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Isolation, Chemical Composition, and Molecular Size of Extracellular Type II and Type Ia Polysaccharides of Group B Streptococci

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Polysaccharides carrying the type II- and type Ia-specific determinants of Lancefield group B streptococci were isolated and purified by anion-exchange chromatography and gel filtration from the supernatant culture medium after growth of strain 18RS21/67/1 (type II) and strain DS/1204/78 (type Ia), respectively. The average molecular weights of these polysaccharides were 97,000 (type II) and 94,000 (type Ia), as determined by reducing end group analyses. These molecular weights were in reasonably good agreement with molecular weights determined by gel filtration at high ionic strength on calibrated columns. The polysaccharides did not cross-react with antisera specific for the other type-specific determinants or with group B-specific antisera. Their content of galactose, glucose, glucosamine, and neuraminic acid (the last two calculated as *N*-acetyl derivatives) accounted for over 96% of their dry weight. The two polysaccharides differed from each other (and from type III polysaccharide) in their relative content of these monosaccharides. The molar ratios of galactose, glucose, and neuraminic acid to glucosamine were 3.3:2.3:1.35:1.0 for the type II polysaccharide and 2.0:0.8:1.4:1.0 for the type Ia polysaccharide. The results obtained indicate that these extracellular type II and Ia polysaccharides contain larger amounts of neuraminic acid than can be accounted for by previously proposed structures of their repeating units.

Acquired immunity to natural group B streptococcal infection in human neonates (3, 7) and to experimental infection in mice (9, 12, 30, 31) and in neonatal rats (14, 19) has been associated with antibody to the type-specific polysaccharides or proteins of these organisms. The opsonic value of antibodies with these specificities has been demonstrated in in vitro assays (1, 8, 18, 19, 21).

Vaccines consisting of the purified type polysaccharides are now being developed for use in humans. The Lancefield procedure of hot hydrochloric acid extraction of whole cells is the classical method for preparing the type-specific polysaccharides of group B streptococci. However, these products are now known to be partially degraded, since they completely or partially lack neuraminic acid residues (5, 20, 23, 26, 27, 30, 37, 38). Data exist which suggest that sialic acid-containing type-specific polysaccharides obtained by milder extraction procedures are better immunogens in terms of the in vivo protective activity (2, 3, 7) and in vitro opsonic activity (27) of the antibody populations induced.

More recently, several investigators (4, 6, 22–26, 30, 38) have used somewhat milder treatments, such as brief cold acid or EDTA extractions (or both), to remove the surface-associated polysaccharides from cells. However, recent observations in two separate laboratories (10, 17) showed that during growth, several type III strains secrete into the culture medium the polysaccharides that carry the type III-specific and the group B determinants, along with a variety of other substances. This secretory process appears to be selective and not associated with gross cellular lysis, since it occurs in the absence of a generalized loss of cytoplasmic proteins and nucleic acids (10, 17).

These observations and our previous experience in isolating and purifying a large-molecular-weight polysaccharide that carries the type III determinants from the culture supernatants of strain M732 (10) prompted us to see whether this was a generalized property of group B streptococci and whether other type-specific polysaccharides could be similarly isolated from culture supernates. We report here that several type II and type Ia strains secrete their respective type-

specific polysaccharides into the culture medium, and that these polysaccharides can be isolated and purified. These extracellular polysaccharides carry their respective type-specific immunodeterminants, do not contain detectable group B determinants, and possess a higher content of neuraminic acid than the amounts that can be accounted for by the reported molar ratios of monosaccharides present in similar antigens obtained from cells by 15 h of exposure to EDTA, phosphate buffer, and sodium chloride (6, 22–25).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The previously reported (10) reference strains of group B streptococcus were used. Clinical isolates of type Ia were obtained from R. Facklam, Centers for Disease Control, Atlanta, Ga. The type polysaccharides were produced by strain 18RS21/67/1 (type II) and DS/1204/78 (type Ia), each grown for 24 h at 37°C without stirring, in the previously described, acid-hydrolyzed casein-containing medium (10). Purity of all cultures was ascertained by streaking onto Trypticase soy blood agar (BBL Microbiology Systems, Cockeysville, Md.) plates at each stage of culture.

Chemical determinations. The methods used to quantify neutral hexoses, protein, nucleic acids, phosphorus, and neuraminic acid were the same as those used previously (10). Gas-liquid chromatography was used to quantify sugars by the method of Clamp et al. (11) with arabinitol, mannitol, and perseitol as internal standards, as described previously (15).

