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Structural Analysis of the Specific Capsular Polysaccharide of *Streptococcus pneumoniae* Type 45 (American Type 72)†

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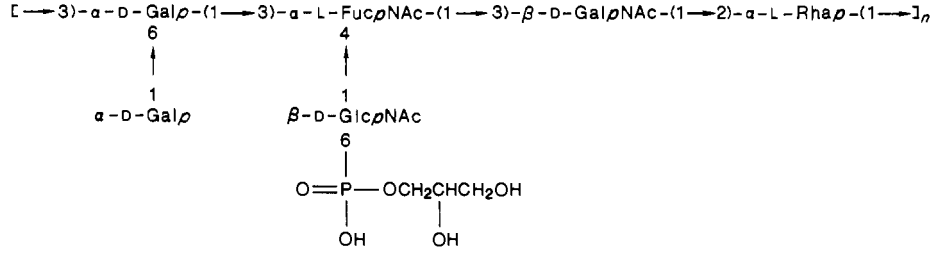
ABSTRACT: The specific capsular polysaccharide of *Streptococcus pneumoniae* type 45 (American type 72) was found to be a high molecular weight polymer composed of D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-L-fucose, L-rhamnose, glycerol, and phosphate (2:1:1:1:1:1:1). Partial hydrolysis, dephosphorylation, methylation analysis, periodate oxidation studies, and one- and two-dimensional ^1H and ^{13}C high-field nuclear magnetic resonance experiments showed the polysaccharide to be a branched polymer of a 1-phosphoglycerol-substituted hexasaccharide repeating unit having the structure:



Investigations on the specific capsular polysaccharides of *Streptococcus pneumoniae* serotypes have increased since the introduction of the wide-scale use of a pneumococcal vaccine composed of 23 of the 84 serologically defined pneumococcal polysaccharides (Lund & Henrichsen, 1978; Robbins et al., 1983). The formulation of the pneumococcal vaccine is based on surveys of the pneumococcal serotypes responsible for the major portion of human infections in a particular geographic region (Austrian, 1978; Austrian et al., 1976).

Serotypes 45 and 46 of *S. pneumoniae*, which appear persistent in infections found in Africa (Bureau of Biologics, 1979), are virtually absent in Europe and North America. It is not known whether this is due to racial or geographic restrictions. While types 45 and 46 pneumococcal poly-

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saccharides are not included in a current 23-polyvalent vaccine, their inclusion in pneumococcal vaccines for recipients in Africa and Southwest Asia has been recommended. In order to understand the immunochemical properties of the capsular polysaccharides, their analysis was undertaken, and this paper records the elucidation of the structure of the type 45 specific pneumococcal polysaccharide, which had previously been purified and characterized by glycose composition (Daoust et al., 1981).

Materials and Methods

Type 45 Pneumococcal Polysaccharide. Type 45 polysaccharide was obtained by ethanol precipitation (45–50% final concentration) from the growth medium of *S. pneumoniae* type 45 (Merck Culture Collection), followed by digestion with trypsin, ribonuclease, and deoxyribonuclease, serological probing, and fractional precipitation with 2-propanol as previously described (Daoust et al., 1981). Polysaccharide samples were purified by precipitation with cetyltrimethylammonium bromide or ion-exchange chromatography on DEAE-Sephacel.

DEAE-Sephacel Ion-Exchange Chromatography. Crude type 45 polysaccharide (80 mg) was decationized with Rexyn 101(H^+) ion-exchange resin (5 mL), and the concentrated eluant was applied to a column of DEAE-Sephacel (3 × 40 cm) (Pharmacia Fine Chemicals) equilibrated with 0.05 M Tris-HCl buffer (pH 7.2). The column was eluted with the same buffer (50 mL) followed by a 0–2 M NaCl gradient in the same buffer (100 mL). Fractions (5 mL) were collected.

Gel Filtration. Polysaccharide samples were subjected to gel filtration on columns of Sephadex G-50 (2.2 × 30 cm) with 0.05 M pyridinium acetate (pH 4.5) as the eluant, while separations of oligosaccharide samples were made on a column (1.5 × 95 cm) of Bio-Gel P-2 (minus 400) eluted with distilled water.

Column eluants were continuously monitored for changes in refractive index by using a Waters R403 differential refractometer, and fractions were assayed colorimetrically for neutral glycose (Dubois et al., 1956), aminoglycose (Gatt & Berman, 1965), and phosphate (Chen et al., 1956). The gel filtration properties of the eluted materials are expressed in terms of their distribution coefficients $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_0 is the void volume of the system, V_e is the elution volume of the specific material, and V_t is the total volume of the system.

Thin-Layer Chromatography. Analytical and preparative TLC was performed on glass plates coated with silica gel 60 (Merck) having a thickness of 0.25 mm developed with 1-propanol–0.88 ammonia–water (6:3:1 v/v/v). The mobilities of compounds are quoted relative to sucrose (R_{suc}).

Gas–Liquid Chromatography. GLC was done with a Hewlett-Packard Model 5710A chromatograph fitted with a hydrogen flame ionization detector and a Model 3380A electronic integrator, and by using a fused silica capillary column (0.3 × 25 mm) containing 3% OV17 employing the following conditions: program A, temperature program, start 180 °C (delay 2 min) to 240 °C at 4 °C/min; program B, temperature program, start 180 °C (delay 2 min) to 320 °C at 10 °C/min; program C, temperature program, start 200 °C (delay 2 min) to 240 °C at 2 °C/min. Development was made with dry nitrogen at 30 mL/min and retention times are quoted relative to D-glucitol hexaacetate (T_{GA}), 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}), and permethylated cellobiitol (T_{CM}).

GLC–mass spectrometry was done by using a Hewlett-Packard 5958B GLC–MS system employing the GLC program conditions A–C by electron impact (EI) employing an ionization potential of 70 eV or by chemical ionization (CI) with methane as the reagent gas. Mass spectra obtained by direct insertion (DI) of samples employed the following temperature program: start 50 °C to 300 °C at 25 °C/min.

Analytical Methods. Quantitative colorimetric methods used were (i) phenol–sulfuric acid for neutral glycoses (Dubois et al., 1956), (ii) modified Elson–Morgan method for aminoglycoses (Gatt & Berman, 1965), and (iii) the method of Chen et al. (1956) for phosphate.

Glycoses were determined by GLC using program A of their alditol acetates (Gunner et al., 1961)) with inositol as internal standard. Samples (2 mg) were hydrolyzed in sealed glass tubes with 2 M aqueous trifluoroacetic acid (1 mL) at 100 °C for 15 h, followed by concentration to dryness in vacuo. The neutral residues were reduced with sodium bordeuteride and acetylated according to the procedure of Henry et al. (1983).

The identity of each glycose was established by comparison of its GLC retention time and mass spectrum with those of an authentic reference specimen.

Methylation Analysis. Polysaccharide samples (2 mg) were methylated by the use of sodium methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide according to the Hakomori procedure (Hakomori, 1964), and the methylated products were recovered from the reaction mixture by filtration through a C18 Sep-pak cartridge (Waters) (Waugh et al., 1983). Oligosaccharide samples were methylated by the same procedure, but the products were recovered by partitioning the reaction mixture between water and methylene chloride followed by concentration of the methylene chloride phase. Permethylated oligosaccharides were analyzed directly by GLC–MS using program B or by direct insertion (DI-MS).

Methylated products were hydrolyzed with 2 M aqueous trifluoroacetic acid at 100 °C for 15 h and, following concentration to dryness, were reduced with sodium borodeuteride, acetylated as previously described (Henry et al., 1983), and analyzed by GLC–MS using program C.

