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Structure of the Complex Polysaccharide C-Substance from *Streptococcus pneumoniae* Type 1[†]

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ABSTRACT: The complex cell-wall polysaccharide, C-substance, was isolated from *Streptococcus pneumoniae* type 1 and purified by DEAE-cellulose (HCO_3^- form) and Sephadex column chromatography. The complete structure of this antigen was obtained by the application of methylation and ^1H NMR and ^{13}C NMR spectroscopic techniques to a series of oligosaccharide fragments obtained by the selective degradation of the N-acetylated antigen. Native C-substance is composed of the following repeating unit: β-D-Glu*p*-1→3-α-AAT-Gal*p*-1→4-α-D-GalNAc*p*-1→3-β-D-GalNH$_2$*p*-1→1′-ribitol-5-phosphate where AATGal is 2-acetamido-4-amino-2,4,6-trideoxygalactose. Phosphocholine substituents are situated at O(6) of the unacetylated galactosamine residues, and the repeating units are linked through a diphosphate ester from ribitol to O(6) of the β-D-glucopyranose residue. This structure has also been shown to be common to C-substances prepared from a number of other pneumococcal types based on the criterion of their identical ^{13}C NMR spectra.

Streptococcus pneumoniae produce a complex species-specific antigenic polysaccharide (PnC)[1] which was first described by Tillett et al. (1930). It is of interest as a common antigen of the various strains of the pneumococci and is a contaminant in most type-specific polysaccharide preparations. PnC contains phosphocholine (Brundish & Baddiley, 1968), and this component is responsible for several properties of the polysaccharide. It is a major antigenic determinant (Heidelberger et al., 1972), and the interactions of PnC with the acute-phase serum protein C-reactive protein (Tillett & Francis, 1930) and with certain mouse myeloma proteins (Potter & Leon, 1968) are both due to phosphocholine (Leon & Young, 1971; Volonakis & Kaplan, 1971). PnC is attached to the cell-wall mucopeptide in the organism, and the phosphocholine plays an important role in its physiology (Tomasz et al., 1975). Mucopeptide fragments are present in PnC preparations (Liu & Gotschlich, 1963; Gotschlich & Liu, 1967).

The structure of the remainder of this complex polysaccharide has been only partly determined, and its role in the above properties is not known. In addition to phosphocholine, Brundish & Baddiley (1967, 1968) identified 2-acetamido-2-deoxy-D-galactose, D-glucose, ribitol, phosphate, and an *N*-acetyldiaminotrideoxyhexose as constituents of PnC and therefore classified it as a ribitol teichoic acid. Further structural studies by Watson & Baddiley (1974) led to a partial structure being proposed. This structure was based on a trisaccharide–ribitol–phosphate repeating unit in which D-glucose was a major constituent. In later work (Poxton et al., 1978) the structure was modified essentially to a disaccharide–ribitol–phosphate repeating unit which did not include D-glucose as a major constituent, despite the fact that the presence of four anomeric signals (of approximately equal intensity) in the ^{13}C NMR spectrum of PnC strongly suggested additional hexose units.

We now report the complete structural analysis of PnC, including the identification of a glucopyranose residue and, in addition, a 2-amino-2-deoxy-D-galactose residue in its repeating unit. On the basis of chemical analyses and assignments made on the ^{13}C NMR spectra of three oligosaccharides obtained from PnC by degradation procedures, a structure for PnC is proposed. This structure is consistent with many of the structural features previously proposed for PnC by Watson & Baddiley (1974).

Experimental Procedures

Purification of PnC. For types 1, 23, and 56, crude lyophilized material, obtained by the further alcohol fractionation of the culture medium (80% ethanol) following removal of the capsular polysaccharide (Krishnamurthy et al., 1978), was kindly supplied by Dr. Thomas Stoudt of Merck Sharp & Dohme, Rahway, NJ. A similar fraction obtained from type 33 organisms was also kindly given by Dr. C. T. Bishop of our laboratories. Cells of the rough strain R36A (which derives from a type 2 organism) were extracted with trichloroacetic acid, and the extract was fractionated with acetone and ethanol as described by Brundish & Baddiley (1968). The yield from R36A cells was greatly increased by lengthening the extraction time to 2 weeks.

PnC was purified from all the above crude fractions by the method of Brundish & Baddiley (1968). Each lyophilized

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[1] Abbreviations used: PnC, C-substance or C-polysaccharide of *S. pneumoniae*; DEAE, diethylaminoethyl.

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extract was suspended in water and treated with RNase and DNase followed by Pronase. The digests were dialyzed against water and applied to columns of DEAE-cellulose (HCO_3^- form of DE52, Whatman Inc.) in water, and the columns were developed with a linear gradient of $(NH_4)_2CO_3$ to 0.15 M. PnC fractions were detected by Ouchterlony immunodiffusion tests using the phosphocholine-specific murine myeloma protein (Leon & Young, 1971), MOPC 167. The PnC eluted in a broad peak which we divided into three fractions in order of elution, the third fraction being heavily contaminated with poly(glutamic acid). The first two fractions were lyophilized, and the PnC in both was purified by gel filtration on a Sephadex G-100 column (90 × 2.5 cm) with water as the eluant. The PnC in the center portion of the fractionation was found to be virtually free of contaminants absorbing at 260 or 280 nm.

N-Acetylated PnC was produced by treating PnC with acetic anhydride in aqueous solution buffered with $NaHCO_3$.

Analytical Methods. Solutions were concentrated under reduced pressure below 40° C. Galactosamine was detected by using a Technicon autoanalyzer and quantified by the method of Strominger et al. (1959), and glucose was determined by the method of Dubois et al. (1956). Ribitol and glycerol were identified (the former being quantified) as their peracetates by gas–liquid chromatography (GLC) analysis (Sawardeker et al., 1965) by using column 1 (see below). Inositol was used as an internal standard, and ribitol was quantified following its removal from PnC by 48% HF. Phosphate was determined by the method of Chen et al. (1956). Paper chromatography was carried out on Whatman No. 1 paper by using 1-butanol–pyridine–water (6:4:3 v/v/v) as the irrigant. Compounds were detected with alkaline silver nitrate (Trevelyan et al., 1950) and periodate–benzidine (Gordon et al., 1956) spray reagents.

