

## STRUCTURAL STUDIES OF THE *Escherichia coli* K93 AND K53 CAPSULAR POLYSACCHARIDES

AD BAX,

*Laboratory of Chemical Physics, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 (U.S.A.)*

MICHAEL F. SUMMERS, WILLIAM EGAN\*,

*Biophysics Laboratory, Office of Biologics Research and Review, 8800 Rockville Pike, Bethesda, Maryland 20892 (U.S.A.)*

NABIL GUIRGIS, RACHEL SCHNEERSON, JOHN B. ROBBINS,

*National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892 (U.S.A.)*

FRITS ØRSKOV, IDA ØRSKOV,

*WHO Collaborative Center for Reference and Research on Escherichiae, Statens Serum Institut, Copenhagen (Denmark)*

AND WILLIE F. VANN

*Bacterial Polysaccharides Laboratory, Office of Biologics Research and Review, 8800 Rockville Pike, Bethesda, Maryland 20892 (U.S.A.)*

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### ABSTRACT

The structures of the *Escherichia coli* K93 and K53 capsular polysaccharides have been investigated by chemical and spectroscopic methods. The repeating unit of both polymers was found to be  $\rightarrow 3$ )- $\beta$ -D-Galf-(1 $\rightarrow$ 4)- $\beta$ -D-GlcAp-(1. The O-5 and O-6 atoms of D-galactose are acetylated in the repeating unit of the K93 polymer, but only O-2 is acetylated in the K53 polymer. The K93 polysaccharide is cross-reactive with the *Neisseria meningitidis* Group A capsular polysaccharide (of known structure). The K53 polysaccharide, although structurally similar to that from K93 organisms, does not cross-react with the Group A polymer.

### INTRODUCTION

Guirgis *et al.*<sup>1</sup> have recently reported that the capsular polysaccharides from *Escherichia coli* K93 and K51 organisms are cross-reactive with that obtained from Group A *Neisseria meningitidis*, a serogroup responsible for severe, invasive disease in humans. Group A meningitis occurs annually with high frequency in central Africa<sup>2</sup> and as 1-2-year epidemics in other parts of the world<sup>3</sup>. In the United States, during the past 30 years, Group A meningococci have been detected only rarely in either patients or asymptomatic carriers<sup>4</sup>. Despite this virtual absence of

\*To whom correspondence should be addressed.

exposure, most adults in the U. S. have protective levels of Group A capsule-directed antibodies<sup>5</sup>, the antigenic stimulus for which has been postulated to derive from non-pathogenic, cross-reacting bacteria of either the respiratory or gastrointestinal tracts<sup>6</sup>. *E. coli* K93 and K51 organisms are probable sources for this "natural immunity". It is unlikely that other bacteria that cross-react with the meningococcal Group A polysaccharide capsule, and which include *Bacillus pumilis* (strain Sh17), or *Streptococcus faecalis* species, or rare strains of *S. faecum*, could serve as this antigenic stimulus<sup>7</sup>.

The structure of the Group A meningococcal capsular polysaccharide has been shown to be a partially *O*-acetylated,  $\alpha$ -(1 $\rightarrow$ 6)-phosphoric diester-linked homopolymer of 2-acetamido-2-deoxy-D-mannose<sup>8,9</sup>. We sought to determine the structures of the *E. coli* K93 and K51 capsular polysaccharides with a view to establishing more firmly the molecular basis for the cross-reactivity between these polysaccharides and that of meningococcal Group A. During the course of this study, it was found that the *E. coli* K53 capsular polysaccharide cross-reacts with K93, but not the Group A meningococcal polysaccharide. We now report the structures of the K93 and K53 capsular polysaccharides.

## RESULTS

*K93 Capsular polysaccharide.* — The *E. coli* K93 polysaccharide is negatively charged at basic pH (8.6), as evidenced by its anodic migration during immunoelectrophoresis, and by titration. Following acid-catalyzed hydrolysis, D-galactose is the only sugar observed in appreciable quantities (plus a small proportion of D-glucose; *vide infra*). Following reduction of the carboxyl groups (by carbodiimide and sodium borohydride), and acid-catalyzed hydrolysis, D-galactose and D-glucose occur in the molar ratio of  $\sim$ 1.0:0.9. These sugars were identified as alditol acetates by using g.l.c.; their absolute configurations were determined enzymically (D-glucose oxidase and D-galactose oxidase) and gas-liquid chromatographically, following glycosidation with optically active 2-octanol. Other sugars were not observed. The small amount of glucose detected (as the alditol) in the first-mentioned hydrolyzate (wherein the carboxylic acid groups were not reduced prior to acid-catalyzed hydrolysis) probably arises from borohydride reduction (during the post-hydrolysis step to form alditols) of a glucuronic acid lactone that forms during acid-catalyzed hydrolysis of the native polysaccharide (or that had been present to a small extent in the native polysaccharide).

