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Structural Studies on the Sialic Acid Polysaccharide Antigen of *Escherichia coli* Strain Bos-12†

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ABSTRACT: A polysaccharide, antigenically related to group C meningococcus, has been isolated from *Escherichia coli* strain Bos-12 (016; K92; NM). Like groups B and C meningococcal polysaccharide, the Bos-12 antigen is a pure polymer of sialic acid. ^{13}C NMR studies on the meningococcal group B and C polysaccharides have indicated that the former consists of sialic acid units linked 2→8-α, whereas the latter contains the sialic acid residues linked 2→9-α (Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A., and Smith, I. C. P. (1975), *J. Biol. Chem. 250,* 1926). Comparison of natural abundance ^{13}C NMR spectra of the Bos-12 polysaccharide with group B and C meningococcal polysaccharides established that Bos-12 was either (a) an equimolar mixture of 2→8-α linked sialic acid homopolymers or (b) a 2→8-α/2→9-α heteropolymer. These possibilities were distinguished in the following manner. The fact that Bos-12 polysaccharide precipitated with anti-group C serum but not with anti-group B serum would seem to exclude a. Further, chemical studies (periodate oxidation followed by tritiated $NaBH_4$ reduction) gave saccharides with a radioactive-labeling pattern expected for alternating 2→8-α/2→9-α sialic acid linkages. Bos-12 is thus an 2→8-α/2→9-α heteropolymer.

It has been shown that injection of formaldehyde-treated *Escherichia coli* strain Bos-12 (016; K92; NM) antigen into rabbits induces precipitating meningococcal group C antibodies which fail to react with the group B polysaccharide (Robbins et al., 1975). Preliminary chemical studies revealed, however, that the Bos-12 antigen shares some properties of both the group B and C meningococcal polysaccharides. Both group B and Bos-12 polysaccharides are susceptible to neuraminidase cleavage, whereas the group C polysaccharide is resistant. Unlike the group B polysaccharide, both group C and Bos-12 polysaccharides are susceptible to acid-catalyzed methanolysis (Liu et al., 1971a,b).

^{13}C NMR spectroscopy has, in recent years, been applied to the structural determination of a number of biologically important polysaccharides, including the group B and C meningococcal polysaccharides (Bhattacharjee et al., 1975). These studies have shown that the group B polysaccharide consists of sialic acid units linked 2→8-α, whereas the group C polysaccharides contain the sialic acid residues linked 2→9-α. The present report presents evidence for the structure of the Bos-12 antigen based on ^{13}C NMR spectroscopy and chemical studies.

Experimental Procedure

Materials. The cells of *E. coli* strain Bos-12 were grown for

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6 h and transferred to a 15-L fermenter containing Davis minimal media supplemented with 0.1 M dialyzed yeast extract (Difco). Following incubation for 8 h at 35 °C with aeration, hexadecyltrimethylammonium bromide (Sigma), 0.1% w/v, was added and the culture centrifuged. The polysaccharides were purified by cold-phenol extraction and ultracentrifugation (Gotschlich et al., 1969). Meningococcal group B and C polysaccharides were prepared as previously described (Gotschlich et al., 1969).

Neuraminidases (*Vibrio cholerae* and influenzae virus) were obtained from Mann Research Laboratory. Neuraminidase (*Clostridium perfringens*) was obtained from Pierce Chemical Co.

Analytical Methods. Sodium and calcium content of the polysaccharides was determined by atomic absorption with a Perkin-Elmer Model 303 absorption spectrophotometer. For the analyses of carbon, hydrogen, and nitrogen, a Perkin-Elmer Model 450 gas chromatograph was used. Sialic acid was determined by the thiobarbituric acid procedure of Warren and Odin (1952), by the ion-exchange chromatographic procedure (Liu, 1972) and by a modified Svennerholm procedure (1957), in which the isoamyl alcohol extraction step was omitted. The color which developed after heating with the resorcinol reagent was read at 580 nm.

Moisture content was determined by the Karl–Fischer titration method (Auerbach et al., 1961). Acetyl groups were determined by a modification of the method of Eleck and Harte (1936). The method of Hestrin (1949) was used for the *O*-acetyl determination.