GLC was performed on a Hewlett-Packard 5830A instrument equipped with a flame ionization detector and a Model 18850A electronic integrator. The glass columns (180 × 0.15 cm) used contained the following liquid phases on Gas-Chrom Q: (1) 3% ECNSS-M at 200 °C, (2) 3% OV-225 at 200 °C, and (3) 3% OV-17 at 200 °C. Glass-capillary chromatography was performed by using SP-1000 W.C.O.T. columns. Combined gas chromatography–mass spectrometry (GC–MS) was carried out on a Finnigan 3100D instrument with columns 1, 2, and 3 and an ionization potential of 70 eV.

^{13}C NMR spectra were recorded at 37 °C on a Varian XL-100-15 spectrometer (in 12-mm tubes) operating at 25.16 MHz and a Varian CFT 20 spectrometer (in 10-mm tubes) operating at 20 MHz, both in the pulsed Fourier transform mode with complete proton decoupling. Chemical shifts are reported in parts per million (ppm) downfield from external tetramethylsilane, and the ^2H resonance of deuterium oxide was used as a field-frequency lock signal. The PnC and derived oligosaccharides were run as deuterium oxide solutions at concentrations of 25–80 mg/mL. ^1H NMR spectra were recorded on a Varian CFT 20 spectrometer operated at 80 MHz at 85 °C in the pulsed Fourier transform mode with a 30° pulse angle and an acquisition time of 1.5 s. These parameters ensured that saturation of the resonances, the integrals of which were utilized for analytical purposes, was avoided. Samples were lyophilized (twice) from 99.7% D_2O and run in the same solvent, and 3-(trimethylsilyl)propanesulfonic acid (Na^+ salt) was used as an internal standard.

Methylation Analysis. Oligosaccharides were reduced with sodium borodeuteride and methylated according to the method of Hakomori (1964), and the methylated oligosaccharides were purified, hydrolyzed, and acetylated as previously described (Jennings et al., 1980). The specifically methylated alditol acetate derivatives were then analyzed by GC–MS with column 1 for glycose derivatives and column 3 for hexosamine derivatives (Lindberg, 1972).

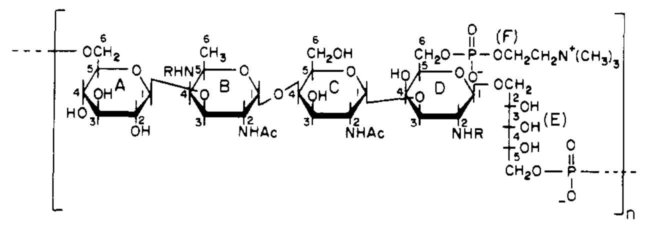
Deamination of PnC. PnC (20 mg) in water (1 mL) was treated with 33% acetic acid (1.5 mL) and 5% sodium nitrite (1.5 mL) at room temperature for 40 min, diluted with water (20 mL), and lyophilized. The deaminated PnC was dissolved in water (1 mL) and desalted by passage through a Sephadex G-25 column (2.6 × 90 cm). Deaminated PnC was reduced with sodium borohydride and hydrolyzed with 0.5 M trifluoroacetic acid at 100 °C for 16 h, and its components were analyzed as their alditol acetate derivatives by GC–MS with columns 1, 2, and 3. Glass-capillary columns were also used in this analysis.

Preparation of Oligosaccharide II. N-Acetylated PnC (200 mg) was treated at 4 °C with 48% HF (2 mL), a reagent which has been shown to promote the facile cleavage of phosphate esters (Lipkin et al., 1969). Samples (10 μL) were withdrawn and analyzed for the release of inorganic phosphate and ribitol. When the amounts of these components had reached a maximum, after 96 h of hydrolysis, excess HF was removed under reduced pressure in a desiccator over solid NaOH, and the residue was dissolved in water and fractionated on a Sephadex G-25 column (2.6 × 90 cm). The major oligosaccharide product (60 mg) is described below as oligosaccharide II, and an earlier eluting higher molecular weight material was shown to contain muramic acid.

Preparation of Oligosaccharides I and III. A Smith degradation was carried out as described by Goldstein et al. (1965). N-Acetylated PnC (200 mg) was treated with 0.01 M $NaIO_4$ (200 mL) and the mixture kept at room temperature in the dark. Samples (0.02 mL) were withdrawn at intervals and diluted to 4 mL with water, and the periodate uptake was measured spectrophotometrically (Aspinall & Ferrier, 1957). Oxidation was complete at 120 h and, following a further incubation period of 48 h, the excess $NaIO_4$ was destroyed by the addition of ethylene glycol (250 mg). Then 2 h after this addition, sodium borohydride (150 mg) was added and the reaction was left for 16 h at room temperature. Excess sodium borohydride was destroyed by the addition of Dowex 50 (H^+ form) ion-exchange resin, and, following removal of the resin by filtration, the boric acid was removed by repeated (3 times) evaporation from methanol under vacuum. The residue was then hydrolyzed with 0.005 M sulfuric acid at 100 °C for 2 h, neutralized with NaOH solution, and lyophilized. The products were fractionated on a Sephadex G-25 column (2.6 × 90 cm). The major oligosaccharide product (62 mg) is described below as oligosaccharide III, and treatment of this material with HF as described above yielded a smaller fragment (34 mg) referred to as oligosaccharide I. Prior to the elution of oligosaccharide III, a higher molecular weight material was obtained which contained muramic acid.

