Carbohydrate Research, 156 (1986) 123–135
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ANALYSIS OF THE CHAIN LENGTH OF OLIGOMERS AND POLYMERS OF SIALIC ACID ISOLATED FROM *Neisseria meningitidis* GROUP B AND C AND *Escherichia coli* K1 AND K92

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(Received March 10th, 1986; accepted for publication, June 9th, 1986)

ABSTRACT

A series of (2→8)-α-, (2→9)-α-, and alternate (2→8)-α- and (2→9)-α-linked oligomers of sialic acid (*N*-acetylneuraminic acid, NeuNAc) was prepared by digestion with bacteriophage or by partial hydrolysis at pH 7.0 and 100° of polymers of sialic acid produced by *Neisseria meningitidis* and *Escherichia coli*. The oligosaccharides were purified by gel filtration or by anion-exchange chromatography, and their chain lengths were determined by (*a*) colorimetric measurement of the formaldehyde released from the non-reducing end residue after periodate oxidation, (*b*) radiolabelling of the reducing end residue by reduction with borotritiide, and (*c*) determination of the ratio of the non-reducing end and internal residues by g.l.c. of the trimethylsilyl derivatives of the methyl ester methyl β-ketosides. ^{1}H-N.m.r. spectroscopy was used to confirm the chain length of two oligosaccharides. These methods were used to determine the average chain-length of the sialic acid polysaccharides produced by *N. meningitidis* and *E. coli* and the percentage of chains with covalently bound lipid moieties at the reducing end.

INTRODUCTION

The serogroup B and C capsular polysaccharides produced by *N. meningitidis* are homopolymers of sialic acid (NeuNAc) linked (2→8)-α and (2→9)-α, respectively[1], whereas the *E. coli* K92 polysaccharide is a heteropolymer containing alternate (2→8)-α- and (2→9)-α-linked NeuNAc residues (see Scheme 1). The polysaccharide from *E. coli* K1 (colominic acid) is structurally identical to the *N. meningitidis* serogroup B polysaccharide[2], and each polymer is poorly immunogenic in humans[3,4]. In contrast, the meningococcal serogroup C polysaccharide is a relatively good immunogen and protective against *N. meningitidis* serogroup C disease[5]. Antibodies directed against the group C polysaccharide recognise a

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0008-6215/86/$ 03.50

structural or linear determinant consisting of not more than the hexasaccharide[6,7], whereas antibodies against group B polysaccharide recognise an, as yet unknown, conformational determinant. In order to probe the nature of the conformational determinant(s) on the B polysaccharide, it is necessary to have both structurally defined antigens and antibodies. Anti-B monoclonal antibodies[8] are poorly cross-reactive with low-molecular-weight colominic acid[6]. We now describe the purification and characterisation of (2→8)-α- and (2→9)-α-linked NeuNAc oligosaccharides, and the determination of their chain lengths.

Poly-saccharide	*Number and structure of oligosaccharides*	*Oligomer name*
B, K1	[α-NeuNAc-(2→8)]$_n$-β-NeuNAc $n = 0$–13	B1–B9 B′1–B′14
C	[α-NeuNAc-(2→9)]$_n$-β-NeuNAc $n = 0$–6	C1–C7
K92	α-NeuNAc-(2→9)-[α-NeuNAc-(2→8)-α-NeuNAc-(2→9)]$_n$-β-NeuNAc $n = 0$–2	K2, K4, K6

Scheme 1. Repeating units of the *N. meningitidis* group B and C polysaccharides, and the *E. coli* K1 (colominic acid) and K92 polysaccharides. The reducing NeuNAc residue is assumed to be β since free NeuNAc is ~95% β and ~5% α.

The *N. meningitidis* group B and C polysaccharides and the *E. coli* K92 polysaccharide possess a phosphatidyl diglyceride moiety which is covalently bound to the reducing ends of some of the polysaccharide chains[9], resulting in hydrophobic aggregation and an apparent high molecular weight as indicated by gel filtration. This lipid moiety has also been found in other *E. coli* polysaccharides[10]. The high-molecular-weight fraction of these polysaccharides may be disrupted by detergents[9,10] or by treatment with alkali[10,11]. We have determined their average chain-length and the extents to which phospholipids are attached at the reducing end of the polysaccharide.

EXPERIMENTAL

Materials. — The B, (*O*-Ac$^+$)-C* (0.5 mol *O*-acetyl/mol NeuNAc), (*O*-Ac$^-$)-C, and K92 polysaccharides were prepared as described previously[6,12]. Colominic acid (Koch–Light), *N*-acetylneuraminic acid (Sigma), 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole (AHMT, "Purpald", Aldrich), DEAE-Sephadex A25 (Pharmacia), and Bio-Gel P4 (−400 mesh) (Bio-Rad) were commercial products. NaB^3H_4 (25 mCi; specific activity, 341 mCi/mmol) was obtained from New England Nuclear. Bacteriophage Ø92, together with its host strain *E. coli* Bos 12 (O16:K92:H$^-$), was a kind gift from Professor S. Stirm.

