*Carbohydrate Research*, 143 (1985) 191–205
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# STRUCTURAL AND CONFORMATIONAL FEATURES OF THE *Escherichia coli* K92 CAPSULAR POLYSACCHARIDE

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(Received February 12th, 1985; accepted for publication, May 9th, 1985)

ABSTRACT

*Escherichia coli* K92 polysaccharide, an alternating (2→8)-α- and (2→9)-α-linked heteropolymer of sialic acid, undergoes lactonisation (10–20%) at low pH or on treatment with a water-soluble carbodi-imide (>90%). Under the latter conditions, the formation of *O*-acylisourea is a minor pathway. The antigenicity of the K92 polysaccharide is unaffected by lactonisation of ~10% of the residues, but is abolished by lactonisation of ~90% of the residues. The unexpected, relative resistance of the K92 polysaccharide to periodate oxidation contrasts with the behaviour of the meningococcal non-*O*-acetylated (*O*-Ac$^-$) C polysaccharide, a (2→9)-α-linked homopolymer of sialic acid. Two-dimensional $^{13}C$–$^1H$-n.m.r. correlation spectroscopy has been used to assign $^1H$ chemical shifts which have aided the interpretation of a resolution-enhanced one-dimensional spectrum. This has led to a determination of conformational features of the K92 polysaccharide in solution. The side-chain adopts a conformation such that H-7 and H-8 are *gauche* in the (2→8)-α-linked residues (**1a** or **1b**), but antiperiplanar in the (2→9)-α-linked residues (**2**). Molecular correlation times have been calculated and some aspects of internal motion elucidated.

## INTRODUCTION

The capsular polysaccharide from *E. coli* K92, a heteropolymer containing alternate (2→8)-α- and (2→9)-α-linked sialic acid residues[1], is structurally similar to the *Neisseria meningitidis* serogroup B and C capsular polysaccharides, which are homopolymers of sialic acid linked (2→8)-α- (**1a** or **1b**) and (2→9)-α- (**2**), respectively[2,3]. However, although the K92 polysaccharide cross-reacts immunologically with the C polysaccharide, it does not do so with the B polysaccharide[4]. This situation is compatible with the hypothesis[3,5] that antibodies against C polysaccharide recognise a linear (*i.e.*, structural) determinant, whereas antibodies against B polysaccharide recognise a conformational determinant. During our studies of the meningococcal B and C polysaccharides, we found[5,6] that, by lowering the pH or by treatment with a water-soluble carbodi-imide, a δ-lactone was formed by

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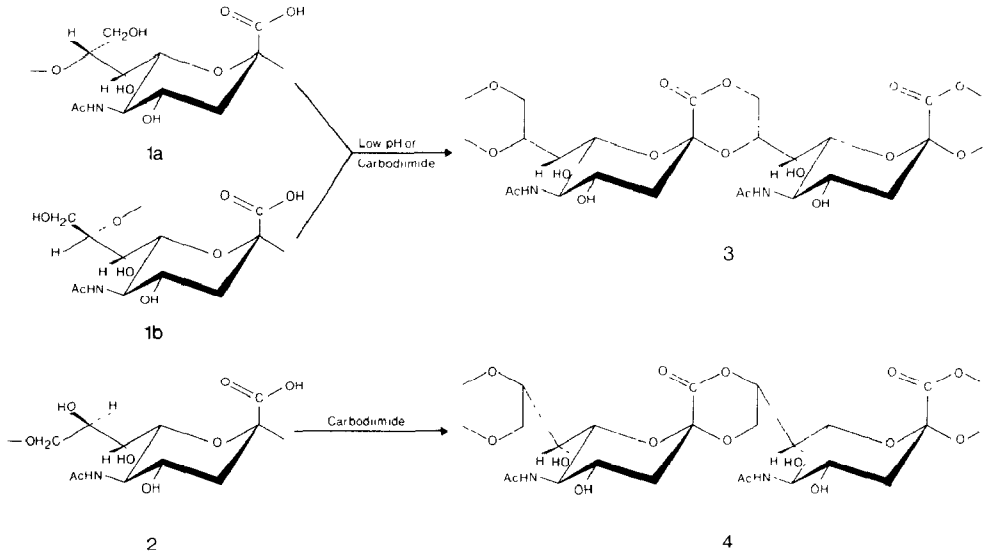
condensation of the carboxyl group of one residue with HO-9 (for the B polysaccharide; **3**) or HO-8 (for the C polysaccharide; **4**) of an adjacent residue. Lactonisation of <20% of the residues markedly reduced the antigenicity of the B polysaccharide, but not of the C polysaccharide, indicating the importance of the three-dimensional structure of the B polysaccharide with regard to its immunological recognition.




1a 1b 2 3 4

The conformation and solution dynamics of the B and C polysaccharides have also been investigated by n.m.r. spectroscopy[3], and H-7,8 of the side-chain were shown to be antiperiplanar in the C polysaccharide (**2**) and *gauche* in the B polysaccharide (**1a** or **1b**). Furthermore, $^{13}$C-n.m.r. spin–lattice relaxation-times have shown that segmental motion occurs in the entire side-chain of the C polysaccharide, whereas the B polysaccharide has a much more rigid molecule with internal rotation of only the pendant C-9 group. In order to gain a better insight into the conformation and immunology of the K92, B, and C polysaccharides, we have investigated the K92 polysaccharide with regard to ease of lactonisation and the resulting antigenicity, and also conformation and flexibility in solution.

## EXPERIMENTAL

*Materials.* — *E. coli* K92 polysaccharide and *N. meningitidis* serogroup B and (*O*-Ac$^-$)-C polysaccharides were prepared[7] from strains CN7873, CN7630, and CN7869, respectively.

*General methods.* — Sialic acid was determined by the Svennerholm method[8], and i.r. spectroscopy, solid-phase radioimmunoassay, and methanolysis of polysaccharides were performed as described previously[5]. Molecular-weight-

distributions were determined[6] by gel filtration on a column (1.5 × 90 cm) of Sepharose CL-4B at 4°.

