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Structure of Native Polysaccharide Antigens of Type Ia and Type Ib Group B *Streptococcus*†

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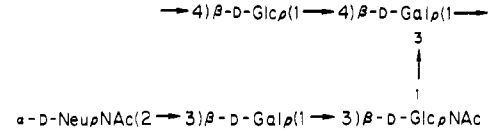
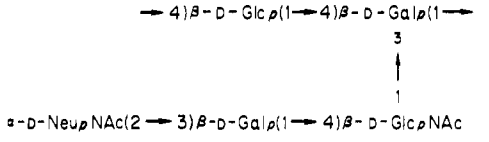
ABSTRACT: The native polysaccharide antigens isolated from the types Ia and Ib group B *Streptococcus* both contain D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and sialic acid in the molar ratio of 2:1:1:1. The structure of the native Ib antigen has been elucidated and is composed of the following repeating unit in which all the side-chain β-D-galactopyranosyl residues are masked by terminal sialic acid residues:



The structure of the native Ia antigen has also been elucidated and is composed of the following repeating unit:



The two native antigens are isomeric, differing only in the linkage of the side-chain β-D-galactopyranosyl residues to their respective 2-acetamido-2-deoxy-β-D-glucopyranosyl residues.

In early immunological studies Lancefield characterized the type-specific antigens (Ia, Ib, II, and III) of group B *Streptococcus* (Lancefield, 1933, 1934, 1938). An additional serotype (Ic) can also be defined on the basis of a type-specific protein antigen (Wilkinson & Eagon, 1971). The type-specific polysaccharide antigens were isolated by hot hydrochloric acid extraction of the streptococcal organisms (Lancefield, 1934, 1938) and thus resulted in structurally incomplete antigens. While still retaining type specificity, these acid-extracted antigens are immunologically incomplete and form a lower molecular weight core to their respective complete native antigens, which all contain additional terminal sialic acid

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residues (Lancefield & Freimer, 1966; Russell & Norcross, 1972; Kane & Karakawa, 1978; Tai et al., 1979; Kasper et al., 1979; Jennings et al., 1980a).

The structure of the type III native antigen has been elucidated (Jennings et al., 1980a, 1981), and the roles of sialic acid in the conformational control of determinants critical to its immunospecificity (Jennings et al., 1981) and in the pathogenicity (Edwards et al., 1982) of the type III organism have been defined. A structure was also previously proposed (Jennings et al., 1980b) for the native type Ia polysaccharide antigen, which, although consistent with the previously published experimental data (Jennings et al., 1980b), has proven to be the incorrect one of two possible structures. This is based on more extensive and definitive degradation studies described in this paper. A revised structure for the type Ia native polysaccharide is proposed, and the same degradation procedures have been used in the structural determination of the type Ib native antigen. The types Ia and Ib native antigens proved to be structural isomers differing from the native type III antigen in the arrangement of their identical glycose constituents.

Materials and Methods

Growth of the Organism. Prototype strain H36B type Ib group B *Streptococcus* was kindly supplied by the late Dr. Rebecca Lancefield, Rockefeller University. The lyophilized strain was rehydrated in Todd–Hewitt broth (Difco) and incubated overnight at 37 °C on a blood agar plate. Strain H36B was inoculated from the blood agar plate into a flask containing 200 mL of Todd–Hewitt broth and incubated for 4 h at 37 °C with stirring (Jennings et al., 1980b). The organisms were checked to document purity at the end of the growth cycle and then inoculated in 20-L volumes of the medium described by Terleckyj et al. (1975) and Carey et al. (1980). The organisms grown in this medium were maintained at pH 7.2 by neutralization of acidic metabolic products with 5 M NaOH, using a pH titrator (Radiometer, Copenhagen) to control additions of base. These cultures were killed with 2% formalin at the end of the growth cycle and then were allowed to stand at 4 °C overnight prior to centrifugation.

Preparation of Type Ib Core Antigen. The core type-specific polysaccharide of group B *Streptococcus* was extracted from strain H36B organisms after overnight growth as described above. The pelleted bacteria were suspended in 0.2 M HCl and boiled for 10 min according to the method of Lancefield (1934). Debris was removed by centrifugation at 100000*g* for 15 min at 4 °C. The supernatant was then titrated to pH 7 with NaOH and extracted with cold 8% trichloroacetic acid at 4 °C for 4 h to remove proteins and nucleic acids. After centrifugation, to remove insoluble debris, the supernatant was neutralized with 1 M NaOH. The crude polysaccharide was precipitated from the extract supernate with 80% ethyl alcohol (v/v). After centrifugation, the precipitate was dissolved in 0.05 M Tris-HCl[1] buffer, pH 7.4. The solution was chromatographed on a 2.6 × 85 cm column of Sephacryl S-200 equilibrated in the same buffer. The serologically active fractions having type Ib or group B activity, excluding UV-absorbable material that was of small molecular size, were combined and concentrated on a PM 30 membrane (Amicon), precipitated with 4 volumes of ethyl alcohol, and suspended in 0.05 M Tris-HCl, pH 8.4. Final purification of the type Ib core antigen devoid of group B antigen was achieved on a column of DEAE-Sephacryl (Pharmacia) equilibrated in 0.05 M Tris-HCl, pH 8.4.