Reducing-Group Analyses. On reduction with $NaBH_4$, sialic acid consumes 1 mol of hydrogen, the keto group obviously being reduced to a secondary alcohol. The number of reducing groups present in the Bos-12 polysaccharide was determined by the tritiated sodium borohydride method (Liu et al., 1971a,b).

Periodate Oxidation. To 6.0 mL of a solution containing 60 mg (0.2 mmol) of Bos-12 polysaccharides in 0.1 M sodium acetate, pH 5.5, was added 60 mg of $NaIO_4$. After incubation at 25 °C for 18 h, the oxidation was stopped by the addition of an excess of ethylene glycol (0.2 mL) and the solution was applied to a column of Sephadex G-25 (1.5 × 194 cm) and eluted with 0.1 M NH_4HCO_3 (Figure 3A). The sialic acid content of the eluate was determined by the modified procedure of Svennerholm (1957). Fractions (300–325 mL) containing the sialic acid were combined, lyophilized, and treated with 10 mg of tritiated $NaBH_4$ dissolved in 0.4 mL of 0.01 M NaOH. After mixing, the solution was warmed to room temperature and the reduction allowed to continue for 30 min with stirring. To ensure completeness of reduction, 20 mg of nonradioactive $NaBH_4$ was then added and the incubation continued for another 60 min. Excess borohydride was destroyed by the addition of acetic acid (50 μL) and lyophilized. The product was placed on a Sephadex G-25 column (1.5 × 194 cm) in 0.1 M NH_4HCO_3. The material in the major radioactive fraction (Figure 3A) was recovered by lyophilization. A yield of 42 mg of a disaccharide derivative (see Results and Discussion) was obtained with a specific activity of 3.75×10^6 dpm mg^{-1}.

The above procedure was repeated with 60 mg of a sample of non-O-acetylated group C meningococcal polysaccharide (Apicella, 1972), and yielded 32 mg of AcNeu[7] [1] derivative with a specific activity of 7.25×10^6 dpm mg^{-1}.

An authentic sample of the tritiated 7-carbon derivative of sialic acid, AcNeu[7], was prepared by a method described by Lenten and Ashwell (1971) from the crystalline β-methylglycoside of AcNeu synthesized according to Yu and Ledeen (1969).

The tritiated products were treated with Dowex 50 × 8 resin (10 mg suspended in 0.5 mL of H_2O) at 100 °C for 60 min to effect the hydrolysis of the glycosidic bonds. Removal of the resin by filtration and lyophilization of the filtrate yielded monosaccharides which were characterized by paper chromatography as described by Lenten and Ashwell (1971) (Figure 3B).

Neuraminidase Treatment. The polysaccharides and the periodate-oxidized samples were digested with neuraminidases at 37 °C as described (Cassidy et al., 1965; Liu et al., 1971a,b). Thiobarbituric acid assays were performed on aliquots of the digestion mixture to measure the release of free sialic acid.

Immunological Procedure. Quantitative precipitin analyses, using equine meningococcal group B and C and rabbit Bos-12 antisera, were performed by published methods (Glode et al., 1977).

^{13}C NMR. ^{13}C Nuclear magnetic resonance spectra were recorded at ca. 35 °C on a Bruker WH-270 spectrometer operating at 67.9 MHz in the pulsed Fourier transform mode using either broad-band proton noise decoupling or single frequency off-resonance decoupling (for retention of ^{13}C–^1H spin coupling). The polysaccharides were run as deuterium oxide solutions at concentrations of ca. 100 mg/mL (at pH 7.0); the solvent, D_2O, served as an internal lock signal. The ^{13}C spectrum of the group B polysaccharide determined in this study was found to be identical to that presented by Bhattacharjee et al. (1975); the group B polysaccharide NHCO resonances from the present and Bhattacharjee's study were taken to have the same chemical shift and all remaining signals were referenced accordingly, thus avoiding the geometric correction necessary in cross-referencing both to external Me_4Si (Becconsall et al., 1970).

^1H NMR. ^1H Nuclear magnetic resonance spectra were recorded on a Varian HR-220 spectrometer operating in the continuous-wave mode at a frequency of 220 MHz. Spectra were recorded at ca. 20 °C using sample concentrations of ca. 100 mg/mL; chemical shifts are reported in parts per million on the δ scale and are relative to internal sodium trimethylsilyl-2,2,3,3-d_4 propionate.