*Lactonisation of the K92 polysaccharide.* — The $Na^+$ salt of the K92 polysaccharide was prepared[5] from the $Ca^{2+}$ salt. Aliquots (12.9 μmol) of separate solutions of the $Na^+$ and $Ca^{2+}$ salts (64.5 μmol) in water (4 mL, pH 6.9) were mixed with HCl to give molar ratios of $CO_2^-$:$H^+$ of 1:0.1, 1:0.25, 1:0.5, 1:0.75, and 1:1. The volume was made up to 3 mL with water and the pH was recorded. After storage for 24 h at room temperature, aliquots (2.25 mL) were incubated with $KBH_4$ (10 mg) at pH 9 for 3 h at room temperature, then dialysed for 48 h at 4° against 0.01M ammonium carbonate (4 × 2 L), and freeze-dried.

The free-acid form of the K92 polysaccharide was prepared, typically, by passing a solution of the $Ca^{2+}$ salt (5–10 mg) in water (0.5–1.0 mL) through a short column of Dowex 50 ($H^+$) resin (X8, 50–100 mesh). The solution (pH 2.6) was stored at room temperature for 24 h and then freeze-dried. A sample of the partially lactonised polysaccharide was reduced with borohydride, and the solution was dialysed and then freeze-dried, as described above. Another sample was incubated with 0.25M NaOH at room temperature for 2 h, neutralised, and desalted on a column (0.9 × 60 cm) of Sephadex G-25, eluted with 0.05M ammonium carbonate. Fractions (2 mL) were analysed for sialic acid. The void volume ($V_0$), which contained the only sialic acid-positive material, was freeze-dried.

Large-scale lactonisation of the K92 polysaccharide was effected[6] with excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC), and the product was saponified as described above.

*Periodate oxidation.* — Solutions (1 mg/mL) of the K92 polysaccharide and meningococcal B and (*O*-$Ac^-$)-C polysaccharides in 10mM $NaIO_4$ were stored in the dark at 4°. Aliquots (30 μL) were removed at intervals and added to water (2.97 mL), and the absorbance at 223 nm was determined. The amount of periodate reduced was determined by the method of Dixon and Lipkin[9] with the correction factor introduced by Aspinall and Ferrier[10].

*Solid-phase radioimmunoassay.* — This technique[5] involved rabbit polyclonal anti-C polysaccharide serum showing high reactivity for K92 polysaccharide. Plates were sensitised with K92 polysaccharide (1 μg/mL in phosphate-buffered saline), followed by appropriate dilutions of rabbit antiserum with or without various concentrations of antigens. Rabbit immunoglobulin bound to the plates was estimated with $^{125}$I-goat anti-rabbit immunoglobulin.

*N.m.r. spectroscopy.* — The $^{13}$C-n.m.r. spectrum of the carbodi-imide-treated K92 polysaccharide was recorded for a solution in $Me_2SO$-$d_6$ at ~50 mg/mL with a Bruker WM-360 spectrometer operating at 90 MHz and 50°. Similarly, the spectrum of the K92 polysaccharide was recorded for a solution in $D_2O$ at ~200 mg/mL at 40°. Spin–lattice relaxation-times ($NT_1$ values) were measured by using the inversion–recovery pulse sequence. $^{13}$C-N.m.r. spin–lattice relaxation-times ($NT_1$ values): (2→8)-α-linked residues, 0.27 s (C-3), 0.21 (C-4), 0.21 (C-5), 0.22 (C-6), 0.21 (C-7), 0.22 (C-8), 0.33 (C-9), 2.31 (*N*-$AcCH_3$); (2→9)-α-linked

residues, 0.25 s (C-3), 0.21 (C-4), 0.22 (C-5), 0.22 (C-6), 0.24 (C-7), 0.23 (C-8), 0.28 (C-9), 2.34 (*N*-Ac$CH_3$). The assignments for C-4 and C-7 of the (2→9)-α-linked residues may be reversed, although that shown is preferred. The molecular correlation time and internal flexibility of the polysaccharide were investigated through the interpretation[3] of the N$T_1$ values, using the models of Doddrell *et al.*[11].

A $^1$H-n.m.r. spectrum of the K92 polysaccharide was measured for a solution in $D_2O$ at 360 MHz and 70° which did not alter the coupling constants, and hence conformation, but did allow the line-width to be narrower such that resolution enhancement was easier. $^1$H-N.m.r. data: (2→8)-α-linked residues, δ 1.79 (H-3*a*), 2.70 (H-3*e*), 3.73 (H-4), 3.85 (H-5), 3.87 (H-6), 3.85 (H-7), 4.18 (H-8), 4.13 (H-9), 3.75 (H-9′), $J_{6,7}$ <1, $J_{7,8}$ ~4, $J_{8,9}$ ~4, $J_{8,9'}$ ~4, $J_{9,9'}$ ~12 Hz; (2→9)-α-linked residues, δ 1.82 (H-3*a*), 2.88 (H-3*e*), 3.84 (H-4), 3.86 (H-5), 3.64 (H-6), 3.67 (H-7), 4.00 (H-8), 3.92 (H-9), 3.77 (H-9′), $J_{6,7}$ <3, $J_{7,8}$ 8.4, $J_{8,9}$ 5.2, $J_{8,9'}$ 3.1 Hz. Deconvolution of spectra determined at ambient temperature showed coupling constants in the same ranges. Resolution enhancement of the time-domain data was performed by using Lorentzian–Gaussian transformation.

The $^1$H spectrum was assigned through the use of a two-dimensional $^{13}$C–$^1$H correlation experiment, the correlation between the $^1$H and $^{13}$C chemical shifts being determined by their mutual one-bond spin-coupling constants[12]. In all, 242 $^{13}$C FIDS were collected into 4k data points with a spectral width of 7246 Hz. After the first Fourier-transform, the interferrograms were zero-filled to 512 points prior to the second transform.