Preparation of Type Ib Native Antigen. After centrifugation to remove organisms, the supernatant of formalin-fixed type Ib group B streptococci that had been grown in the modified Terleckyj's medium was concentrated by ultrafiltration on a PM 30 membrane in a TCE concentrator (Amicon Corp., Lexington, MA). The concentrated supernate was dialyzed against 0.15 M NaCl. Nucleic acids and proteins were partially removed by fractionation with ethyl alcohol at 30% v/v. The resulting supernatant was precipitated with 80% v/v ethyl alcohol, and after centrifugation, the precipitate was suspended in 0.01 M Tris-HCl buffer, pH 7.3, with 0.001 M $MgCl_2$ and 0.001 M $CaCl_2$ added. The solution was treated with DNase, RNase, and Pronase as previously described (Tai et al., 1979). The enzyme-treated supernate was then chromatographed on a 2.6 × 90 cm column of Sephacryl S-200 equilibrated in 0.05 M Tris-HCl buffer, pH 7.4. Four-milliliter fractions were collected and assayed for type Ib and group B serologic activity by capillary precipitin tests to specific antisera (Tai et al., 1979). The fractions containing only type Ib activity were pooled and concentrated to 5 mL in an ultrafiltration cell on a PM 30 membrane. Final purification took place by ion-exchange chromatography with DEAE-Sephacryl equilibrated in 0.05 M Tris-HCl buffer, pH 8.6. The antigen was eluted with a gradient of 1.0 M NaCl. The type Ib native antigen was eluted at approximately 0.3 M NaCl. The fractions having type Ib activity were pooled, concentrated on a PM 30 membrane, and exhaustively dialyzed in distilled water. The final material was lyophilized.

Instrumental Methods. Solutions were concentrated under reduced pressure below 50 °C. Gas/liquid chromatography (GC) 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 (all from Supelco, Bellefonte, PA) on 100–120-mesh gas chrom Q: (i) 3% (w/w) Sp 2340 at 160 → 240 °C at 4 °C/min (partially methylated alditol acetates); (ii) 3% (w/w) OV-17 at 190 °C (partially methylated amino alditol acetates) and at 230 °C (methylated sialic acid derivative); (iii) 3% (w/w) OV-1 at 230 °C (methylated trisaccharide). Combined gas/liquid chromatography–mass spectrometry (GC–MS) was carried out on a Finnigan 3100 D instrument in the above columns and with an ionization potential of 70 eV.

^{13}C NMR spectra were recorded in 10-mm tubes at 37 °C in the pulsed Fourier-transform mode with complete proton decoupling. Chemical shifts are reported in ppm downfield from external tetramethylsilane, and the ^{2}H resonance of deuterium oxide was used as a field-frequency lock signal. The polysaccharides were run as deuterium oxide solutions at concentrations of approximately 50 mg/mL.

Glycose Analysis. Analyses for glycose constituents were carried out essentially by the method of Dmitriev et al. (1975). The polysaccharide (2–3 mg) was hydrolyzed with 0.25 M sulfuric acid at 100 °C for 16 h. The hydrolysate was neutralized with barium carbonate, filtered, and lyophilized. The residue in water (0.6 mL) was then treated with 33% (w/v) acetic acid (1 mL) and 5% sodium nitrite (1 mL) for 1 h at room temperature. Under these conditions, 2-amino-2-deoxy-D-glucose is converted to 2,5-anhydro-D-mannose. The solution was deionized with Dowex 50 (H^+) ion-exchange resin and lyophilized, and the residue was analyzed by GC–MS in column i after conversion of the glycoses into their alditol acetates (Sawardeker et al., 1965). The D configuration was

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance.

assigned to both the galactose and glucose residues by GC analysis of their (+)-2-octyl glycosides as described by Leontein et al. (1978).

Free sialic acid was determined by the thiobarbituric acid method of Aminoff (1961). The sialic acid was removed from the native Ib antigen by mild acid hydrolysis (0.1 M sulfuric acid at 80 °C for 80 min).