*(*O*-Ac$^-$) and (*O*-Ac$^+$) connote *O*-acetyl negative and positive, respectively.

Analytical methods. — NeuNAc in the polysaccharides was determined by the Svennerholm method[13] prior to analysis of the chain length, since all of the polymers had a moisture content of ~20%. Formaldehyde was determined by the chromotropic acid[14], tryptophan[15], or 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole (AHMT)[16] method. The last method was modified slightly in order to allow for different concentrations of periodate and times of oxidation, and for centrifugation (1,000*g*, 5 min) to remove precipitated polysaccharides when necessary. The reaction was performed in glass vials [15 (i.d.) × 45 mm], the size of which was important for the intensity of the final colour and the linearity of the standard curve. ^{1}H-N.m.r. spectra (360 MHz) were recorded for solutions in D_2O at 23°, using a Bruker WM-360 spectrometer. Resolution enhancement was performed as described previously[17].

Radiolabelling with NaB^3H_4. — A solution of NeuNAc (10 μg), oligo- (10–100 μg), or poly-saccharide (100–400 μg) in water (100 μL) was treated with NaB^3H_4 (10 μL; 50 μCi in 0.1M NaOH) for 24 h at 30° in order to effect complete reduction. The solution was then acidified with 0.1M acetic acid (20 μL) and concentrated to dryness with methanol–4M HCl (20:1; 100 μL). Subsequent concentrations did not reduce the background count; methanol–glacial acetic acid (20:1) was not as efficient at reducing the background count as was methanolic HCl. The residue was dissolved in water (100 μL), and aliquots (20 μL), in Fiso fluor "1" (Fisons) Scintillation grade fluid (5 mL), were counted in a Packard PLD Tri-count Scintillation Counter.

G.l.c. — Solutions of (2→8)-α-linked oligosaccharides (200 μg) or polysaccharides (1 mg) in water (0.4 mL) were incubated with 0.1M $NaIO_4$ (100 μL) for 5 min at room temperature in order to cleave the C-7–C-8 and C-8–C-9 bonds of the non-reducing end residue. Excess of periodate was then reduced by the addition of aqueous 25% glycerol (50 μL), and after 5 min, a solution of $NaBH_4$ (10 mg) in water (50 μL) was added. After incubation for 3 h at room temperature, the mixture was neutralised with acetic acid and desalted on a column (0.9 × 60 cm) of Sephadex G-10 (elution with 0.05M ammonium carbonate). The sialic acid-positive fractions eluted at the void volume were combined and freeze-dried.

Methanolysis and g.l.c. of the periodate-oxidised and borohydride-reduced oligo- and poly-saccharides[6] and g.l.c.–m.s.[18] were performed as described previously. The relative retention time of the trimethylsilyl derivatives of the methyl ester methyl β-ketosides of the C-7 analogue of NeuNAc (5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid; C-7-NeuNAc) and NeuNAc on 3% of OV-225 on Chromosorb W-HP (80–100 mesh) at 220° were 0.80 and 1.00, respectively. A mass spectrum unequivocally identified the former through prominent fragment ions at *m/z* 406 (M^+ – Me), 389 (M^+ – MeOH), 362 (M^+ – CO_2Me), 299 (M^+ – MeOH – Me_3SiOH), 259 (M^+ – CH_2OSiMe_3 – NH_2COCH_3), 227 (M^+ – CH_2OSiMe_3 – NH_2COCH_3 – MeOH), 186 ($AcN^+H{=}CH{\cdot}COSiMe_3{=}CH_2$), and 173 ($AcNH{\cdot}CH{\cdot}CH{=}O^+SiMe_3$).

Chain lengths were calculated by determining the ratio of internal (NeuNAc)

to non-reducing end (C-7-NeuNAc) residues. The relative response factors were estimated from a direct comparison of the areas of the two peaks from the trisaccharide B3, which had an equimolar ratio of NeuNAc and C-7-NeuNAc. Thus,

$$\text{Area (NeuNAc)} = \text{Area (C-7-NeuNAc)} \times 1.3,$$

and, since the reducing-end (NeuNAc) residue was destroyed by periodate oxidation and borohydride reduction,

$$\text{Chain length} = \text{Area (NeuNAc)}/[\text{Area (C-7-NeuNAc)} \times 1.3] + 2.$$

Large-scale propagation and purification of bacteriophage Ø92. — This was performed as described previously[19,20]. The final yield of 3.5×10^{13} PFU of purified phage from 1 L of lysate was in close agreement with the yield (4×10^{13} PFU) of purified virions reported.