## RESULTS

*Lactonisation at low pH.* — The degrees of lactonisation of the $Na^+$ and $Ca^{2+}$ salts of the K92 polysaccharide were determined by incubating solutions in dilute HCl at room temperature for 24 h, followed by borohydride reduction, methanolysis of the partially carboxyl-reduced polysaccharides, and analysis of the trimethylsilylated derivatives by g.l.c.[13] (see Table I); the degree of lactonisation increased as the pH was lowered for both the $Na^+$ and $Ca^{2+}$ salts, being marginally higher in the former.

The K92 polysaccharide was also incubated in the free-acid form at room temperature for 24 h, conditions under which the meningococcal B polysaccharide, a (2→8)-α-linked homopolymer of sialic acid, undergoes 70–80% lactone formation (**1**→**3**), and the meningococcal C polysaccharide, a (2→9)-α-linked homopolymer of sialic acid, undergoes no lactone formation (**2**↛**4**). The degree of lactonisation (10–20%) for the K92 polysaccharide was lower than expected.

*Lactonisation with carbodi-imide.* — The i.r. spectra of the K92 polysaccharide before, and after treatment with water-soluble carbodi-imide are shown in Fig. 1. The latter spectrum contained a strong C=O band near 1745 $cm^{-1}$ and a strong C–O band near 1190 $cm^{-1}$, indicative of ~70% lactonisation. In addition, a very weak band near 1695 $cm^{-1}$ showed that <5% of *O*-acylisourea formation had

TABLE I

CORRELATION BETWEEN DEGREE OF LACTONISATION AND ANTIGENICITY OF $Na^+$ AND $Ca^{2+}$ SALTS OF THE K92 POLYSACCHARIDE AT LOW pH

| *K92 polysaccharide* | *Molar ratio* ($CO_2^-$:$H^+$) | *Incubation conditions (pH)*[a] | *Degree of lactonisation (%) by g.l.c.* | *Relative concentration giving 50% inhibition (μg/mL)* |
|---|---|---|---|---|
| $Ca^{2+}$ | 1:0 | 6.9 | 0 | 0.1 |
| | 1:0.1 | 5.2 | 0 | 0.1 |
| | 1:0.25 | 3.9 | 0.6 | 0.1 |
| | 1:0.5 | 3.1 | 3.3 | 0.4 |
| | 1:0.75 | 2.7 | 5.3 | 0.6 |
| | 1:1 | 2.5 | 9.0 | 1.0 |
| $Na^+$ salt | 1:0 | 6.9 | 0 | 0.1 |
| | 1:0.25 | 4.2 | 2.0 | 0.4 |
| | 1:0.5 | 3.3 | 5.7 | 0.8 |
| | 1:0.75 | 2.75 | 8.5 | 0.4 |
| | 1:1 | 2.4 | 9.9 | 0.7 |
| $H^+$ form | 1:1 | 2.6 | 12.0 | 1.9 |
| $H^+$ form/alkali-treated | — | — | N.d.[c] | 1.4 |
| EDC-treated[b] | — | — | 94 | >50 |
| EDC-treated/alkali-treated | — | — | N.d.[c] | 0.1 |

[a]Also for 24 h at room temperature. [b]1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide. [c]N.d. = not detected.

occurred[5]. Following borohydride reduction and methanolysis, g.l.c. suggested that ~94% of the carboxyl groups had been reduced.

The $^{13}$C-n.m.r. spectrum of the native K92 polysaccharide is a composite of the spectra obtained for the meningococcal B and (*O*-Ac$^-$)-C polysaccharides[1], and the chemical shifts are therefore easily assigned. Similarly, the $^{13}$C-n.m.r. spectrum of carbodi-imide-treated K92 polysaccharide (Fig. 2), as a solution in $(CD_3)_2SO$, is a composite of the spectra of the carbodi-imide-treated B and (*O*-Ac$^-$)-C polysaccharides. These latter spectra have been interpreted[5,6] to show that (2→8)-α-linked (B) residues lactonise through $CO_2H$ and HO-9 in adjacent residues (**1→3**), and that (2→9)-α-linked (C) residues lactonise through $CO_2H$ and HO-8 in adjacent residues (**2→4**). Essentially complete lactonisation of the K92 polysaccharide was apparent (Fig. 2) from the large upfield shifts of the signals for the two carboxyl (C-1) carbons (~10 p.p.m.) and the two anomeric (C-2) carbons (~6 p.p.m.), in agreement with data for the degree of lactonisation (~94%) by g.l.c. analysis of the products obtained after carboxyl-reduction of the polysaccharide followed by methanolysis and trimethylsilylation.

*Molecular size of the K92 polysaccharide following lactonisation.* — Table II shows the degree of lactonisation of the K92 polysaccharide before and after treatment in the free-acid form or with a carbodi-imide. Both the g.l.c. and i.r. methods revealed that lactonisation did not occur in the salt form of the polysaccharide.