Results and Discussion

Chemical Properties of E. coli Bos-12 Polysaccharide. The results shown in Table I indicate that the Bos-12 polysaccharide is nearly a pure polymer of sialic acid. The material contains less than 1% protein or mucopeptide and is free of amino sugars. The polysaccharide is also devoid of *O*-acetyl or glycolic groups. Acid-catalyzed methanolysis with 1 N HCl in anhydrous methanol at 65 °C for 20 h followed by the identification of the product by ion-exchange chromatography as methoxyneuraminic acid methyl ketoside yielded 26% (w/w) of the polysaccharide. When the Bos-12 polysaccharide was treated with anhydrous methanolic methanesulfonic acid followed by analysis on an ion-exchange column, 86 to 92% (w/w) was recovered as methoxyneuraminic acid. The resistance of the 2→8-α linkage of sialic acid to methanolysis with methanolic HCl was noted earlier (Liu et al., 1971). Enzymatic hydrolysis of the polysaccharide with neuraminidases from *V. cholerae* and *C. perfringens* followed by the thiobarbituric acid assay indicated that the sialic acid released accounted for 82 to 89% of the weight of Bos-12 polysaccharide. The analytical data also indicate that nearly all the nitrogen in the preparation can be accounted for as sialic acid. The results of these analyses

[1] Abbreviations used are: AcNeu, N-acetylneuraminic acid; AcNeu[7], 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid.

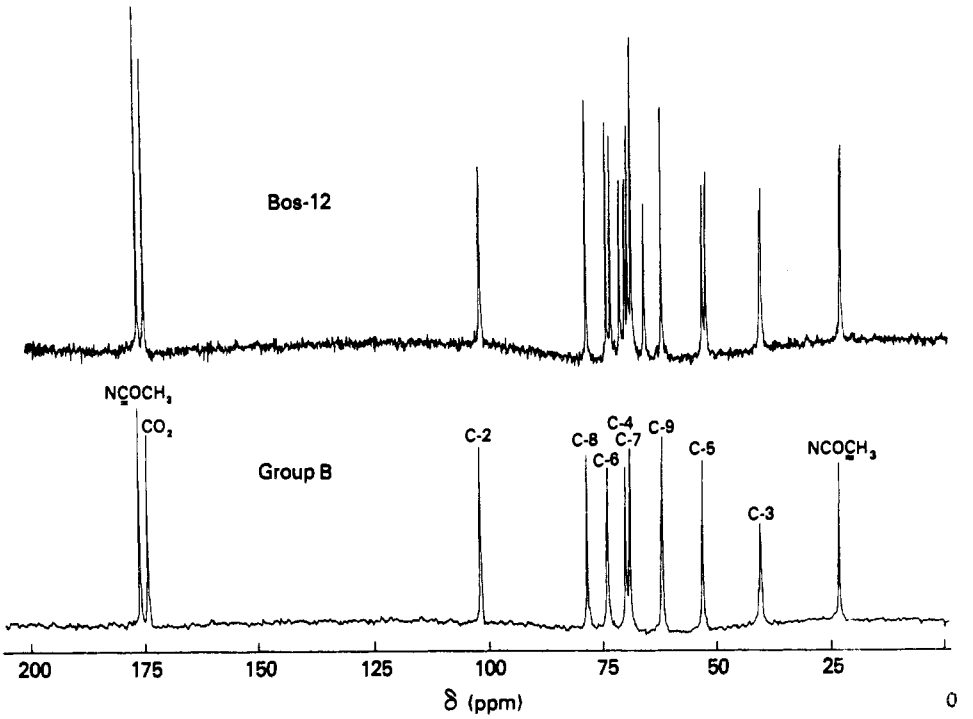

FIGURE 1: The proton noise-decoupled ^{13}C NMR spectra (68 MHz) of the Bos-12 and meningococcal group B polysaccharides; the scale is in parts per million and relative to external tetramethylsilane. Each spectrum results from approximately 20 K accumulations; a 90° pulse angle (~25 μs) and 2.5-s recycle time were employed. The spectral width is 15 151 Hz. The numbering of the carbon atoms is given in Figure 3.

