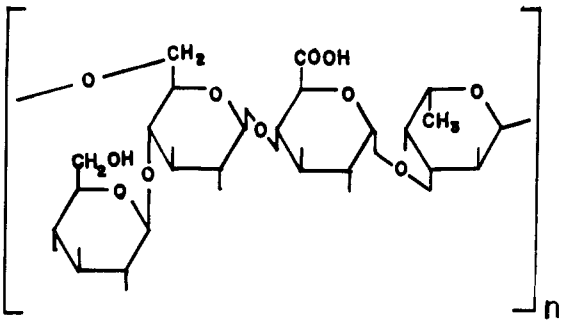

FIGURE 4: The repeating sequence of the *A. aerogenes* A3(S1) polysaccharide.

quantitatively to D-glucose and the aldotriouronic acid. L-Fucose is the reducing terminal of both oligosaccharides as indicated (1) by stoichiometric yields of acetaldehyde on periodate oxidation, and (2) by recovery of quantitative yields of D-glucose (Table II) and reduced glucuronosylfucose from the borohydride-reduced oligosaccharides. The methylation data show that L-fucose is not a branch point in the polysaccharide and that all of the D-glucuronic acid is linked through C-4. Therefore, since all of the L-fucose in the polysaccharide is found as glucuronosylfucose, the cellobiose of the aldotetraouronic acid and the D-glucose of the aldotriouronic acid must be glycosidically linked to C-4 of the D-glucuronic acid residue of the glucuronosylfucose.

The anomeric configuration at the linkage between cellobiose and aldobiouronic acid is determined to be β by comparison of the proton magnetic resonance spectrum of the borohydride-reduced aldotetraouronic acid with those of reduced cellobiose and maltose. The latter show τ values at 4.2–4.3 ppm for a proton at an α linkage and 4.9 ppm for a proton at a β linkage. The tetrasaccharide has a single proton at 4.4 ppm, attributed to the glucuronosylfucose linkage, and 2.2 β protons at 5 ppm, which we attribute to the anomeric protons of the two D-glucose residues. The tetrasaccharide is therefore *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyluronic acid-(1→3)-L-fucose, while the trisaccharide is *O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyluronic acid-(1→3)-L-fucose. Since the oligosaccharides described here are the only ones found in A3(S1) hydrolysates, the sequence analysis taken together with the previous methylation studies indicate that the A3(S1) polysaccharide has the repeating structure shown in Figure 4.

Hydrolysis Studies. In the sequence analysis two striking features of the hydrolysis of this polysaccharide are noted: (1) the glucuronic acid–fucose bond is extremely stable under the hydrolytic conditions used; thus, very little free D-glucuronic acid or L-fucose are obtained on hydrolysis and the aldobiouronic acid can be recovered from hydrolysates almost quantitatively, and (2) L-fucose is at the reducing terminal

in all of the acidic oligosaccharides recovered. These two facts indicate a marked variance in the rate of cleavage of the different glycosidic bonds of the polymer, the glucuronic acid–fucose bond being quite stable while the fucose–glucose bond is extremely labile. Using the data presented in Figure 3 which show the rate of release of free D-glucose-, L-fucose, and fucosyl reducing terminals during hydrolysis it is possible to calculate first-order rate constants for hydrolysis of the individual bonds in the polysaccharide. The data are presented in Table III.

TABLE III: Rate Constants for Hydrolysis of Glycosidic Bonds in the A3(S1) Polysaccharide.

Bond Cleaved[a]	Rate Constant (sec^{-1}),[b] $\times 10^3$
F $\frac{1\ ?\ 6}{}$ G	1.3
G $\frac{1\ \beta\ 4}{}$ G	0.13
G $\frac{1\ \beta\ 4}{}$ GA	0.05
GA $\frac{1\ \alpha\ 3}{}$ F	0.004

[a] F = L-fucose, G = D-glucose, GA = D-glucuronic acid. [b] Hydrolysis in 1 N sulfuric acid at 100°. Values calculated using assumptions described in Discussion.

The value for the fucosyl bond was calculated directly from the data in Figure 3 using the final fucose-reducing terminal (acetaldehyde) value as C_0. Values determined for hydrolysis of cellobiose and aldobiouronic acid under identical hydrolysis conditions were assumed to be essentially equivalent to the constants for the same glycosidic bonds in the polysaccharide. The rate constant for the glucosyl–glucuronic acid bond was calculated from the rate of total glucose release (Figure 3) using data obtained after all of the fucosyl–glucose bonds had been cleaved (1 hr). At this time the rate of total glucose release was the sum of the rates for cleavage of the nonreducing glucosyl bond and glucosyl–glucuronic acid bond. The rate of glucose release from the nonreducing terminal, calculated from the cellobiose rate constant, was subtracted from the rate of total glucose release to obtain the rate of cleavage of the glucosyl–glucuronic acid bond. For this calculation C_0 was taken as one-half the final glucose value at the completion of hydrolysis (1 equiv of glucosyl–glucuronic acid bond/anhydrotetrasaccharide unit).