Dephosphorylation of the Type 45 Polysaccharide. Type 45 polysaccharide (50 mg) was dissolved in 48% aqueous hydrofluoric acid (3 mL), and the solution was kept at 4 °C for 72 h. Following removal of the HF in vacuo at 4 °C, the dephosphorylated polysaccharide was recovered as the void volume fraction from the Sephadex G-50 gel filtration system.

Nuclear Magnetic Resonance Spectroscopy. Spectra were obtained by using Bruker AM-500 or AM-200 spectrometers equipped with Aspect 3000 computers, operating in the pulsed Fourier transform mode with quadrature detection using standard Bruker DISNMRP software. Samples were twice lyophilized from deuterium oxide and dissolved at concentrations of 10–50 mg/mL. Proton and carbon-13 measurements were made on solutions (0.4 mL) of samples (20 mg) of the original (sodium salt, pD ~ 7) and the dephosphorylated type 45 polysaccharide in 5-mm tubes at 350 K.

Proton spectra at 500 MHz were recorded by using a spectral width of 2.5 kHz, a 16K data block for a digital resolution of 0.3 Hz/point, an aquisition time of 3.2 s, and a 90° pulse. Resolution enhancement was achieved by using a Gaussian line shape transformation (Ferrige & Lindon, 1978) with typical values of −2.0 to −4.0 Hz for the line broadening factor and a Gaussian broadening factor of +0.1 or +0.2. Chemical shifts are expressed relative to internal acetone (0.1%) (2.225 ppm). NOE difference spectra (Richarz & Wuthrich, 1978) were obtained by using a selective low-power presaturation pulse, typically 44 dB below 0.2 W, ap-

plied to the proton resonance for 100 or 200 ms followed by an 90° observation pulse.

Broad-band proton-decoupled carbon-13 spectra were obtained at 125 MHz with a 25-kHz spectral width by using a 32K data block for a digital resolution of 1.5 Hz/point and a 90° pulse (8.0 μs) employing WALTZ decoupling (Shaka et al., 1983). DEPT spectra (Doddrell et al., 1982) were obtained with broad-band proton decoupling, a 135° proton pulse, and a 3.3-ms delay [$1/2(^1J_{C,H})$] between pulses. Chemical shifts are referenced to external 1,4-dioxane (67.40 ppm).

Two-dimensional homonuclear proton correlation experiments, COSY (Bax et al., 1981), and relay COSY (Wagner, 1983; Bax & Drobny, 1985) were measured with solvent supression by employing the conventional pulse sequences. The experiments were carried out by using data sets ($t_1 \times t_2$) of 256 × 1024 or 512 × 2048 points that were zero filled in t_1 to 512 × 1024 or 1024 × 2048 points, respectively. Spectral widths of either 2500 or 1200 Hz and a recycle delay of 1.0–1.5 s were used, and either 48 or 64 transients were collected for each value of the incrementable delay. Resolution enhancement was implemented in both dimensions by means of a nonshifted sine bell window function (de Marco & Wuthrich, 1976), and the doubly transformed data were processed to give magnitude spectra (Lindon & Ferrige, 1979) with symmetrization (Baumann et al., 1981).

Heteronuclear carbon–proton chemical shift correlations were achieved by using the CHORTLE technique described by Pearson (1985). Four "cosine"/"sine" pairs of ^{13}C spectra were acquired for the τ values 0.16, 1.00, 2.40, and 3.20 ms by using 8K data sets. The 90° pulses were 20 μs for the ^1H decoupler and 12.5 μs for the ^{13}C transmitter, and a 1.5 recycle delay was employed. The number of transients acquired was 3584 for each of the four spectra. The ^1H resonance offsets were calculated from the ^{13}C intensities by using a nonlinear least-squares calculation (J.-R. Brisson, NRC, Ottawa).

Phosphorus-31 spectra were measured at 202 MHz with 10 mm tubes for a spectral width of 10 kHz by using a 90° pulse (48 μs) and a 16K data set for a digital resolution of 1.2 Hz/point. Chemical shifts were referenced to external 85% phosphoric acid. Spectral simulations were performed by using the Bruker Panic program.

Periodate Oxidation. Polysaccharide (30 mg) was treated in the dark with 0.05 M sodium metaperiodate (30 mL) at 4 °C for 4 days, and following destruction of excess periodate by the addition of ethanediol (0.4 mL), the oxidized polymer was reduced by treatment with sodium borohydride (300 mg) at 4 °C for 15 h. The solution was neutralized with acetic acid, and the product was obtained by lyophilization after extensive dialysis against distilled water.

Smith-type (Goldstein et al., 1970) hydrolysis of the periodate-oxidized and reduced polysaccharide was effected with 1% (v/v) aqueous acetic acid (7 mL) at 100 °C for 5 h followed by concentration to dryness. The Smith degradation product mixture was treated with sodium borodeuteride prior to fractionation by gel filtration on the Bio-Gel P-2 column.

N-Deacetylation and Deamination. The dephosphorylated type 45 polysaccharide (8 mg) in 2 M sodium hydroxide solution (1 mL) containing sodium borohydride (1 mg) was heated at 100 °C for 2 h. The cooled solution was neutralized with 2 M HCl, and the partially N-deacetylated polysaccharide was recovered as the void volume fraction on the Sephadex G-50 column.

The partially N-deacetylated polysaccharide in 16% (v/v) aqueous acetic acid (0.8 mL) was treated with a freshly prepared 5% (w/v) sodium nitrite solution (0.4 mL). After standing at 25 °C for 30 min, the reaction mixture was decationized with a Rexyn 101 (H^+) (2-mL) resin, lyophilized, and fractionated on the Sephadex G-50 gel filtration system.

Immunodiffusion. Double diffusion studies (Ouchterlony, 1958) were made at 4 °C in 1% agarose gels by using polysaccharide concentrations of 1 mg/mL. Precipitin lines were allowed to develop over 3 days. Type 45 pneumococcal antiserum was supplied by Dr. J. Henrichsen, Statens Serum Institute, Copenhagen.

General Methods. Commercial reagents and solvents were analytical grade. Concentrations were made under reduced pressure at bath temperatures below 40 °C. Optical rotations were determined at 22 °C in 10-cm microtubes with a Perkin-Elmer Model 243 polarimeter.

Results and Discussion

Type 45 pneumococcal-specific polysaccharide was obtained from the spent growth medium of *S. pneumoniae* serotype 45 and was purified via its insoluble cetyltrimethylammonium salt as previously described (Daoust et al., 1981). The type 45 polysaccharide had $[\alpha]_D$ −7° (*c* 1.1, water) and was composed of D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-L-fucose, L-rhamnose, glycerol, and phosphate in the molar ratios 2:1:1:1:1:1:1. Treatment of the type 45 polysaccharide with cold 48% (w/v) aqueous hydrofluoric acid liberated glycerol and phosphate and left a phosphate-free polysaccharide (68%) that had a high molecular weight, as evidence by its elution at the void volume of a Sephadex G-50 gel filtration system, and that had the same glycose composition as the original polysaccharide, indicating that the original glycerophosphate component did not form part of a backbone structure but was probably present as a phosphodiester substituent of a glycose residue.

The ^{31}P NMR spectrum of the original 45 polysaccharide showed one signal at 1.21 ppm, indicating the presence of a single phosphate group in a repeating oligosaccharide unit. The ^1H NMR spectra of both the original and dephosphorylated 45 polysaccharides (Figure 1) showed six anomeric proton resonances (4.6–5.4 ppm), indicating that the polysaccharides each possessed regular repeating hexasaccharide units, a conclusion consistent with the determined glycose composition. Other characteristic features present in the ^1H NMR spectra were two high-field doublets ($J_{5,6}$ ~6 Hz) centered at 1.3 ppm, corresponding in relative signal area to six protons arising from the methyl protons of the two 6-deoxyhexosyl residues, and three equal-area singlets (1.9–2.1 ppm), corresponding in chemical shifts to the methyl protons of *N*-acetyl groups located on the three 2-acetamido-2-deoxyglycosyl residues.