Digestion of polysaccharides with bacteriophage Ø92. — This was performed essentially as described previously[20]. To a solution of *E. coli* colominic acid or the K92 polysaccharide (80 mg) in 0.05M ammonium carbonate were added 0.1M ammonium acetate adjusted to pH 7.2 with M acetic acid (ACAA buffer; 1.5 mL) and a suspension of bacteriophage Ø92 (2.5 mL; 0.6×10^{13}PFU). The mixture was incubated at 37° for 24 h, and then eluted at 14 mL/h from a column (2.6 × 100 cm) of Sephadex G-75 with ACAA buffer. The broad sialic acid-positive fractions were combined and freeze-dried.

A solution of the digested polysaccharide (20 mg) in water (0.8 mL) was applied in a column (1.6 × 100 cm) of Bio-Gel P-4 (−400 mesh) and eluted at 2.5 mL/h with ACAA buffer. Fractions (1 mL) were collected and aliquots (20 μL) were analysed for sialic acid.

Autohydrolysis of polysaccharides. — A solution of *E. coli* colominic acid or *N. meningitidis* (*O*-Ac$^-$)-C polysaccharide (30 mg) in water (3 mL), adjusted to pH 7.0 where necessary, was kept at ~100° for 1 and 9 h, respectively, and then freeze-dried. The products of hydrolysis of the (*O*-Ac$^-$)-C polysaccharide were redissolved in water (1 mL), and the oligosaccharides were purified by chromatography on Bio-Gel P-4 (−400 mesh) as described above.

A solution of the products of colominic acid in 0.01M Tris-HCl (pH 7.6, 1 mL) was applied to a column (1.5 × 10 cm) of DEAE Sephadex A25 (Cl^- form), and the oligosaccharides were fractionated by the method of Nomoto *et al.*[21]. The column was washed with starting buffer, followed by a linear gradient (500 mL) of 0–0.6M NaCl in 0.01M Tris-HCl (pH 7.6) at 12 mL/h. Fractions (2 mL) were collected and aliquots (20 μL) were analysed for sialic acid. Fractions corresponding to peaks were combined and freeze-dried, and a solution of each residue in water (1 mL) was desalted on a column (0.9 × 60 cm) of Sephadex G-10. The purified oligosaccharides were isolated by freeze-drying and stored at −20°.

Alkaline hydrolysis of polysaccharides. — Solutions of the *N. meningitidis* B and (*O*-Ac$^+$)-C polysaccharides in 0.1M NaOH were incubated at 37° for 4 h, and then neutralised with M acetic acid. Aliquots were analysed for chain length by the AHMT method either directly or after precipitation of the polysaccharide by the addition of ethanol to a final concentration of 75%, centrifugation, and redissolving the pellet in water.

RESULTS AND DISCUSSION

Purification of oligosaccharides. — *(a) Obtained by digestion with Bacteriophage Ø92.* A series of linear oligosaccharides obtained from *E. coli* K92 polysaccharide and colominic acid was fractionated on Bio-Gel P-4 (−400 mesh) (Figs. 1a and 1b, respectively). Since bacteriophage Ø92 specifically cleaves (2→8)-α-linked NeuNAc residues, the three major peaks obtained from the K92 digest correspond to the di-, tetra-, and hexa-saccharides, K2, K4, and K6, respectively (see Scheme 1), and the seven peaks from the colominic acid digest to the mono- to hepta-saccharides (B1–B7) (Scheme 1). In addition, oligosaccharides B8 and B9 were obtained by chromatography of colominic acid, without prior hydrolysis, on Bio-Gel P-4 (−400 mesh) (Fig. 1c).

(b) Obtained by hydrolysis at pH 7 and 100°. The products of hydrolysis of the meningococcal (*O*-Ac$^-$)-C polysaccharide at pH 7 and 100° for 9 h were fractionated on Bio-Gel P-4 (−400 mesh) (Fig. 2). A series of oligosaccharides, C1–C7 (mono- to hepta-saccharide, Scheme 1), was isolated. Under these conditions of hydrolysis, the B polysaccharide is degraded essentially to the monosaccharide[22], indicating that (2→8)-α-linked NeuNAc residues are much more sensitive than those which are (2→9)-α-linked. However, at pH 2, the C polysaccharide is more acid-labile than the B polysaccharide[7]. At this pH, the B polysaccharide undergoes complete lactonisation[23].

The products of hydrolysis of colominic acid at pH 7 and 100° for 1 h were fractionated on DEAE-Sephadex A-25[21] (Fig. 3). A series of oligosaccharides, B′1–B′14 (mono- to tetradeca-saccharide, Scheme 1), was isolated and each was desalted using Sephadex G-10.

Analysis of chain length. — *(a) By determination of formaldehyde.* Attempts to determine the formaldehyde released from the non-reducing terminus of NeuNAc oligosaccharides by the chromotropic acid[14] or tryptophan[15] method were unsuccessful probably due to interference from non-specific acid-catalysed side-reactions. There were no such problems with the AHMT method[16] which involves an alkaline pH. Using this method, the experimental and predicted chain-lengths were in reasonably good agreement (Table I) for the oligosaccharides B2–B9, C2–C7, and K2–K6. However, the oligosaccharides (B′1–B′14) purified by DEAE-Sephadex A-25 chromatography were not examined by this method, because of interference which gave consistently higher intensities of colour (hence, lower chain-length) than expected. This may have been due to traces of Tris which does not seem to be removed completely during desalting on Sephadex G-10.

