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SIALIC ACID POLYSACCHARIDE ANTIGENS OF *Neisseria meningitidis* AND *Escherichia coli*: ESTERIFICATION BETWEEN ADJACENT RESIDUES

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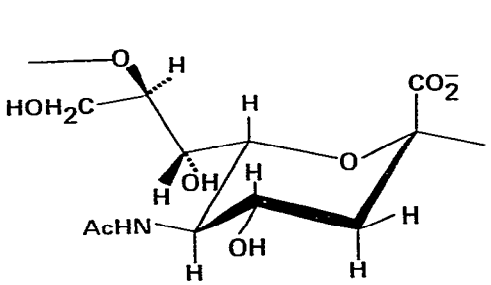
ABSTRACT

Colominic acid and meningococcal Goup B polysaccharide, both (2→8)-α-linked homopolymers of sialic acid, are made water-insoluble either by reaction with a carbodiimide in aqueous solution at pH 4.75 or by treatment with 48% aqueous hydrofluoric acid at 0° for 48 h. I.r. spectra of the products show a major band near 1750 cm^{-1}, consistent with ester formation; this band virtually disappears after mild, alkali treatment. Esterification also occurs by incubating the native polysaccharides below pH 6.0, and their i.r. spectra show that the degree of esterification increases as the pH is lowered. The relatively low molecular weight of these partially esterified, water-soluble polymers is consistent with intra- rather than inter-molecular ester formation. Counter-current immunoelectrophoresis shows that esterification of ~9% is sufficient to abolish immunoprecipitation. ^{13}C-N.m.r. spectroscopy of fully esterified colominic acid provides strong evidence for cross-linking between the carboxyl group of one residue and HO-9 of an adjoining residue. Meningococcal serogroup C polysaccharide, a (2→9)-α-linked homopolymer of sialic acid containing *O*-acetyl groups at C-7 and/or C-8, does not undergo intramolecular esterification after carbodiimide treatment. However, upon *O*-deacetylation of the polysaccharide, esterification occurs.

INTRODUCTION

The capsular polysaccharides from *Escherichia coli* K1 (colominic acid) and *Neisseria meningitidis* serogroup B are (2→8)-α-ketosidically linked homopolymers (**1**) of sialic acid, which are structurally[1] and serologically[2] indistinguishable. Both organisms are causative agents of meningitis in humans and there is much evidence to suggest that the capsular polysaccharide conveys invasiveness[3,4]. In contrast to many encapsulated bacteria, the capsular polysaccharides are poor immunogens when purified and injected into humans[3,5]. However, the capsular polysaccharide of *N. meningitidis* serogroup C (**2**) confers protection against meningitis caused by this organism, as does an *O*-acetyl-negative, serogroup C variant[6] (**3**). The difference in immunogenicity between these structurally related polysaccharides is at present un-

explained. We now report the varying ability of these sialic acid polysaccharide antigens to form intramolecular esters under mild conditions.



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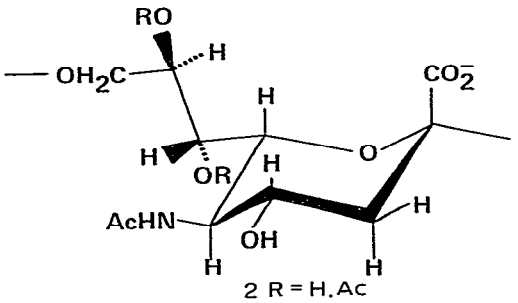


2 R = H.Ac
3 R = H

EXPERIMENTAL

Materials. — Colominic acid (**1**; Na^+ form) and *Vibrio cholerae* neuraminidase were obtained from Koch–Light Laboratories, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC), *Clostridium perfringens* neuraminidase Type V, and *N*-acetylneuraminic acid from the Sigma Chemical Co. *N. meningitidis* serogroup B and C polysaccharides were prepared from strains CN 7630 and CN 7038, respectively, and purified as previously described[7].

General methods. — C polysaccharide was *O*-deacetylated as previously described[1]; the *O*-acetyl content was determined by the method of Hestrin[8] as modified by Ludowieg and Dorfman[9], free sialic acid by the thiobarbituric acid method of Aminoff[10], and polymeric sialic acid by the Svennerholm method[11].