Periodate Oxidation. Upon periodate oxidation of the A3(S1) polysaccharide there is a rapid (12 hr) consumption of 2 moles of periodate/tetrasaccharide unit with the simultaneous formation of 1 mole of acid indicating rapid oxidation of the nonreducing glucose terminals. The rate of oxidation of the remaining 1,2-glycol groupings proceeds extremely slowly by comparison to the initial rate and drops to zero only after 300 hr when a total of 4 moles of periodate tetrasaccharide has been consumed. The fact that the rate of periodate consumption finally does drop to zero and the consistency of these results with the methylation and the sequence analyses indicate that this slow rate is not due to overoxidation. As expected, fucose was recovered quantitatively from the oxidized polysaccharide as determined by quantitative vapor phase chromatography of the trimethysilyl derivative of the fucose formed on hydrolysis of the oxidized polysaccharide. When the periodate oxidation was run in unbuffered solution, there was a rapid consumption of 3 moles of periodate/tetrasaccharide unit and then the oxidation proceeded at a similar slow rate. However, the pH dropped to 2, the periodate consumption at 400 hr was in excess of 4.5 moles/tetrasaccharide unit and had not leveled off, and 2 moles of formic acid was found. This suggests that overoxidation took place in the unbuffered solution.

Discussion

The structure of the slime polysaccharide produced by *A. aerogenes* A3(S1) is established by the previous methylation studies (Sandford and Conrad, 1966) and by the sequence analysis described here. The polysaccharide is composed of repeating D-glucose-D-glucose-D-glucuronic acid-L-fucose sequences. Since the only oligosaccharides present at intermediate stages of hydrolysis of the polysaccharide are derived from this tetramer and since the pattern of changes in oligosaccharide concentrations during hydrolysis show a precursor–product relationship consistent with the rate constants for hydrolysis of the individual glycosidic bonds of the tetrasaccharide (see Figure 3 and Table III), the repeating unit illustrated in Figure 4 appears to be the only structural sequence in the polymer. With the exception of the fucosyl linkage, the anomeric configurations in this repeating sequence have been established. The glucopyranosyl–glucuronate bond of the aldotriouronic acid is shown by proton magnetic resonance data to be β linked. The failure of β-glucosidase to cleave this bond is apparently a function of the aglycon specificity of the almond enzyme (for a discussion of the specificity of this enzyme, see Baumann and Pigman, 1957). Periodate oxidation of the polysaccharide in pH 3.9 buffer gives periodate uptake, formic acid production, and fucose recovery which are quantitatively consistent with this repeating tetrasaccharide structure.

For estimation of the rate constants for hydrolysis of the four glycosidic bonds in the polysaccharide several simplifying assumptions had to be made since it is impossible to evaluate all of the factors which affect the rate of hydrolytic cleavage of a glycosidic bond in a polymeric species. The assumptions, based on current knowledge and theories of the mechanism of glycoside hydrolysis (Edward, 1955; Semke *et al.*, 1964; Feather and Harris, 1965), place emphasis on the major factors known to affect hydrolysis rates,

namely, the ring size and conformation, the orientation and substitution at the hydroxyls of the glycosyl ring, and the anomeric configuration at the glycosidic bond. Thus, in these studies the rate of cleavage of cellobiose is considered to approximate the rate of hydrolysis of the glucose residues at nonreducing terminals of the polysaccharide since the anomeric configuration, the configurations and degree of substitution (none) at the glycosyl hydroxyls, and the ring conformations (presumably) of the glycosyl residues are identical. In the case of the fucosyl bond in the polysaccharide the only factor which might be expected to give rise to variation in the rate of cleavage during hydrolysis, namely, the removal of substitution by D-glucuronic acid at C-3 of the L-fucose as a result of hydrolysis of the glucuronosyl bond, need not be considered since less than 1.5% of the glucuronosyl bonds was cleaved by the time all of the fucosyl bonds were hydrolyzed. The rate of hydrolysis of the glucuronosyl bond in the polysaccharide may be taken as the rate or hydrolysis of free aldobiouronic acid since the effect of substitution on the D-glucuronic acid moiety in the polysaccharide is a minor effect compared to that of the C-5 carboxyl group (Semke *et al.*, 1964; Feather and Harris, 1965). The rate constant calculated for the glucosyl residue in the main chain of the polysaccharide is subject to the greatest error. This residue is doubly substituted by fucosyl and glucosyl residues which are being cleaved from its C-6 and C-4 positions, respectively, during hydrolysis of the polysaccharide, causing rather significant changes in the rate of cleavage of the glucosyl–glucuronic acid bond. The effect of removal of the fucosyl residue is eliminated in calculations of the rate constant by estimating the rate of glucosyl–glucuronic acid bond hydrolysis using values obtained after most of the fucosyl residues had been cleaved.

