Capsular Polysaccharides of *Clostridium perfringens* Hobbs 5*

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ABSTRACT: One of the principal immunochemical determinants of *Clostridium perfringens* Hobbs 5 was isolated by mild hot water extractions of intact highly encapsulated cells. Approximately 10% of the dry weight was solubilized by this technique without apparent extensive damage to the cells. Purification by precipitation with alcohol, treatment with RNase, chloroform–butanol, and fractionation on Dowex 1-X2 (Cl^-) resulted in the isolation of an acidic polysaccharide containing glucose, galactose, mannose, glucosamine, galactosamine, and glucuronic acid in the molar ratio of 1.2:1.2:1.2:2:2:2.6 and less than 0.4% peptide. The acidic heteropolysaccharide represented 85% of the isolated carbohydrate. It exhibited a molecular weight of 9300 g/mole and had a tendency to aggregate with the remaining carbohydrate, or neutral polysaccharide which contained mostly mannose. The acidic polysaccharide formed a distinct precipitation band only with its homologous antisera in gel diffusion.

The principal cell envelope antigens of gram-positive organisms appear to be the one or more heteropolysaccharides commonly found associated with peptidoglycan of the cell envelope. Most strains of *Clostridium perfringens* isolated from their normal habitats possess a serologically and chemically heterogeneous capsular material, that covers the cell surface, masking the common somatic antigens (Smith and Holdeman, 1968; Willis, 1969). Occasionally some strains become heavily encapsulated and are found to be phage resistant (Smith, 1959). Though a number of studies have been reported concerning the soluble antigens of *C. perfringens*, none have dealt with homogeneous capsular polysaccharides free of contaminating material (Svec and McCoy, 1944; Izumi, 1962; Meisel-Mikolajczyk, 1963; Pickering, 1966; Johnson *et al.*, 1969; Darby *et al.*, 1970).

The complexity of the capsular antigens of *C. perfringens* and their relationship to pathogenicity have been amply illustrated (Smith and Holdeman, 1968; Willis, 1969). Apparently several hundred serological types of *C. perfringens* exist. However, definitive data concerning the chemical and immunochemical properties of these organisms and their relation to the various serotypes are almost completely lacking.

The primary objective of this work was to initiate the characterization of the capsular polysaccharide of a serologically defined strain of *C. perfringens* Hobbs 5. This and future information could be used to develop methods which would enable the classification and relationships of the various serotypes to be based on composition and structure of the capsular polysaccharides.

Materials and Methods

Analytical Procedures. Glucose and galactose were assayed with glucostat and galactostat (Worthington Biochemical), respectively. Vapor-phase chromatography columns of SE-52 operated at 180° according to Sweeley *et al.* (1963) were utilized in determining the carbohydrate composition of the neutral fraction. The general detection of carbohydrate was accomplished by the phenol–sulfuric acid method of Dubois *et al.* (1956). Uronic acid was estimated by the carbazole reaction (Dische, 1947). Reducing sugar was determined by the method of Park and Johnson (1949). Hexosamine was determined by the method of Elson and Morgan as modified by Boas (1953), and with a Beckman amino acid analyzer. Hexosamines were isolated by Dowex 50 H^+ chromatography according to Gardell (1953) and identified by the method of Stoffyn and Jeanloz (1954). Protein was determined with the Folin–Ciocalteau reagent according to Lowry *et al.* (1951).

Molecular weight was determined by the method of Yphantis (1964) according to Roark (1970) in 2.25 M KCl at 34,000 rpm.

Samples (5 mg/ml) were hydrolyzed in 2 N HCl in a sealed tube at 100° for 6 hr for the analytical determination of the carbohydrate moieties and for paper chromatography. In some instances samples hydrolyzed for 20 hr were used for the determination of hexosamines. The polysaccharide (14 mg) was also hydrolyzed with 100 mg of Dowex 50 (H^+)–1 ml of 0.05 N HCl for the isolation of basic (fraction A), acidic (fraction B), and neutral (fraction C) components (Jeffrey and Rientis, 1967). Hydrochloric acid was removed by repeated evaporations with absolute alcohol *in vacuo*.