The ^{13}C NMR spectra of the original and the dephosphorylated 45 polysaccharides (Figure 2) were also consistent with the proposed hexasaccharide repeating unit in the polymeric structure. The spectrum of the original polysaccharide showed anomeric carbon resonances at 104.26 (1 C), 102.03 (2 C), 100.66 (1 C), 100.45 (1 C), and 99.12 ppm (1 C), two signals at 18.27 and 17.21 ppm from the methyl carbons of the two 6-deoxyhexopyranosyl residues, and three signals at 57.35, 53.10, and 50.09 ppm arising from the C-2 atoms of the three 2-acetamido-2-deoxyglycosyl residues, together with characteristic resonances at 175.62, 175.58, 175.00 ($-NHCOCH_3$), and 23.5 ppm ($-NHCOCH_3$).

Methylation analysis of the original and dephosphorylated 45 polysaccharides afforded essentially identical results (Table I), giving, after appropriate corrections for aminoglycose de-

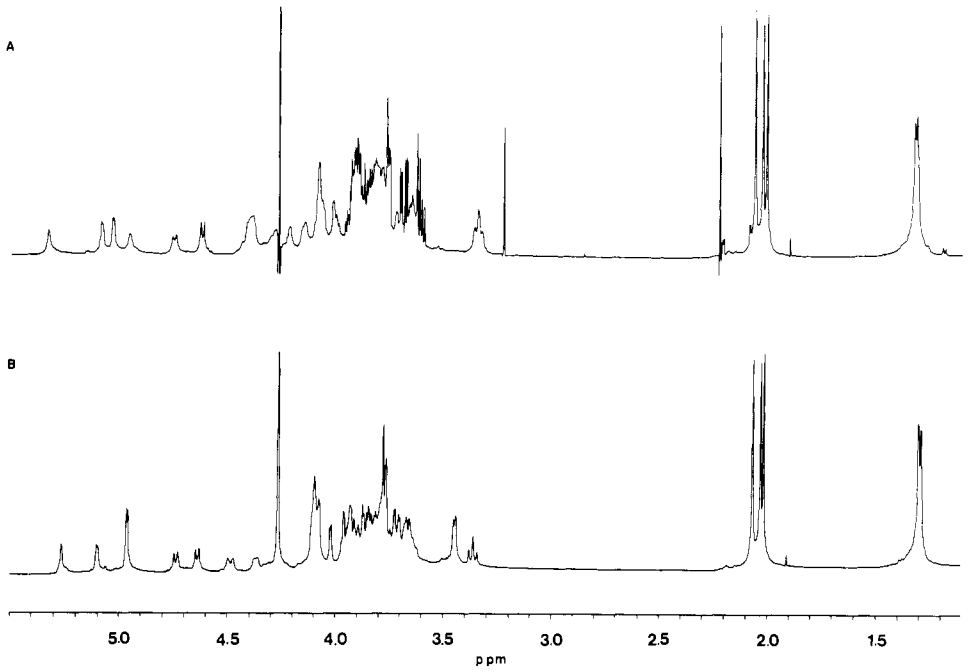


FIGURE 1: ^{1}H NMR spectra of the type 45 *S. pneumoniae* specific capsular polysaccharide recorded at 350 K: (A) original polysaccharide; (B) dephosphorylated polysaccharide.

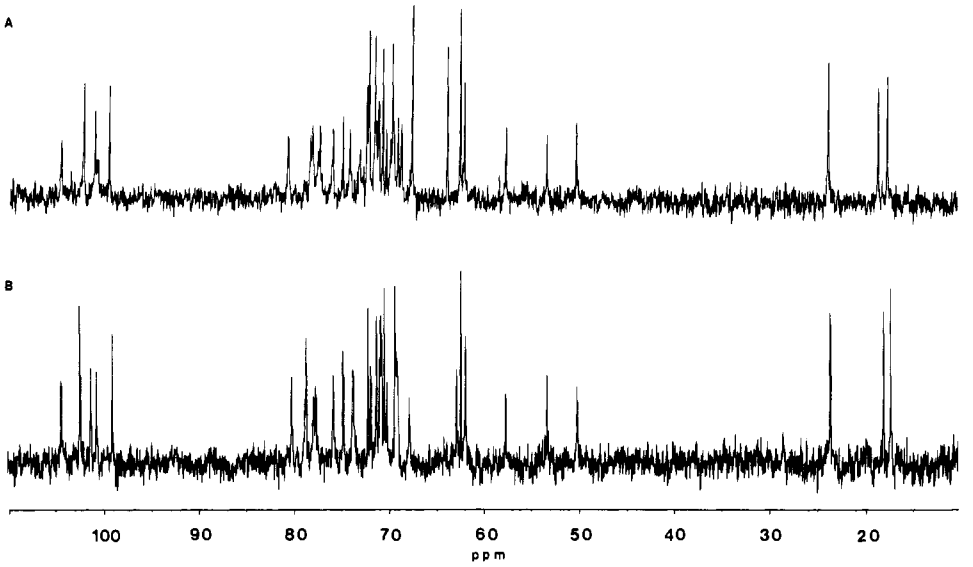


FIGURE 2: Proton-decoupled ^{13}C NMR spectra of the type 45 *S. pneumoniae* specific capsular polysaccharide recorded at 350 K: (A) original polysaccharide; (B) dephosphorylated polysaccharide.

Table I: GLC–MS Methylation Analysis of Original and Dephosphorylated Type 45 Polysaccharides and of Partially N-Deacetylated and Deaminated Product Derived from Dephosphorylated 45 Polysaccharide

		mole ratio[a]		
derivative	T_{GM}[b]	original polysaccharide	dephosphorylated polysaccharide	partially N-deacetylated deaminated dephosphorylated polysaccharide
1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol-*1-d*	0.89	0.8	0.6	0.7
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-*1-d*	1.05	1.0	1.0	1.0
1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol-*1-d*	1.58	1.0	1.4	1.3
1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)-D-glucitol-*1-d*	1.72	0.2	0.4	tr
1,3,5-tri-*O*-acetyl-2-deoxy-4-*O*-methyl-2-(methylacetamido)-L-fucitol-*1-d*	1.73			0.7
1,3,4,5-tetra-*O*-acetyl-2-deoxy-2-(methylacetamido)-L-fucitol-*1-d*	1.78	0.6	1.1	tr
1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(methylacetamido)-D-galactitol-*1-d*	2.01	0.3	0.5	0.9

[a] Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-*1-d* and aminoglycose detector response factors determined from relative response of authentic reference samples. [b] GLC, program C.