Methylation Analysis. The polysaccharides and oligosaccharides were methylated with methyl iodide in the presence of methyl sulfinyl anion according to the method of Hakomori (1964). The methylated polysaccharides were recovered by dialysis against water and concentration of the aqueous solution; and the methylated oligosaccharides were recovered by chloroform extraction of an aqueous solution of the methylation mixture with subsequent concentration of the chloroform solution. The residue was dissolved in 90% (w/v) formic acid and heated at 100 °C for 2 h and then concentrated to dryness. The residue was further hydrolyzed with 0.25 M sulfuric acid at 100 °C for 16 h, and the hydrolysate was neutralized (barium carbonate), filtered, and reduced with $NaBH_4$. The resultant partially methylated glycitols were converted to their alditol acetate derivatives and analyzed by GC–MS (Lindberg, 1972) in column ii. The same method of analysis in column ii could also be used to identify the methylated sialic acid residue (Bhattacharjee & Jennings, 1976). In this analysis, the methylated residue was first isolated as its methyl ester methyl glycoside by methanolysis of the permethylated polysaccharide (Bhattacharjee & Jennings, 1976).

Removal of Terminal β-D-Galactopyranose Residues from Type Ia Core Polysaccharides. The type Ia core polysaccharide (50 mg) in 0.05 M sodium acetate buffer (50 mL) at pH 4.5 was treated with sodium metaperiodate (500 μM) for 20 h at 4 °C. Consumption of periodate was measured spectrophotometrically at 225 nm and was found to be 2.6 μM/mg of the type Ia core polysaccharide. The excess periodate was destroyed by the addition of ethylene glycol (0.33 mL), and the oxidized polysaccharide was reduced with $NaBH_4$. Aqueous acetic acid (50% v/v) was added to the solution, and the solution was dialyzed against water to remove excess boric acid. Lyophilization of the dialyzate yielded 43.5 mg (87%) of the reduced oxidized Ia core polysaccharide. The reduced polysaccharide (43 mg) was hydrolyzed with 0.025 M sulfuric acid (8 mL) at 80 °C for 1 h, and following its adjustment to pH 7.0 with 0.5 M sodium hydroxide, the hydrolysate was dialyzed against water. Lyophilization of the hydrolysate yielded 25 mg (60%) of the degalactosylated Ia core polysaccharide. When subjected to the same degradation procedure, the Ib core polysaccharide also gave an equivalent yield of degalactosylated Ib core polysaccharide.

Deamination of Types Ia and Ib Core Antigens. This was accomplished by the prior N-deacetylation of the Ia and Ib core antigens according to the procedure of Kenne & Lindberg (1980). The N-deacetylated core antigens were then deaminated essentially by the method of Dmitriev et al. (1975) as described for type Ib core antigen. The N-deacetylated type Ib core antigen (35 mg) was dissolved in water (1.2 mL), and following the addition of 33% aqueous acetic acid (2.0 mL), the solution was treated with 5% aqueous sodium nitrite (2.0 mL) for 1.5 h at room temperature. The reaction mixture was passed through Dowex 50 (H^+) ion-exchange resin after which sodium borodeuteride (60 mg) was added to the solution, and it was allowed to stand for 16 h. The excess sodium borodeuteride was destroyed by the addition of glacial acetic acid, and a further addition of acetic acid was made to acidify the solution (pH 4.0). The aqueous acetic acid was evaporated off to leave a residue from which methanol was evaporated 6 times in order to remove boric acid. The products of the deamination were fractionated on a Sephadex G-25 column (2.5 × 80 cm) with a 0.02 M pyridinium acetate buffer at pH 5.4, and a Waters Associates differential refractometer (Model R405) was used for monitoring the eluate. Two major fractions were identified that on lyophilization yielded the polymeric backbone of the type Ib antigen (19.4 mg) and a disaccharide (8.0 mg) containing galactose and 2,5-anhydromannose derived from the branches of the type Ib core antigen.

Results

Structure of the Core Type Ib Antigen. The type Ib acid-extracted core antigen of group B *Streptococcus* contains galactose, glucose, and 2-acetamido-2-deoxyglucose in the molar ratio of 2:1:1 (Tai et al., 1979). The D configuration was assigned to the galactose and glucose constituents by virtue of the characteristic retention times of their (+)-2-octanol glycosides (Leontein et al., 1978). The D configuration of the remaining 2-acetamido-2-deoxyglucose constituent was deduced from the low value of the optical rotation $[\alpha]_D$ −8° (*c* 1.0, water) of the Ib core antigen and the fact that all its constituent sugars had the β-D configuration. This was determined from the ^{13}C NMR spectrum of the Ib core antigen, which exhibited only one signal in the region of the spectrum associated with the signals of anomeric carbons (Jennings & Smith, 1978) at 104.0 ppm. Thus, as in types Ia (Jennings et al., 1980b) and II (Jennings et al., 1980a) core antigens, all the sugar components gave overlapping anomeric carbon signals, which is consistent with them all having the β-D configuration.