Spectroscopic methods. — I.r. spectra (KBr discs) were recorded on a Perkin–Elmer 580 spectrophotometer interfaced to a PDP 11/34 mini-computer running time-sharing software. The degree of esterification was estimated by using computer subtraction to remove any residual carboxylate absorption, followed by comparing the intensity of the ester C=O stretching band near 1750 cm^{-1} with the Amide I and II bands. The ^{13}C-n.m.r. spectrum (external Me_4Si) of a water-insoluble sample of carbodiimide-treated colominic acid was recorded for a solution in $(CD_3)_2SO$ at a concentration of ~70 mg/ml with a Bruker WM-250 spectrometer at 62.9 MHz and 30°.

Esterification with carbodiimide (EDC)[12]. — In a typical experiment, the polysaccharide (salt form, 10 mg) was dissolved in water (4 ml), and EDC (100 mg) was added with stirring at room temperature. The pH was adjusted and maintained at 4.75 and the reaction allowed to continue for 6 h. With B polysaccharide and colominic acid (**1**), a precipitate formed within 2 h, which was collected by centrifugation at 10,000*g* for 10 min. The pellet was washed twice with water and freeze-dried. C polysaccharide (**2**) (*O*-acetyl content, 1.7 μmol/mg) and its *O*-deacetylated analogue (**3**) remained water-soluble during the reaction and were dialysed for 48 h at 4° against distilled water (4 × 2 litres) and freeze-dried.

Esterification with dilute acid. — Solutions of B polysaccharide and colominic acid (**1**, salt form; 4 mg) in water (1.5 ml) were adjusted to pH values between 3.0 and 6.0 with dilute HCl, incubated at room temperature for 6 h, and freeze-dried. In a similar manner, B polysaccharide (**1**), colominic acid (**1**), C polysaccharide (**2**), and its *O*-deacetylated analogue (**3**) (salt form, 6 mg) were converted into the free-acid form by passage through a short column of Dowex 50 (H^+ form, X8, 50–100 mesh) resin, incubated (pH 2.9) at room temperature for 6 h, and freeze-dried.

Esterification with hydrofluoric acid. — B polysaccharide (**1**, salt form; 5 mg) was incubated with 48% aqueous HF (BDH, AristaR) for 48 h at 0° with stirring in a capped, polyethylene, centrifuge tube. HF was removed *in vacuo* over KOH pellets, using[13] a polypropylene/polycarbonate desiccator and a copper trap containing NaF.

Neuraminidase. — Samples of polysaccharide (0.5 mg) were incubated with neuraminidase from *V. cholerae* (100 μl) or from *Cl. perfringens* (100 μg in 100 μl) at 37° in 0.05M sodium acetate buffer (pH 6.0) containing 1% of NaCl, 0.1% of $CaCl_2$, and 0.005% of NaN_3 (0.5 ml). Aliquots were removed at timed intervals and analysed for free sialic acid[10].

Counter-current immunoelectrophoresis (*c.i.e.*). — This was performed as previously described for determination of meningococcal polysaccharides[14,15]. Summarily, standard agarose plates prepared as for immunoelectrophoresis were used. Small wells (7-μl capacity and 1 cm apart) were filled either with undiluted, rabbit, anti-B polysaccharide serum (Wellcome Reagents, Hither Green, London) or with various concentrations of partially esterified or native B polysaccharide (0.01–100 μg/ml in 0.01M phosphate-buffered saline, pH 7.4). Electrophoresis for 30 min at 4° and 14 V/cm was performed, keeping the serum closer to the anode. Plates were inspected for precipitation lines in the agar. Inhibition of c.i.e. was performed in an identical manner after preincubating sera for 30 min at 4° with several concentrations of polysaccharide and testing with a standard concentration of native B polysaccharide (1 μg/ml).