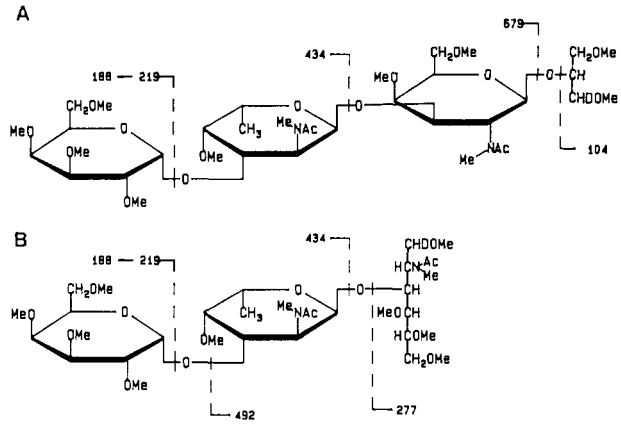


FIGURE 3: Primary MS fragmentation patterns of the Smith degradation products, oligosaccharide **1** (A) and oligosaccharide **2** (B).

rivatives, equal molar amounts of 3,4-di-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2-deoxy-2-(methylamino)-L-fucose, 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylamino)-D-glucose, and 2-deoxy-4,6-di-*O*-methyl-2-(methylamino)-D-galactose. The results show that the type 45 polysaccharide contains six structural units: two nonreducing end groups, D-Gal*p*-(1→ and D-Glc*p*NAc-(1→; two branch point units, a 3,6-di-*O*-glycosylated D-Gal*p* unit, and a 3,4-di-*O*-glycosylated L-Fuc*p*NAc unit; and the monoglycosylated units →2)-L-Rha*p*-(1→ and →3)-D-Gal*p*-NAc-(1→. The sequence and anomeric configurations of these units were subsequently established from the analysis of oligosaccharides obtained by periodate oxidation methods, and by the use of NMR techniques.

Smith-type hydrolysis (Goldstein et al., 1970) of the reduced ($NaBH_4$) periodate-oxidized dephosphorylated type 45 polysaccharide with 1% (v/v) acetic acid (5 h, 100 °C) followed by fractionation of the reduced ($NaBD_4$) products on a column of Bio-Gel P-2 gave approximately equal amounts of two oligosaccharides, **1** and **2**, having K_{av} 0.83 and 0.77, respectively.

Oligosaccharide **1** gave a single spot on TLC (R_{suc} 0.85) and was composed of D-galactose, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxy-L-fucose, and glycerol-*1-d* (1:1:1:1). The ^1H NMR spectrum of **1** showed three equal-intensity anomeric proton signals at 5.12 (d, 1 H, $J_{1,2}$ = 3.1 Hz), 5.01 (d, 1 H, $J_{1,2}$ = 3.9 Hz), and 4.64 ppm (d, 1 H, $J_{1,2}$ = 8.6 Hz) characteristic of two α-linked and one β-linked hexopyranosyl residues, two singlet *N*-acetyl methyl proton signals at 2.06 and 2.03 ppm, and a 6-deoxyhexosyl methyl proton doublet at 1.26 ppm (d, 3 H, $J_{5,6}$ = 6.7 Hz). The anomeric configuration assignments of the component glycosyl residues in **1** were made by 2D homonuclear shift correlation (COSY) techniques which allowed the ^1H resonances to be grouped into subspectra corresponding to the α-D-galactopyranosyl, 2-acetamido-2-deoxy-α-L-fucopyranosyl, and 2-acetamido-2-deoxy-β-D-galactopyranosyl residues as well as those of the 2-*O*-substituted terminal glycerol residue (Table II).

Methylated **1** gave a molecular ion m/z = 800 (M + 1, CI) in its MS having a fragmentation pattern (Figure 3A) that was consistent with **1** being characterized as *O*-α-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-L-fucopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→2)-D-glycerol-*1-d*.

Oligosaccharide **2** gave a single spot on TLC (R_{suc} 0.82) and was composed of D-galactose, 2-amino-2-deoxy-L-fucose, and 2-amino-2-deoxy-D-galactitol-*1-d* (1:1:1). The ^1H NMR spectrum of **2** showed α-anomeric proton signals at 5.10 (d, 1 H, $J_{1,2}$ = 3.0 Hz) and 5.08 ppm (d, 1 H, $J_{1,2}$ = 3.0 Hz)

Table II: Proton Chemical Shift Assignments and Coupling Constants for the Glycose Residues of Oligosaccharide **1**[a]

proton resonance (coupling constant)	glycose residue		
	α-D-Gal*p*-(1→	→3)-α-L-Fuc*p*NAc-(1→	→3)-β-D-Gal*p*NAc-(1→
H-1	5.12	5.01	4.64
($J_{1,2}$)	(3.1)	(3.9)	(8.6)
H-2	3.79	4.36	4.08
($J_{2,3}$)	(10.4)	(10.8)	(10.5)
H-3	3.90	4.06	3.85
($J_{3,4}$)	(3.5)	(3.0)	(3.0)
H-4	4.02	4.05	3.97
($J_{4,5}$)	(1.0)	(0.9)	(0.8)
H-5	4.06	4.17	3.73
H-6	3.77	1.26	3.84
($J_{5,6}$)	(5.6)	(6.7)	(7.6)
H-6′	3.77		3.80
($J_{5,6'}$,$J_{6,6'}$)	(5.6, −11.0)		(4.2, −12.0)

[a] Measured at 310 K relative to internal acetone (2.225 ppm). The data are refined by spectral simulation. Simulation of the 2-*O*-substituted glycerol residue was complicated due to partial replacement by deuterium of H-1 (3.74 ppm, ca. 80% $^2H_1$) or H-1′ (~3.67 ppm, ca. 20% $^2H_1$): H-2, 3.87 ppm; H-3, 3.68 ppm ($J_{2,3}$ = 4.0 Hz); H-3′, 3.65 ppm ($J_{2,3'}$ = 5.6 Hz, $J_{3,3'}$ = −11.6 Hz).

together with high-field signals corresponding to the methyl protons of *N*-acetyl groups at 2.10 ppm (s, 6 H) and of a 6-deoxyglycosyl residue at 1.28 ppm (d, 3 H, $J_{5,6}$ = 6.0 Hz). Methylated **2** on GLC–MS gave a single peak with a molecular ion m/z 728 (M^+ + 1, CI) and the EI fragmentation pattern shown in Figure 3B. GLC (program C) of the reduced ($NaBD_4$) and acetylated hydrolysis products of methylated **2** gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol-*1-d* (T_{GM} 1.05), 3-*O*-acetyl-2-(methylamino)-1,4,5,6-tetra-*O*-methyl-D-galactitol-*1-d* (T_{GM} 2.18), and 1,3,5-tri-*O*-acetyl-2-deoxy-2-(methylamino)-4-*O*-methyl-L-fucitol-*1-d* (T_{GM} 2.76) (1:1:1). The combined NMR and methylation evidence leads to **2** being assigned the structure *O*-α-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-L-fucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactitol-*1-d*. The characterization of oligosaccharides **1** and **2** indicates that part of the type 45 polysaccharide structure contains the unit →3)-α-D-Gal*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→2)-L-Rha*p*-(1→. The proposed terminal L-rhamnopyranosyl residue in the above unit is consistent with the finding of the 2-O-substituted glycerol moiety in **1**. The absence of the glycerol end group in oligosaccharide **2** is probably a result, as previously observed (Smith et al., 1985), of hydrolysis of the glycosyl glyceraldehyde linkage in the reduced periodate-oxidized polysaccharide, a conclusion supported by the observation that extended hydrolysis conditions [2% (v/v) acetic acid, 6 h, 100 °C] resulted in the almost exclusive production of **2**.