TABLE I: Chemical Composition of *E. coli* Strain Bos-12 Polysaccharide.[a]

Composition	Wt/100 mg of sample (mg)	Amount per 100 mg of sample (μmol)
Sialic acid[b]		
(1) Methanolic HCl: ion-exchange column	26.2	117
(2) Methanesulfonic acid: ion-exchange column	87.5	301
(3) Enzymatic hydrolysis	89.2	307
Acetyl[b]	0	0
Sodium	2.7	117
Moisture[c]	0.5	
Total recovery	92.4	
Nitrogen	4.35	310

[a] Amino acids and amino sugars were determined by ion-exchange chromatography. The values for sialic acid were determined by two different procedures. For the calculation of the weight recovery, the value obtained by the methanesulfonic acid method was used. Acetyl and nitrogen were determined as described in the text. Not included in the total recovery are ninhydrin-negative carbohydrate determined by ion-exchange chromatography, total amino acids, and nucleic acids, all of which were present in less than 1% (w/w). Moisture was determined by the Carl-Fisher titration procedure. [b] Calculated as *N*-acetylneuraminic acid-H_2O; molecular weight, 291. [c] Only *O*-acetyl groups are included here. [d] Dried at 40 °C over P_2O_5 in a vacuum to constant weight.

TABLE II: ^{13}C Chemical Shifts[a] of Serogroups B and C and Bos-12 Polysaccharides.

	Group B[b]	Group C de-O-acetylated[b]	Bos-12	No. of covalently bound H's[c]
C-1	174.3	174.9	174.5	0
			101.9	
C-2	102.1	101.4	102.0	0
			40.9	
C-3	40.9	41.2	41.1	2
C-4	69.4[d]	69.5	69.8[d]	
			52.8	
C-5	53.6	53.0	53.5	1
			73.5	
C-6	74.3	73.6	74.5	
			69.0[d]	
C-7	70.4[d]	69.5	70.4	
			71.5	
C-8	78.8	71.4	78.8	1
			62.4	
C-9	62.4	66.3	66.2	2
			175.9	
*C*PNH	176.1	176.1	176.0	0
			23.3	
CH_3CO	23.6	23.3	23.5	3

[a] Chemical shifts are in ppm (δ) downfield from external Me_4Si. [b] Assignments are taken from Bhattacharjee et al. (1975). [c] Determined by off-resonance decoupling. [d] Assignments may be reversed.

indicate that the sum of sodium and sialic acid accounts for close to 90% of the weight of the material.

The Bos-12 polysaccharide was eluted in the void volume of the Sephadex G-150 column in 0.1 M ammonium acetate, suggesting a molecular weight in excess of 100 000. The result of the reducing group analyses indicated the presence of 1 reducing group per 130 ± 10 residues of AcNeu. The molecular weight of an average chain length of a polymer with 130 residues of AcNeu is 136 × 290 = 37 700. Thus, the Bos-12 polysaccharide, as isolated by the above procedure, is a kinetically stable aggregate consisting of several chains with an average chain length of 130 residues of AcNeu. The physical properties of several of these polysaccharides will be published elsewhere (Liu et al., 1977).