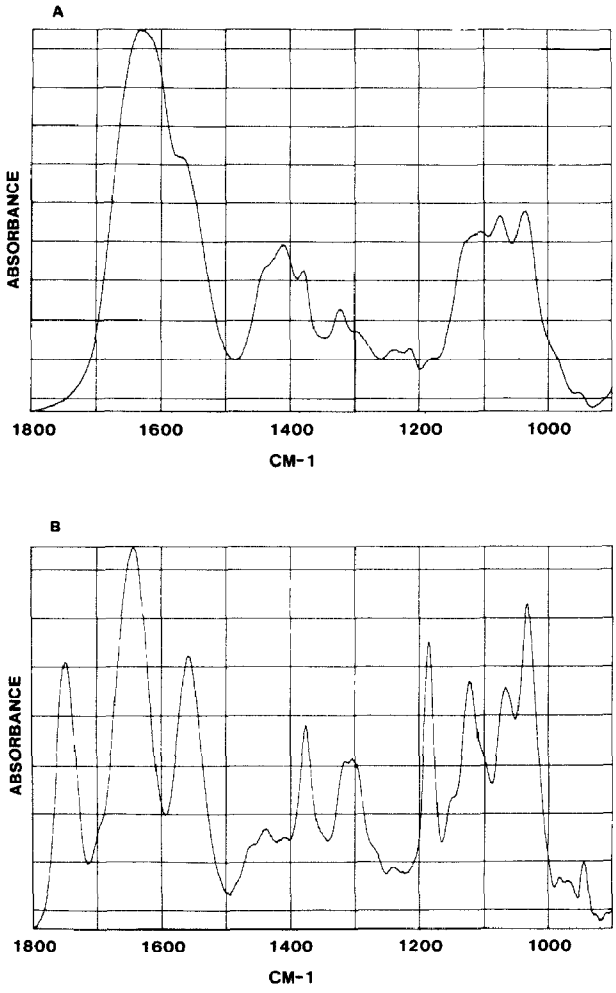



Fig. 1. I.r. spectrum of the K92 polysaccharide ($Ca^{2+}$ salt) before (A) and after (B) treatment with carbodi-imide.

The results of gel filtration (on Sepharose CL-4B) of the native, carbodi-imide-treated, and free-acid forms of K92 polysaccharide, either with or without subsequent borohydride reduction, are shown in Table II. Only the salt form of the polysaccharide was eluted to a significant extent before $K_D$ 0.5. Carbodi-imide treatment of the polysaccharide reduced the apparent molecular weight ($K_D$ 0.59), suggesting that intermolecular cross-linking did not occur, and the molecular size was not altered significantly ($K_D$ 0.61) upon borohydride reduction. Following incubation of the free-acid form of the K92 polysaccharide at room temperature for 24 h (pH 2.6), the product had a low apparent molecular weight ($K_D$ 0.81), which, again, was unaffected ($K_D$ 0.82) by subsequent borohydride reduction.

*Inhibition of antigenicity.* — Following treatment of the K92 polysaccharide

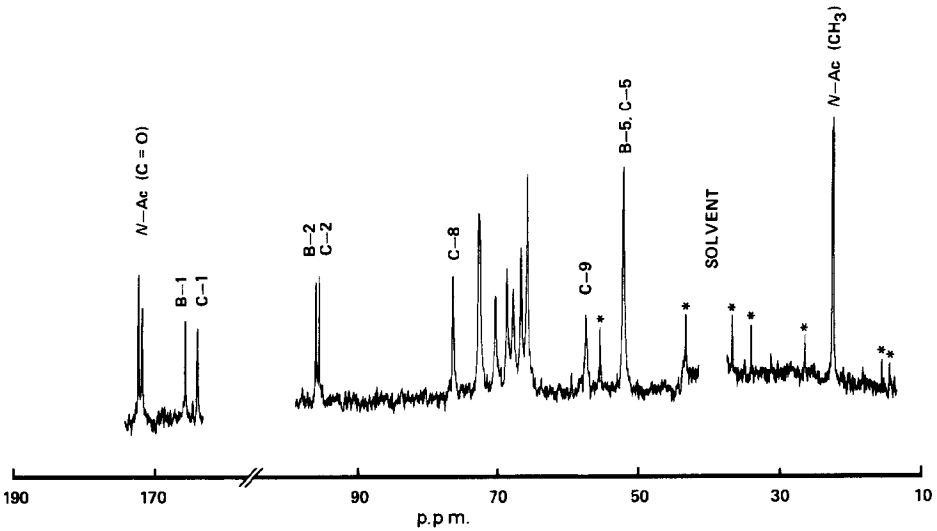



Fig. 2. $^{13}$C-N.m.r. spectrum of the carbodi-imide-treated K92 polysaccharide; *, minor products derived from the carbodi-imide (urea or *O*-acylisourea).

at low pH or with a carbodi-imide, and subsequent borohydride reduction, the partially carboxyl-reduced derivatives were tested for inhibition of antigenicity in a radioimmunoassay. The results in Table I show that the product obtained by incubation of the free-acid form (pH 2.6) for 24 h at room temperature, followed by borohydride reduction (12% lactonisation), had a ~10-fold decrease in antigenicity. However, prior removal of lactonised residues from the free-acid form of the K92 polysaccharide by alkaline hydrolysis also resulted in a ~10-fold decrease in antigenicity. Thus, loss of antigenicity at low pH is determined by a decrease in molecular size (see Table II) rather than by lactonisation. As expected, borohydride reduction of the carbodi-imide-treated K92 polysaccharide (~90% lactonisation) resulted in no detectable antigenicity. However, by prior removal of lactonised residues by alkaline hydrolysis, antigenicity was restored, thus implicating lactone formation, and not molecular size, as being responsible for loss of antigenicity.

*Periodate oxidation.* — The periodate consumption of the K92 and meningococcal B and (*O*-Ac$^-$)-C polysaccharides over 7 days is shown in Fig. 3. The (*O*-Ac$^-$)-C polysaccharide consumed ~0.9 μmol of periodate/μmol of NeuNAc in 96 h, due to vicinal HO-groups at C-7,8 whereas the B polysaccharide, which is linked through C-8, consumed only 0.1 μmol of periodate/μmol of NeuNAc during the same time. Unexpectedly, however, the K92 polysaccharide was resistant to periodate oxidation, and consumed only 0.22 μmol of periodate/μmol of NeuNAc (44% of the theoretical value) in 96 h. Furthermore, since overoxidation of the B polysaccharide occurs, oxidation of the K92 polysaccharide is probably not confined to the C-7,8 vicinal-diol group. Thus, the (2→9)-α-linked sialic acid residues of the