Molecular size. — The molecular weight distribution of the native or partially esterified, water-soluble polysaccharides was determined by gel filtration on a column (2.6 × 95 cm) of Sepharose 4B equilibrated and eluted with 0.1M ammonium acetate buffer (pH 7.0) containing 0.01% of NaN_3 at a flow rate of 10 ml/h. Fractions (2.5 ml) were analysed for sialic acid[11]. The column was calibrated by using the following Dextran T markers of defined molecular weight (Pharmacia Fine Chemicals): T-20, $\overline{M}_w$ 2×10^4; T-70, $\overline{M}_w$ 7×10^4; T-150, $\overline{M}_w$ 1.5×10^5; T-500, $\overline{M}_w$ 5×10^5; void volume (V_O), Blue Dextran; total volume (V_T), D-glucose.

RESULTS AND DISCUSSION

Esterification with carbodiimide. — Both colominic acid and B polysaccharide with repeating structure **1** have very similar i.r. spectra that are strikingly different from those of the two polysaccharides after carbodiimide treatment. Fig. 1 shows

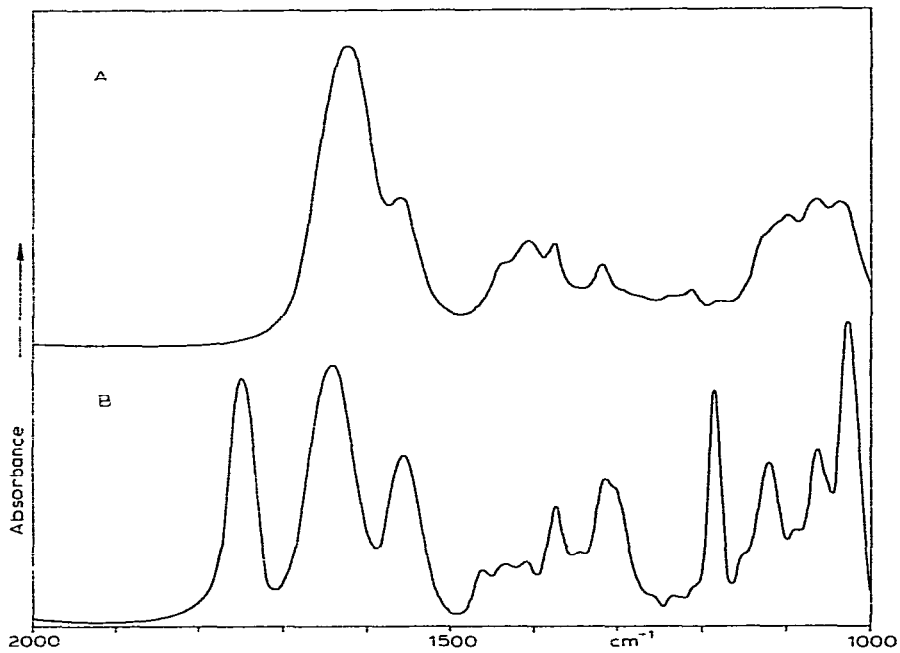


Fig. 1. I.r. spectrum of Group B polysaccharide before (A) and after (B) carbodiimide treatment.

the region between 2000–1000 cm^{-1} for both the native and carbodiimide-treated B polysaccharide.

The spectrum from the native polysaccharide shows a strong carboxylate peak near 1610 cm^{-1} that overlaps the Amide I and Amide II bands. After carbodiimide treatment, however, the latter bands can clearly be seen at ~1645 and 1555 cm^{-1}, respectively, whilst a symmetric peak near 1750 cm^{-1} can be assigned to the C=O stretching vibration of an ester or lactone. The disappearance of the 1610 cm^{-1} band and the absence of any bands near 1700 cm^{-1} (C=O group of carboxylic acid) or between 2600 and 2400 cm^{-1} (OH group of carboxylic acid) imply that no significant amount of carboxylic acid or carboxylate is present. Alkali treatment (0.2M NH_4OH, 30 min, 25°) decreased the ester peak near 1750 cm^{-1} to below 10% of its original, relative height and re-established the carboxylate peak. These results are consistent with ester or lactone formation involving all carboxyl groups of the carbodiimide-treated polysaccharides and may explain their insolubility in water. After, but not before, carbodiimide treatment, the i.r. spectrum of *O*-deacetylated C polysaccharide exhibits a symmetric peak near 1750 cm^{-1} and a decreased carboxylate peak at ~1610 cm^{-1}. These results are consistent with less than half (~44%) of the carboxyl groups being esterified.