Results and Discussion

Composition of PnC. The analytical results indicate that PnC contains galactosamine, glucose, phosphate, ribitol, choline, and a trideoxydiaminohexose in the molar ratio of 2.4:0.9:1.8:0.8:0.9:1.0, thus confirming glucose as a major consituent of the repeating unit of PnC (Watson & Baddiley, 1974) and also indicating the presence of an additional galactosamine residue. The analyses for choline and the trideoxydiaminohexose were obtained from the ^1H NMR spectrum of PnC. Integration of the CH_3 signals of the trideoxydiaminohexose (δ 1.35), acetamido (δ 2.12), and choline



Ia) R = H
Ib) R = $COCH_3$

FIGURE 1: Repeating unit of PnC (a) and N-acetylated PnC (b).

(δ 3.28) residues gave an intensity ratio of 1.0:2.0:2.8, thus indicating a mole ratio of 1.0:2.0:0.9 for these respective constituents. That only two acetamido groups were detected despite the presence of two galactosamine residues and a diamino sugar is only consistent with the presence of two free amino groups in the repeating unit of PnC. This was confirmed by the ^1H NMR spectrum of N-acetylated PnC which gave an intensity ratio of 1.0:4.0:2.9 for the above constituents. The above component analyses accounted for only 70% of the total weight of the antigen, thus indicating the presence of substantial amounts of other material. The identification of muramic acid (amino acid analyzer) in this material, as with a previous PnC preparation reported by Gotschlich & Liu (1967), indicated that it contained peptidoglycan.

When PnC was deaminated with HNO_2 and hydrolyzed and the monomers were reduced and peracetylated, GC–MS analysis showed two major additional components to those previously identified in PnC. The first was identified as 2,5-anhydrotalitol acetate which must have been derived from a 2-amino-2-deoxygalactose residue (Williams, 1975) of PnC. The second component had the same retention time by GC analysis as an authentic sample of 2-acetamido-2,6-dideoxyglucose (quinovosamine) alditol acetate (Perry & Daoust, 1973). The MS of the second component also gave a fragmentation pattern identical with that given by the authentic compound. The identification of this component in the gluco configuration indicated that it was derived from the 2-acetamido-4-amino-2,4,6-trideoxyhexose originally proposed as a constituent of PnC by Distler et al. (1966) and confirmed by Brundish & Baddiley (1967, 1968) and that this latter constituent of PnC must have the galacto configuration. This is established by the isolation of 2-acetamido-2,6-dideoxyglucose in similar deamination studies on the *Shigella sonnei* phase 1 lipopolysaccharide (Kenne et al., 1980) and the *S. pneumoniae* type 1 capsular polysaccharide (Lindberg et al., 1980). These authors demonstrated that it was the major product of the deamination of 2-acetamido-4-amino-2,4,6-trideoxygalactose residues in the above polysaccharides and that replacement of the 4-amino group by the hydroxyl group occurred with inversion of configuration.

The mode of linkage and the sequence of the components of the repeating unit of PnC were determined by using data obtained from the methylation and ^{13}C NMR analyses of some constituent oligosaccharide fragments obtained by selective degradations of N-acetylated PnC. The oligosaccharide fragments were prepared in the following way. Native PnC, the repeating unit of which is shown in Figure 1a, was treated with acetic anhydride in $NaHCO_3$ solution to yield N-acetylated PnC (Figure 1b). A portion of N-acetylated PnC was then degraded by periodate oxidation (Smith degradation) to a nonreducing trisaccharide component containing choline phosphate (III). Oligosaccharide III was then dephosphorylated with 48% HF for 96 h at 4 °C to yield a further degraded, reducing trisaccharide (I). Direct treatment of another portion of N-acetylated PnC with 48% HF yielded a reducing tetrasaccharide (II).

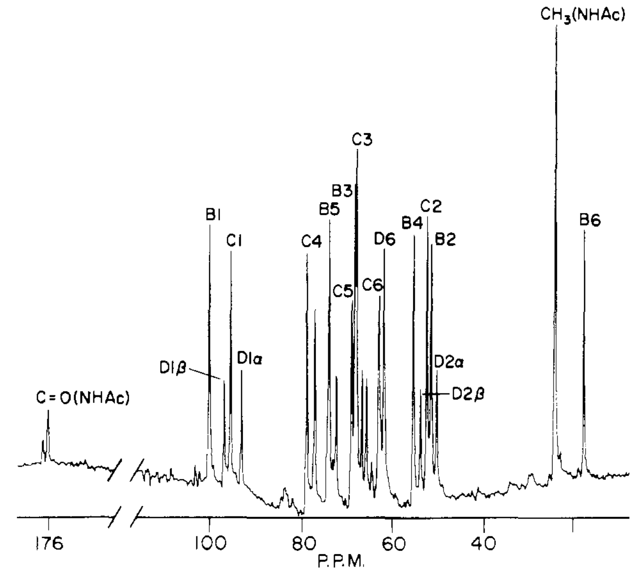
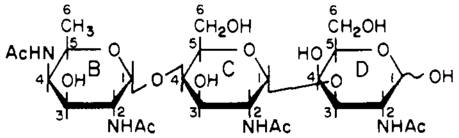


FIGURE 2: Fourier-transformed ^{13}C NMR spectrum of oligosaccharide I at 20 MHz taken with an aquisition time of 0.45 s and a spectral width of 5 kHz. The number of free induction decays was 140 000.