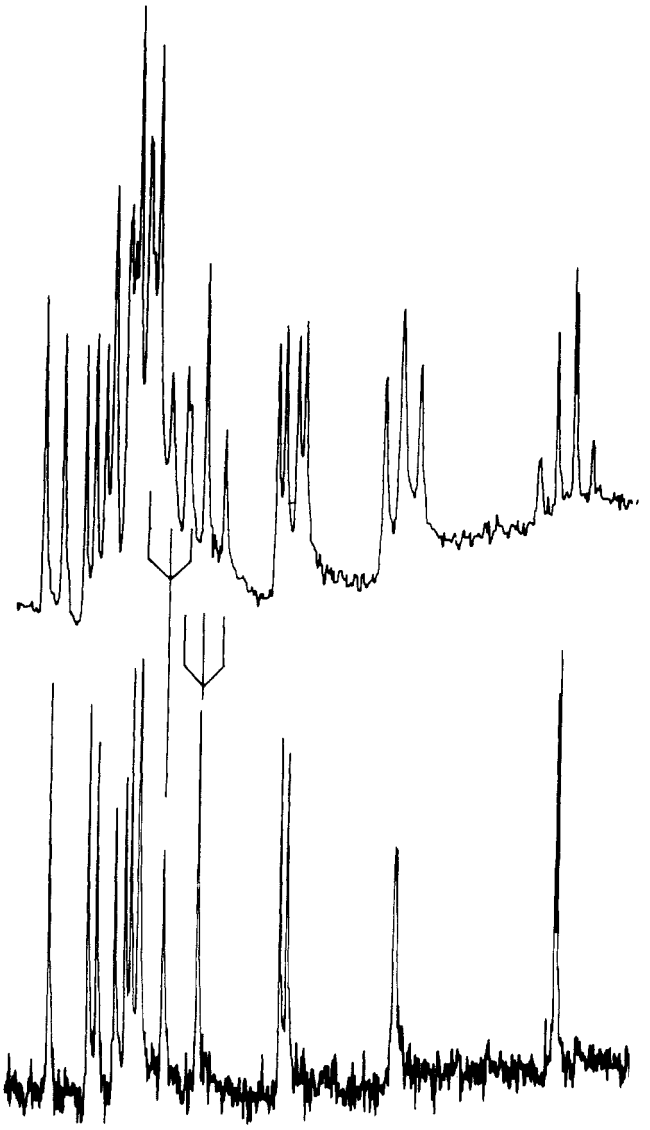

FIGURE 2: The off-resonance decoupled (upper trace) and broad-band proton decoupled (lower trace) ^{13}C NMR spectra of the Bos-12 polysaccharide; the spectral region from ca. δ 15 to 85 ppm is shown.

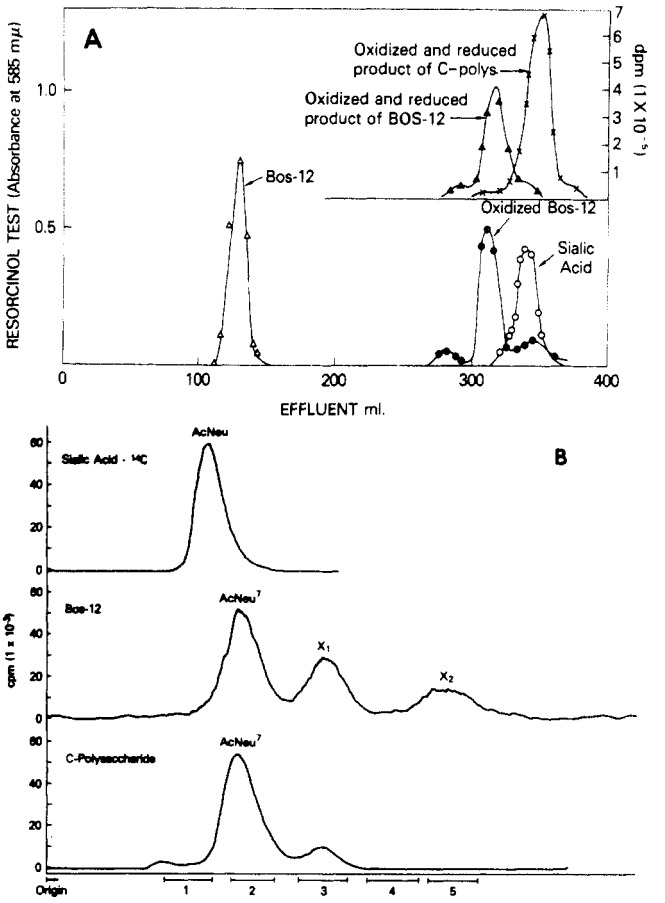

FIGURE 3: (A) Sephadex G-25 gel filtration of the Bos-12 polysaccharide (△); sialic acid (○); oxidized Bos-12 polysaccharide (●); tritiated oxidized product of Bos-12 (▲); tritiated oxidized product of meningococcal group C. (B) Paper chromatographic tracing of the radioactive reaction product. Tritiated polysaccharides were acid hydrolyzed and an aliquot was chromatographed overnight in *n*-butyl acetate–acetic acid–water (3:2:1) and a radioactive tracing was made on a Vanguard autoscanner. The chromatogram was sliced into 1-cm strips, extracted with H_2O, and assayed by the thiobarbituric acid procedure as described by Warren and Odin (1952) with the exception that the periodate oxidation was concentrated at 37 °C (Lenten and Ashwell, 1971).