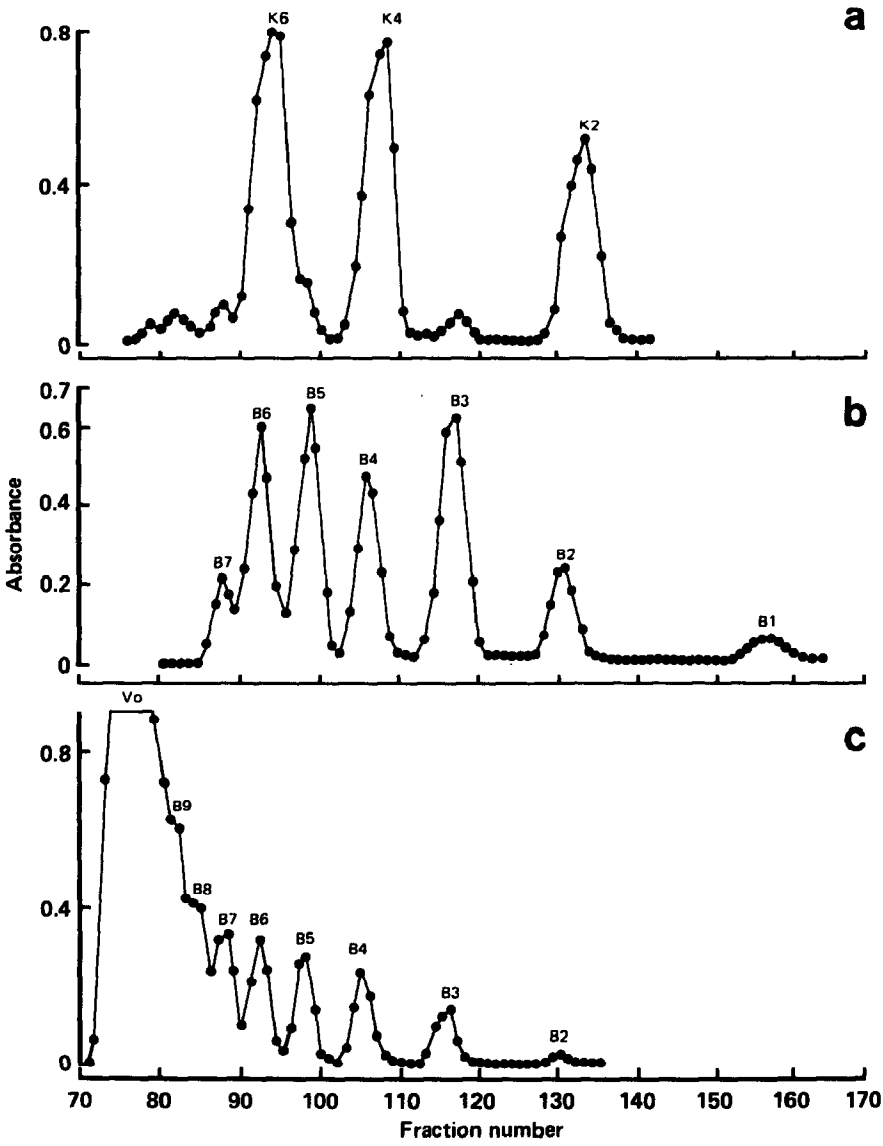

Fig. 1. Chromatography on Bio-Gel P-4 (−400 mesh) of the products of digestion of K92 polysaccharide (*a*) and colominic acid (*b*) with bacteriophage Ø92, and (*c*) colominic acid. Peaks are labelled as shown in Scheme 1.

(b) By radiolabelling with NaB^3H_4. Chain lengths, measured by radiolabelling by borotritiide reduction of the oligosaccharides B2–B9, B′6–B′14, C1–C-7, and K2–K6 in comparison to free NeuNAc, showed (Table I) generally good agreement between experimental and predicted values.