Fig. 1 also shows that esterification of B polysaccharide generated an additional band near 1190 cm^{-1} which can be assigned to a C–O vibration of the ester group. This is also true of the *O*-deacetylated C polysaccharide after carbodiimide treatment.

TABLE I

DEGREE OF ESTERIFICATION OF B POLYSACCHARIDE (1), COLOMINIC ACID (1), C POLYSACCHARIDE (2), AND *O*-DEACETYLATED C POLYSACCHARIDE (3), AS DETERMINED BY I.R. SPECTROSCOPY

Incubation conditions	*Degree of esterification (%)*	
	B Polysaccharide	*Colominic acid*
pH 3.0	53	50
pH 3.5	35	29
pH 4.0	30	25
pH 4.5	19.3	15.7
pH 5.0	8.6	8.0
pH 5.5	2.5	2.7
pH 6.0	<1	<1
Salt form	<1	<1
Free-acid form (pH 2.9)	90	80
HF-treated	81	—
EDC-treated	96	100
Glacial acetic acid-treated	—	41.5
	C Polysaccharide	*O-Deacetylated C polysaccharide*
Salt form	47[a]	<1
EDC-treated	[b]	44
Free-acid form (pH 2.9)	[b]	6.7

[a]Due to *O*-acetyl groups near 1725 cm^{-1}. [b]Not determined due to the presence of *O*-acetyl and carboxylic acid groups near 1725 cm^{-1}.

The i.r. spectrum from native C polysaccharide shows that the *O*-acetyl groups have a C–O stretching band at 1255 cm^{-1} and a symmetric C=O band near 1725 cm^{-1} which, following esterification, would overlap a C=O ester band near 1750 cm^{-1}. However, carbodiimide treatment of the C polysaccharide did not affect the band near 1725 cm^{-1} and, as the characteristic peak of the esterified B and *O*-deacetylated C polysaccharides near 1190 cm^{-1} did not become apparent, this indicates that ester formation was either minor (<5%) or absent.

Esterification with mild acid. — When colominic acid was originally isolated[16] (as the free acid), it was suspected that ester linkages were present in some of the residues. However, it was not known whether they were artefacts from the purification procedure or were endogenously formed in the bacterium. The results presented here confirm that the free-acid form of the molecule is sufficient for ester formation to occur. Table I shows the degree of esterification of B polysaccharide between pH 3.0 and 6.0 (colominic acid gave very similar results). In all cases, the i.r. spectra show a symmetric ester band near 1750 cm^{-1} and a decreased carboxylate band at ~1610 cm^{-1}. The characteristic carboxylic acid bands near 1700 cm^{-1} and between 2600

and 2400 cm^{-1} are not observed in any of the spectra, suggesting that the free-acid form of the polysaccharide is sufficient for ester or lactone formation to occur. Certainly, as the pH was lowered and the acid–salt equilibrium changed in favour of the acid, there was a marked increase in the ester content (Table I). Furthermore, B polysaccharide (free-acid form) in aqueous solution (pH 2.9) formed an almost completely (~90%) esterified polymer.

Colominic acid had previously[17] been treated with glacial acetic acid for 5 days at room temperature to form a "polylactone". In our hands, an esterified product with an i.r. band near 1750 cm^{-1} was obtained, although only ~41% of the carboxyl groups were esterified (Table I). Similar, acid-catalysed esterification of sialic acid residues occurs[18] in brain gangliosides in which the sialic acid glycosidically bound to O-3 of a galactopyranosyl residue forms an ester linkage with a hydroxyl group of this residue; at present, the position of linkage is not known. These intramolecular esters can be endogenously formed[19].