The <sup>13</sup>C-n.m.r. spectrum of the K93 capsular polysaccharide displays eleven resonances of approximately equal integrated absorption intensity in the 60–115-p.p.m. region of the spectrum (see Table I), and is in accord with the chemical composition already presented; resonances that could be attributable to other sugars were not noted. Several resonance assignments in the carbon spectrum of the K93 polysaccharide can be readily made on the basis of chemical-shift comparisons with spectra derived from model compounds<sup>10</sup>. Anomeric carbon

resonances observed at 110.4 and 105.7 p.p.m. indicate that C-1 of each sugar unit is involved as a glycoside<sup>10,11</sup>. The signal at 66.2 p.p.m. corresponds to the C-6 atom on the D-galactosyl ring, its assignment being confirmed by obtaining the spectrum under <sup>1</sup>H-coupled conditions and observing this resonance as a widely-spaced triplet ( $J \sim 150$  Hz); C-6 of D-galactose is the only carbon atom in the polysaccharide bearing two hydrogen atoms, and, thus, the only one that could give rise to a triplet in the <sup>1</sup>H-coupled spectrum. The signal at 78.8 p.p.m., which shifts upfield to 76.9 p.p.m. on lowering the solution pH to 2.5, most probably corresponds to the C-5 atom of the D-glucuronic acid residue (*vide infra*); similar changes in chemical shift with change in the pH value of the solution have been noted<sup>10</sup> for uronic acids. In addition to these eleven signals in the 65–110-p.p.m. region, an *O*-acetyl methyl group resonance, with intensity corresponding to two carbon atoms, was seen at 23.2 p.p.m. Three carbonyl carbon resonances were observed at 176.2, 176.6, and 177.5 p.p.m., corresponding to the two *O*-acetyl carbonyl carbon atoms and the carboxylate carbon atom of the glucuronic acid, respectively; on lowering the solution pH value to 2.5, the 177.5-p.p.m. signal shifted upfield to 175.5 p.p.m. Following mild treatment of the polysaccharide with sodium hydroxide, neutralization of the base with HCl, and dialysis against distilled water, the signals at 23.2, 176.2, and 176.6 p.p.m. were no longer observable. The accumulated data indicate a di-*O*-acetylated, regular polymer having a glucuronic acid–galactose repeating unit. To complete the characterization of the polymer, it remained to establish: (i) sugar ring-forms (pyranoid or furanoid), (ii) anomeric

TABLE I

<sup>13</sup>C-N.M.R. CHEMICAL SHIFTS OF THE *Escherichia coli* K93, *O*-DEACETYLATED K93, AND K53 CAPSULAR POLYSACCHARIDES<sup>a</sup>

Carbon atom <sup>b</sup>	Native K93 <sup>c</sup>	<i>O</i> -Deacetylated K93	K53
C-1	110.4 (110.2)	110.7	108.0
C-1'	105.7 (105.9)	105.1	104.8
C-3	88.7 (88.8)	87.9	86.0
C-4	84.6 (84.4)	85.4	85.7
C-2	82.4 (82.5)	82.9	84.3
C-4'	81.2 (80.7)	81.0	80.4
C-5'	78.8 (76.9)	78.9	78.8
C-3'	76.4 (76.4)	76.7	76.7
C-2'	75.6 (75.6)	75.8	75.7
C-5	72.7 (72.3)	73.3	72.9
C-6	66.2 (66.2)	66.0	65.7
C(O)CH <sub>3</sub>	23.1 (23.2)		22.8
C(O)CH <sub>3</sub>	176.2 (176.2)		175.3
C(O)CH <sub>3</sub>	176.7 (176.6)		
CO <sub>2</sub> H	177.5 (175.5)	178.1	177.3