I

Oligosaccharide I. This trisaccharide constitutes components B, C, and D of the repeating unit of N-acetylated PnC (Figure 1b). Analyses indicated that oligosaccharide I contained galactosamine and diacetamidotrideoxygalactose in the molar ratio of 2:1. The former component was estimated by using the Elson–Morgan method (Strominger et al., 1959), and the latter was estimated by using the intensity ratio (1:4) of its B6 protons with all the acetamido CH_3 protons by ^1H NMR spectroscopy. Direct treatment of the unhydrolyzed trisaccharide I with the Elson–Morgan reagents indicated a galactosamine content exactly half that given by the normal Elson–Morgan method involving prior hydrolysis of the trisaccharide I. This result suggests that one of the galactosamine residues constitutes the reducing end group of I and that it is probably linked at O(3) (Jeanloz & Trémège, 1956). This was confirmed by methylation analysis of I which yielded equal amounts of 3,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)galactitol and 4,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)galactitol as determined by GC–MS analysis using column 3. When I was reduced with sodium borohydride prior to the methylation analysis, instead of the latter derivative, 1,4,5,6-tetramethyl-2-deoxy-2-(*N*-methylacetamido)galactitol was detected. A 3-linked galactosamine residue was therefore present as the reducing end group of I, while the 4-linked residue was present as an interior residue, both residues being in the pyranose form. Thus, the diacetamidotrideoxygalactose residue, the methylated derivative of which was not detected by GLC analysis, must be situated at the nonreducing end of I. These results were confirmed by ^{13}C NMR spectroscopy which also revealed the anomeric configuration of the linkages. The ^{13}C NMR spectrum of I is shown in Figure 2, and some of the assigned chemical shifts are listed in Table I. The

Table I: Carbon-13 Chemical Shifts of Oligosaccharide Fragments Obtained from N-Acetylated PnC[a]

fragment	constituent	^{13}C chemical shift (ppm) of constituent carbon 1	2	3	4	5	6
I	B	99.4	51.7	67.5[b]	54.6	73.1	16.6
	C	94.8	50.7	67.2[b]	78.0	68.2[b]	62.4
	D(β)	96.1	53.0	78.0	65.9	76.2	61.2[c]
	D(α)	92.3	49.4	76.2	64.9	71.2	62.4[c]
II	A	105.0	76.4[d]	76.9[d]	70.7	76.9[d]	62.1
	B	99.6	50.3	74.1[e]	54.5	73.1[e]	16.6
	C	94.8	50.7	67.4[f]	78.0	68.2[f]	62.1
	D(β)	96.1	53.1	78.0	65.9	76.3	61.2
	D(α)	92.4	49.4	76.3	64.9	71.6	61.2
III	B	99.6	51.7	67.6[g]	54.7	72.4	16.7
	C	94.5	50.7	67.3[g]	77.7	68.1[g]	61.8
	D	102.4	51.7	75.8	67.3[g]	75.0	65.1[i]
	E	64.3	60.5[h]				
	F	55.2	65.1[i]	60.8[h]			
phosphocholine		55.3	67.4	59.3			

[a] In parts per million from external tetramethylsilane. [b-h] Due to the closeness of their chemical shifts, the assignments within each of these groups could be interchanged. [i] Overlapping doublets.

spectrum exhibits both resonances attributed to the carbons of residues B and C and resonances of lower intensity attributed to the carbons of D by virtue of the presence of both α and β anomers in solution. Thus, the less intense anomeric signals at 92.3 and 96.1 ppm were assigned to D1(α) and D1(β), respectively, by comparison with previous assignments made on galactosamine (Bundle et al., 1973). The two anomeric resonances at 94.8 and 99.4 ppm were assigned to C and B, respectively, because on reduction of I only the former signal was displaced (to 99.6 ppm), thus indicating it was the anomeric carbon of the residue (C) adjacent to D. In comparison with the chemical shifts of α-D-galactosamine residues in another bacterial polysaccharide (Jennings et al., 1979), residue C can be assigned the α-D configuration, and, because it is a configurational analogue of C, residue B can also be assigned in a similar manner to the α-D configuration. Confirmation of this latter assignment is established by the recent finding in other bacterial polysaccharides of both the α-D (Lindberg et al., 1980) and β-D (Kenne et al., 1980) anomers of residue B. In comparison with the chemical shifts of the anomeric carbons of the above polysaccharides that of residue B is only consistent with its assignment to the α-D configuration.

Reduction of I also caused the D1(α) and D1(β) signals to disappear from the anomeric region of the ^{13}C NMR spectrum and caused a downfield displacement (+0.4 ppm) in only one of the signals of the acetamido-substituted carbons of residues B and C. On the basis of residue C being adjacent to D, the signal which was displaced from 50.7 to 51.1 ppm was assigned to C2. The C3, C5, and C6 signals were assigned by comparison with previous assignments made on α-D-galactosamine (Bundle et al., 1973), allowing for an 8.3-ppm downfield shift on the linked C4 position and an accompanying smaller upfield shift on the vicinal C3 and C5 positions (Jennings & Smith, 1978). The characteristic signal at 16.6 ppm was assigned to B6, and those at 51.7 and 54.6 ppm were assigned respectively to B2, in comparison with the configurationally analogous C residue, and to B4, as the remaining unassigned acetamido-substituted carbon. Further evidence for this latter assignment can be obtained from ^{13}C NMR data on PnC and N-acetylated PnC (vide infra). By use of the configurationally similar C residue as a model for B, the resonance at 67.5 ppm can be tentatively assigned to B3, and by elimination the remaining intense resonance at 73.1 ppm was assigned to B5.