When C polysaccharide and *O*-deacetylated C polysaccharide were incubated for 6 h in the free-acid form (pH 2.9), their i.r. spectra showed a strong band near 1725 cm^{-1} which, together with absorptions between 2600 and 2400 cm^{-1}, imply that free carboxylic acid was present. In the former case, the C=O stretching bands from carboxylic acid and *O*-acetyl groups were superposed and, since the characteristic C–O stretching band near 1190 cm^{-1} was absent, this indicated that esterification did not occur. In the latter case, removal of the carboxylic acid C=O stretching band near 1725 cm^{-1} by passage through a short column of Dowex 50 (Na^+) resin revealed a weak ester band near 1750 cm^{-1}, suggesting that less than 7% esterification had occurred.

Esterification with hydrofluoric acid. — When B polysaccharide was treated with 48% aqueous HF at 0° for 48 h (conditions used to cleave phosphodiester linkages)[13], the polymer became water-insoluble. An i.r. spectrum showed a symmetric ester band near 1750 cm^{-1} and an asymmetric band near 1645 cm^{-1} (Amide I) with a shoulder at ~1610 cm^{-1} (carboxylate) consistent with extensive ester formation (~81%; see Table I). Although the mechanism of HF action is complex and dependent upon the reaction conditions[20], it is likely that the free-acid form of B polysaccharide predominated in the acidic environment, thus enabling esterification to occur.

Neuraminidase action. — Previous studies[16] have suggested that partially esterified colominic acid is resistant to neuraminidases, although the alkali-treated polysaccharide is readily degraded. However, we have found that both polymers are susceptible to hydrolysis by the neuraminidases from *Vibrio cholerae* and *Clostridium perfringens*. B polysaccharide, after treatment at pH 3.0 (~53% ester formation; see Table I), released 29% of free sialic acid after 24 h and 62% after 96 h when incubated with neuraminidase from *V. cholerae*. In comparison, the native polysaccharide released 46 and 60% after 24 and 96 h, respectively.

O-Deacetylated C polysaccharide released 70% of free sialic acid after 24-h incubation with neuraminidase from *V. cholerae*. However, after treatment with

TABLE II

CORRELATION BETWEEN DEGREE OF ESTERIFICATION AND THE ANTIGENICITY OF B POLYSACCHARIDE

Incubation pH	*Degree of esterification (%)*	*Minimal concentration giving positive band by c.i.e. (μg/ml)*	*Minimal concentration giving full inhibition by c.i.e. (μg/ml)*
3.0	53	>100	[a]
4.0	30	>100	[a]
5.0	8.6	>100	400
5.5	2.5	0.4	80
6.0	<1	0.4	40
Salt form	<1	0.4	20

[a]Not determined.

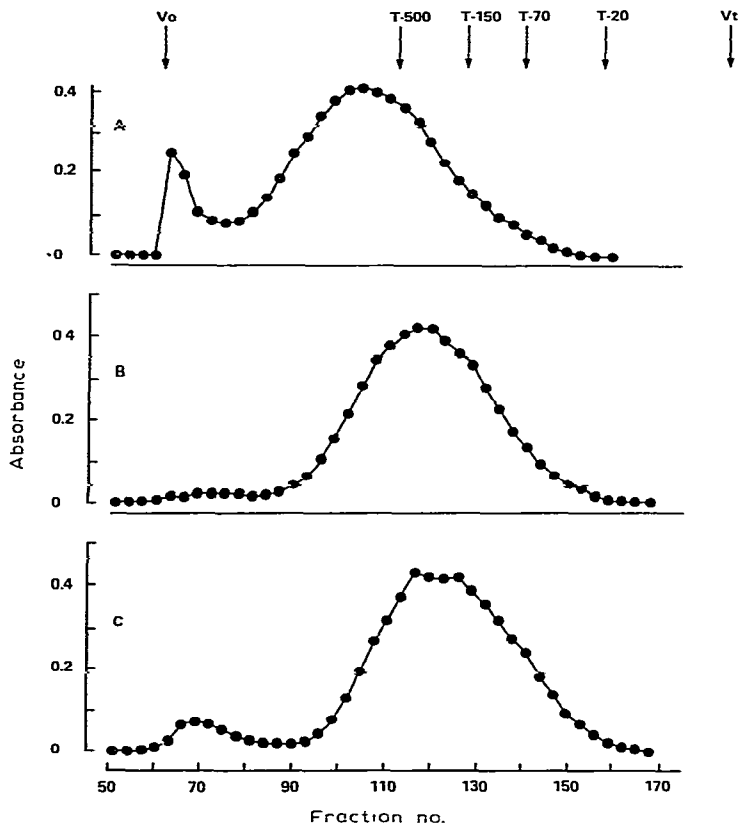


Fig. 2. Sepharose 4B chromatography of native (A), ~30% esterified (B), and ~90% esterified (C) Group B polysaccharide. Conditions of chromatography and Dextran T markers are as described in the Experimental section.