<sup>a</sup>Chemical shifts are relative to internal sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-*d*<sub>4</sub>. The solution pH was 7.0, unless otherwise noted. <sup>b</sup>Primed numbers for carbon atoms refer to the glucosyluronic acid residue, whereas unprimed carbon atoms refer to the galactosyl residue. <sup>c</sup>Numbers in parentheses refer to chemical shift values at a solution pH of 2.5.

stereochemistries, (iii) linkage sites, and (iv) *O*-acetylation sites. Assigning, for the moment, the 105.9-p.p.m. signal to C-1 of the glucuronic acid residue<sup>10</sup>, the remaining anomeric carbon resonance (at 110 p.p.m.) arises from the galactosyl residue. The unusually low-field shift of this resonance indicated that the sugar is in the furanoid form and, moreover, has<sup>12</sup> the  $\beta$ -stereochemistry at C-1 as the chemical shift for the anomeric carbon atom of a galactosyl residue is  $\sim 100$  p.p.m. for both anomers when in the pyranoid form<sup>10,12</sup>. A <sup>13</sup>C-chemical shift of  $\sim 105$  p.p.m. for the anomeric carbon atom of a glucosyl residue is characteristic of the  $\beta$  anomer; the one-bond, <sup>1</sup>H-<sup>13</sup>C-1 coupling constant, obtained from the <sup>1</sup>H-coupled spectrum, was 165 Hz, also indicating the  $\beta$ -stereochemistry<sup>13</sup>.

The <sup>1</sup>H-n.m.r. spectrum was next examined in order to gain further structural information about the K93 polymer. A two-dimensional (2D), proton-proton correlated spectrum (COSY spectrum)<sup>14</sup> of the polysaccharide is shown in Fig. 1. The proton connectivities (proton resonances connected through three-bond scalar couplings) for the two individual sugar rings were readily established from this spectrum. The signal for the anomeric proton of the glucosyluronic residue was observed at 4.45 p.p.m., a value characteristic of the  $\beta$ -stereochemistry at C-1, the

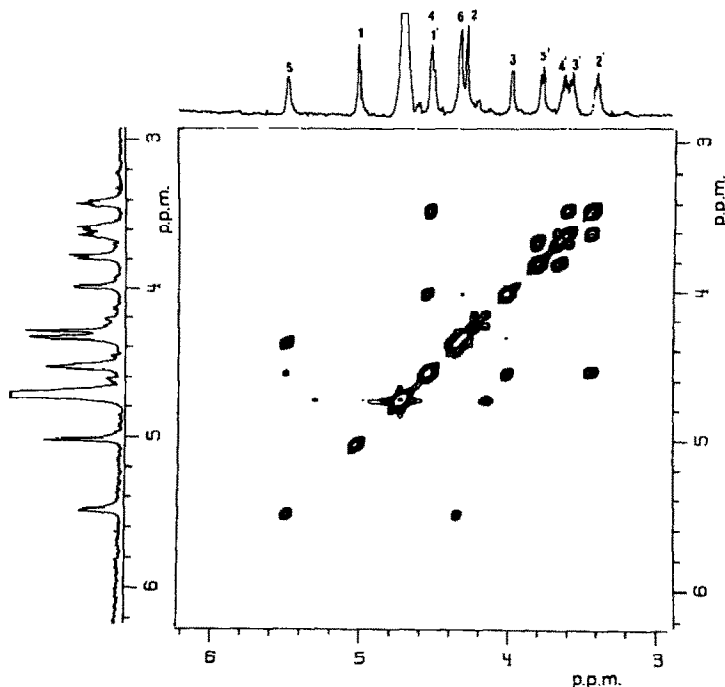


Fig. 1. Two-dimensional COSY spectrum (500 MHz) of the K93 capsular polysaccharide, at a concentration of  $\sim 10$  mg in 0.5 mL of D<sub>2</sub>O at a solution pH value of 7.0. The spectrum results from a  $512 \times 1024$  data matrix; only one-quarter of the 2D spectrum is displayed in the Figure. The total acquisition time was  $\sim 4$  h. A non-shifted sine-bell filtering function was used in both dimensions. In order to lessen spectrometer instabilities that generate "t<sub>1</sub>-noise", the sample was not spun.

corresponding value for the  $\alpha$ -stereochemistry being<sup>15</sup>  $\sim 5.1$  p.p.m. A cross-peak (off-diagonal peak) was observed between this resonance and that at 3.4 p.p.m., and it established that the latter resonance derived from H-2 of the glucosyluronic residue. The H-3, H-4, and H-5 resonances of the glucosyluronic residue were sequentially located in a similar fashion. Beginning with H-1 of the galactosyl residue (at 5.05 p.p.m.), the various proton resonances for this ring were similarly assigned. [The small value of the coupling constant between H-1 and H-2 of the galactosyl ring required the use of a contour level lower than that displayed in Fig. 1 in order to determine the H-1-H-2 connectivity.] The proton assignments for the two sugar rings, as derived from the COSY spectrum, are provided in Fig. 1.