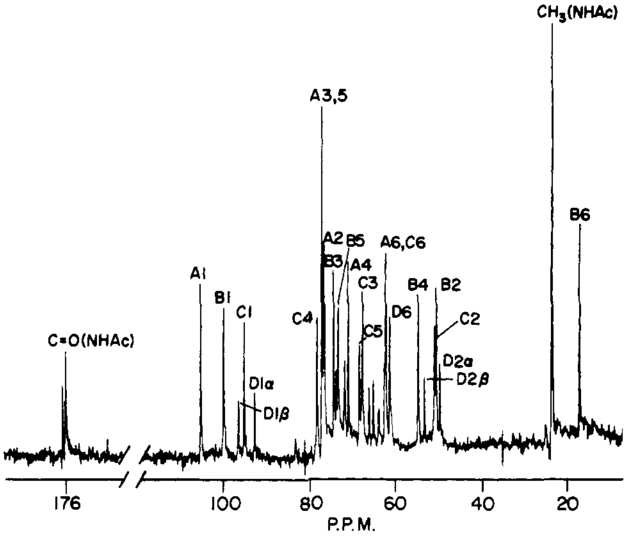
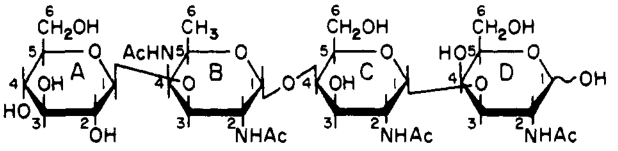


FIGURE 3: Fourier-transformed ^{13}C NMR spectrum of oligosaccharide II at 20 MHz taken with an aquisition time of 0.5 s and a spectral width of 5 kHz. The number of free induction decays was 250 000.

From the proposed structure of N-acetylated PnC (Figure 1b), treatment of its periodate oxidized product (III) with HF would not have been expected to yield a reducing trisaccharide. This finding is discussed below.

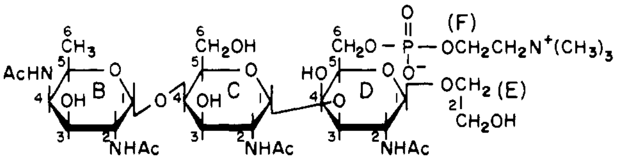


II

Oligosaccharide II. Direct treatment of N-acetylated PnC with 48% HF yielded tetrasaccharide II which was shown by analytical methods to contain glucose, galactosamine, and diacetamidotrideoxygalactose in the molar ratio of 1.1:2.0:1.0. Thus, fragment II has all the hexose constituents (residues A, B, C, and D) of the repeating unit of PnC (Figure 1a). Methylation analysis using columns 1 and 3 indicated the presence of the same 3,6- and 4,6-di-*O*-methyl(*N*-methylacetamido)galactitol derivatives as obtained previously from oligosaccharide I plus an additional 2,3,4,6-tetra-*O*-methylglucitol derivative. This evidence also indicated that the glucopyranose residue must constitute the nonreducing end group of II and is therefore linked to the diacetamidotrideoxygalactose residue (B). The fact that it is linked to O(3) of B is evident because the only alternative possibility, an N-glycosidic linkage, can be discounted by the analytical proof of the presence of four acetamido groups in N-acetylated PnC. The anomeric configuration of the glucopyranose residue (A) was confirmed by ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of II is shown in Figure 3 and the assigned chemical shifts are listed in Table I. A comparison of these chemical shifts with those previously assigned in I indicates that most of the resonances of trisaccharide I are unaltered in the ^{13}C NMR spectrum of II except for those of B2 and B3 which underwent characteristic shifts (−1.4 and +6.5 ppm, respectively) associated with A being linked to B3 (Jennings & Smith, 1978). The additional resonances in this ^{13}C NMR spectrum as compared to the spectrum of I are those associated with the glucopyranose residue (A) which was shown to have the β-D configuration by the similarity of the chemical shifts of the resonances of A, except for the anomeric resonance, with those previously assigned to β-D-glucopyranose (Colson et al., 1975). In addition, the anomeric resonance of A at 105.0 ppm is also consistent with the glucopyranose residue (A) having the β-D configuration (Colson et al., 1974).

Treatment with 48% HF of both N-acetylated PnC and oligosaccharide III, obtained by periodate oxidation of N-acetylated PnC, yielded reducing oligosaccharides II and I, respectively. On this evidence one might suspect the presence of a phosphodiester group between the galactosamine (D) and ribitol (E) residues (Lipkin et al., 1969). However, such a linkage would not be consistent with the findings of Watson & Baddiley (1974), who established that ribitol was linked to galactosamine by an O-glycosidic linkage. We concur with their findings and have provided further evidence for the presence of this linkage by isolating fragment III from N-acetylated PnC (vide infra) which contains an ethylene glycol aglycon. Thus, the O-glycosidic bonds between galactosamine and both ethylene glycol in III and ribitol in N-acetylated PnC are broken by treatment with 48% HF. The facile hydrolysis of an O-glycosidic bond by HF has been observed previously in the case of a rhamnose–erythritol linkage (Bennett & Glaudemans, 1979), and recently we have accumulated additional evidence to indicate that it is due to the higher sensitivity of certain types of glycosidic linkages to hydrolytic cleavage (H. J. Jennings and C. Lugowski, unpublished experiments).

Oligosaccharide III. This substituted trisaccharide was



III

obtained from the Smith degradation of N-acetylated PnC and was principally composed of the same constituents (B, C, and D) as I. This became evident when treatment of III with 48% HF afforded fragment I. However, in contrast to I, fragment III did not give a color with the Elson–Morgan reagents directly, indicating that D was glycosidically linked in this case. This evidence was consistent with the ^{13}C NMR spectrum of III (Figure 4) in which the less intense signals in the ^{13}C NMR spectrum of I (Figure 2) attributed to residues D(α) and D(β) were replaced by other more intense signals. Thus, the D1(α) and D1(β) signals in the ^{13}C NMR spectrum of I were replaced by a single anomeric resonance (D1) at 102.4 ppm. The large displacement (+7.9 ppm) of the D1 signal in relation to that of the anomeric signal of the other galactosamine residue of III (previously assigned the α-D configuration) indicates that the glycosidic linkage of D is in the β-D configuration. The fact that ethylene glycol formed the aglycon of residue D in III was established by the identification of ethylene glycol in the low molecular weight products of the 48% HF hydrolysate of III. Ethylene glycol was detected in both underivatized and peracetate forms by GLC analysis using a glass-capilliary column at 220 and 150 °C, respectively. It was also detected by paper chromatographic analysis.