The location of the 2-acetamido-2-deoxy-D-glucopyranosyl end group in the 45 polysaccharide was established by its selective deamination. As expected from the known resistance to N-deacetylation of 3-O-glycosidically substituted 2-acetamido-2-deoxyglycosyl residues (Lindberg & Lonngren, 1978), treatment of the dephosphorylated 45 polysaccharide with 2 M sodium hydroxide (2 h, 100 °C) resulted in the complete N-deacetylation of the 2-acetamido-2-deoxy-D-glucose units without saponification of the 2-acetamido-2-deoxy-L-fucose or 2-acetamido-2-deoxy-D-galactose residues. Nitrous acid deamination (Williams, 1975) of the selectively N-deacetylated polysaccharide left a high molecular weight polymer that eluted at the void volume of a Sephadex G-50 gel filtration system. Methylation analysis of the latter polymer (Table I)

Table III: Proton Chemical Shifts[a] and Coupling Constants[b] of Dephosphorylated *S. pneumoniae* Type 45 Polysaccharide[c]

proton resonance (coupling constant)	hexopyranosyl residue					
	residue **a**, →2)-α-L-Rha*p*-(1→	residue **b**, →3,6)-α-D-Gal*p*-(1→	residue **c**, α-D-Gal*p*-(1→	residue **d**, →3,4)-α-L-Fuc*p*NAc-(1→	residue **e**, β-D-Glc*p*NAc→-(1→	residue **f**, →3)-β-D-Gal*p*-NAc-(1→
H-1	5.26 [5.33]	5.09	4.96 [5.03]	4.96	4.73 [4.75]	4.63
($J_{1,2}$)	(s)[d]	(4.0)	(4.0)	(3.4)	(8.1)	(8.1)
H-2	4.10 [4.08]	3.82	3.86 [3.83]	4.48	3.76 [3.78]	4.09 [3.68]
($J_{2,3}$)	(3.5)	(10.1)	(10.7)	(10.1)	(10.1)	(10.7)
H-3	3.91	3.88	3.71	4.07	3.63	3.80
($J_{3,4}$)	(9.6)	(3.1)	(3.0)	(3.0)	(10.4)	(3.1)
H-4	3.36 [3.34]	4.08	4.02	~4.08 [4.21]	3.45 [3.33]	3.93 [4.15]
($J_{4,5}$)	(9.4)	(~1)	(~1)	(-)[e]	(~10)	(~1)
H-5	3.79	4.36	3.96 [3.99]	4.08	3.44 [3.64]	3.68
H-6	1.30	3.89	~3.77 [~3.75]	1.30 [1.31]	3.95 [~4.41]	3.82
($J_{5,6}$)	(6.1)	(~7)	(-)[e]	(6.0)	(~4)	(-)[e]
H-6′		3.66 [3.88]	~3.77 [~3.75]		3.71 [3.86]	~3.78
($J_{5,6'}$,$J_{6,6'}$)		(~1, ~11)	(-, -)[e]		(7.0, 10.5)	(-, -)[e]

[a] Measured at 350 K with acetone as internal standard. [b] Observed first-order couplings (±0.5 Hz) measured from a resolution-enhanced spectrum or the appropriate COSY cross-peak. [c] Chemical shifts in square brackets are for proton resonances shifted in the spectrum of the original (Na^+ salt) polysaccharide. [d] $w_{1/2}$ = 3 Hz. [e] Unresolved.

gave 3,4-di-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2-deoxy-4,6-di-*O*-methyl-2-(methylamino)-D-galactose, and 2-deoxy-4-*O*-methyl-2-(methylamino)-L-fucose (1:1:1:1:1). The identification of the latter glycose which was not present in the products of the hydrolysis of the methylated original polysaccharide shows that the 2-acetamido-2-deoxy-D-glucopyranosyl end groups are linked to the O-4 position of the 2-acetamido-2-deoxy-L-fucopyranosyl branch point units in the 45 polysaccharide. The D-galactopyranosyl end groups in the 45 polysaccharide can now be inferred to be linked to either the O-3 or O-6 positions of the α-D-galactopyranosyl branch units. The location of this latter substitution, as well as the verification of the glycose sequences, linkage positions, and anomeric configurations, was made by high-field NMR analyses which also served to establish the location of the phosphoglycerol substituent in the original polysaccharide.

A detailed analysis of the two-dimensional proton homonuclear shift-correlated (COSY and relay COSY) spectra of the original and dephosphorylated type 45 polysaccharides permitted the ^1H resonances to be grouped into subspectra corresponding to each of the six component monosaccharide residues in the repeating unit and to the phosphoglycerol substituents. Examination of the COSY spectrum of the dephosphorylated type 45 polysaccharide (Figure 4) led to the assignment of almost all the ^1H resonances. The anomeric proton resonances present in the low-field region (4.5–5.3 ppm) of the spectrum were readily identified by their single vicinal ^1H–^1H couplings and thereby provided a convenient starting point for spectral analysis. These resonances were arbitrarily assigned the notations H-1**a**, H-1**b**, H-1**c**, H-1**d**, H-1**e**, and H-1**f** in order of decreasing chemical shift. Although the proton resonances assigned to the anomeric protons of residues **c** and **d** showed coincident chemical shifts (4.96 ppm, relative area of 2 H), the respective spin systems were identified from the corresponding cross-peaks, 1,2**c** and 1,2**d**, which indicated distinct chemical shift values for H-2**c** (3.86 ppm) and H-2**d** (4.48 ppm). In the spectrum of the original polysaccharide (Figure 1A), the corresponding proton resonance for H-1**c** (5.03 ppm) was completely resolved from that of H-1**d** (4.96 ppm). Analysis of the connectivities between cross-peaks led to the unambiguous assignment of most of the proton resonances corresponding to each of the component monosaccharide units (**a**–**f**) (Table III). In this way, the L-rhamnosyl unit was readily associated with the connectivity pathway corresponding to residue **a** on the basis of the small

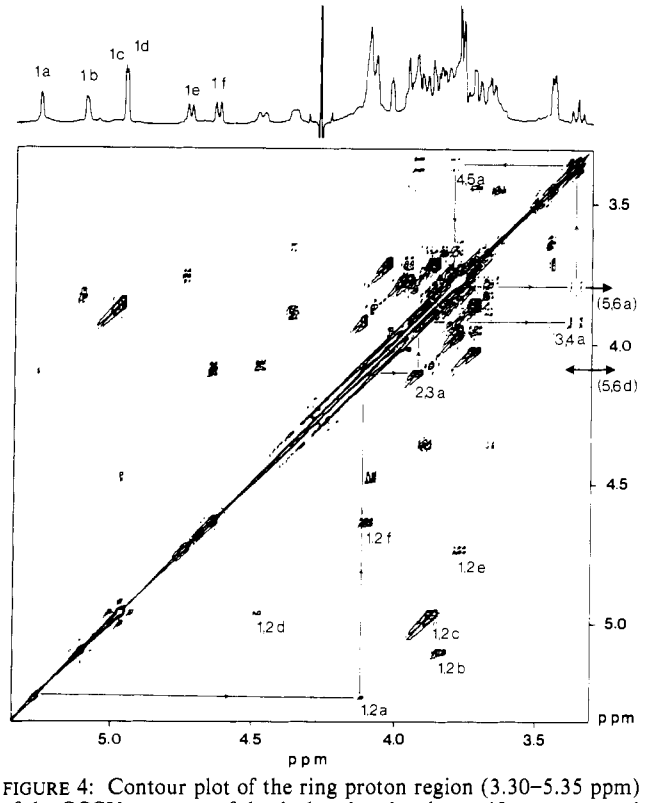


FIGURE 4: Contour plot of the ring proton region (3.30–5.35 ppm) of the COSY spectrum of the dephosphorylated type 45 pneumococcal polysaccharide. Resonance assignments are shown for H-1 and H-2 of each of the component monosaccharides, and the connectivity pathway corresponding to the residue **a** subspectrum is indicated by the solid line.

values of the vicinal coupling constants $J_{1,2}$ (≤3 Hz) and $J_{2,3}$ (~3 Hz) and the occurrence of cross-peaks relating H-5**a** (3.79 ppm) to one of the 6-deoxy proton doublets centered at 1.30 ppm.