The type Ib core antigen was permethylated, and on subsequent hydrolysis, yielded a number of partially methylated sugars that were quantified by GC analysis and identified by GC–MS (Lindberg, 1972; Stellner et al., 1973). The individual methylated sugars detected in this analysis and their respective molar ratios are shown in Table I. The methylation analysis indicates the presence of terminal β-D-galactopyranosyl residues, interchain β-D-glucopyranosyl and 2-acetamido-2-deoxy-β-D-glucopyranosyl residues linked at *O*-4 and *O*-3, respectively, and one branched β-D-galactopyranosyl residue linked at *O*-3 and *O*-4. The linkage analysis was confirmed, and some evidence of the sequence of the sugar components in the type Ib core antigen was elucidated by a modification of the Smith degradation procedure (Jennings et al., 1980a,b). In this degradation, the polyalcohol obtained after periodate oxidation and sodium borohydride reduction of the polysaccharide was methylated, and the permethylated product was partially hydrolyzed. This yielded a partially methylated oligosaccharide that was further methylated with trideuteriomethyl iodide. By this procedure, labeled methyl groups were introduced at exposed hydroxyl groups: previous sites of linkage positions in the original type Ib core antigen. The resultant methylated oligosaccharide is shown in Figure 1. Hydrolysis of the methylated oligosaccharide gave the individual methylated sugars, which were analyzed as their alditol acetate derivatives by GC-MS analysis with sodium borodeuteride to label their reducing hemiacetal carbons (C-1). The detection of 2,4,6-tri-*O*-methylgalactitol, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol, and 1,3,4-tri-*O*-methylerythritol having trideuteriomethyl groups in their respective O-4, O-3, and O-3 positions is consistent with the original linkage (methylation) analysis of the type Ib core antigen (Table I). When subjected to direct GC–MS analysis in column iii at 230 °C, the oligosaccharide gave the following major fragments: mass-to-charge ratio (*m/e*) 45, 71, 74, 92,

Table I: Methylation Analysis of Native Ib and Modified Ia and Ib Group B Polysaccharides

methylated glycose derivative	molar ratios of polysaccharides[a]						
	Ia core	Ia degalactosylated	Ia backbone	Ib native	Ib core	Ib degalactosylated	Ib backbone
2,3,4,6-tetra-*O*-methyl-galactose[b]	0.8	–	–	–	0.9	–	–
2,4,6-tri-*O*-methyl-galactose[b]	–	–	–	0.9	–	–	–
2,3,6-tri-*O*-methyl-glucose[b]	1.0	1.0	1.0	1.0	0.9	1.0	1.0
2,3,6-tri-*O*-methyl-galactose[b]	–	–	0.7	–	–	–	0.7
2,6-di-*O*-methyl-galactose[b]	0.9	0.9	–	1.1	1.0	0.8	–
3,4,6-tri-*O*-methyl-*N*-methyl-*N*-acetyl-glucosamine[c]	–	+	–	–	–	+	–
3,6-di-*O*-methyl-*N*-methyl-*N*-acetyl-glucosamine[c]	+	–	–	–	–	–	–
4,6-di-*O*-methyl-*N*-methyl-*N*-acetyl-glucosamine[c]	–	–	–	+	+	–	–
4,7,8,9-tetra-*O*-methyl-*N*-methyl-*N*-acetyl-neuraminic acid[d]	–	–	–	+	–	–	–

[a] +, slight nonquantitative response; –, not detected. [b] Identified and quantitated as alditol acetates on column i. [c] Identified as alditol acetates on column ii. [d] Identified as the methyl ester methyl glycoside on column ii.

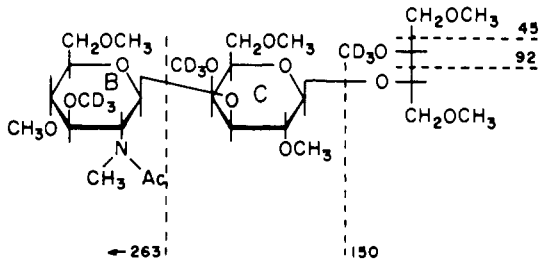

FIGURE 1: Permethylated reduced trisaccharide from type Ib core antigen obtained after Smith degradation, methylation, mild acid hydrolysis, and remethylation with trideuteriomethyl iodide (D, deuterium). Some characteristic fragments in its mass spectrum are shown by broken lines.

101, 104, 111, 115, 150, 210, 231, and 263. The sequence of sugars in the methylated oligosaccharide was elucidated by masses 263 and 150, the erythritol residue of the oligosaccharide being derived from the original β-D-glucopyranose residue of the type Ib core antigen. This evidence indicated that the original type Ib core antigen contained a β-D-GlcNAc*p*(1→3)β-D-Gal*p*(1→4)β-D-Glc*p* trisaccharide.

The above evidence is consistent with the structure of the type Ib core antigen being represented by the repeating unit shown in Figure 2d. However, this structure could not be verified definitively until further degradation procedures involving the removal of terminal residues from the type Ib core antigen had been carried out.