In addition to ethylene glycol and constituents B, C, and D, fragment III also contained a phosphocholine substituent. Analysis indicated that B, C, D, phosphate, and choline were all present in molar ratios, the analysis for choline content being carried out by ^{1}H NMR as explained previously for PnC.

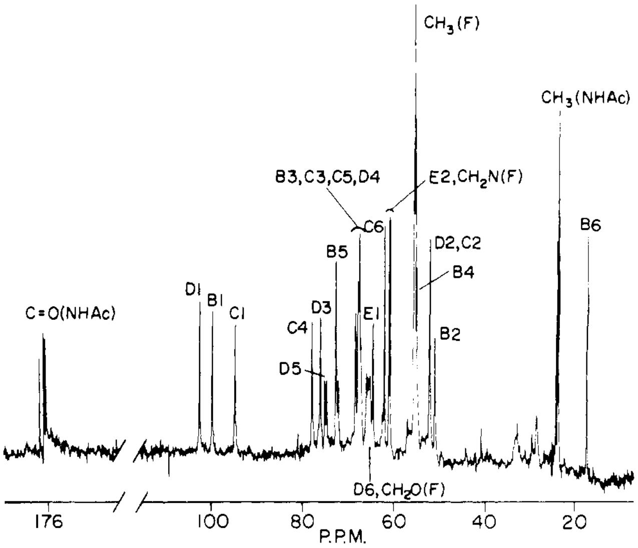


FIGURE 4: Fourier-transformed ^{13}C NMR spectrum of oligosaccharide III at 20 MHz taken with an aquisition time of 0.5 s and a spectral width of 5 kHz. The number of free induction decays was 161 000.

In contrast to the GC–MS methylation analysis of I, analysis of III yielded only the 3,6-di-*O*-methyl(*N*-methylacetamido)galactitol derivative (originating from constituent C), thus indicating that the charged phosphocholine residue was linked to the terminal 3-linked galactosamine residue (D). This charged substituent would prevent the passage of the methylated derivative through the column. The ^{13}C NMR spectrum of III shown in Figure 4 is also consistent with the above evidence and in addition enables the location of the phosphocholine substituent on D to be established. A comparison of the chemical shifts associated with III with those associated with I (Table I) indicated that, except for a small shift on B5, all the other resonances of constituents B and C remained essentially at the same chemical shifts. Model studies using phosphocholine enabled the signals at 55.2 and 60.8 ppm and one of the two overlapping ^{31}P–^{13}C coupled signals centered on 64.3 ppm to be assigned to the CH_3, CH_2N, and CH_2O carbons of the phosphocholine substituent (F), respectively [Table I and Poxton et al. (1978)]. The signals at 64.3 and 60.5 ppm were assigned to the ethylene glycol aglycon (E1 and E2, respectively). Thus all the unsubstituted hydroxymethyl signals in the ^{13}C NMR spectrum of III were accounted for which implied that the phosphocholine substituent of III was linked at O(6) of D. In this event the D6 signal would be a doublet ($^{2}J_{^{31}P-^{13}C}$) at lower field (Jennings & Smith, 1978) and is probably the second of the two overlapping coupled (^{31}P–^{13}C) signals centered at 65.1 ppm. The doublet at 75.0 ppm was assigned to the vicinally coupled D5 signal, and, when allowance for characteristic shielding (−1.3 ppm) on D5 by the adjacent phosphate substituent (Jennings & Smith, 1978) was made, its chemical shift was consistent with that expected for D being in the β-D configuration (Bundle et al., 1973). The remaining D3, D2, and D4 signals were assigned by comparison with previous assignments made on 2-acetamido-2-deoxy-β-D-glucopyranose (Bundle et al., 1973) when allowance for a downfield displacement (+3.5 ppm) on the signal of the linked D3 position and other characteristic displacements (−1.7 and −3.2 ppm) on the signals of the respective D4 and D2 positions was made.

Structure of PnC. The greater part of the structure and sequence of the repeating unit of PnC obtained from *S. pneumoniae* type 1 was established from the structural data accumulated on its oligosaccharide fragments (I, II, and III).

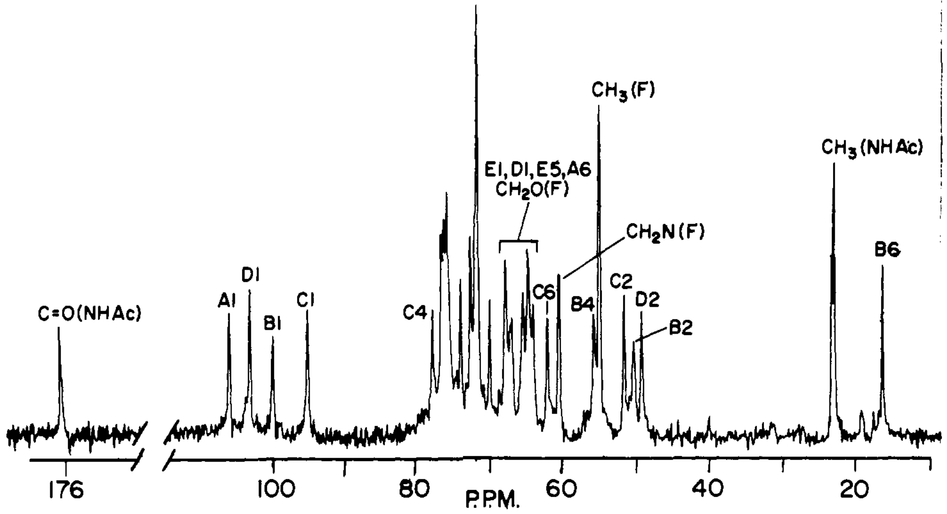


FIGURE 5: Fourier-transformed ^{13}C NMR spectrum of PnC at 25.16 MHz taken with an aquisition time of 0.4 s and a spectral width of 5 kHz. The number of free induction decays was 192 000.