carbodiimide, the esterified polysaccharide (~44% ester formation; Table I) was resistant to the enzyme, only 6% of free sialic acid being released after 24-h and 15% after 96-h incubation. C polysaccharide, due to the presence of *O*-acetyl groups, was resistant to neuraminidase action both before and after treatment with carbodiimide.

Counter-current immunoelectrophoresis (c.i.e.). — Several partially esterified, water-soluble preparations of B polysaccharide were tested against antiserum to the native polysaccharide by c.i.e. Table II shows that esterification of only 9% is sufficient to abolish immunoprecipitation, implying that the antibody binding-sites recognise a relatively long part of the molecule rather than individual residues. From the same data, however, it can be concluded that 2–3% esterification is insufficient to alter the antigenic sites. Inhibition of c.i.e. (*i.e.*, neutralisation of anti-B antiserum by different polysaccharides; Table II) indicates that the capacity to react with antibody is not completely lost upon esterification above 9%, although binding capacity is decreased to a level that does not allow the formation of insoluble antigen–antibody complexes.

Molecular size. — Native B polysaccharide shows a broad distribution of molecular weight (Fig. 2A) due to varying degrees of aggregation of individual chains in solution[21]. After incubation for 6 h at room temperature, at pH 4 (~30% esterification; Fig. 2B) or in the free-acid form (~90% esterification; Fig. 2C), there was no evidence of an increase in the molecular size of the polysaccharide. Furthermore, hydrolysis of glycosidic linkages is not suspected, since both of the partially esterified polymers have an apparent molecular weight of $\sim 5 \times 10^5$. Similarly, *O*-deacetylated C polysaccharide had a broad distribution of molecular weight, which was essentially unaltered after esterification with carbodiimide. We conclude that intra- rather than inter-molecular esterification occurs in these preparations.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. data [$(CD_3)_2SO$] for carbodiimide-

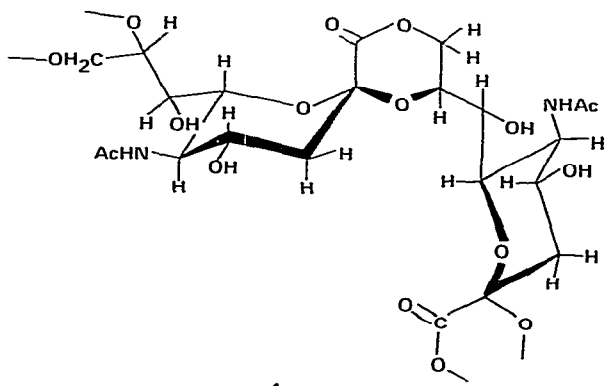
TABLE III

^{13}C-N.M.R. CHEMICAL SHIFT VALUES[a] ASSIGNED TO COLOMINIC ACID AND ITS ESTERIFIED ANALOGUE

Carbon atom	*Colominic acid (1)*[b]	*Esterified colominic acid*	*Difference (p.p.m.)*
C-1	174.4	167.4	−7.0
C-2	102.2	97.9	−4.3
C-3	41.0	40–43	—
C-4	69.4	69.8	+0.4
C-5	53.7	53.7	0.0
C-6	74.4	74.5	+0.1
C-7	70.6	71.4	+0.8
C-8	78.6	71.4	−7.2
C-9	62.5	68.1	+5.6
N-Ac (CH_3)	23.7	24.3	+0.6
N-Ac (C=O)	176.1	174.1	−2.0

[a]P.p.m. from Me_4Si. [b]Ref. 1.