Removal of Terminal β-D-Galactopyranosyl Residues from Types Ia and Ib Core Antigens. Removal of the terminal β-D-galactopyranosyl residues from both types Ia and Ib core antigens could be achieved by the method previously described for the removal of these residues from the type III core antigen (Jennings et al., 1981). In this procedure, the primary hydroxyl groups of the terminal β-D-galactopyranosyl residues were enzymatically oxidized to aldehyde groups, thus rendering these terminal residues susceptible to basic elimination and in the eliminated form to their subsequent facile removal under mild acidic conditions. However, this method proved to be inconsistent in that sometimes the complete removal of the terminal galactopyranosyl residues was not achieved and often the procedure produced low yields of the degalactosylated antigens together with large quantities of insoluble product. Therefore, an improved method was developed on the basis of the selective periodate oxidation of the terminal β-D-galactopyranosyl residues of types Ia and Ib core antigens as described for the type Ia core antigen under Materials and Methods. The oxidation of the terminal β-D-galactopyranosyl residues could be followed analytically to completion, and the subsequent reduction of the aldehyde groups and mild acid hydrolysis of the polyalcohol invariably led to much improved yields of the water-soluble types Ia and Ib degalactosylated antigens. The degalactosylated types Ia and Ib antigens contained D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose in the molar ratio of 1:1:1, and methylation analysis demonstrated the presence of the same methylated component sugars (2,3,6-tri-*O*-methyl-D-glucose, 2,6-di-*O*-methyl-D-galactose, and 3,4,6-tri-*O*-methyl-2-acetamido-2-deoxy-D-glucose) in both the permethylated types Ia and Ib degalactosylated antigens. This evidence indicated that both types Ia and Ib degalactosylated antigens had identical structures and that on this evidence, the structure is consistent with the repeating unit shown in Figure 2e. However, confirmatory evidence for the above structure required the removal of the terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl residues from the degalactosylated antigen.

Removal of the 2-Acetamido-2-deoxy-β-D-glucopyranosyl Residues from Types Ia and Ib Degalactosylated Antigens. The formation of types Ia and Ib backbone antigens was most easily accomplished by the direct removal of the branch disaccharides from their more readily available core antigens as described for the type Ib backbone antigen under Materials and Methods. Following removal of the *N*-acetyl groups from the 2-acetamido-2-deoxy-D-glucosyl residues of types Ia and Ib core antigens, the de-N-acetylated core antigens were deaminated with nitrous acid (Dmitriev et al., 1975). This procedure yielded a structurally identical polymeric backbone antigen from both the Ia and Ib core antigens containing D-glucose and D-galactose in the molar ratio of 1:1. Meth-