Table II: Some Carbon-13 Chemical Shifts of PnC and N-Acetylated PnC[a]

	^{13}C chemical shift (ppm) of										
compd	A1	D1	B1	C1	C4	C6	B4	C2	B2	D2	D6
PnC	105.2	102.4	99.4	94.7	77.8	62.2	56.1	51.8[b]	50.6[b]	49.4[b]	16.6
N-acetylated PnC	104.4	102.3	99.5	94.7	77.4	62.2	53.9	51.8	50.4	50.4	16.7

[a] In parts per million from external tetramethylsilane. [b] Tentative assignments.

The structure of the repeating unit is shown in Figure 1a and, except for the ribitol–phosphate and phosphocholine residues, has a backbone consisting of oligosaccharide II. Studies on fragment III established that phosphocholine was attached to O(6) of the 3-linked galactosamine residue (D) and that this latter residue was linked glycosidically to ribitol in the β-D configuration. In previous studies, Liu & Gotschlich (1963) and Brundish & Baddiley (1968) had identified galactosamine 6-phosphate as a product of the acid hydrolysis of PnC. The identification of ethylene glycol as the aglycon of D in III also established that ribitol was linked via its O(1′) position (E1). The determination of the remaining phosphate diester linkage from the ribitol (E) to the β-D-glucopyranose residue (A) was accomplished by using ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of PnC is shown in Figure 5, and, although it is more complex than those of its fragments due to overlapping signals, some resonances essential to the structural elucidation can be assigned. These are listed in Table II. The ^{13}C NMR spectrum of PnC manifests only one signal in the region of its spectrum associated with an unsubstituted primary hydroxyl carbon of a hexapyranose ring (62.0 ± 0.5 ppm) (Bundle et al., 1973; Colson et al., 1974) at 62.5 ppm which could be readily assigned to C6 of the repeating unit by virtue of the studies on fragment III, provided that C6 itself is not involved in the interchain phosphate diester linkage of PnC. This possibility was eliminated when only phosphate associated with choline remained on fragment III following the Smith degradation of N-acetylated PnC. Thus, in agreement with Watson & Baddiley (1974), the interchain phosphate diester of PnC must be attached to its oxidizable β-D-glucopyranose residue (A). The failure to detect any additional unsubstituted primary hydroxymethyl resonance in the ^{13}C NMR spectrum of PnC implied that all the remaining five primary hydroxymethyl groups of the repeating unit were substituted, indicating that the interchain phosphate diester linkage must be situated between O(5) of ribitol (E5) and O(6) of the β-D-glucopyranose residue (A). The presence of five signals in the region of the ^{13}C NMR spectrum associated with substituted primary hydroxymethyl groups (Jennings & Smith, 1978) confirms this assignment. Although these resonances at 64.1, 64.8, 65.5, 67.2, and 67.9 ppm could not be assigned individually without some ambiguity, they could be collectively assigned to E1, D6, E5, A6, and CH_2O– of the choline moiety (F). This analysis is also only consistent with the quantitative results of the periodate oxidation of N-acetylated PnC (Figure 1b) which yielded an uptake of 4.32 mol of periodate/2.00 mol of phosphorus, which is close to the 4 mol of uptake predicted from the proposed structure. This analysis could be complicated by the presence of peptidoglycan (30%), but the fact that the latter remained in a high molecular weight form following periodate oxidation and HF treatment indicates that its contribution to the uptake of periodate and release of inorganic phosphate would probably be minimal.

On the basis of previous assignments made on the fragments (I, II, and III) of PnC, the following signals in the ^{13}C NMR spectrum of PnC were assigned. The four anomeric signals, indicative of a tetrasaccharide repeating unit at 105.2, 102.4, 99.4, and 94.7 ppm, were assigned to A1, D1, B1, and C1, respectively. The absence of ^{31}P–^{13}C coupling on these signals contraindicates the presence of glycosidic phosphate ester bonds in the repeating unit of PnC. In addition, the first three of the four nitrogen-substituted ring carbon resonances at 56.1, 51.8, 50.6, and 49.4 ppm were similarly assigned to B4, C2, and B2, respectively, the remaining resonance at 49.4 ppm being tentatively assigned to D2. This latter assignment and that of the B4 signal were substantiated by comparison of the ^{13}C NMR spectrum of PnC with that of its N-acetylated derivative. Both the B4 and D2 signals in the latter spectrum were displaced, the B4 signal upfield (−2.2 ppm) and the D2 signal downfield (+1.0 ppm). Similar displacements have been observed on the N-acetylation of hexosamines (Bundle et al., 1973). More recently, Kenne et al. (1980) have reported a similar large upfield shift (−2.8 ppm) on the N-acetylation of the 4 position of a residue identical with B in the β-D configuration.

It is of interest to note that N-acetylation of PnC also resulted in an upfield displacement (−0.8 ppm) in the anomeric signal of the β-D-glucopyranose residue (A1), and a small displacement (−0.4 ppm) on C4 was also observed. Because neither of these residues contain free amino groups and because of their remoteness from the free amino groups of PnC (B4 and D2), these displacements can be attributed to conformational factors. Certainly the magnitude of the former displacement is unusually large for shielding by a δ substituent (Bhattacharjee et al., 1975). The masking of the free amino groups at B4 and D2 would cause changes in the ionic associations within the PnC molecule which could conceivably result in changes in the torsion angles of some of the bonds linking the different residues. In terms of the displacement of anomeric signals, it would appear that the bonds vicinal to residue B are the most affected by the N-acetylation of PnC. A similar conformational dependency has been proposed to account for the large chemical shift displacements which occur on the linkage carbons of a linear (1→4)-α-D-glucan (amylose) when it is cyclized to hexaamylose (Colson et al., 1974).