Paper chromatography was carried out by the descending technique using Whatman No. 1 paper with the following solvent systems: I, 1-butanol–pyridine–H_2O (6:4:3, v/v); II, 1-butanol–ethanol–H_2O (4:1:1, v/v); III, acetone–ethanol–2-propanol–0.5 M, pH 10 sodium borate buffer (3:1:1:2, v/v); IV, ethyl acetate–pyridine–H_2O–acetic acid (5:5:3:1, v/v). Sugars were detected with alkaline silver nitrate and hexosamines with ninhydrin.

Ouchterlony plates for immunodiffusion were prepared with 1% special noble agar in 0.0025 M sodium borate buffer, pH 8.4 containing 0.85% saline (Crowle, 1961; Ochterlony, 1968).

Cultures. C. perfringens, Hobbs strains were obtained through the courtesy of Dr. V. R. Dowell, National Center for Disease Control, Atlanta, Ga.

Encapsulated strains were selected under anaerobic conditions on blood agar plates fortified with 5% glucose. Mucoid

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TABLE I: Isolation and Purification of Capsular Polysaccharide: *Clostridium perfringens* Hobbs 5.

Step	Isolation: Fraction Isolated	Isolation: Fraction Treated	Isolation: Treatment	Composition: % by Wt of Isolated Polysaccharide: Recov % by Wt	Carbohydrate[a]	Protein	Phosphate	Hexosamine
1	Supernatant I	100 g of cells	Hot water extraction I					
	Supernatant II		Hot water extraction II					
2	Precipitate I	Supernatant I	1% acetic acid–2 volumes of 95% alcohol	6.3	17	12	12	4.5
	Precipitate II	Supernatant II		2.3				
3	R-S I	Precipitate I 8.0 g	RNase–Sevag	27	30	1.3	1.5	13.0
	R-S II	Precipitate II 1.1 g		R-SI + R-S II combined				
4	Peak I	R-S (combined)	Dowex 1-X2 (Cl)–H_2O	10	51	5	0.3	0.6
	Peak II	2.4 g	Dowex 1-X2 (Cl)–0.2 M NaCl	66	34	0.4	0.3	16.0 (21)[b]
5	Peak IG	Peak II	Dowex 1-X2 (Cl)	4.8[c]				
	Peak IIG	0.201 g	0–0.5 M NaCl	48[c]				21

[a] Determined as glucose by the phenol–H_2SO_4 method. [b] Value in parenthesis determined after 20-hr hydrolysis. [c] 19% loss in phenol–H_2SO_4 and 20% gain in carbohydrate per milligram of sample.

cultures were maintained on cooked meat medium (Difco). Growth characteristics were determined in fluid thioglycollate broth (Difco) or sterility test broth (BBL). Selected strains were preserved in brain media (Dowell and Hawkins, 1968) and/or lyophilized in NIH thioglycollate broth. Preparative isolation of cells was accomplished by incubating freshly prepared fluid media, (NIH thioglycollate broth or sterility test broth) previously innoculated with a 5%, 8-hr seed culture, for 16 hr at 37°. The cells were harvested at 41,000*g* by continuous-flow centrifugation, washed twice with saline, triturated with acetone, dried *in vacuo*, and stored over $CaCl_2$ (yield 8–9 g).

Isolation of Crude Polysaccharide. Dried cells (25 g) were dispersed by gentle grinding with a mortar and pestle. The pulverized cells were suspended in 300 ml of water and heated in a boiling-water bath for 5 min with vigorous stirring. The suspension was cooled in an ice bath and stirring continued for 1 hr to ensure complete dispersal and hydration of the cells. The cooled suspension was then heated in a boiling-water bath for 30 min with continuous stirring. After cooling, the suspension was centrifuged at 36,000*g* for 20 min and the residue extracted a second time with 200 ml of H_2O for 30 min in the same manner. The supernatant fractions were adjusted to 1% acetic acid and crude polysaccharide precipitated by the addition of two volumes of 95% alcohol. After allowing the suspensions to stand at 0° overnight the precipitates were collected by centrifugation. The crude polysaccharide fractions were washed with increasing concentrations of alcohol and finally with acetone. Residual acetone was removed *in vacuo* and the precipitates stored over $CaCl_2$.