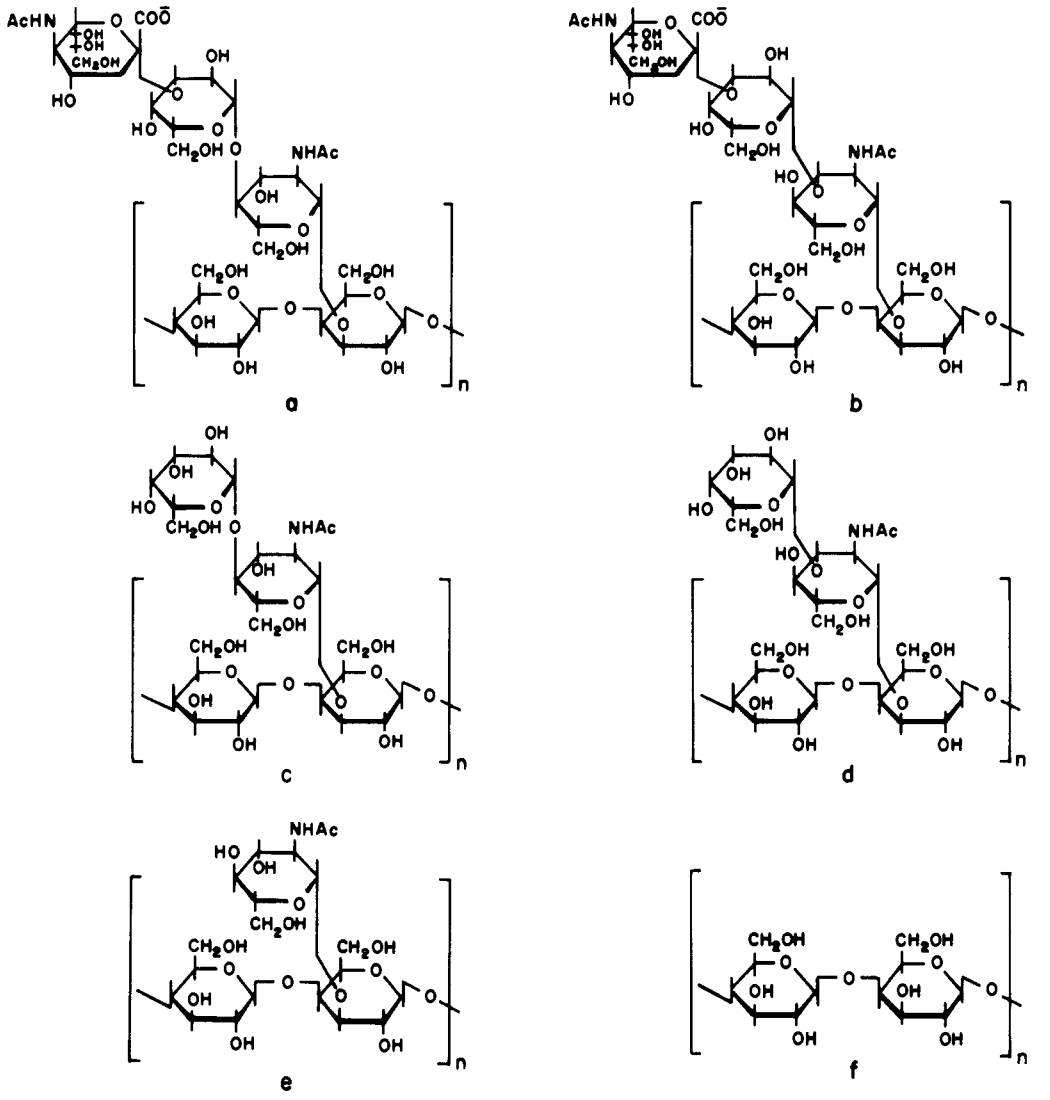

FIGURE 2: Structure of repeating units of native Ia (a) and Ib (b) group B streptococcal antigens and some of their modified structures. These include the types Ia (c) and Ib (d) core antigens and the common degalactosylated (e) and backbone (f) antigens.

ylation analysis of both backbone antigens (Table I) yielded the same methylated component (2,3,6-tri-*O*-methyl-D-galactitol and 2,3,6-tri-*O*-methyl-D-glucitol), thus indicating that the common backbone was composed of a sequence of 4-*O*-β-D-linked lactose units as shown in Figure 2f. A comparison of the methylation analysis of the common backbone antigen with that of the common degalactosylated antigen indicates that the 2,3,6-tri-*O*-methylgalactose derivative in the backbone antigen is replaced by a 2,6-di-*O*-methylgalactose derivative in the degalactosylated antigen (Table I). Thus, the terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl residues are linked to O-3 of the backbone's β-D-galactopyranosyl residues. The deamination of types Ia and Ib core antigens also yielded different disaccharides originating from the branch structures of the Ia and Ib core antigens. This was shown by methylation analyses of these disaccharides and identification of their individual components as their alditol acetates by GC–MS analysis in column i (Table I). The methylated Ia disaccharide contained 2,3,4,6-tetra-*O*-methyl-D-galactose and 1,3,6-tri-*O*-methyl-2,5-anhydro-D-mannose while the methylated Ib disaccharide contained the same tetra-*O*-methylgalactose derivative and 1,4,6-tri-*O*-methyl-2,5-anhydro-D-mannose. These methylated derivatives are consistent with the proposed repeating units of types Ia (Figure 2c) and Ib (Figure 2d) core antigens.

Structure of Native Type Ib Polysaccharide Antigen. Having established the structure of the type Ib core antigen as represented by the repeating unit shown in Figure 2d, it now only remained to locate the additional terminal sialic acid residues on this core structure. Methylation analysis of the native type Ib antigen established that all the sialic acid residues were present as single nonreducing end groups because in the methanolysis of the permethylated native antigen, only fully methylated derivatives of sialic acid (Jennings & Bhattacharjee, 1977) were detected (Table I). Hydrolysis of the methylated antigen and reduction and acetylation of the products yielded the partially methylated alditol acetates shown in Table I. The difference between these partially methylated derivatives and those yielded by the core antigen clearly reflect the structural relationship between the type Ib native and core antigens. In addition to fully methylated sialic acid residues, the methylated native type Ib antigen contained 2,4,6-tri-*O*-methylgalactosyl residues that replaced the 2,3,4,6-tetra-*O*-methylgalactosyl residues of the type Ib core antigen (Table I). This is indicative of the sialic acid residues being linked to O-3 of the peripheral end-group β-D-galactopyranosyl residues of the core antigen. The fact that no 2,3,4,6-tetra-*O*-methylgalactitol could be detected in the permethylated type Ib native antigen is consistent only with its being composed of the repeating unit shown in Figure 2b, in which all the

peripheral β-D-galactopyranosyl residues of the core antigen (Figure 2d) are substituted. The α-D configuration of the terminal sialic acid residues of the type Ib native antigen could be determined by the neuraminidase sensitivity of the type Ib native antigen (Tai et al., 1979) and by the characteristic chemical shift (174.8 ppm) of their carboxylate carbon signals (Bhattacharjee & Jennings, 1976; Jennings & Bhattacharjee, 1977) in the ^{13}C NMR spectrum of the type Ib native antigen.