Residues **b**–**d** were each identified as hexopyranosyl residues having the *α-galacto* configuration from the magnitude of the vicinal ring proton couplings $J_{1,2}$ (~4 Hz), $J_{2,3}$ (~10 Hz), and $J_{3,4}$ (~3 Hz) (Altona & Haasnoot, 1980). The chemical shifts of H-3**d**, H-4**d**, and H-5**d** were almost identical (4.07–4.08 ppm), and the assignment of these resonances was verified by a relay COSY experiment (Wagner, 1983; Hughes et al., 1985). Since the high-field methyl resonances centered at 1.30 ppm could be associated with this group of resonances, both

Table IV: Carbon-13 Chemical Shifts[a] for the Dephosphorylated Type 45 Pneumococcal Polysaccharide[b,c]

	hexopyranosyl unit					
carbon resonance	residue **a**, →2)-α-L-Rha*p*-(1→	residue **b**, →3,6)-α-D-Gal*p*-(1→	residue **c**, α-D-Gal*p*-(1→	residue **d**, →3,4)-α-L-Fuc*p*NAc-(1→	residue **e**, β-D-Glc*p*NAc-(1→	residue **f**, →3)-β-D-Gal*p*-NAc-(1→
C-1	101.23 (100.66)[c]	102.39 (102.03)	99.05 (99.12)	100.62 (100.45)	101.39 (102.03)	104.35 (104.26)
C-2	80.15	~69.3[d]	69.38 (69.38)	50.11 (50.09)	57.57 (57.35)	53.26 (53.10)
C-3	71.00	77.84[d]	71.28 (71.25)	78.76[d]	74.76	77.60
C-4	73.73	69.22	70.44 (70.37)	73.73[d]	71.85	69.10
C-5	70.19	70.79[d]	72.18 (71.82)	70.80	78.65	75.78
C-6	17.26 (17.21)	67.87[e] (68.26)[e]	62.36 (62.28)	18.01 (18.27)	62.85 (67.47)[d,e]	61.89 (61.77)

[a] Measured at 350 K and expressed in ppm relative to external 1,4-dioxane. [b] Unless indicated, assignments were determined from the heteronuclear ^1H-^{13}C chemical shift correlation experiment. [c] Values in parentheses are for ^{13}C resonance assignments determined for the original type 45 pneumococcal polysaccharide by ^1H–^{13}C chemical shift correlation. [d] Tentative assignment, made by comparison with reference compounds. [e] Identification confirmed by DEPT experiment.

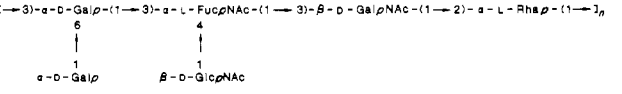
in the COSY and relay COSY experiments, via the respective cross-peaks to H-5**d** (4.07 ppm) and to H-5**d** and H-4**d** (4.08 ppm), residue **d** was identified as the 2-acetamido-2-deoxy-α-L-fucopyranosyl unit. On the basis of the chemical shift data (Bock & Thogersen, 1982), residues **b** and **c** were identified as the 3,6-di-O-substituted and the terminal nonreducing α-D-galactopyranosyl units, respectively. In agreement with this conclusion, the spin system corresponding to residue **c** gave rise to the most intense cross-peaks in the COSY spectrum (Figure 4), an indication of the relative mobility of the terminal residues (Gorin & Mazurek, 1979). Comparison of chemical shift data and the ring proton coupling constants of residues **e** and **f** (Table III) with those in the literature (Bock & Thogersen, 1982) permitted assignment of these residues as the 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-2-deoxy-β-D-galactopyranosyl units, respectively. The identity of residues **e** and **f** as amino sugars was unequivocally established by direct correlation of H-2**e** (3.76 ppm) and H-2**f** (4.09 ppm) with the corresponding ^{13}C signals, since the resonances attributed to C-2**e** (57.57 ppm) and C-2**f** (53.26 ppm) occurred in a region of the ^{13}C spectrum characteristic of the amino-substituted carbon of 2-acetamido-2-deoxy-glycoses (Bundle et al., 1973). Similarly, a ^1H–^{13}C correlation was observed between H-2 (4.48 ppm) and C-2 (50.11 ppm) of the 2-acetamido-2-deoxy-α-L-fucopyranosyl residue (**d**).

The heteronuclear ^1H–^{13}C chemical shift correlation experiment was effected by using the CHORTLE pulse sequence (Pearson, 1985) and led to a definitive assignment of the ^{13}C resonances in uncrowded regions of the ^{13}C spectrum (Figure 2B) and of those associated with the nonreducing α-D-galactopyranosyl and 2-acetamido-2-deoxy-β-D-glucopyranosyl end groups (Table IV). The ^{13}C chemical shift data determined via the appropriate ^1H–^{13}C connectivities for residues **c** and **e** (Table IV) were not substantially different from data reported for the respective methyl glycosides of α-D-galactopyranose (Gorin & Mazurek, 1975) and 2-acetamido-2-deoxy-β-D-glucopyranose (Perkins et al., 1977), respectively.

The sequence and the positions of linkages determined from chemical analyses were verified by ^1H–^1H NOE measurements (Dabrowski et al., 1981; Prestegard et al., 1982; Bernstein & Hall, 1982). NOE experiments were carried out on both the original and the dephosphorylated type 45 polysaccharides, and the difference spectra obtained for saturation of each of the anomeric proton resonances of the original polysaccharide are shown in Figure 5. This set of experiments (Figure 5) readily provided the overall sequence information since overlap of the anomeric resonances, H-1**c** and H-1**d**, and of the H-3, H-4, and H-5 resonances of residue **d** in the ^1H spectrum of the dephosphorylated polymer rendered the interpretation of the corresponding NOE experiments difficult. Assignment of the relevant ^1H resonances in the spectrum of the original type 45 polysaccharide was effected, as above, by analysis of the connectivity relationships among the ^1H resonances observed in 2D COSY and relay COSY spectra, and those protons that showed a significant change in chemical shift (≥0.01 ppm) from the values observed in the spectrum of the dephosphorylated polysaccharide are indicated in Table III.

Saturation of the anomeric proton resonance of the L-rhamnopyranosyl residue (**a**) (Figure 5A) showed an *inter*residue NOE at H-3**b**, together with a single *intra*residue NOE at H-2**a** indicating that the L-rhamnosyl unit (**a**) is α-linked to the O-3 position of the 3,6-di-O-substituted α-D-galactopyranosyl residue (**b**). Small indirect *inter*residue effects observed at the other ring proton resonances of residue **b** also accompanied saturation of H-1**a** and were probably due to spin diffusion (Noggle & Schirmer, 1971). Similar effects were observed in the difference spectrum obtained on saturation of H-1**d** (Figure 5D). These secondary effects were not observed in the NOE difference spectrum obtained by saturation of the corresponding proton in the dephosphorylated polysaccharide.

The occurrence of *inter*residue NOE's between H-1**b** and H-3**d**, H-1**d** and H-3**f**, and H-1**f** and H-2**a** (Figure 5), together with the aforementioned effect between H-1**a** and H-3**b**, establishes the linear sequence of glycoses within the main chain of the repeating unit as (→**b**→**d**→**f**→**a**→), consistent with conclusions reached from the periodate oxidation studies. The NOE data clearly point to residues **c** and **e** as the terminal nonreducing end groups. From the occurrence of the *inter*residue NOE between the H-1**c** and the H-6**b**/H-6′**b** pair (Figure 5C) it is evident that the terminal α-D-galactopyranosyl unit (**c**) is linked to the O-6 position of the 3,6-di-O-substituted α-D-galactopyranosyl residue (**b**), while the effect observed between H-1**e** and H-4**d** (Figure 4E) indicates that the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue (**e**) must be 1,4-linked to the branching 3,4-di-O-substituted 2-acetamido-2-deoxy-α-L-fucopyranosyl residue (**d**). Thus the combined evidence permits the partial structure of the repeating hexasaccharide unit to be deduced as



The nature and location of the phosphoglycerol substituent was determined from a comparison of the ^1H and ^{13}C NMR data of the original and dephosphorylated polysaccharides, in conjunction with the results from periodate oxidation studies.