(c) By periodate oxidation, borohydride reduction, and methanolysis. Application of this sequence to B2–B9 was followed by trimethylsilylation of the

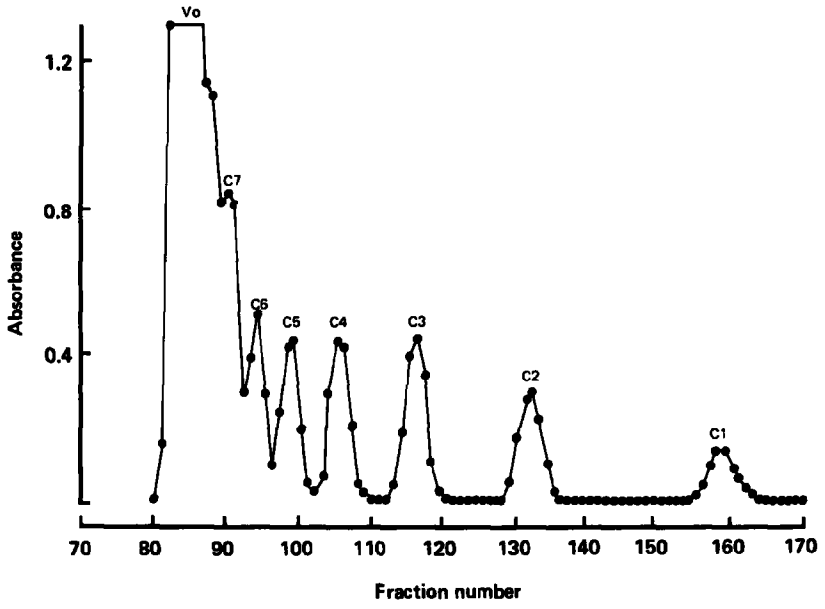

Fig. 2. Chromatography on Bio-Gel P-4 (−400 mesh) of the products of hydrolysis of the (*O*-Ac⁻)-C polysaccharide at pH 7 and 100° for 9 h. Peaks are labelled as shown in Scheme 1.

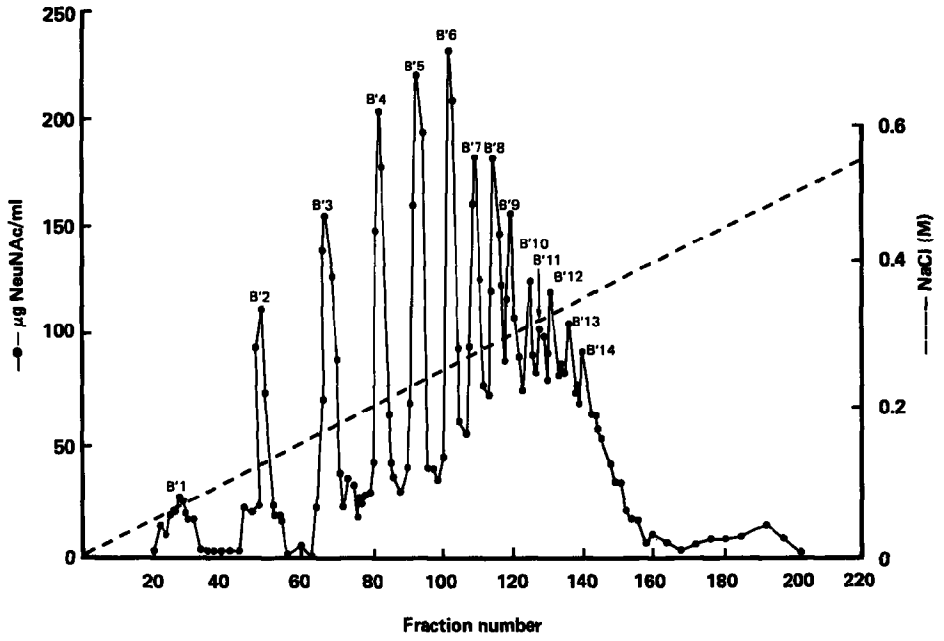

Fig. 3. Chromatography on DEAE-Sephadex A-25 of the products of hydrolysis of colominic acid at pH 7 and 100° for 1 h. Peaks are labelled as shown in Scheme 1.

TABLE I

ESTIMATED CHAIN-LENGTHS OF OLIGOSACCHARIDES PURIFIED FROM DEPOLYMERISED COLOMINIC ACID, (*O*-Ac⁻)-C POLYSACCHARIDE, AND K92 POLYSACCHARIDE

Oligosaccharide[a]	*Chain length*		
	AHMT[b]	*NaB³H₄*[c]	*G.l.c.*[d]
B2	1.9	1.9	2
B3	2.8	2.9	3.0
B4	3.4	4.1	4.1
B5	4.3	4.9	5.1
B6	5.7	6.1	6.1
B7	6.7	8.6	6.0
B8	9.0	8.3	9.2
B9	9.3	9.5	9.2
B'6	[e]	5.9	N.d.
B'7	[e]	6.6	N.d.
B'8	[e]	7.8	N.d.
B'9	[e]	9.0	N.d.
B'10	[e]	9.9	N.d.
B'11	[e]	10.4	N.d.
B'12	[e]	12.1	N.d.
B'13	[e]	13.3	N.d.
B'14	[e]	14.1	N.d.
C1	N.d.	1.1	[f]
C2	2.0	2.3	[f]
C3	2.9	3.3	[f]
C4	N.d.	5.4	[f]
C5	5.4	5.2	[f]
C6	4.9	6.1	[f]
C7	6.4	7.6	[f]
K2	1.8	2.3	[f]
K4	3.6	4.1	[f]
K6	5.3	6.2	[f]