Purification of the Crude Polysaccharide. Crude polysaccharide (1.25 g) was dissolved in 100 ml of 0.01 M Tris–0.1 M NaCl buffer (pH 7.5). RNase (21,000 units, Worthington Biochemical Corp.) was added and the mixture dialyzed against 0.01 M Tris–0.1 M NaCl at room temperature. The dialysate was changed frequently and half again as much RNase was added after 16 hr. The dialysis was continued until the absorbancy (260 nm) of the dialysate failed to increase.

An equal volume of chloroform–butanol (9:1, v/v) was added to the RNase-treated polysaccharide solution which was subsequently blended for 10 min at low speed. The resulting emulsion was separated by centrifugation and the aqueous phase extracted with fresh solvent in the same manner until denatured protein failed to appear at the interface. The aqueous layer was then dialyzed against running tap water for 24 hr and then against distilled water. The dialyzed polysaccharide was adjusted to 1% acetic acid and the polysaccharide recovered by precipitation with two volumes of alcohol. The precipitate was washed and dried with alcohol and acetone as described above.

The RNase–chloroform–butanol-treated polysaccharide (200 mg) was dissolved in 30 ml of H_2O and the pH adjusted to 7. The sample was applied to a Dowex 1-X2 chloride (200–400 mesh) column (1.5 × 30 cm) and the column washed with 150 ml of H_2O followed by elution with 0.2 M NaCl. An elution rate of 24 ml/hr was maintained with a constant volume pump. Selected samples were refractionated in the same manner except a linear gradient was employed (0–0.5 M NaCl). Individual fractions were assayed by the phenol–H_2SO_4 reaction and individual peaks were combined, dialyzed, and lyophilized.

Results

Strain Selection. Initial observations of smooth colony morphology on blood agar–5% glucose and high viscosity when cultured in fluid media demonstrated that *C. perfringens* Hobbs 5 was the most highly encapsulated strain investigated (Figure 1).

Isolation and Purification of Polysaccharide. Hot water treatment of Hobbs 5 cells and alcohol precipitation of the supernatant fractions yielded 6.3% (precipitate I) and 2.3% (precipitate II), dry weight, from the first and second extractions respectively (Table I). Acid hydrolysis of precipitate I and precipitate II followed by paper chromatography in solvent I demonstrated the probable presence of uronic acid, galactosamine, glucosamine, galactose, glucose, mannose,

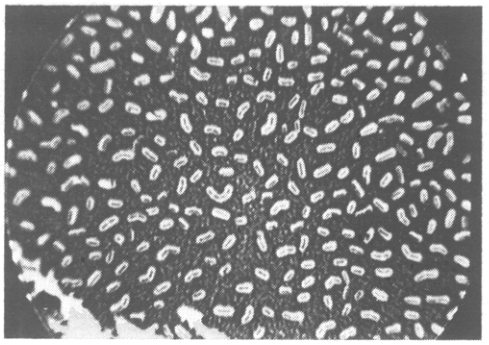

FIGURE 1: *Clostridium perfringens* Hobbs 5 special capsule stain according to Maneval (1956). Light areas are indicative of the extent of capsular material and disappears after treatment with hot water.

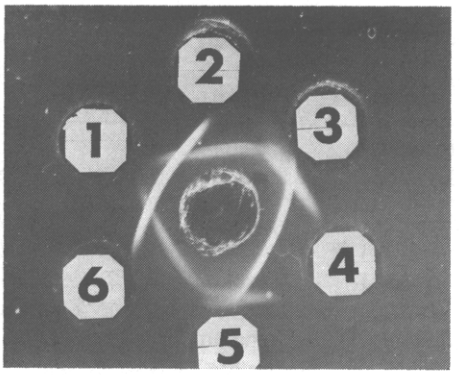

FIGURE 3: Immunodiffusion pattern of Hobbs 5 fractions. Antisera were kindly supplied by Dr. V. R. Dowell, National Center for Disease Control. Center well Hobbs 5 antisera. Hole 1, 4, peak I; hole 2, 6, peak II; hole 3, 5, R-S.