In the ^1H NMR spectrum of the original type 45 polysaccharide (Figure 1A), the two intense double doublets of narrow line width observed at 3.685 (^3J = 4.4 Hz, ^2J = 11.3 Hz) and 3.603 ppm (^3J = 6.0 Hz, ^2J = 11.3 Hz) are indicative of the methylene protons at the unsubstituted end of a 1-phosphoglycerol substituent. This conclusion was reinforced

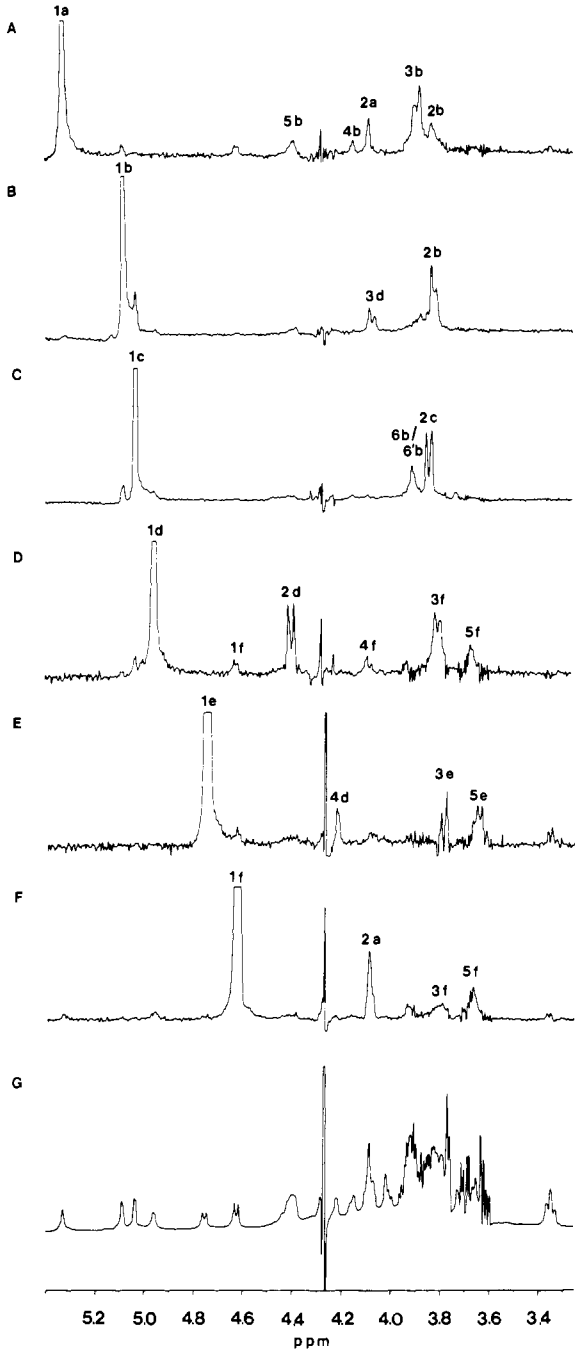


FIGURE 5: NOE difference spectra of the original type 45 pneumococcal polysaccharide for irradiation of the respective anomeric proton resonances H-1**a** to H-1**f**: (A–F) difference spectra; (G) off-resonance control spectrum.

by identification of the 1-phosphoglycerol spin system in the COSY spectrum of the original polysaccharide (Figure 6). Intense cross-peaks clearly showed the connectivity relationships between the H-3 (3.685 ppm) and H-3′ (3.603 ppm) proton resonances (3,3′**g**) and between these resonances and the methine resonance (3,2**g** and 3′,2**g**) at 3.890 ppm (H-2**g**), which, in turn, showed cross-peaks to the methylene proton resonances at the phosphate-substituted end of the substituent (1,2**g** and 1′,2**g**) at 3.934 (H-1**g**) and 3.845 ppm (H-1′**g**).

The original, 1-phosphoglycerol-containing type 45 polysaccharide was subjected to Smith degradation employing the mild acid hydrolysis conditions (1% acetic acid) that had given optimal product yield with the dephosphorylated polymer (100 °C, 5 h). Gel filtration of the reduced ($NaBH_4$) products on Bio-Gel P-2 afforded a mixture of oligosaccharide **1** and **2**,

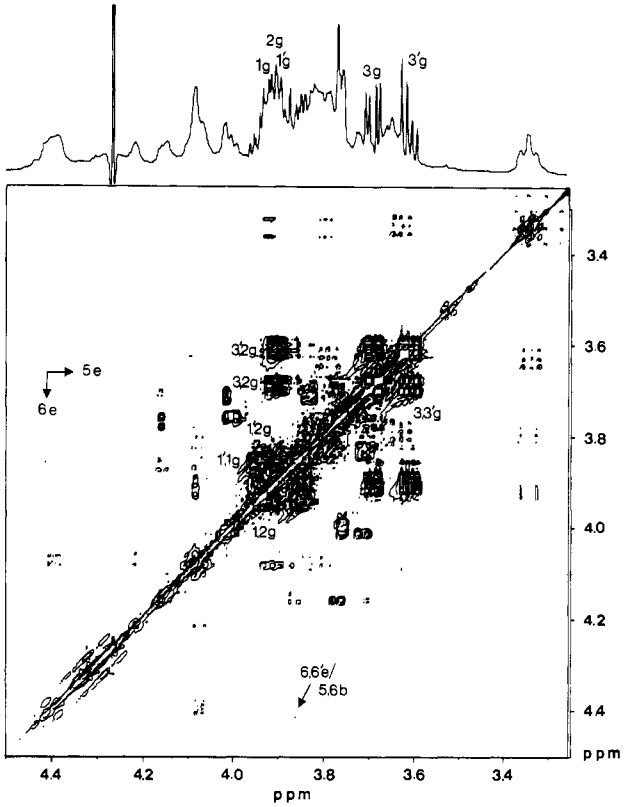


FIGURE 6: Contour plot of part of the COSY spectrum of the original type 45 pneumococcal polysaccharide. Resonance assignments are indicated for the protons of the 1-phosphoglycerol substituent.

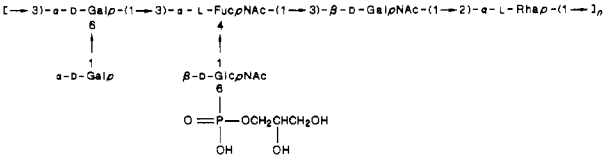
which were readily identified from their respective ^1H NMR spectra, together with a low molecular weight phosphate-containing product, **3**, having K_{av} 1.1.

The phosphate-containing product **3** showed five signals in its ^{13}C NMR spectrum (310 K) at 71.58 (d, $^3J_{C,P}$ = 7.6 Hz), 67.73 (d, $^2J_{C,P}$ = 5.7 Hz), 67.14 (d, $^2J_{C,P}$ = 5.7 Hz), 62.98 (s), and 62.04 ppm (d, $^3J_{C,P}$ = 7.6 Hz) and on hydrolysis with 2 M NaOH gave molar equivalents of glycerol and ethanediol as identified as GLC–MS. Interpretation of the ^{13}C NMR spectrum, accomplished by comparison with the chemical shift data for reference 1-phosphoglycerol (C-1, 65.60, $^2J_{C,P}$ = 4.9 Hz; C-2, 72.04, $^3J_{C,P}$ = 7.5 Hz; C-3, 63.14) and 1-phosphoethanediol (C-1, 68.42, $^2J_{C,P}$ = 5.2 Hz; C-2, 61.95, $^3J_{C,P}$ = 7.6 Hz) (Richards et al., 1985), permitted **3** to be identified as the phosphodiester ethanediol–1-phosphoglycerol. The ethanediol moiety of the diester is undoubtedly derived by the direct oxidation of the 1-phosphoglycerol substituent, while the occurrence of a O-1-substituted glycerol moiety as part of the phosphodiester indicates that the 1-phosphoglycerol substituent must be substituted at the C-6 position of one of the two terminal nonreducing residues in the original polysaccharide, a conclusion further confirmed by the fact that oligosaccharides **1** and **2** were the only glycose-containing products obtained by Smith degradation.