A second 2D-correlated n.m.r. experiment was used to assign the <sup>13</sup>C resonances. In this heteronuclear correlated 2D experiment<sup>14</sup>, a one-to-one correspondence is established between the resonances from particular protons and the resonances of the carbon atoms to which they are *directly* attached. The 2D-heteronuclear correlated spectrum, and the resulting carbon resonance assignments, are shown in Fig. 2.

The proton and carbon resonance assignments, deriving from the 2D experiments, were consistent with the resonance assignments made solely on the basis of analogies of chemical shift to those of model compounds, and of

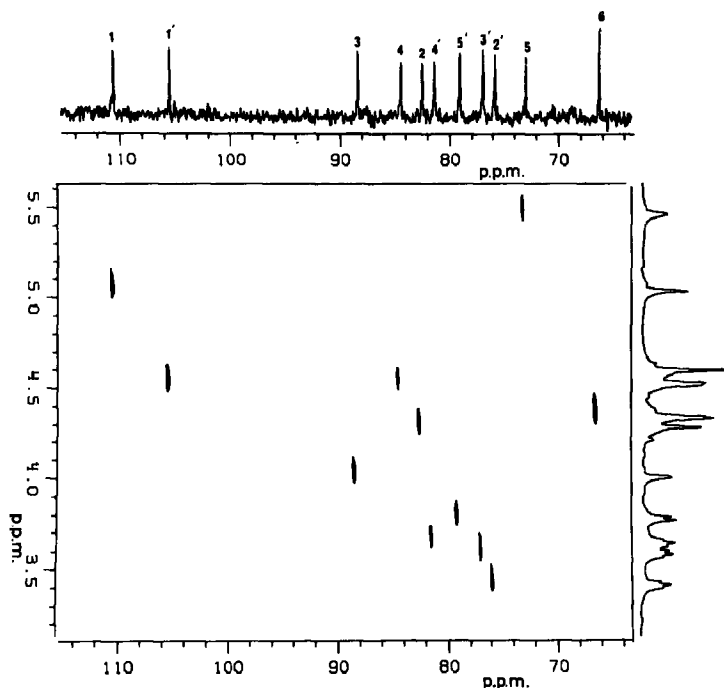


Fig. 2. Two-dimensional proton-carbon correlated spectrum of the K93 capsular polysaccharide (500 MHz proton frequency). The spectrum resulted from a  $128 \times 1024$  data matrix. Moderate Gaussian to Lorentzian resolution enhancement was used in both directions. The total measuring time was  $\sim 2$  h.

multiplicities in the  $^1\text{H}$ -coupled  $^{13}\text{C}$ -spectrum (*vide supra*). The value for the H-1–H-2 coupling constant for the glucosyluronic residue ( $\sim 8$  Hz) confirmed the assignment of the  $\beta$ -stereochemistry to this residue, a value of  $< 3$  Hz being expected for the  $\alpha$  anomer. The downfield chemical shifts in the carbon spectrum, relative to corresponding chemical-shift values for model compounds, indicated that C-3 of the galactosyl residue and C-4 of the glucosyl residue were alkoxyated.

A previously presented<sup>16</sup>, selective INEPT n.m.r. experiment (based on 3-bond  $\text{H}-\text{C}-\text{O}-\text{C}$  scalar couplings) was applied to the study of the K93 polysaccharide, in order to establish a connectivity between an anomeric proton and the carbon atom, three bonds removed, of the aglycon. Connectivities between H-1 of the glucosyluronic ring and C-3 of the galactosyl residue, as well as between H-1 of the galactosyl ring and C-4 of the glucosyluronic residue, were thereby established; see Fig. 3. These results were in accord with expectations based on considerations of chemical-shift values (*vide supra*). The selective INEPT experiments were consistent, moreover, with the glucosyluronic residue's being in the pyranoid form and having the  $\beta$ -stereochemistry at C-1, and the galactosyl residue's being in the furanoid form; thus, a transfer of polarization was observed from the galactosyl H-1 to the galactosyl C-3 and C-4, while no transfer was observed from the glucosyluronic H-1 to any of the glucosyluronic carbon atoms; *cf.* ref. 16.