[a]See Scheme 1 for structures. [b]Colorimetric determination of formaldehyde. [c]Radiolabelling of reducing end. [d]Estimation of ratio NeuNAc:C-7-NeuNAc. [e]Not determined due to interference from unknown impurity (probably Tris buffer). [f]Not determined due to susceptibility of internal linkages to periodate oxidation.

products, g.l.c., and g.l.c.–m.s. The ratio of the non-reducing terminal C-7-NeuNAc to the internal NeuNAc residues (allowing for the relative response factors and the loss of the reducing-end residue) was calculated to give the chain lengths shown in Table I. There was reasonably good agreement between the results obtained by this method and methods (*a*) and (*b*).

This method is only suitable for (2→8)-α-linked oligosaccharides since even mild treatment of (2→9)-α-linked oligomers with periodate causes some internal cleavage which markedly reduces the apparent chain-length.

^{1}H-N.m.r. spectroscopy. — Further confirmation of the chain lengths of the oligosaccharides in Table I was obtained from the ^{1}H-n.m.r. data for B'7 and B'11. A comparison of the intensities of the H-3*e* signals for the non-reducing (α-NeuNAc), internal (α-NeuNAc), and reducing (β-NeuNAc) residues at δ 2.78, 2.69, and 2.21, respectively, gave ratios of 1:5:1 for B'7 and 1:7.8:1 for B'11. Although the reducing-end residue is easily identified because it is mainly β, the non-reducing (α-NeuNAc) residue can be distinguished from, and therefore has a different conformation than, the internal (α-NeuNAc) residues.

Average chain-lengths of the polysaccharides. — The average chain-lengths of the *N. meningitidis* group B, (*O*-Ac$^+$)-C, and (*O*-Ac$^-$)-C polysaccharides and of the *E. coli* K1 (colominic acid) and K92 polysaccharides were investigated using the methods described above.

The polysaccharides were treated (2.5–80 min) with 50mM periodate, and the formaldehyde released from the non-reducing end of the polymer chains was determined by the AHMT method. The results (Fig. 4) showed that maximum colour (measured as nmol of formaldehyde) occurred after treatment of the B polysaccharide for $\not>$2.5 min, which is consistent with rapid release of formaldehyde from the non-reducing end with the internal (2→8)-α-linked residues being resistant to periodate. However, the colours produced from the (2→9)-α-linked polymers, (*O*-Ac$^-$)-C, (*O*-Ac$^+$)-C, and K92 polysaccharides, which have various degrees of susceptibility to periodate[12], increased in intensity with time of incubation. This suggested that formaldehyde was released from the non-reducing end and also after cleavage of internal residues. The (*O*-Ac$^-$)-C polysaccharide, the most susceptible of the three polymers to periodate cleavage of internal residues[12], produced the highest intensity of colour, whereas both the (*O*-Ac$^+$)-C polysaccharide, which is 25–50% *O*-acetylated at C-7 and/or C-8 (and therefore resistant to periodate at these sites), and the K92 polysaccharide gave lower intensities of colour with time of oxidation.

In order to minimise oxidation of internal residues of the (*O*-Ac$^-$)-C, (*O*-Ac$^+$)-C, and K92 polysaccharides, milder conditions (*i.e.*, 0.5–5mM periodate for 2.5–80 min) were used. The B polysaccharide underwent complete cleavage of non-reducing residues [*i.e.*, produced maximum intensity of colour on treatment with periodate (mM) as follows: 0.5 for 80 min, 1.0 for 40 min, 2.5 for 20 min, or 5 for 10 min; Fig. 5a]. Similarly, the K92 (Fig. 5b) and (*O*-Ac$^+$)-C polysaccharides (Fig. 5c) gave maximum releases of formaldehyde and there was no overoxidation. With 2.5 and 5mM periodate only, there was some oxidation of the internal residues (Fig. 5d) of the (*O*-Ac$^-$)-C polysaccharide, and calculation of the formaldehyde released, and hence the average chain-length, was based on the periodate concentrations and times determined for the B polysaccharide to be sufficient for complete cleavage of non-reducing residues. The average chain-length of the (*O*-Ac$^+$)-C polysaccharide, however, was adjusted to take account of *O*-acetylation (0.5 mol of *O*-acetyl/mol of NeuNAc) which appears to occur randomly at C-7 and C-8 along the chain[1]. If random *O*-acetylation occurs for the non-reducing end