Discussion

The incomplete core polysaccharide antigens of group B *Streptococcus* types Ia (Kane & Karakawa, 1978), Ib (Tai et al., 1979), II (Lancefield & Freimer, 1966), and III (Russell & Norcross, 1972; Kasper et al., 1979; Jennings et al., 1980a) are composed of the same sugars (galactose, glucose, and 2-acetamido-2-deoxyglucose), and those of types Ia, Ib and III core antigens retain their serological specificity although minor cross-reactions involving types Ia and Ib have been reported attributed to the Iabc determinant (Lancefield, 1934). All the polysaccharides have two common structural features in the form of terminal β-D-galactopyranosyl residues and a β-D-Glc*p*NAc(1→3)β-D-Gal*p*(1→4)β-D-Glc*p* trisaccharide unit, and in addition, the presence of both these structural features has also been identified in the type II core antigen (Jennings et al., 1983). A structure was previously proposed (Jennings et al., 1980b) for the type Ia core antigen in which, as in the structure of the type III core antigen (Jennings et al., 1980a), the common trisaccharide constituted the repeating unit of its backbone. This proposal was made on the basis of the identification by GC–MS analysis of an identical methylated trisaccharide, except for differences in deuteriomethyl ether labeling, obtained from the permethylated Smith degradation products of both types III (Jennings et al., 1980a) and Ia (Jennings et al., 1980b) core antigens. While more extensive degradation studies confirmed the presence of the trisaccharide repeating unit in the backbone of the type III antigen (Jennings et al., 1981), they were not consistent with its presence in the backbone of the type Ia core antigen. The identification of terminal 2-acetamido-2-deoxyglucopyranosyl residues in the type Ia degalactosylated core antigen (Figure 2e) necessitated the proposal of an alternate structure for the type Ia core antigen (Figure 2c), still consistent with all the previously published experimental data (Jennings et al., 1980b) but in which the common trisaccharide was not linearly disposed. This structure was confirmed when methylation analysis of the type Ia backbone antigen indicated that it had a repeating unit composed of (1→4)β-D-linked lactose units (Figure 2f). A comparison of the methylation analysis of the type Ia backbone antigen with that of the degalactosylated type Ia core antigen indicated that the terminal 2-acetamido-2-deoxyglucopyranosyl residues of the latter were linked to O-3 of the backbone β-D-galactopyranosyl residues (Figure 2e). Having established the structure of the type Ia core antigen (Figure 2), it was now only necessary to use previously published methylation and ^{13}C NMR analyses (Jennings et al., 1980b) to establish the structure of the native type Ia antigen, the repeating unit of which is shown in Figure 2a. These analyses demonstrated that the terminal sialic acid residues had the α-D configuration and were linked to O-3 of all the terminal β-D-galactopyranosyl residues of the type Ia core antigen.

The type Ib core antigen contained an identical trisaccharide sequence in its structure with that of the types Ia (Jennings et al., 1980b) and III (Jennings et al., 1980a) core antigens. This was demonstrated by the identification, by GC–MS analysis, of a methylated trisaccharide identical in all respects except for the different deuteriomethyl ether labeling of the 2-acetamido-2-deoxyglucose residue. In the type Ib methylated trisaccharide (Figure 1), these residues are labeled at position O-3, identifying a linkage in this position in the original type Ib core antigen. Removal of the terminal β-D-galactopyranosyl residues from the type Ib core antigen yielded a degalactosylated antigen structurally identical with that of the degalactosylated type Ia core antigen (Figure 2e). This was confirmed when removal of the terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl residues from the type Ib degalactosylated antigen also yielded an identical backbone antigen (Figure 2f) as shown by methylation analysis. Therefore, the common trisaccharide unit of the type Ib core antigen, like that of the type Ia core antigen (Jennings et al., 1980b) and unlike that of the type III core antigen (Jennings et al., 1980a), is disposed nonlinearly. This evidence indicates that the internal structures of the two former core antigens are identical. Methylation analyses of types Ia and Ib core antigens demonstrate that they only differ structurally in the linkage of their terminal β-D-galactopyranosyl residues to the branch 2-acetamido-2-deoxy-β-D-glucopyranosyl residues. In the type Ia core antigen they are linked to O-4 (Figure 2c), and in the type Ib core antigen they are linked to O-3 (Figure 2d). This evidence is also consistent with the methylation analyses of their respective deaminated core antigens and the deuteriomethyl ether labeling of their respective partially hydrolyzed methylated Smith degradation products. Methylation analysis of the native type Ib antigen indicated that its additional sialic acid residues were all terminal and were linked to O-3 of all the terminal β-D-galactopyranosyl residues of the type Ib core antigen. Thus, types Ia (Figure 2a) and Ib (Figure 2b) native antigens also have identical terminal α-D-Neu*p*NAc(2→3)β-D-Gal*p* units, differing only in structure in the linkage between the β-D-galactopyranosyl and 2-acetamido-2-deoxy-β-D-galactopyranosyl residues of their trisaccharide branches. It is of interest to note that the same terminal α-D-Neu*p*NAc(2→3)β-D-Gal*p* units are also a structural feature of fetuin (Spiro & Bhoyroo, 1974) and the human M and N blood group substances (Sadler et al., 1974).