Structure of Bos-12 Polysaccharide. ^{13}C NMR spectroscopy has, in recent years, been applied to the structure determination of a number of biologically interesting polysaccharides (for examples, see Bundle et al., 1974; Bhattacharjee et al., 1975). The sensitivity of the ^{13}C chemical-shift values to structural and conformational properties provides the experimental basis for this method.[2] Of particular significance for the present investigation is the report by Bhattacharjee et al. (1975) of the ^{13}C spectrum of the group B and C meningococcal polysaccharides. Robbins et al. (1975) have demonstrated that the *E. coli* bacterial strain Bos-12 elicits antibody formation against the group C meningococcal polysaccharide. Since the group C meningococcal polysaccharide antigen is known to be a pure 2→9-α linked homopolymer of sialic acid, it was of interest to know if the cross-reactivity of Bos-12 was due to a close structural or conformational similarity between the polysaccharides or, alternatively, to some other factor.

The proton noise-decoupled ^{13}C NMR spectrum (68 MHz) of Bos-12 is shown in Figure 1. From the appearance of the spectrum, two conclusions may be drawn. First, as 20 major resonances are observed, the Bos-12 polysaccharide is neither a simple 2→8-α linked homopolymer of sialic acid as is the de-O-acetylated group C meningococcal polysaccharide (Bhattacharjee et al., 1975). In agreement with chemical analysis (Table I), no *O*-acetyl resonances (either *C*O or *C*H_3) were observed for the Bos-12 polysaccharide (Table II).

A possible explanation for the number of resonances observed for the Bos-12 polysaccharide, consistent with its chemical and immunological properties, is that this material is a 2→8-α/2→9-α linked sialic acid heteropolymer. A peak-by-peak comparison of the ^{13}C spectrum of Bos-12 with the group B and totally de-O-acetylated group C polysaccharides provides evidence for this interpretation. Thus, the spectrum of Bos-12 is nearly a composite of the individual group B and C spectra (see Table II). The small displacements in chemical shift (<0.5 ppm) which do occur for several Bos-12 resonances relative to their homopolymer counterparts are understandable in terms of environmental changes which accompany heteropolymer formation; that is, a strict transferral of chemical shifts is not expected. Off-resonance decoupling results (Figure 2) are consistent with this assignment. Thus, carbon atoms assigned as possessing either three, two, or one directly bonded hydrogen atom displayed the expected mul-

[2] The limited range in chemical shifts, in concert with a complex pattern of spin–spin couplings, renders, in general, ^1H NMR studies unsuitable for polysaccharide structure determinations.