Serological methods. All serological methods were the same as those used previously (10).

Molecular weight estimations. The average molecular weight of the polysaccharides was determined by estimating reducing end groups (35) with glucose as a standard. Calculation of molecular weight was then based on the monosaccharide composition as determined by gas-liquid chromatography, assuming a single reducing end per polysaccharide chain.

Molecular weights were also estimated by gel filtration on Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.) in high-ionic-strength buffer (0.6 M NaCl in 0.01 M Tris-hydrochloride, pH 7.3). The column was calibrated with Dextran T70 (Pharmacia). Elution profiles were monitored by neutral hexose determinations.

RESULTS

Production, isolation, and purification of the type II polysaccharide. In preliminary experiments, four type II isolates were examined for production of extracellular polysaccharide-carrying type II determinants. Material that precipitated with type II antiserum was found in the supernatant culture medium after growth of all of the four strains examined. However, strain 18RS21/67/1 produced more extracellular type II antigen than did the other strains, as determined by rocket immunoelectrophoresis, and was therefore used for antigen production. Production of type II reactive polysaccharide by this strain was highest when it was grown for 24 h in the casein hydrolysate medium containing 4% glucose.

The purification procedure, in general, was very similar to that previously used to purify the extracellular type III polysaccharide (10). After growth of a 20-liter culture for 24 h at 37°C, bacteria were removed by filtration through a 0.45-μm filter in a Pellicon cassette system (Millipore Corp., Bedford, Mass.) at 4°C. The supernatant culture medium was concentrated 40-fold, and low-molecular-weight components were removed by dialysis through a PGT 00005 (10,000-dalton) filter in the Pellicon cassette, also at 4°C. The retentate was then exposed to 60°C for 30 min to inactivate enzymes that might alter the type II polysaccharide. The concentrated retentate was then mixed with 100 ml of DEAE-Sephacel (Pharmacia) in 0.01 M Tris-hydrochloride (pH 7.0) and allowed to stand overnight at 4°C. The mixture was poured into a column (2.4 by 20 cm). Because the type II polysaccharide was more weakly bound to the column than was the type III antigen, the column was washed with 6 volumes of 0.05 M NaCl in 0.01 M Tris (pH 7.0) and then eluted with a shallow, linear NaCl gradient (0.05 to 0.20 M in 0.01 M Tris, pH 7.0). This resulted in the partial separation of two major carbohydrate-containing peaks (Fig. 1B). Lowry-reactive protein was found throughout the elution profile (Fig. 1C). Capillary precipitins and counterimmunoelectrophoresis showed that the early portion of the first major carbohydrate peak contained only group B reactivity, that fractions 45 to 63 contained both group B and type II reactivities, and that fractions 64 to 74 contained type II reactivity, but not group B reactivity (Fig. 1A). These latter fractions were pooled, concentrated, dialyzed against 0.01 M Tris (pH 7.3), and then treated with 1 mg of trypsin (Calbiochem, La Jolla, Calif.) for 18 h at 37°C to hydrolyze residual protein present. This material was then filtered on a column of Sepharose 4B (Pharmacia; 2.5 by 90 cm), in 0.01 M Tris (pH 7.3) (Fig. 2). Carbohydrate and type II reactivity coeluted in a single, reasonably symmetrical, broad peak at a K_{av} of 0.29 (Fig. 2). Fractions 58 to 110 were pooled, dialyzed against distilled water, and lyophilized. This product, which contained 1.5% Lowry-reactive protein, was dissolved in water, and ethanol was added to a final concentration of 50%. After removal of a small precipitate by centrifugation, the alcohol was removed by evaporation, and the type II polysaccharide was lyophilized. The overall yield per liter of culture was about 4 mg of type II polysaccharide con-