The assumptions thus permit an assessment of the order of magnitude of the rate constants for hydrolysis of the glycosidic bonds. The data obtained indicate that the order of stability to acid of the different glycosidic linkages in the polysaccharide is similar to that previously observed for hexosides, hexuronosides, and 6-deoxyhexosides (Feather and Harris, 1965). The great difference in magnitude of the rate constants, however, is quite striking and explains several aspects of the data obtained in the structural analysis of this polysaccharide. For example, $t_{1/2}$ for acid hydrolysis of the fucosyl bond is less than 10 min and for the glucuronosyl bond is 47 hr! The lability of the fucosyl bond results in the conversion of more than 50% of the starting polysaccharide to aldotetraouronic acid after a 30-min hydrolysis period (Figure 3). The stability of the uronic acid bond, on the other hand, permits recovery of almost 50% of the polysaccharide as aldobiouronic acid after a 4–5-hr hydrolysis period. In addition, this marked stability of the glucuronosyl bond precludes the complete and quantitative hydrolysis of the aldobiouronic acid to its monosaccharide components without extensive degradation of the sugars.

The demonstration that this polysaccharide is a repeating sequence of a relatively simple tetrasaccharide allows the consideration of much less complex biosynthetic mechanisms for this species-specific slime polysaccharide than required by the earlier structural studies (Aspinall *et al.*, 1958). For example, it would be possible for this organism to biosynthesize the polysaccharide by the action of four specific transfer enzymes acting sequentially. Alternatively, the tetrasaccharide could be preformed on a specific acceptor molecule and transferred as a tetrasaccharide moiety to the growing chain of the polysaccharide (Anderson *et al.*, 1965; Weiner *et al.*, 1965; Wright *et al.*, 1965; Struve *et al.*, 1966). This latter process would require only five specific enzymes for the polymerization process. In either case a relatively small proportion of the organism's enzymatic machinery is required for capsule or slime biosynthesis. Whether reevaluation of structural studies of other polysaccharides will lead to similar results cannot be stated at present. However, the currently emerging mechanisms for heteropolysaccharide biosynthesis (Weiner *et al.*, 1965; Wright *et al.*, 1965) would require considerable amplification to account for biosynthesis of many of the complex polysaccharides described in the literature. It appears, therefore, either that a unique mechanism for polymer biosynthesis is required to account for the literature data or that the methods which have suggested the complex structures are subject to errors of considerable magnitude.

The experiments described here have been greatly facilitated by the adoption of radiochemical methods in the analytical procedures. The classical copper reduction method for reducing carbohydrates is a nonstoichiometric reaction in which the amount of Cu^{2+} reduced is a function of the structural and stereochemical features of the reducing monosaccharide as well as the degree and mode of substitution at the hydroxyls. Thus, when the nature of the reducing carbohydrates being analyzed is unknown, as in hydrolysates of heteropolysaccharides, the amount of copper reduced by a sample is of limited quantitative significance. On the other hand, carbohydrate-reducing groups are stoichiometrically reduced with borohydride to the corresponding alcohol with the incorporation of one hydrogen from the borohydride into the product. Recognition of this fact has led to the use of borohydride for quantitative estimation of reducing groups (Lindberg and Theander, 1954; Skell and Crist, 1954; Peat *et al.*, 1956; Bragg and Hough, 1957; Schiffman *et al.*, 1960). These methods have involved manometric measurement of the amount of borohydride remaining after reduction or colorimetric assay for the reduced products. The radiochemical adaptation of this method avoids the difficulties associated with manometric or colorimetric measurements. It can also be usefully extended to allow quantitation of chromatographically separated components of a mixture as illustrated by the radiochromatographic analyses described here.

The low specific activity sodium [^{3}H]borohydride for the reducing value method was obtained as a

custom-prepared, uniform sample (11.5 g, 170 mc). Each analysis used 2 mg of borohydride. The reduction was run at alkaline pH to minimize the amount of borohydride required. In strongly basic solutions (0.1 N sodium hydroxide), however, greater than stoichiometric amounts of tritium were incorporated into the products, presumably as a result of alkaline degradation at the reducing group. This was especially noticeable with D-fructose and with the 3-*O*-α-D-glucopyranosyluronic acid-L-fucose. Reduction in highly buffered solution was not satisfactory due to reduction of the buffers themselves or to rapid catalytic destruction of the borohydride by oxyanion buffers. At 50° monosaccharides were completely reduced in 5–10 min. With unknown oligosaccharide mixtures the reaction period was extended to 1.5 hr to ensure complete reaction of slowly reduced groups (Bragg and Hough, 1957).