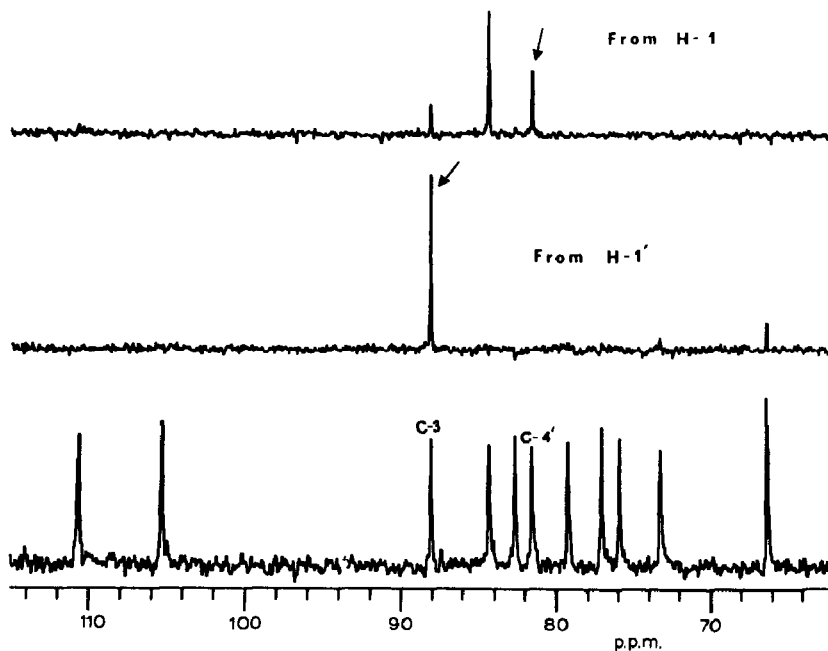


Fig. 3. Selective INEPT spectra (500 MHz proton frequency) of the K93 capsular polysaccharide. The spectrum resulting from selective pulsing of the galactosyl H-1 atom is shown in the top tracing, and that from selective pulsing of the glucosyluronic proton in the middle tracing. The normal  $^{13}\text{C}$ -n.m.r. spectrum is displayed in the bottom tracing. Four thousand transients were accumulated for each of the selective INEPT spectra and 400 transients were collected for the normal spectrum.

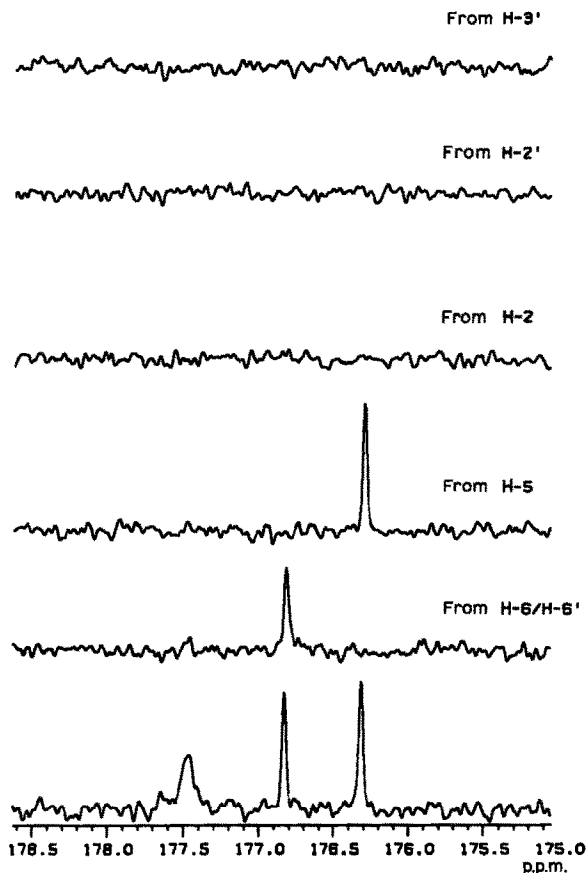


Fig. 4. Selective INEPT experiment (500 MHz proton frequency) showing the carbonyl region of the  $^{13}\text{C}$ -n.m.r. spectrum of the K93 polysaccharide. The normal  $^{13}\text{C}$ -n.m.r. spectrum is shown in the bottom trace; the spectra resulting from the selective pulsing of H-5 and H-6/6' of the galactosyl residue are shown in the indicated traces. For comparison, the results of pulsing H-2, H-2' and H-3' are shown in the top three traces.