TABLE II

PROPERTIES OF THE K92 POLYSACCHARIDE BEFORE AND AFTER TREATMENT AT LOW pH OR WITH A CARBODI-IMIDE (EDC)

| *Incubation conditions* | *Formation of lactone/O-acylisourea (%)* | | | | *Molecular size*[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | *Gl.c.* | *I.r.* | | | *%$K_D$ 0* | *%$K_D$ <0.5* | *Secondary peak* | |
| | | *C=O (1745 cm$^{-1}$)* | *C=O (1695 cm$^{-1}$)* | *C–O (1190 cm$^{-1}$)* | | | *$K_D$ value* | *Percent* |
| Salt form | 0 | 0 | 0 | 0 | 2 | 74 | 0.44 | 97 |
| EDC-treated | — | 66 | <5 | 76 | 0 | 16 | 0.59 | 96 |
| EDC-treated/$NaBH_4$-reduced | 94 | — | — | — | 0 | 14 | 0 61 | 100 |
| $H^+$ form, 24 h, room temp. | — | [a] | — | [b] | 0 | 0 | 0.81 | 100 |
| $H^+$ form, 24 h, room temp., $NaBH_4$-reduced | 12 | — | — | — | 0 | 0 | 0.82 | 100 |

[a]Not determined due to the presence of a COOH C=O band near 1725 cm$^{-1}$. [b]Not determined due to the presence of a COOH C–O band near 1180 cm$^{-1}$.
[c]$K_D = (V_e - V_0)/(V_t - V_0)$, where $V_0$ = void volume, $V_t$ = total volume, and $V_e$ = elution volume.

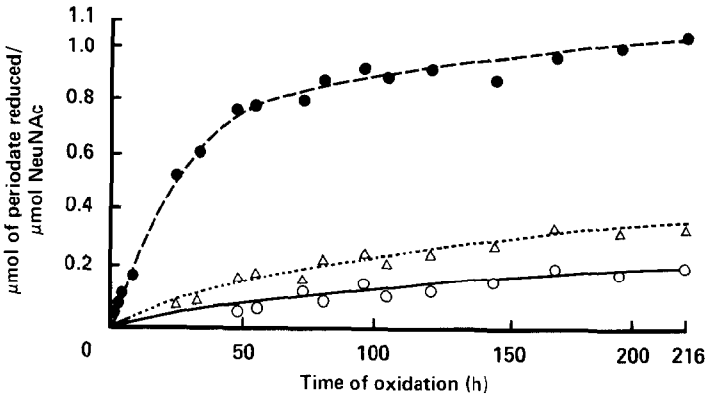



Fig. 3. Periodate oxidation of the B (—○—), (*O*-Ac⁻)-C (– –●– –), and K92 polysaccharides (---△---).

K92 polysaccharide are considerably more resistant to periodate oxidation than are those of (*O*-Ac⁻)-C polysaccharide.

*Conformation of the K92 polysaccharide.* — The $^1$H-n.m.r. spectra of the meningococcal B and (*O*-Ac⁻)-C polysaccharides have been assigned[3]. The side chain of the B polysaccharide adopts a conformation such that H-7,8 are *gauche* (**1a** or **1b**), whereas they are antiperiplanar (**2**) in the (*O*-Ac⁻)-C polysaccharide. The chemical shifts and coupling constants in the $^1$H-n.m.r. spectrum of the K92 polysaccharide have now been assigned by means of two-dimensional $^{13}$C–$^1$H correlation spectroscopy, since the $^{13}$C chemical shifts are known[1]. The results are plotted as a contour map (Fig. 4). A peak appears where the $^{13}$C chemical shift of the signal of a $CH_n$ fragment intersects with its $^1$H chemical shifts. An expansion of the region containing C-4/9 for both (2→8)-α- (B) and (2→9)-α-linked (C) sialic acid residues is shown in Fig. 5. In the following argument, H-4(B) and C-4(B) will, for example, represent H-4 and C-4, respectively, of the (2→8)-α-linked residues in the K92 polysaccharide.

The *N*-acetyl methyl and C-3 methylene groups are trivially assigned (see Fig. 4), and the expanded contour plot (Fig. 5) enables the assignment of the other $^1$H chemical shifts (see Experimental). The digital resolution in the projection of the $^1$H spectrum precludes further analysis, but recourse to a resolution-enhanced one-dimensional spectrum (Fig. 6) allows the relevant coupling constants to be extracted. In addition, the assignments and, particularly, the $^1$H shifts of the signals of the hydrogens attached to C-4(B), C-4(C), and C-7(C) which have very similar $^{13}$C shifts, were confirmed by double-resonance difference experiments.

Fig. 6 shows that the resonances at δ 4.18 and 4.00, assigned to H-8(B) and H-8(C), respectively, each have three coupling constants, as expected. The resonance due to C-9(B) at δ 4.13 showed a large coupling constant (~12 Hz) consistent with the expected geminal H-9(B)–H-9′(B) coupling, and a smaller coupling, with second-order effects, to the resonance assigned to H-8(B). Analysis of the H-8(B) multiplet showed three couplings, *viz.* $J_{7,8}$(B), $J_{8,9}$(B), and $J_{8,9'}$(B), each