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Unexpected Similarity of the Structures of the Weakly Toxic Amanitin (*S*)-Sulfoxide and the Highly Toxic (*R*)-Sulfoxide and Sulfone As Revealed by Proton Nuclear Magnetic Resonance and X-ray Analysis†

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ABSTRACT: The three-dimensional structures of the slightly toxic diastereomeric (*S*)-sulfoxide of 6′-*O*-methyl-α-amanitin [6′-*O*-Me-α-ama (*S*)-sulfoxide, **4**] and of the corresponding highly toxic sulfone **5** have been determined by X-ray diffraction analysis. The same derivatives along with 6′-*O*-methyl-α-amanitin [*O*-Me-α-ama (*R*)-sulfoxide, **3**] and the corresponding thioether (*O*-Me-α-ama sulfide, **6**) have been investigated in dimethyl sulfoxide solutions by 360-MHz ^{1}H NMR spectroscopy including nuclear Overhauser effects (NOE). In addition α-amanitin (**2**) has been reinvestigated by this high-resolution method involving the identification of the ABMX systems of the tryptophan, cysteine, and asparagine and discrimination between the glycine residues. The structures of compounds **2**–**6** are compared with the structure of β-amanitin which was solved previously by X-ray structure analysis. The results are (1) the structures in the crystalline state of the (*S*)-sulfoxide **4** and sulfone **5** are practically identical and (2) in dimethyl sulfoxide solution the structures of compounds **4** and **5** are likewise identical with each other and with those of the (*R*)-sulfoxide **3** and the thioether **6**. The general structure of the peptide backbone of the α-amanitin derivatives investigated here almost corresponds to that of β-amanitin (**1**), the main difference being a rotated plane of the peptide bond between the asparagine and cysteine residue. In order to explain the lack of high toxicity in the (*S*)-sulfoxide **4** we tentatively suggest alternative hydrogen bonding of a donor from the protein, or displacement of the *R* oxygen to the *S* oxygen of a hydrogen bond donor. This alternative bonding or displacement might not occur in the sulfoxide **4**. Other explanations which include local conformational changes in the inhibitors or a difference between the SO and SO_2 local dipoles are also possible.

In 1941 Wieland et al. (1941) reported on the isolation from the toxic mushroom *Amanita phalloides* of the slow-acting toxin and called it amanitin. Many years later this component was recognized as a mixture of a neutral compound, called α-amanitin, and an acidic one, β-amanitin (Wieland et al., 1949). Later on β-amanitin was found to be the carboxylic acid (**1**) whose carboxamide derivative represents α-amanitin (**2**) (Wieland & Boehringer, 1960). The three-dimensional structure of **1** in the solid state was determined by X-ray diffraction analysis (Kostansek et al., 1977, 1978) whereas a three-dimensional structure of **2** was suggested from NMR data by Tonelli et al. (1978). There is rather good agreement between the crystal structure of **1** and the solution structure of **2**.

Compounds **1** and **2** are (*R*)-sulfoxides (Faulstich et al., 1968). The question arose as to whether the (*R*)-sulfoxide moiety of the amatoxins is a structural feature essential for their toxicity. Hence, the modification of this structural element was undertaken. The sulfoxide oxygen atom of 6′-*O*-methyl-α-amanitin (**3**), an equally toxic derivative of **2**, was removed by treatment either with Raney nickel (Buku et al., 1974) or with K_3MoCl_6 [according to Nuzzo et al. (1977); A. Buku, unpublished results]. Reoxidation of the thioether **6** yielded the (*R*)-sulfoxide **3** along with the diastereomeric (*S*)-sulfoxide **4** and the sulfone **5** (Buku et al., 1974). The formulas of the amatoxins mentioned are given in Figure 1 and in Table I.

Interestingly, thioether **6** and sulfone **5** show high toxicity like the (*R*)-sulfoxides **1**–**3**, whereas the (*S*)-sulfoxide **4** is at least 20 times less toxic. The inhibitory constants of DNA-

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