The sites of *O*-acetylation of the K93 polysaccharide were next studied. Evidence that O-5 of the galactosyl residue was acetylated came from the unusually low-field chemical shift of H-5 in the  $^1\text{H}$ -n.m.r. spectrum (see Fig. 1). [When the K93 polymer was treated with NaOH to remove *O*-acetyl groups, this low-field resonance disappeared (data not shown).] The site of attachment of the second *O*-acetyl group was not, however, apparent from changes in the  $^1\text{H}$ -n.m.r. spectrum. Changes in the  $^{13}\text{C}$ -n.m.r. spectrum following *O*-deacetylation were too minor to permit determining sites of acetylation (see Table I). The attachment sites of the acetyl groups were established with a second series of selective INEPT spectra, wherein polarization was transferred from specific sugar protons to the carbonyl carbon atom of the acetyl group [ $\text{H}-\text{C}-\text{O}-\text{C}(\text{O})\text{CH}_3$ ]. This set of experiments (see Fig. 4) demonstrated unequivocally that O-5 and O-6 of the galactosyl residue are acetylated.

*The K53 capsular polysaccharide.* — The sugar composition of the K53 capsular polysaccharide was found to be identical to that of K93, containing nearly equimolar amounts of D-glucuronic acid and D-galactose. The  $^1\text{H}$ - and  $^{13}\text{C}$ -n.m.r. spectra of the K53 polysaccharide (see Table I) were consistent with this chemical analysis. The  $^1\text{H}$ - and  $^{13}\text{C}$ -n.m.r. spectra of the K53 polysaccharide (data not shown\*) differed from the corresponding K93 spectra, and, moreover, showed that the K53 polysaccharide is mono-*O*-acetylated. A set of 2D and selective INEPT n.m.r. experiments was conducted on the K53 polysaccharide.

The COSY spectrum (data not shown) allowed the proton resonances for both the galactosyl and glucosyluronic residues to be assigned in the manner already described for the K93 polysaccharide. Carbon resonances were then assigned relative to the proton resonances, using a heteronuclear correlated 2D-n.m.r. experiment (data not shown). Once again, there was agreement between carbon resonances that were readily assigned on the basis of chemical shift and resonance multiplicity (in the  $^1\text{H}$ -coupled  $^{13}\text{C}$ -n.m.r. spectrum) arguments (the anomeric carbon atoms, C-5 of the glucuronic acid residue, C-6 of the galactosyl residue) and those obtained from the 2D-n.m.r. correlation experiments. The remaining carbon and proton resonance assignments formed a self-consistent set. The  $\beta$ -anomeric stereochemistry is assigned to the glucosyluronic residue on the basis of its H-1–H-2 coupling constant, the chemical shift of its H-1 resonance, the chemical shift of its C-1 resonance, and its  $^{13}\text{C}$ -1– $^1\text{H}$ -1 coupling constant ( $\sim 165$  Hz); *vide supra*. On the basis of  $^{13}\text{C}$  chemical-shift considerations, it appeared that the glucosyluronic residue is alkoxyated at C-4, as in the K93 polymer. With the exception of C-1, the glucosyluronic resonances of the K93 and K53 polysaccharides were the same to within  $\sim \pm 0.2$  p.p.m.; the anomeric resonance differed by 0.8 p.p.m. The  $^{13}\text{C}$  chemical shifts of the galactosyl residue could be rationalized by postulating that O-2 of this residue was acetylated; such an acetylation pattern would cause the C-2 signal to shift downfield by  $\sim 2$ –5 p.p.m., and those of C-1 and C-3 to shift upfield  $\sim 0$ –3 p.p.m.<sup>10</sup>, relative to the *O*-deacetylated K93 polysaccharide; see Table I.