and ribose. The crude polysaccharide, precipitate I and precipitate II, was then treated with RNase followed directly by chloroform–butanol (9:1, v/v). After these treatments the identical carbohydrate constituents found in precipitate I and II were observed in the ribonuclease–solvent (R-S)-treated fractions except for a great diminution in the ribose content. Proceeding from step 2 (precipitate I) to step 3 (R-SI) (Table I) a concomitant decrease in protein and phosphate, 2-fold increase in neutral sugar and a 3-fold increase in amino sugar was observed analytically and by paper chromatography. Dowes 1-X2 (Cl^-) chromatography yielded two major fractions, a minor neutral fraction which appears in the water wash, peak I, and a major fraction eluted with 0.2 M NaCl, peak II (Figure 2A, Table I). Peak II contained the usual distribution of carbohydrate moieties, less than 0.4% protein and exhibited zero absorption at 260 nm. An increase in the per cent carbohydrate and hexosamine in going from step 3 (R-SI) to step 4 (peak II) and paper chromatography indicated an increase in the purity of the sample (Table I and Figure 3). No additional heteropolysaccharides were eluted with 0.5 M or 2 M NaCl. Refractionation of peak II by a linear NaCl gradient yielded two fractions; a small peak eluted with the water wash, containing mostly mannose, and a second larger peak with a carbohydrate distribution representative of the original peak II (Figure 2B). Approximately 85% of the phenol–H_2SO_4-positive material was recovered after refractionation on Dowex 1-X2 (Cl^-), while 50% of the original weight of the sample was isolated (Table I). In addition, the hexosamine increased from 16% in step 4, peak II to 21% in step 5, peak IIG (Table I).

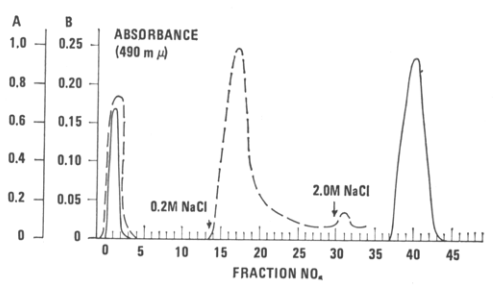

FIGURE 2: Ion-exchange chromatography. (A) Fractionation of R-SI on Dowex 1-X2 (Cl) resin (1.5 × 30 cm). Sample eluted with 0.2 M NaCl at fraction 13 and with 1.0 M NaCl at fraction 30 (broken line). (B) Refractionation of peak II on Dowex 1-X2 (Cl^-) resin (1.5 × 30 cm). Sample eluted with NaCl gradient (0–0.5 M NaCl) (solid line).

Identification and Analysis of the Carbohydrate Constituents of the Acid Polysaccharide, Peak II and Peak IIG. URONIC ACID. The carbohydrate constituents of peak II were separated into three fractions basic (fraction A), acidic (fraction B), and neutral (fraction C), after hydrolysis with Dowex 50 (H^+)–0.05 N HCl. Chromatography of fraction B in solvent I revealed a single spot corresponding to glucuronic acid. This component was identified as glucuronic acid by chromatography in solvents III and IV. The carbazole:orcinol ratio of isolated uronic acid was 1, as would be expected for glucuronic acid.

HEXOSAMINE. Acid hydrolysis of the acid polysaccharide fractions (peak II and peak IIG), analyzed by paper chromatography in solvent I, indicated the presence of galactosamine and glucosamine. Similar hydrolysates which were applied to a column of Dowex 50-X8H^+, gave two equal peaks corresponding to glucosamine and galactosamine standards. The individual peaks were identified by ninhydrin degradation of the isolated hexosamine to the corresponding pentoses. As expected, fraction I (glucosamine) and fraction II (galactosamine) yielded arabinose and lyxose, respectively. A third sample of peak II hydrolyzed in 6 N HCl for 24 hr was analyzed by automated amino acid analysis. This technique confirmed the presence of glucosamine and galactosamine in 1:1 ratio and that the sample contained trace amounts of acidic and hydroxyamino acids.

NEUTRAL SUGARS. Fraction C, the neutral fraction discussed earlier, contained three constituents which were identified as glucose, galactose, and mannose by paper chromatography in solvents I and IV. Vapor-phase chromatography demonstrated the presence of the three monosaccharides in the approximate ratio of 1:1:1. In addition, each monosaccharide was isolated by paper chromatography in solvent I and subsequently identified in solvent IV. The neutral fraction was susceptible to both glucostat and galactostat reagents.