treated colominic acid are given in Table III, together with the assignments for the native and the esterified polysaccharide. The latter showed 11 simple resonances (one of which, C-3, was obscured by the solvent peak between 40 and 43 p.p.m.). This is indicative of esterification of the same hydroxyl group on each residue rather than random esterification of all hydroxyl groups. The upfield shift of one of the resonance signals at ~174 to 167.4 p.p.m. is consistent with esterification of the C-1 carboxyl group[22]. Of the three possible sites for ester formation (C-4, C-7 or C-9), two may be discounted (C-4 and C-7) by comparing the chemical shifts of the native and the esterified polysaccharide (Table III). Chemical shift differences of 0.4 (C-4) and 0.8 p.p.m. (C-7) are not significant. However, the chemical shift of C-9 of the esterified polymer is displaced downfield by 5.6 p.p.m., consistent with ester formation through the carboxyl group at C-1 of one residue and HO-9 of an adjacent residue (**4**) to form a six-membered ring. This structure would be involved in some strain, since an sp^2-hybridised carbon atom (C=O) is normally accommodated within a five-membered ring more readily than in a six-membered ring[23]. In accord with this view, the chemical shifts at C-2 and C-8 of the esterified polysaccharide are displaced upfield by 4.3 and 7.2 p.p.m., respectively (see Table III). However, the large upfield shift of the latter resonance may be due to factors other than ring strain alone. It is known, for instance, that *O*-acetyl substitution results in a modest downfield shift (~3 p.p.m.) of the signal for the α-carbon atom, whereas the β-carbon atom experiences an upfield shift of similar magnitude, the effect being attributed to shielding by the *O*-acetyl group[24]. It may be expected, therefore, that esterification at O-9 of colominic acid would result in a significant upfield shift of the adjacent carbon atom (C-8).



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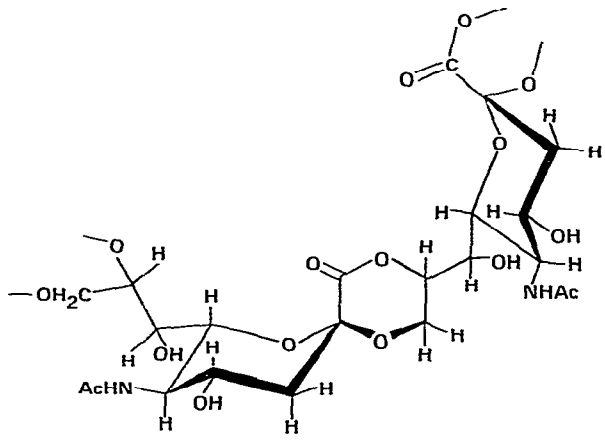
C polysaccharide (**2**) and its *O*-deacetylated analogue (**3**) cannot be esterified in this manner, since O-9 is blocked through the glycoside linkage. We envisage a structure (**5**), analogous to **4**, in which the carboxyl group of one residue and HO-8 of an adjacent residue form a six-membered ring, although this awaits confirmation. The postulated structure would, however, explain why *O*-acetyl groups in the native



5

C polysaccharide inhibit, and their removal aids, ester formation. Structures **4** and **5** differ in that the former is esterified through a primary (C-9) and the latter through a secondary (C-8) hydroxyl group. This may explain, sterically, why structure **1** can be rapidly and completely esterified, whereas structures **2** and **3** are less reactive. The difference between the two structures (**4** and **5**) may also explain why **4** is susceptible, and **5** resistant, to neuraminidase action.

There are two possible consequences from the ease of esterification of B polysaccharide and colominic acid. Firstly, esterification may control the functioning of *N. meningitidis* serogroup B and *E. coli* K1 by regulating the charge of the surface, anionic polysaccharide. Analogously, brain gangliosides containing terminal sialic acid residues form internal esters upon mild treatment with acid[18], and it has been postulated that control of the negative charge density may play a role in membrane interactions[19]. Secondly, modification of B polysaccharide and colominic acid at pH 5 (~9% esterification) is sufficient to abolish antigenicity (see Table II). These two polysaccharides are poor immunogens in humans[3,5], and it is conceivable that esterification *in vivo* is a causative factor.

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