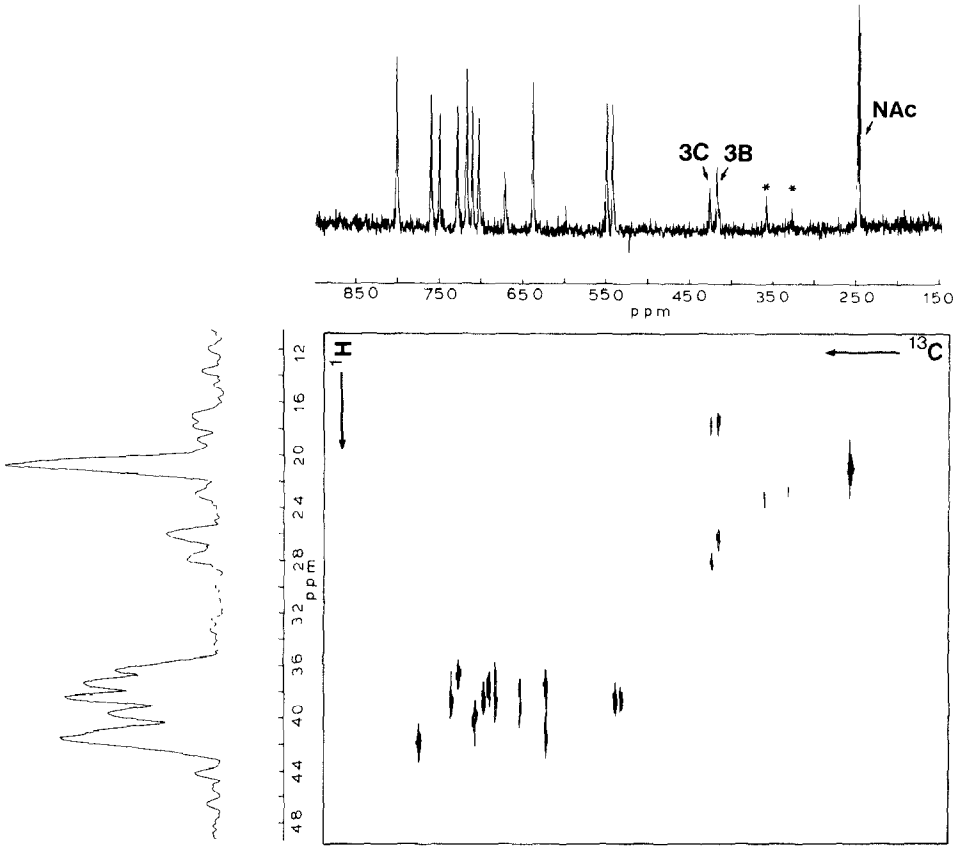



Fig. 4. Two-dimensional contour plot of the correlation between $^{13}$C and $^{1}$H chemical shifts of the K92 polysaccharide, with assignments as marked; *, minor impurity peaks.

of ~4 Hz. Irradiation of the H-8(B) resonance collapsed the adjacent H-9(B) resonance to a geminally coupled doublet, and perturbed the spectrum at $\delta$ 3.85 and 3.75. Using double-resonance difference spectroscopy, the resonance at $\delta$ 3.75 showed the geminal coupling constants, thus confirming it as that of H-9′(B). It follows that the resonance at $\delta$ 3.85 is due to H-7(B), which, in the decoupled spectrum, appears as a broad singlet, showing that $J_{6,7}$(B) is <1 Hz. All assignments for the side-chain protons of the (2→8)-$\alpha$-linked (B) residues are in agreement with the two-dimensional contour plot and, because of the values $J_{6,7}$(B) <1 and $J_{7,8}$(B) ~4 Hz, the conformation of the side-chain is identical to that found in the meningococcal B polysaccharide[3], *i.e.*, H-6,7 and H-7,8 are *gauche* (**1a** or **1b**).

Confirmation of the assignments for the (2→9)-$\alpha$-linked (C) residues was more difficult. The H-8(C) resonance has been assigned to the peak at $\delta$ 4.00 and irradiation at this frequency showed perturbations at $\delta$ ~3.7 and ~3.9; the former appeared as a single line when decoupled, thus confirming it as the H-7(C)