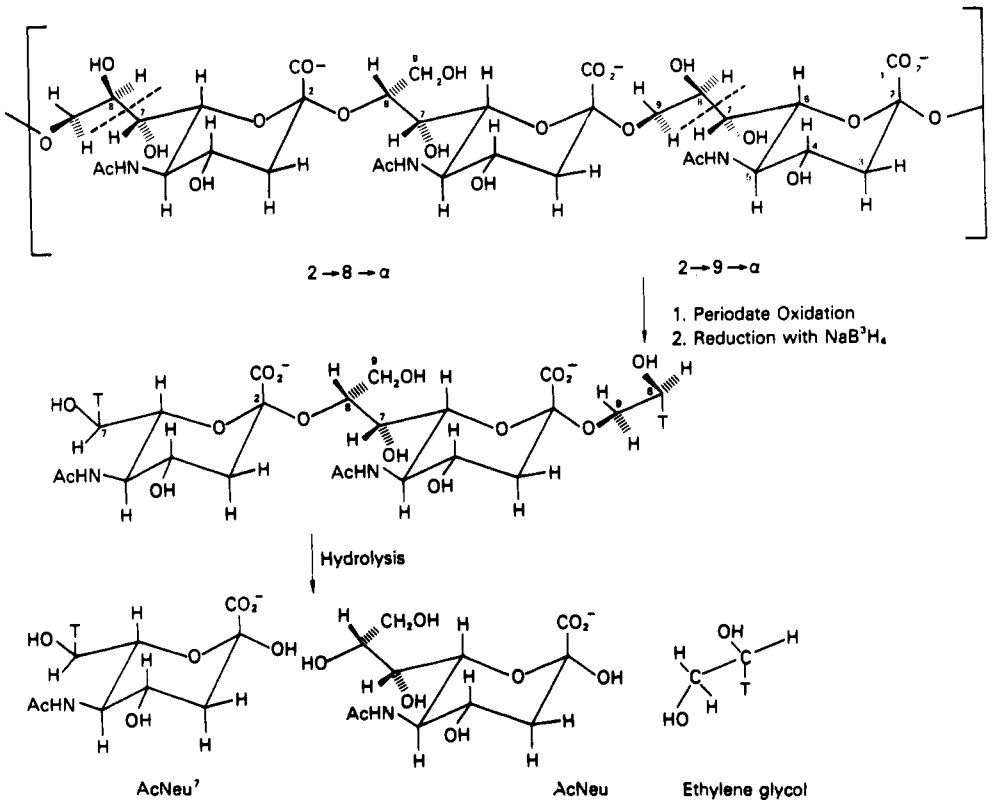

FIGURE 4: Proposed structure of the Bos-12 polysaccharide.

tiplicity when subjected to an off-resonance decoupling field; the resonances assigned to the anomeric and carbonyl carbon atoms demonstrated no splitting.

Alternatively, the ^{13}C spectrum of Bos-12 might be interpreted as arising from a mixture of 2→8-α and 2→9-α linked sialic acid homopolymers. While the NMR method does not allow a distinction between a heteropolymer and a mixture of homopolymers, the fact that Bos-12 failed to precipitate with anti-group B serum would seem to exclude this latter possibility. Periodate, which cleaves C-C bonds having vicinal hydroxyl groups (e.g., the C_7-C_8 bond of the 2→9-α linked homopolymer), was employed to further distinguish between these alternatives. Thus, if Bos-12 was a mixture of the two homopolymers, periodate oxidation would yield a mixture containing intact group B polysaccharide. In contrast, if Bos-12 was a heteropolymer, periodate oxidation would result in a mixture of small-molecular-weight fragments, the exact composition of which would depend on the distribution of the 2→8-α linkages (^{13}C and ^1H NMR spectra demonstrate that the 2→8-α/2→9-α ratio is approximately unity; vide infra).

The results shown in Figure 3A,B indicate that, upon periodate oxidation followed by reduction, the Bos-12 polysaccharide yielded essentially a disaccharide derivative of AcNeu⁷ [³H]AcNeu-[³H]ethylene glycol (Figure 4), whereas the group C polysaccharide yielded a monosaccharide derivative of [³H]AcNeu⁷-[³H]ethylene glycol. As would be expected from the proposed structures (Figure 4), the specific activity of the tritiated product obtained from the Bos-12 polysaccharide was about half that of the product obtained from the group C polysaccharide. Upon acid hydrolysis, the tritiated Bos-12 products yielded two thiobarbituric acid positive components on the paper chromatogram (Figure 3B), a product with radioactivity which cochromatographed with AcNeu⁷ (region 2) and a nonradioactive component which cochromatographed with AcNeu (region 1). The tritiated products from the group C polysaccharide gave rise to a single thiobarbituric acid reactive area which coincides with radioactivity, and cochromatographed with AcNeu⁷. A third region (region 3) with radioactivity but thiobarbituric acid negative was present in both samples. This region should represent the [³H]ethylene glycol. When the group B polysaccharide (2→8-α) was treated with periodate, it remained intact as shown by gel filtration. These results are in agreement with the proposed structures of group C polysaccharide as a 2→9 linked copolymer of sialic acid, of group B polysaccharide as 2→8 linked copolymer of sialic acid, and supports the alternating 2→8/2→9 heteropolymeric linkage proposed for the Bos-12 polysaccharide. Only if the polymer exists as a repeating unit of 2→8/2→9 heteropolymer linkages of sialic acid would one obtain the disaccharide derivative of [³H]AcNeu⁷-AcNeu-[³H]ethylene glycol.