Analysis. Peak II and peak IIG were analyzed for the various carbohydrate constituents. The per cent weight of most of the constituents increased while the molar ratio of each remained approximately the same. Glucose:mannose:galactose:glucosamine:galactosamine:glucuronic, 1.2:1.0:1.2:2:2:2.6 (peak IIG, Table II).

Molecular weight. The weight-average molecular weight determined as 9300 g/mole. A tendency to aggregate was observed.

Immunodiffusion. Peak I and peak II reacted with Hobbs

TABLE II: Analytical Composition of Capsular Polysaccharide.

	Fraction	Glucose	Mannose	Galactose	Glucosamine	Galactosamine	Glucuronic Acid
Peak II	% by weight	3.9	2.8[a]	6.8	8	8	12
	Molar ratio	1.0	1.2	1.7	2	2	3
Peak IIG	% by weight	6.4		6.2	10.5	10.5	14
	Molar ratio	1.2	1.2[b]	1.2	2	2	2.6

[a] Glucose + galactose subtracted from reducing value of neutral fraction C. [b] Estimated value based on gas chromatography.

5 antisera but did not cross react with other specific antisera (Figure 3).

Discussion

In order to avoid the problems inherent in isolating and characterizing capsular material from culture media, we sought a serologically defined strain of *C. perfringens*, which possessed a well-developed capsule, from which the capsular polysaccharide could be extracted directly from isolated cells. Our investigations demonstrated that Hobbs 5 was well suited for further study. The extensive capsule of this serotype transformed the liquid media into a highly viscose mass and prohibited the formation of a discrete pellet even after centrifugation at 40,000*g* for 1 hr. Several other recently isolated serotypes behaved similarly except that a more compact pellet formed upon high-speed centrifugation.

While investigating the gross nature of Hobbs 5 capsule, we discovered that it could easily be removed from the cell envelops of washed cells by treatment with hot water. This procedure removed a great deal of material; the cells appeared intact and gram positive though we could account for 10–15% of the dry weight after hot water extraction. In addition to polysaccharide, the extract also contained protein and nucleic acids. The major portion of RNA and protein was removed by treatment with RNAse dialysis and chloroform–butanol, respectively. The partially purified polysaccharide (R-S) was recovered by precipitation. Fractionations on Dowex 1-X2 (Cl^-) resulted in the removal of residual RNA and isolation of a small neutral fraction (peak I) containing predominately mannose, and a large acidic fraction (peak II) containing glucose, mannose, galactose, glucosamine, galactosamine, and glucuronic acid in the ratio 1.0:1.2:1.7:2:2:3. Peak II could be refractionated further by rechromatography on Dowex 1-X2 (Cl^-) utilizing a linear gradient (0–0.5 M NaCl). The equivalent fraction (peak IIG) essentially exhibited the identical analysis of peak II (Table II). Comparison of the hexosamine content of the initial alcohol precipitate (precipitate I) with that of peak IIG showed an approximate 5-fold purification based on hexosamine. However, a minor neutral fraction (peak IG), corresponding to peak I was obtained. The reappearance of the neutral polymer by refractionation of peak II could be due to aggregation with the acidic polymer since this phenomenon was indicated by the molecular weight determination. Considering the possibility of aggregation we cannot rule out the possibility that the neutral polymer is an associated mannan picked up from the yeast extract of the media.

The carbohydrate content and composition of the acidic polysaccharide remained constant during each phase of the purification while the ribose, protein, phosphate, and 280-nm absorption disappeared (Table I). The best indication of homogeneity was the appearance of a single acidic fraction obtained by changing the column eluent from 0.2 M NaCl (Figure 2A) to 0–0.5 M NaCl linear gradient (Figure 2B). Both peak I and peak II formed precipitin bands with Hobbs 5 antisera but were unreactive to other specific antisera tested. The detailed immunochemical properties of these fractions will be presented elsewhere (R. Cherniak and B. G. Henderson, in preparation).