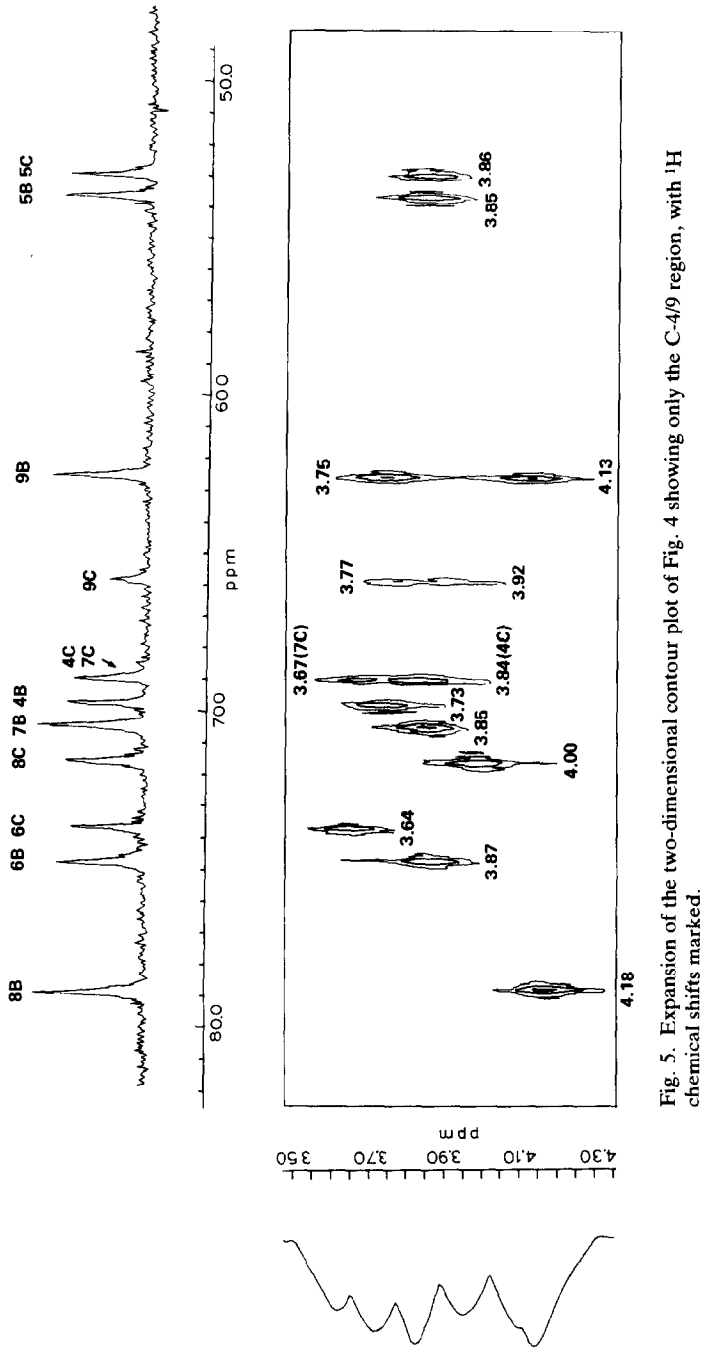



Fig. 5. Expansion of the two-dimensional contour plot of Fig. 4 showing only the C-4/9 region, with $^1H$ chemical shifts marked.

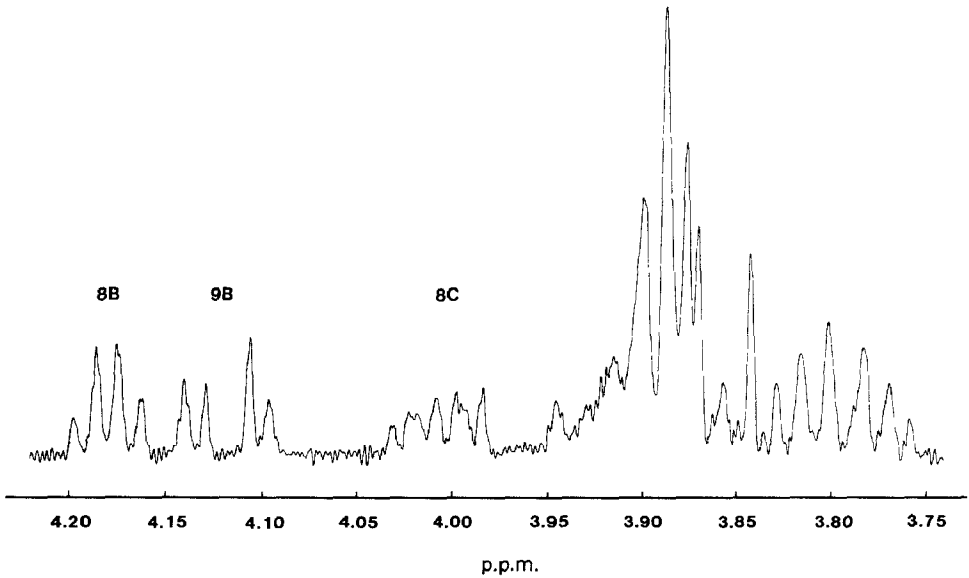



Fig. 6. Resolution-enhanced 360-MHz $^1$H-n.m.r. spectrum of the K92 polysaccharide, with assignments of chemical shifts as shown.

resonance, since H-9(C) would be expected to show geminal coupling. The two shifts due to the signals for H-9(C) and H-9′(C) were difficult to distinguish from those of H-7(B) ($\delta$ 3.85) and H-9′(B) ($\delta$ 3.75). Analysis of the H-8(C) spin-coupling pattern on a first-order basis showed three couplings; irradiation of the resonance due to H-7(C) collapsed the $J_{7,8}$ coupling; and inspection of the H-8(C) resonance showed $J_{7,8}$ to be 8.4 Hz. Since $J_{6,7}$ was hidden within the line-width of H-7(C), the coupling constant must be <3 Hz. These results show that the conformation of the side-chain of the (2→9)-$\alpha$-linked (C) residues is such that H-6,7 are close to *gauche*, and H-7,8 are antiperiplanar (**2**) as found in meningococcal (*O*-Ac$^-$)-C polysaccharide[3].

*Solution dynamics of the K92 polysaccharide.* — Dynamic information is derived from interpretation of the $^{13}$C spin–lattice relaxation-times ($NT_1$) (see Experimental).

The (2→8)-$\alpha$-linked (B) residues of the K92 polysaccharide showed a constancy of $^{13}$C relaxation-times for C-4/8, which indicates that there is no segmental motion for the backbone of these residues. However, C-9(B), the pendant $CH_2OH$ group, had a longer $NT_1$ than the rigid backbone, demonstrating some extra degree of motion besides the overall molecular tumbling. Similar results were found for the meningococcal B polysaccharide[3].

The (2→9)-$\alpha$-linked (C) residues of the K92 polysaccharide also showed a constancy of $NT_1$ values for C-4/8, and only C-9 showed any significant additional flexibility. These results differ from those obtained for the meningococcal (*O*-Ac$^-$)-C polysaccharide[3] where the $NT_1$ values indicated flexibility along the whole of the

C-7,8,9 side-chain. Use of the model of isotropic reorientation[3], assuming an average $NT_1$ value of 0.22 s, leads to a correlation time, $\tau_R$ of $4.0 \times 10^{-9}$ s, and an internal rotation correlation time for the C-9(B) $CH_2OH$ group, $\tau_G$ (B), of $0.6 \times 10^{-10}$ s. Furthermore, since it appears that only C-9 of the (2→9)-α-linked (C) residues showed any significant additional flexibility, it is possible to use the model of Doddrell *et al.*[11], allowing one degree of internal motion, on the C-9(C) group, giving $\tau_G(C) = 0.8 \times 10^{-10}$ s. Thus, the C-9 groups of both (2→8)-α-linked (B) and (2→9)-α-linked (C) residues have an internal rotational correlation time ~50 times faster than the overall molecular tumbling.

## DISCUSSION

The K92 polysaccharide cross-reacts immunologically with the meningococcal C polysaccharide but not with the meningococcal B polysaccharide[4], consistent with our findings[5] that antibodies against the latter recognise a conformational determinant. The results presented here suggest that the K92 polysaccharide has both similarities to, and differences from, the B and C polysaccharides.