The ^{13}C spectrum of the periodate oxidation product of Bos-12 polysaccharide (Fx I, II) was complex, consisting of approximately 50 major resonances, and not readily amenable to detailed analysis. One noticeable feature of the spectrum, however, was the markedly reduced relative intensity of the carbonyl resonances when a short-pulse recycle time (ca. 0.5 s) was used; lengthening the pulse recycle time to ca. 2.5 s led to a significant increase in the relative intensity of the carbonyl resonances (the carrier frequency was positioned approximately 2 KHz from the carbonyl resonances). As a point of reference, the carbonyl absorptions for either Bos-12 or the group B polysaccharide were the most intense in the spectrum under comparable conditions of a short- or long-pulse recycle time. The dependence of the carbonyl absorption intensity on the pulse recycle time indicates the presence of predominantly low-molecular-weight fragments, that is, fragments possessing correlation times short in comparison to Bos-12. The chemical data taken together with the ^{13}C spectrum of the oxidation product thus indicated that Bos-12 is a heteropolymer (Figure 4).

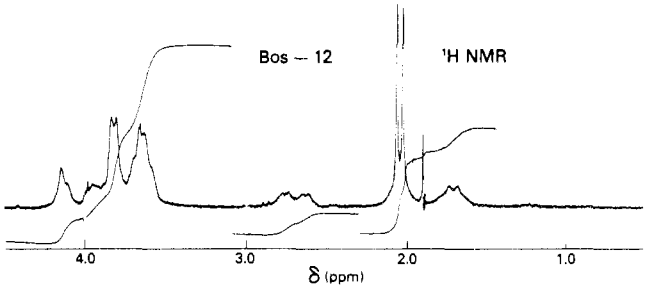

FIGURE 5: The continuous-wave ^{1}H NMR spectrum (220 MHz) of the Bos-12 polysaccharide; the scale is in parts per million and relative to internal sodium trimethylsilyl-2,2,3,3-α_4-propionate. The spectrum was recorded on a Varian HR 220 spectrometer at an operating temperature of 20 °C.

Given the identity of Bos-12 as a heteropolymer two questions arise: first, the relative proportion of 2→8-α and 2→9-α linkages; second, the primary sequence of the polymer. With reference to the first of these questions, the ^{13}C spectrum indicated an approximately equal number of 2→8-α and 2→9-α linkages. However, as Fourier transform spectroscopy can, in certain instances, misrepresent relative concentrations, Bos-12 was examined by continuous-wave proton NMR spectroscopy. The 220 MHz ^{1}H NMR spectrum of Bos-12, shown in Figure 5, exhibited distinct 2→8-α and 2→9-α *N*-acetyl methyl group resonances. By comparison at 60 and 220 MHz, these signals were shown to be chemically shifted singlets centered at ca. 2.06 ppm downfield from sodium trimethylsilyl-2,2,3,3-d_4 propionate. The integrated intensities of these signals (at 220 MHz) were, to within experimental error, equal. Accordingly, Bos-12 is compositionally an equimolar mixture of the 2→8-α and 2→9-α moieties. The available data do not enable us to specify the sequence of the Bos-12 polysaccharide.

Two other poly(sialic acid)-producing *E. coli* strains, N-67 (013:K92:H4) and MT 411 (023:K92:H4), were investigated. Their ^{13}C NMR spectra, with regard to peak position and relative signal intensities, were virtually identical to that observed for Bos-12. These polysaccharides, therefore, are compositionally and structurally 2→8-α/2→9-α sialic acid heteropolymers. Slight variations in the chemical shift of several corresponding carbons in the three strains were observed. These small differences (<0.1 ppm) may be due to the effects of varying chain length and/or sample conditions and do not alter our structural conclusions. The ^{1}H NMR spectra of N-67 and MT-411 were identical to Bos-12 and, therefore, likewise have a 2→8-α/2→9-α ratio of approximately unity.

The results we have obtained from the ^{13}C spectrum of Bos-12, MT-411, and N-67 meningococcal polysaccharides provide cogent evidence for the 2→8-α/2→9-α linked sialic acid heteropolymer structure (Figure 4). This structure satisfactorily explains the characteristic immunological and chemical properties of these *E. coli* strains discussed in the introductory section. The proposed K 92 antigen structure, however, fails to explain the non-cross-reactivity with group B meningococcal antibodies. A more detailed investigation, with regard to higher-order structure, is needed in order to shed more light on this aspect of the problem; such studies are currently in progress.

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