Comparison of the ^1H chemical shift data of the original and the dephosphorylated type 45 polysaccharides (Table III) showed that the chemical shifts associated with the protons of the terminal nonreducing α-D-galactopyranosyl end groups (residue **c**) were unaffected by the presence or absence of the 1-phosphoglycerol substituent. By contrast, several of the ^1H resonances corresponding to the terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl end groups (residue **e**) showed significant differences (≥0.1 ppm) in chemical shift. Although the connectivity relationships among the resonances corresponding to H-5**e** (3.64 ppm), H-6**e** (~4.41 ppm), and H-6′**e**

(~3.86 ppm) were weak due to the very low intensity of the respective cross-peaks in the COSY spectrum of the original type 45 polysaccharide (Figure 6), the substantial downfield shift (0.2 ppm) of H-5e, together with the observed invariance of the ^1H shifts of residue **c** and the periodate oxidation evidence, would suggest that it is the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue which is substituted at the O-6-position by the 1-phosphoglycerol substituent.

Analysis of the ^{13}C chemical shift data (Table IV) provided corroborative evidence for this conclusion. The assignments of the ^{13}C resonances corresponding to residue **c** in the spectra of both the dephosphorylated and the original polysaccharides, as determined from the appropriate ^1H–^{13}C correlation, were found to be almost identical (Table IV) and correlated well with those reported for methyl α-D-galactopyranoside (Gorin & Mazurek, 1975). The ^{13}C resonances arising from methylene carbon atoms were identified by DEPT experiments (Doddrell et al., 1982), which were carried out on both the dephosphorylated and original polysaccharides. Three signals, observed in the region characteristic of primary hydroxyl carbons (60–64 ppm) (Bock & Pedersen, 1983) in the ^{13}C NMR spectrum of the dephosphorylated polysaccharide (Figure 2B), were readily assigned to C-6**c** (62.36 ppm), C-6**e** (62.85 ppm), and C-6**f** (61.89 ppm) from the respective ^1H–^{13}C correlation with the directly attached H-6 protons. The downfield-shifted signal (67.87 ppm) assigned to C-6 of residue **b** was indicative of O-substitution at that position. Interpretation of the DEPT and ^1H–^{13}C chemical shift correlation experiments carried out on the original polysaccharide indicated that only the signals corresponding to C-6**c** (62.28 ppm) and C-6**f** (61.77 ppm), together with the resonance attributed to the terminal methylene carbon (C-3**g**) of the 1-phosphoglycerol substituent (63.54 ppm), were present in the unsubstituted hydroxymethylene region of the spectrum (Figure 2A): the C-6**e** resonance was no longer observable in that region (60–64 ppm) and most probably corresponded to a methylene signal at 67.47 ppm. A ^1H–^{13}C connectivity relating to the resonance at 67.47 ppm could not be detected due to the proximity of the intense doublet ($^2J_{C,P}$ = 5.7 Hz) identified as the C-1 resonance of the 1-phosphoglycerol substituent at 67.32 ppm, but since substitution by phosphate is expected to lead to downfield shifts of ca. 3–5 ppm at the attached carbon resonance (Gorbach et al., 1980) and C-6**b** (68.26 ppm) could be readily identified, it would appear that the downfield-shifted resonance (67.32 ppm) corresponded to C-6 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl unit. Thus, the accumulated evidence permits the structure of the repeating hexasaccharide unit of the *S. pneumoniae* type 45 polysaccharide to be deduced as



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Quantitative Analysis of Linkage in Macromolecules When One Ligand Is Present in Limited Total Quantity†

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ABSTRACT: We present a general framework for analysis of two closely related problems in biochemical studies: (1) The first is analysis of binding data obtained under conditions in which a second, linked ligand is present in limited total quantity. In such conditions the free activity of the second ligand varies throughout the primary ligand binding curve, and the resultant behavior can be quite complex. Analysis of such curves enables one to quantitatively extract detailed information regarding the linkage of the two ligands at intermediate stages of ligation. The treatment is applied in an accompanying paper to oxygen binding in human hemoglobin in the presence of organic phosphates [Robert, C. H., Fall, L., & Gill, S. J. (1988) *Biochemistry* (following paper in this issue)]. (2) The second treatment we outline regards the analogous problem of analyzing differential scanning calorimetry (DSC) data obtained for a macromolecule binding a ligand present in limited quantity. A simple model is presented that accounts for dual transitions like those already seen in DSC data for human serum albumin in the presence of nonsaturating amounts of fatty acids [Ross, P., & Shrake, A. (1987) *Abstracts of the 42nd Calorimetry Conference*, University of Colorado, Boulder, CO].

The cellular environment can influence the function of a heterotropic linkage system (Wyman, 1964) in two major ways. In one case it may provide a buffering capacity for one ligand, as is often seen in the case of hydrogen ions, maintaining that ligand at a particular activity throughout the binding process of another, primary ligand. However, another common situation is where the total amount of a particular ligand in the cell is limited. The free concentration of this ligand, whose binding affinity for the macromolecule is either increased (positive linkage) or decreased (negative linkage) upon the binding of the primary ligand, can then undergo large variations during the primary binding process. The resultant binding curve of the primary ligand can then be far more complex than that seen under conditions where the secondary ligand is buffered at a particular value. A related complication can appear in thermal transition curves when the macromolecule is subjected to limited quantities of ligand. The thermodynamic analysis of the effects of limiting the total amount of a ligand is the subject of the present paper.

In the red blood cell a classic example of variation in a linked ligand's activity is that occurring with the effector compound 2,3-diphosphoglycerate (DPG) during oxygen binding to hemoglobin (Benesch & Benesch, 1968). The affinity of hemoglobin for DPG decreases upon oxygen saturation of the molecule. Since the DPG molecule is not free to pass through the cell membrane, its activity increases with increasing oxygenation, and the result can be a complex, biphasic effect on the oxygen binding curve under low chloride conditions (Imai & Tyuma, 1973; Herzfeld & Stanley, 1974). In 1979 Ackers formulated the dependence of the median of the oxygen binding curve under such situations (Ackers, 1979) for a second ligand binding with one-to-one stoichiometry, later applied to the analysis of overall linkage of inositol hexasulfate to oxygen binding in hemoglobin (Ackers et al., 1982). Imai and Tyuma (1973) described the effects one would see on the Adair oxygen binding curve for one-to-one stoichiometry of binding of the second ligand, and Herzfeld and Stanley (1974) used a detailed allosteric model to show similar effects. However, a general phenomenological treatment of the binding curve and median under limited total effector conditions thus far has been unavailable.

The increased complexity of the binding curve often seen in the case of the fixed, nonsaturating amount of second ligand makes it apparent that experiments undertaken with such constraints can furnish more information than can experiments conducted under buffered or excess ligand conditions. In this paper we outline a thermodynamic description of the various equilibria present in systems of a macromolecule binding two types of ligand, especially when one of the ligands, which we

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