Lactonisation takes place at low pH with ease in the B polysaccharide (**1→3**), but not at all in the C polysaccharide (**2→4**)[5,6]. Lactonisation at low pH in the K92 polysaccharide was expected to occur preferentially between the carboxyl group of a (2→9)-α-linked (C) residue and HO-9 of an adjacent (2→8)-α-linked (B) residue (*i.e.*, potentially 50% of the sialic acid residues in the polymer). In practice, the K92 polysaccharide typically underwent 10–20% lactonisation, which indicates that some of the (2→8)-α-linked (B) residues cannot form lactones, perhaps due to steric constraints imposed by flanking (2→9)-α-linked (C) residues.

Treatment of both the B and K92 polysaccharides under more-forcing conditions with EDC, a water-soluble carbodi-imide, gave a fully lactonised product, with *O*-acylisourea formation as only a minor pathway. Significant *O*-acylisourea formation (~20%) occurs in the (*O*-$Ac^-$)-C polysaccharide[5], which is prevented from proceeding further towards lactone formation, probably because of the unfavourable disposition of the activated carboxyl group towards HO-8 of an adjacent residue. Since *O*-acylisourea formation is only a minor pathway (<5%) in the K92 polysaccharide, the carboxyl group of a (2→8)-α-linked (B) residue and HO-8 of an adjacent (2→9)-α-linked (C) residue are favourably disposed for lactonisation.

Carboxyl-reduction[5] of 10–20% of the residues causes a marked decrease in the antigenicity of the B polysaccharide, but has little or no effect upon the antigenicity of the C polysaccharide. Lactonisation of the K92 polysaccharide followed by borohydride treatment should cause predominantly a reduction of the carboxyl groups of the (2→9)-α-linked (C) residues. In the r.i.a., incubation at low pH followed by borohydride treatment gave 12% carboxyl reduction, which resulted in an ~10-fold decrease in the antigenicity of the K92 polysaccharide. However, this is due to a substantial lowering of the molecular weight following incubation at low pH, as observed by gel filtration on Sepharose CL-4B, rather than to carboxyl-

reduction of the polymer, since the K92 polysaccharide, when incubated at low pH and then alkali-treated to reverse lactonisation, also underwent an ~10-fold decrease in antigenicity. Thus, the K92 polysaccharide, like the C polysaccharide, does not undergo significant loss of antigenicity by lactonisation of ~10% of the residues. This finding suggests that rabbit anti-C antisera, which cross-react with the K92 polysaccharide, recognise a sequential determinant on the molecule, rather than a conformational determinant as seems to be the case for B polysaccharide[5]. Only when ~90% of the carboxyl groups in the K92 polysaccharide have been reduced does the antigenicity markedly decrease, which suggests that the carboxyl group is an important part of the determinant of the polymer.

The (*O*-Ac$^-$)-C polysaccharide consumes 0.9 μmol of periodate/μmol of NeuNAc in 96 h (90% of theoretical), due to the 7,8-diol group. The B polysaccharide is linked through C-8 and, therefore, is resistant to periodate as reflected by the slow uptake of periodate, presumably due to overoxidation. The K92 polysaccharide was expected to consume 0.5 μmol of periodate/μmol of NeuNAc at a rate comparable to that for the (*O*-Ac$^-$)-C polysaccharide. In practice, the rate was much lower, indicating significant differences between the (2→9)-α-linked sialic acid residues of the K92 and (*O*-Ac$^-$)-C polysaccharides, particularly in the C-7,8 part of the molecule. Free NeuNAc consumes[14] the theoretical amount of periodate (2 μmol/μmol of NeuNAc) within 10 min, and 9-*O*-acetylated NeuNAc has a much lower rate of oxidation [although comparable with that of the (*O*-Ac$^-$)-C polysaccharide], ascribed to the antiperiplanar disposition of HO-7,8. The (*O*-Ac$^-$)-C polysaccharide has[3] this antiperiplanar conformation (**2**); the lower oxidation rate in the K92 polysaccharide, therefore, is not explained solely by steric hindrance of the vicinal diol group. Other factors, including hydrogen-bonding[15], hemiacetal formation[15–17], and electrostatic repulsion of carboxyl groups[18,19], have been suggested to explain the low consumption of periodate. The (2→9)-α-linked (C) side-chain of the K92 polysaccharide, as determined by $^{13}C$ relaxation-times, is less flexible than that of the (*O*-Ac$^-$)-C polysaccharide, additional rigidity presumably being conferred by the flanking (2→8)-α-linked (B) residues, and this may also help to explain the low consumption of periodate by the K92 polysaccharide.

The K92 polysaccharide is characterised by internal or segmental motion of only the C-9(B) and C-9(C) parts of the molecule, which rotate ~50 times faster than the overall molecular tumbling. Since C-9(C) is involved in the ketosidic linkage, however, this suggests that the polysaccharide chains will have a considerable degree of freedom of motion, similar to that predicted for the (*O*-Ac$^-$)-C polysaccharide, but different to the relative rigidity envisaged for the B polysaccharide.

The conformation of both the (2→8)-α-linked (B) and (2→9)-α-linked (C) residues of the K92 polysaccharide are very similar to their counterparts in the B and (*O*-Ac$^-$)-C polysaccharide. The lack of cross-reactivity between the K92 and B polysaccharides, therefore, does not seem to be due to differences in the conformation of the individual (2→8)-α-linked sialic acid residues, suggesting that anti-B

antisera recognise a determinant larger than the monosaccharide repeating-unit of B polysaccharide, and consistent with our finding that B polysaccharide has a conformational determinant[5].

ACKNOWLEDGMENTS

We thank Dr. A. S. Gilbert for recording the i.r. spectra, Mr. D. C. Brown for isolating and purifying K92 polysaccharide, and Mrs. J. Esdaile and Miss U. Nowicka for their technical assistance.

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