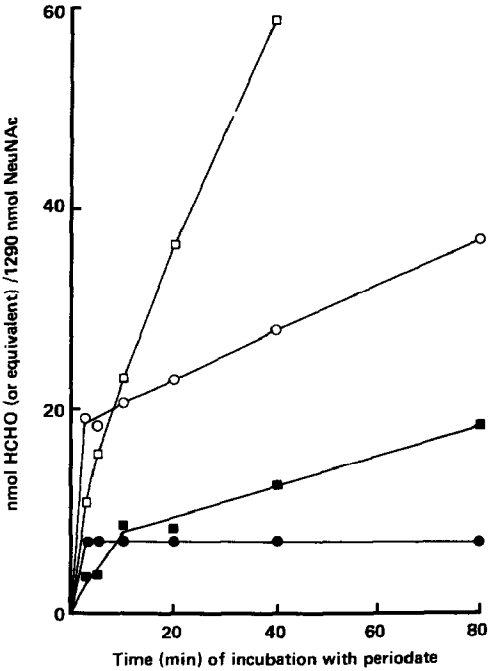

Fig. 4. Rate of formation of formaldehyde (or its equivalent) from the B (—●—), (*O*-Ac$^+$)-C (—■—), K92 (—○—), and (*O*-Ac$^-$)-C polysaccharides (—□—) following incubation with 50mM $NaIO_4$ at room temperature.

residues, then 25% would be mono- (C-8) or di-*O*-acetylated (C-7,8), thus preventing the release of formaldehyde; *O*-acetylation only at C-7 would not prevent the formation of formaldehyde. The experimentally derived, average chain-length of the (*O*-Ac$^+$)-C polysaccharide was therefore multiplied by a factor of 0.75 to account for putative *O*-acetylation at C-8 of non-reducing end residues. The average chain-lengths of the B, (*O*-Ac$^+$)-C, (*O*-Ac$^-$)-C, and K92 polysaccharides, and of colominic acid, determined by the AHMT method, are shown in Table II.

Treatment of the polysaccharides with borotritiide was ineffective (apart from colominic acid; see Table II) due to the putative presence of a lipid moiety[9,10] covalently attached *via* a phosphodiester to the reducing NeuNAc residue of each polymer chain. Some evidence for the proposed structure of a diglyceride as the lipid moiety was obtained by mild alkaline hydrolysis (0.1M NaOH, 4 h, 37°) of the B and (*O*-Ac$^+$)-C polysaccharides, which should be deacylated at positions 2 and 3 of the glycerol moiety and would then be susceptible to periodate and release 1 mol of formaldehyde from each lipid-bound chain. The chain length of the alkali-treated B polysaccharide (Table II) was calculated on the basis of 2 mol of formaldehyde per chain, 1 mol each from the non-reducing end NeuNAc residue and from the exposed glycerol moiety attached to the reducing end. The chain length of the

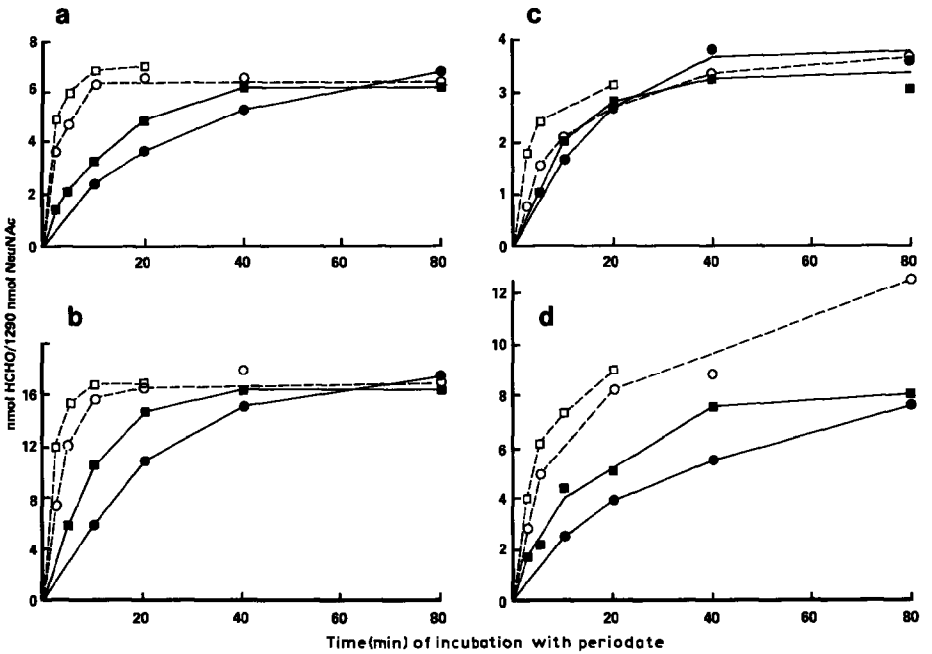

Fig. 5. Rate of formation of formaldehyde (or its equivalent) from B (*a*), K92 (*b*), (*O*-Ac$^+$)-C (*c*), and (*O*-Ac$^-$)-C polysaccharides (*d*) following incubation with $NaIO_4$: 0.5 (—●—), 1.0 (—■—), 2.5 (---○---), or 5mM (---□---).