Corrections were made for residual tritium in the blanks. This correction was approximately 1.5% of the amount of tritium incorporated into 10 μequiv of reducing compound. This blank value reduces the accuracy of determinations made on samples containing less than 1 μequiv of reducing groups but there is essentially no upper limit on the method since sufficient borohydride is present to reduce 400 μequiv of reducing groups, and still more borohydride could be added to the reaction mixture. The standard deviation from the mean was found to be ±1.5% when a series of identical aliquots of a glucose standard were analyzed and ±3.9% when equivalent amounts of a variety of hexoses, pentoses, and oligosaccharide standards were analyzed. The higher deviation in the latter series probably reflects a variation in the purity of the standards used.

The advantages of a stoichiometric reaction in the quantitation of reducing carbohydrates are obvious. In the work described here the ease and precision of the [^{3}H]borohydride method has greatly facilitated the study of hydrolysis of the A3(S1) polysaccharide. Particular utility is found in determination of rate constants since at zero time the method gives the number of reducing equivalents present initially and at any time thereafter the increment in reducing equivalents is equal to the number of glycosidic bonds cleaved. An extension of this method involves chromatographic separation of [^{3}H]borohydride-reduced mixtures. In this procedure 5–10-μl aliquots of the reduced mixture are separated by the usual chromatographic procedures and segments of the paper strip are counted with 3–5% efficiency. It is necessary, therefore, to use [^{3}H]sodium borohydride with 20–30-fold higher specific activity than that used in the reducing value method. Since the specific activity of the borohydride can be determined, the number of disintegrations per minute in a single spot is a measure of the number of micromoles of this substance in the aliquot of the reduced mixture applied to the paper. This procedure combines the versatile qualitative capacities of paper (or thin layer) chromatography with the quantitative capacity of the [^{3}H]borohydride method.

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Polyuronic Acids Produced by *Pseudomonas aeruginosa**

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ABSTRACT: Polysaccharides produced by thirteen "mucoid-type" *Pseudomonas aeruginosa* organisms were compared and all were found to be polyuronic acids. The principal component of each polyuronide preparation was D-mannuronic acid, with variable amounts of L-guluronic acid detectable. The polyuronides were partially reduced to the corresponding hexosans by esterification and reduction of the methyl esters with sodium borohydride.

The uronic acids and their corresponding hexoses were identified by paper chromatography and electrophoresis. D-Mannose was identified in acid hydrolysates of the reduced polymers by preparation of a characteristic phenylhydrazone derivative (mp 198–200°) and by a specific reaction with hexokinase and adenosine triphosphate (ATP). L-Gulose was identified by reduction to a product that was a substrate for D-sorbitol dehydrogenase.

The isolation of a "mucus-producing" *Pseudomonas* from the respiratory tract of patients with cystic fibrosis has been reported by Doggett *et al.* (1964, 1965). Blood agar, covered by a dialysis membrane, was used as the growth medium, and the viscous material elaborated by these organisms was isolated by an ethanol–benzene method (DiSant'Agnese *et al.*, 1957). The product was identified as a polysaccharide containing glucose, mannose, galactose, glucosamine, galactosamine, sialic acid, and two unidentified compounds. Since this material contained no naphthoresorcinol-reactive material, these authors concluded that no uronic acids were present.

Linker and Jones (1964) subsequently reported on the isolation of another polysaccharide produced by a *Pseudomonas*, also isolated from the respiratory tract of a patient with cystic fibrosis. This material was purified by extraction with dilute alkali, precipitation with cetylpyridinium chloride, and ethanol fractionation. The product resembled alginic acid and mannuronic and guluronic acids were the principal components.

The "mucoid-type" *Pseudomonas* reported by Doggett *et al.* and by Linker and Jones were found only in the tracheobronchial tree of cystic fibrosis patients. The possible significance of this, together with the reported differences in polysaccharide constituents of the viscous materials isolated from these organisms, prompted a further investigation of related *Pseudomonas* strains.

A number of "mucoid-type" *Pseudomonas* organisms were available to us, and all had been identified as *Pseudomonas aeruginosa*.[1] The polysaccharides produced by 13 "mucoid-type" organisms, both from cystic fibrosis and noncystic fibrosis sources, were compared and all were found to be polyuronic acids. The principal component of each polyuronide prepara-

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[1] A manuscript is in preparation describing these organisms as *P. aeruginosa*. The authors wish to thank Dr. Bernard Boxerbaum, Department of Pediatrics, University Hospitals, Cleveland, for making these data available.