The properties of hexosamine containing polymers make it difficult to ascertain an exact value for the constituents by simple acid hydrolysis as indicated by the variation in hexosamine values after 6 and 20 hr, and the failure to account for 100% of the sample as carbohydrate. However, a good indication of the molecular composition was attained.

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Disruption of Low- and High-Density Human Plasma Lipoproteins and Phospholipid Dispersions by 1-Anilinonaphthalene-8-sulfonate*

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ABSTRACT: A hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS), at high concentrations was found to alter profoundly the structure of both low-density lipoproteins of the S_f 0–10 class and high-density lipoproteins of d 1.063–1.21. The disruption of these lipoproteins by 0.1 M NH_4ANS yielded essentially lipid-free proteins and phospholipid-rich and neutral lipid-rich fractions. Although all of the fractions obtained by the disruption of the lipoproteins were initially stabilized by association with ANS, the neutral lipid-rich fraction aggregated gradually upon loss of the ANS-stabilized phospholipid components. Lecithin dispersions were also disrupted extensively in 0.1 M ANS yielding an ANS–lecithin complex with an ANS:lecithin molar ratio of 2.4, a density of 1.16, and an approximate particle weight of 5.4×10^4. It appeared that ANS at high concentrations initially displaced the total lipid moiety from the lipoproteins.

The formation of phospholipid-rich and neutral lipid-rich fractions seemed to be effected by the progressive removal of the phospholipid-rich surface layer from the total lipid moiety.

Since Weber and Laurence (1954) introduced hydrophobic probes, 1-anilinonaphthalene-8-sulfonate (ANS)[1] and related organic compounds have been used extensively to study hydrophobic sites of proteins (Edelman and McClure, 1968; McClure and Edelman, 1966; Stryer, 1965; Brand *et al.*, 1967; Turner and Brand, 1968). These small organic anions possess a characteristic property of being practically nonfluorescent in water but highly fluorescent when dissolved in organic solvents or bound to hydrophobic sites of a number of proteins. In view of the hydrophobic character of the constituents of plasma lipoproteins, the study of the nature of the interaction between the lipoproteins and ANS was thought to be of considerable interest.

The present communication describes disruption by ANS of low-density lipoproteins of the S_f 0–10 class (LDL)[1] and high-density lipoproteins of d 1.063–1.21 (HDL) as well as phospholipid dispersions. High concentrations of ANS were found to release essentially lipid-free proteins from the lipoproteins.

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[1] Abbreviations used are: ANS, 1-anilinonaphthalene-8-sulfonate; LDL, low-density lipoproteins of the S_f 0–10 class; HDL, high-density lipoproteins of d 1.063–1.21.

Materials and Methods

ANS. The ammonium salt of ANS (K & K Laboratories, Inc., Jamaica, N. Y.) was purified by crystallizing three times from water as described for the magnesium salt of ANS (Weber and Young, 1964). The recrystallized ANS was further purified by column chromatography on silicic acid–Super Cel (2:1, w/w) employing chloroform–methanol (9:1, v/v) as the solvent. The solvent was evaporated using a Rinco evaporator and the purified ANS was stored over phosphorus pentoxide at 4° in a vacuum desiccator. During the purification, precautions were taken to minimize the exposure of ANS to laboratory lighting. Thin-layer chromatography of the purified ANS on silica gel G with chloroform–methanol–water (65:25:4, v/v) as the developing solvent indicated that the ANS preparation was essentially pure; a trace of slower moving contaminant became detectable only when the thin-layer plate was overloaded with sample.

Buffer. Unless otherwise indicated, experiments were performed with 0.02 M sodium phosphate buffer containing 0.1 M sodium chloride and 0.05% EDTA (pH 7.0).

Isolation of LDL and HDL. Recently outdated human blood containing 0.2 volume of citric acid–sodium citrate–dextrose anticoagulant solution was obtained from a local hospital as the source of plasma. For the separation of plasma lipoproteins pooled samples of the plasma free of turbidity were used. Plasma LDL of the S_f 0–10 class was isolated and purified by ultracentrifugation as previously described (Janado and Nishida, 1965). HDL ($1.063 < d < 1.210$) was isolated from the bottom fraction obtained by centrifugation of plasma