TABLE II

ESTIMATED AVERAGE CHAIN-LENGTHS OF THE B, (*O*-Ac$^+$)-C, (*O*-Ac$^-$)-C, AND K92 POLYSACCHARIDES AND COLOMINIC ACID

Polysaccharide	*Average chain-length*	
	AHMT[a]	*G.l.c.*[b]
B	196	191
Alkali-treated B	167[c]	207
Colominic acid	15.8	17.1
(*O*-Ac$^+$)-C	316[d]	[e]
Alkali-treated (*O*-Ac$^+$)-C	322[c]	[e]
(*O*-Ac$^-$)-C	172	[e]
K92	78	[e]

[a]Colorimetric determination of formaldehyde formed from the non-reducing end residue. [b]From the ratio NeuNAc:C-7-NeuNAc. [c]Calculated on the basis that alkali-treatment (0.1M NaOH, 4 h, 37°) would expose the glycerol moiety at the reducing end, resulting in the formation of 2 mol of formaldehyde per chain. [d]Adjusted to account for random *O*-acetylation (see text). [e]Not determined due to susceptibility of internal linkages to periodate oxidation.

alkali-treated (*O*-Ac$^+$)-C polysaccharide (Table II) was calculated on the basis that, in addition to exposure of the glycerol moiety at the reducing end, *O*-deacetylation of the polymer would expose all non-reducing end NeuNAc residues, thus removing the assumptions made about *O*-acetylation at C-8 of the non-reducing terminus (see above). The fact that the chain lengths calculated for the alkali-treated and for the native (*O*-Ac$^+$)-C polysaccharides agreed closely suggests that assumptions made about *O*-acetylation in the latter are valid. Thus, all of the chains in the B and (*O*-Ac$^+$)-C polysaccharides appeared to have a covalently attached lipid moiety, which explained the non-incorporation of tritium on treatment with borotritiide. This may not be valid for all preparations of the B and (*O*-Ac$^+$)-C polysaccharides or for all *N. meningitidis* and *E. coli* polysaccharides, since the phosphodiester linkage is sensitive to mild acid[10] and may be severed during purification or storage. However, our results agree closely with those of a study[24] of the biosynthesis of (2→8)-α-linked NeuNAc homopolymers in *E. coli* K-235, namely, that endogenously formed polysaccharide chains averaged 165 residues in length and no free reducing-end residues were present.

The above results were obtained when the B and (*O*-Ac$^+$)-C polysaccharides were alkali-treated, neutralised, and analysed directly by the AHMT method. When, in addition, the polysaccharides were recovered by precipitation with ethanol, only 1 mol of formaldehyde per chain was released upon periodate oxidation. This result may be due to blocking of the reducing-end glycerol moiety by the formation of a 1,2-cyclic phosphoglycerol derivative, although this remains to be confirmed.

The g.l.c. method for determining the average chain-length was suitable only for the (2→8)-α-linked B polysaccharide and colominic acid, since even mild periodate oxidation of the (2→9)-α-linked polymers caused cleavage of some internal residues (see Figs. 4 and 5) which markedly reduced the apparent average chain-length. Table II shows the average chain-lengths determined by this method for the B polysaccharide and colominic acid, which agree with the results from the AHMT and radiolabelling methods.

The importance of chain length for the immunogenicity of purified polysaccharides has been known for many years[25,26], whereas the relevance of the length of the oligosaccharide or polysaccharide chain when attached to protein carriers is still being clarified[27,28]. Dextran of low molecular weight (10^3–10^4) was a much better immunogen than dextran of high molecular weight (5–40 × 10^6) when each was coupled to a particular protein and injected into mice. Evaluation of chain length could be important when examining the immunological response to meningococcal polysaccharides coupled or complexed to protein carriers.

Capsular polysaccharides from *N. meningitidis,* intended for use as vaccines for humans, must be analysed by gel-filtration chromatography and this is the only criterion used to estimate the size of the polymer. Meningococcal polysaccharides form[9,29] aggregates of chains with a broad distribution of molecular weights, the high-molecular-weight fraction being eluted at the void volume of Sepharose

CL-4B (mol. wt. of $>5 \times 10^6$ in comparison with dextran molecular weight markers). Since the average chain-length of the B and C polysaccharides used in this study was 200–300 NeuNAc residues, it may be deduced that a polymer with a mass of 5×10^6 daltons could be constituted by 50–100 molecules. It is difficult to envisage stable particles of this size having a central lipidic core, and many of these aggregates probably exist in a lamellar form. The size and stability (and perhaps immunogenicity) of these aggregates would depend on the extent of lipid formation at the reducing end of the polysaccharide and perhaps the nature of the fatty acid constituents within the lipid moiety, factors, until now, not considered when studying vaccination with meningococcal polysaccharides.

ACKNOWLEDGMENTS

We thank Dr. V. Riveros-Moreno for help in bacteriophage purification, and Mrs. J. M. Williams for recording the ^{1}H-n.m.r. spectra.

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