Selective INEPT experiments were performed in order to establish the linkage sites as well as the acetylation site (data not shown). These experiments confirmed the foregoing structural hypothesis. The K93 and K53 polysaccharides thus possess the same backbone structure, and differ only in the number and location of *O*-acetyl groups; K93 is di-*O*-acetylated, at O-5 and O-6 of the galactofuranosyl residue, whereas K53 is mono-*O*-acetylated, at O-2 of the galactofuranosyl residue. The structures of these two polysaccharides are depicted below.

The K93 and K53 capsular polysaccharides are mutually cross-reactive; however, only the K93 capsular polysaccharide is cross-reactive with the capsular polysaccharide from Group A *N. meningitidis* organisms (data not shown). The observable cross-reactivity (double diffusion) between the meningococcal group A and K93 capsular polysaccharides is lost following chemical removal of the *O*-acetyl

\*The spectra are available on request.



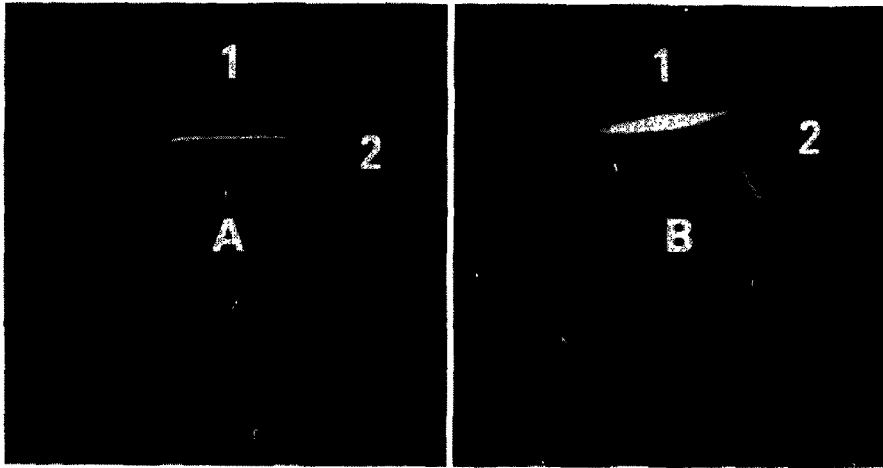
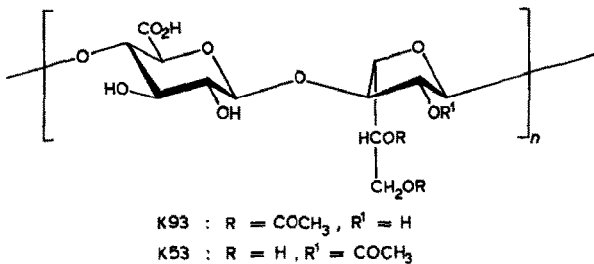


Fig. 5. Double immunodiffusion of antisera from purified polysaccharides. Key: A, meningococcal Group A antiserum (H49); B, *E. coli* K93 rabbit antiserum; 1, the native preparation of the K93 capsular polysaccharide; 2, K93 capsular polysaccharide after *O*-deacetylation.

groups from the K93 polymer (see Fig. 5). The native K93 capsular polysaccharide remains cross-reactive with the *O*-deacetylated K93 polysaccharide, although one or more antigenic determinants have been lost, as evidenced by the presence of a "spur" in the immunodiffusion experiment (see Fig. 5).



DISCUSSION

The close structural similarity between the K93 and K53 polysaccharides furnishes an obvious explanation for their serological cross-reactivity. The surprising findings of this study are, first, that the K93 and meningococcal Group A polysaccharides cross-react, and second, given the K93-Group A cross-reaction, that the K53 polysaccharide does not, as well, cross-react with the Group A polysaccharide. There is no obvious structural relationship between the K93 and Group A polysaccharides, which share no common sugar and differ also in that their negative charges are based on different functionalities: phosphoric diester for the meningococcal polymer and carboxylic acid for the K93 polymer; moreover, the

Group A polymer contains an amino sugar. The unexpected cross-reaction between these two polysaccharides presumably derives from a three-dimensional mimicry, by the K93, of a portion of the Group A capsular polysaccharide. A similarity in three-dimensional structure is also presumably operative in the cross-reactive type 8 and type 19 pneumococcal polysaccharides described by Heidelberger<sup>17</sup>; these polysaccharides have no sugar residue in common, and their negative charges derive from different functionalities. Alteration of the *O*-acetylation pattern, such as occurs on going from K93 to K53, or removal, as in the *O*-deacetylated K93, causes a loss of cross-reactivity with the meningococcal Group A polysaccharide. There is ample precedent for alterations in serological reactivity with changes in *O*-acetylation<sup>18</sup>. It is not yet known whether the *O*-acetyl groups are part of the antigenic determinant (that is, if they are part of the binding site to the antibody) or simply enforce a particular conformation, or set of conformation, on the polysaccharide.