The original component analysis of PnC accounted for only 70% of the total weight of the antigen, and, although muramic acid was detected in our preparation of PnC, indicating that it contained peptidoglycan, no signals characteristic of the presence of muramic acid (H. J. Jennings et al., unpublished experiments) could be detected in its ^{13}C NMR spectrum. This could be due to a line broadening of these signals caused by the restricted motion of the carbons of the peptidoglycan moiety or by the presence of an insufficient number of these carbons to produce a detectable signal.

Conclusion

The complete structure of the repeating unit of PnC obtained from *S. pneumoniae* type 1 has been elucidated. On the basis of the similarity of their ^{13}C NMR spectra, the structural identity of PnC from types 1, 23, 33, 56, and R36A can also be proposed. This provides further physical evidence that PnC is a common antigen of the pneumococci as previously proposed by Brundish & Baddiley (1968) on the basis of degradation studies. A complete structure of the repeating unit of PnC has not been previously reported although many of its individual structural features have been elucidated (Brundish & Baddiley, 1967, 1968; Watson & Baddiley, 1974; Poxton et al., 1978). For the sake of brevity, all these individual structural features have not been extensively reviewed here. However, using conventional techniques, Watson & Baddiley (1974) proposed a partial structure having many of the features of the present one. A more recent attempt to elucidate the structure of PnC by using ^{13}C NMR spectroscopy by Poxton et al. (1978) met with only partial success due mainly to the fact that their analysis of this extremely complex ^{13}C NMR spectrum was based on an insufficient knowledge of the constituent sugars of PnC.

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Inhibition of Ion Permeability Control Properties of Acetylcholine Receptor from *Torpedo californica* by Long-Chain Fatty Acids[†]

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ABSTRACT: The characteristics of fatty acid inhibition of acetylcholine receptor function were examined in membrane vesicles prepared from *Torpedo californica* electroplax. Inhibition of the carbamylcholine-induced increase in sodium ion permeability was correlated with the bulk melting point of exogenously incorporated fatty acids. Above its melting temperature, a fatty acid could inhibit the large increase in cation permeability normally elicited by agonist binding to receptor. Below its melting temperature, a fatty acid was ineffective. None of the fatty acids altered any of the ligand binding properties of the receptor. Inhibitory fatty acids did not induce changes in membrane fluidity, as determined by electron paramagnetic resonance using spin-labeled fatty acids. The spin-labeled fatty acids also acted as inhibitors, and the extent of inhibition depended largely on the position of the nitroxide group along the fatty acid chain. Addition of noninhibitory fatty acid to the vesicle membranes did not protect the receptor from inhibition by spin-labeled fatty acids. The effects of free fatty acids on acetylcholine receptor function are attributed to the disruptions of protein-lipid interactions.

Membrane vesicles enriched in acetylcholine receptor (AcChR)[1] can be prepared from the electric tissue of *Torpedo californica*. The receptors in the vesicles retain important functional properties, such as agonist and antagonist binding and cation permeability control (Heidmann & Changeux, 1978). The AcChR normally exists in a resting but activatable state; pretreatment with agonist converts the AcChR into a desensitized state. This state is characterized by a 20–400-fold increase in affinity for agonist (Quast et al., 1978; Weiland et al., 1977), but agonist binding no longer results in the opening of a transmembrane cation channel. Both in vivo and in the in vitro vesicle system, the transition to the desensitized state is speeded by local anesthetics. [For reviews, see Heidmann & Changeux (1978) and Barrantes (1979).]

We previously reported that treatment of AcChR-enriched vesicles with phospholipase A_2 (EC 3.1.1.4) from *Naja naja siamensis* venom blocked AcChR activation (Andreasen & McNamee, 1977) and converted the AcChR into the desensitized state (Andreasen et al., 1979). Similar results with other phospholipases A_2 have also been reported (Hanley, 1978; Bon et al., 1979; Moody & Raftery, 1978). Further investigation revealed that certain fatty acids could cause an inactivation of AcChR which was apparently different from the desensitization phenomenon. These fatty acids did not affect any of the ligand binding properties of AcChR. However, they totally abolished the channel opening in response to bound agonist (Andreasen et al., 1979). The AcChR appeared to be in a nondesensitized but uncoupled state. The inhibition of AcChR function depended on fatty acid structure and concentration in the membrane. The rate of fatty acid uptake correlated with the rate of onset of inhibition and removal of fatty acids by bovine serum albumin restored AcChR function. Unsaturated fatty acids were much more effective inhibitors of AcChR function than saturated fatty acids.

Fatty acids have been found to modify membrane functions in a number of other systems. For example, they activate adenylate cyclase (Oryl & Schramm 1975; Hanski et al., 1979) and guanylate cyclase (Glass et al., 1977; Asakawa et al., 1976), promote fusion in vesicles (Kantor & Prestegard 1975) and cells (Ahkong et al., 1973), alter permeability control of zymogen granules (Schramm et al., 1967) and mitochondria (Wojtczak, 1976), and inhibit capping in lymphocytes (Klausner et al., 1980).

We report here results indicating that the inhibitory potency of fatty acids is directly correlated with the physical state of the fatty acids. AcChR inhibition is discussed in terms of specific disruption of protein–lipid interactions induced by the various fatty acids.

Materials and Methods

AcChR-Enriched Vesicles. Vesicles were prepared from *T. californica* electroplax as described previously (Andreasen & McNamee, 1977). *T. californica* were obtained live (Pacific Biomarine, Venice, CA), and the electroplax was used immediately or stored in liquid nitrogen. Final pellets were suspended in vesicle diltuion buffer (VDB: 255 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 1.5 mM sodium phosphate, and

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[1] Abbreviations used: AcChR, acetylcholine receptor; VDB, vesicle dilution buffer; carb, carbamylcholine chloride; Mops, 3-(*N*-morpholino)propanesulfonic acid; EPR, electron paramagnetic resonance.