A practical benefit may derive from the discovery of this cross-reactivity between the meningococcal and *E. coli* K93 polysaccharides. The Group A meningococcal polysaccharide is used worldwide as a vaccine, and is licensed in the United States by the Food and Drug Administration. A problem with this vaccine is its tendency to degrade, through hydrolysis of the phosphoric diester linkage, on prolonged storage. The K93 polysaccharide, lacking a labile phosphoric diester linkage, might provide a suitable adjunct or substitute.

#### EXPERIMENTAL

*Isolation of polysaccharide.* — *Escherichia coli* K93 and K53 were separately prepared by transferring 2.0 L of a 6-h growth to a 50-L fermenter containing minimal media, supplemented with 0.1%, dialyzed yeast extract<sup>19</sup>. After 16-h incubation at 37°, with vigorous aeration, the culture was centrifuged, and an equal volume of 0.2% (w/v) cetyltrimethylammonium bromide (Cetavlon, Sigma Chemical Co., St. Louis, MO.) in water was added to the supernatant liquor. The resultant precipitate was separated by centrifugation, and dissociated with 0.9M CaCl<sub>2</sub>. The polysaccharides, purified by treatment with DNase, RNase, cold phenol, and ultracentrifugation<sup>20</sup>, contained <1% (w/w) each of protein, nucleic acids, and LPS; the contaminants were analyzed as described<sup>21</sup>. The purified polysaccharide had a *K<sub>d</sub>* value of 0.04 on CL-4B Sepharose (Pharmacia, Inc., Piscataway, NJ) equilibrated in 0.2M NaCl.

*Antisera.* — Hyperimmune sera against Group A *N. meningitidis* (strain A1) and *E. coli* K93 and K53 were produced by multiple intravenous injections of formalinized cells into rabbits<sup>22</sup>.

*Polysaccharide sugar analysis.* — Sugars were identified as their alditol acetates using gas-liquid chromatography<sup>23</sup>. Hydrolysis and borohydride reduction conditions, as well as chromatographic conditions, have been described<sup>23</sup>. Absolute configurations were established by g.l.c. following glycosidation of the hydrolyzate

with optically active (*R*)-(-)-2-octanol<sup>24</sup> (Aldrich Chemical Co., Milwaukee, WI) and comparing with authentic standards [D-glucose and D-galactose with (*R*)-(-)-2-octanol, and D-glucose and D-galactose with (*S*)-(+)-2-octanol, the latter two being equivalent to L-glucose and L-galactose with (*R*)-(-)-2-octanol with regard to g.l.c.]; the carboxylate groups were reduced with sodium borohydride prior to the hydrolysis step.

Following carboxylic acid reduction and hydrolysis, absolute configurations of the sugars were also determined enzymically by alternately treating aliquots of the hydrolyzate with D-glucose oxidase [EC 1.1.3.4; Boehringer-Mannheim Biochemicals (Indianapolis, IN)] and D-galactose oxidase [EC 1.1.3.9; Sigma Chemical Co. (St. Louis, MO)], and monitoring, respectively, the disappearance of glucose and galactose on an automated sugar analyzer<sup>25</sup>.

*N.m.r. spectroscopy.* — The two-dimensional n.m.r. spectra as well as selective INEPT spectra, for the K93 capsular polysaccharide, were recorded with a Nicolet NT-500 spectrometer<sup>16</sup> (500 MHz). All n.m.r. spectra of the K53 capsular polysaccharide were acquired with a JEOL GX-400 spectrometer (400 MHz). One-dimensional, single-pulse, <sup>13</sup>C-n.m.r. spectra of the K93 and K53 polysaccharides were acquired on either a Bruker WM-300 (300 MHz) or JEOL GX-400 spectrometer. Spectrometer and sample conditions are described in the Figure captions.

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