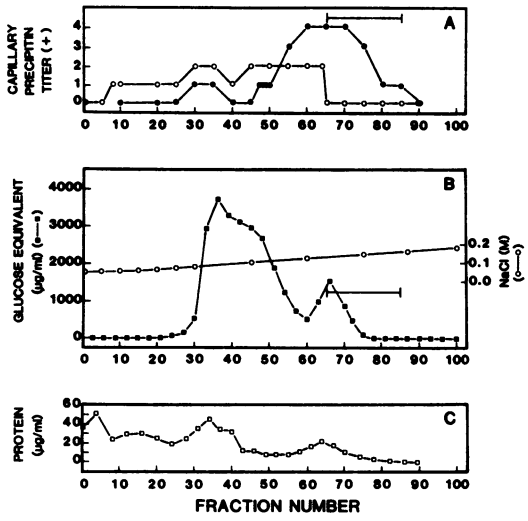

FIG. 1. Isolation of type II polysaccharide from culture supernatant by DEAE-Sephacel chromatography. The concentrated, dialyzed, and heat-inactivated extracellular culture fluid (500 ml in 0.01 M Tris, pH 7.0) was bound to DEAE-Sephacel equilibrated with the same buffer. After the column was washed with 0.05 M NaCl in 0.01 M Tris (pH 7.0), bound components were eluted with a shallow linear sodium chloride gradient in a total volume of 1,200 ml of 0.01 M Tris (pH 7.0), and 8-ml fractions were collected. Vertical axes: (A) capillary precipitin titer (expressed as 1+ to 4+) for group B antigen (○) and for type antigen (●); (B) neutral hexose content (■) and NaCl molarity (○); (C) protein content (□). The bars in A and B indicate the fractions pooled and concentrated for further purification.

taining 0.2% Lowry-reactive protein and undetectable amounts of nucleic acid (determined by absorption at 260 nm).

Production, isolation, and purification of type Ia polysaccharide. In preliminary experiments, four type Ia strains, three clinical isolates and reference strain DS/1204/78, were examined for production of extracellular polysaccharide-carrying type Ia determinants. All strains released the type Ia polysaccharide into the culture medium, but strain DS/1204/78 elaborated the most, as determined by rocket immunoelectrophoresis, and was therefore chosen for antigen production. The conditions used for type Ia antigen production and purification were similar to those used for the type II and type III polysaccharides (see legend to Fig. 3 for details). DEAE-Sephacel chromatography (Fig. 3) resulted in separation of polysaccharide-containing type Ia determinants from group B-specific polysaccharide. Fractions 61 to 80, containing type Ia polysaccharide, were pooled, concentrated, and dialyzed against 0.01 M Tris (pH 7.3). The pooled material contained 6.3% protein. The material was treated with pronase (Calbiochem; 40 μg of enzyme per 20 mg of polysaccharide per ml of 0.01 M Tris, pH 7.3) for 42 h at 37°C. Because the pronase treatment was only partially effective in reducing protein content, trypsin was then added (40 μg/ml), and incubation was continued for 24 h at 37°C. The product was subjected to gel filtration on Sepharose 4B in 0.01 M Tris (pH 7.3) containing 0.6 M NaCl (Fig. 4). Salt was added to reduce ionic interactions to help remove contaminating proteins. Fractions 85 to 132, containing the type polysaccharides (K_{av} of 0.61), were pooled, dialyzed against water, and lyophilized. The overall yield of type Ia polysaccharide was 8.5 mg per liter of culture; the preparation contained 0.75% Lowry-reactive material and undetectable amounts of nucleic acids (determined by absorption at 260 nm).

Chemical composition of the purified type polysaccharides. The purified type II and type Ia polysaccharides were found to contain galactose, glucose, glucosamine, and neuraminic acid in the amounts and molar ratios shown in Table 1. These data are representative of duplicate

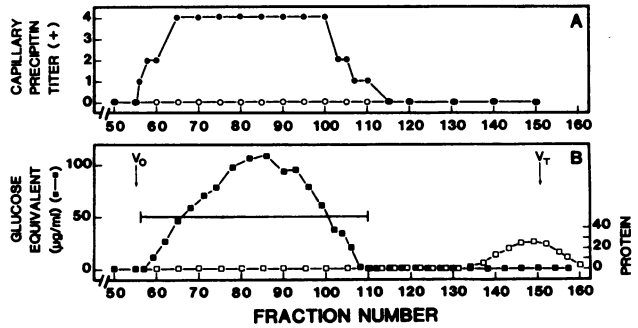

FIG. 2. Sepharose 4B filtration of type II polysaccharide. The trypsin-treated type II polysaccharide (fractions 64 to 84, Fig. 1) was applied to and eluted from a Sepharose 4B column (2.5 by 90 cm) in 0.01 M Tris (pH 7.3), and 2.9-ml fractions were collected. V_o (160 ml) and V_t (450 ml) were determined with blue dextran 2000 (Pharmacia) and glucose, respectively. Symbols are as in Fig. 1.

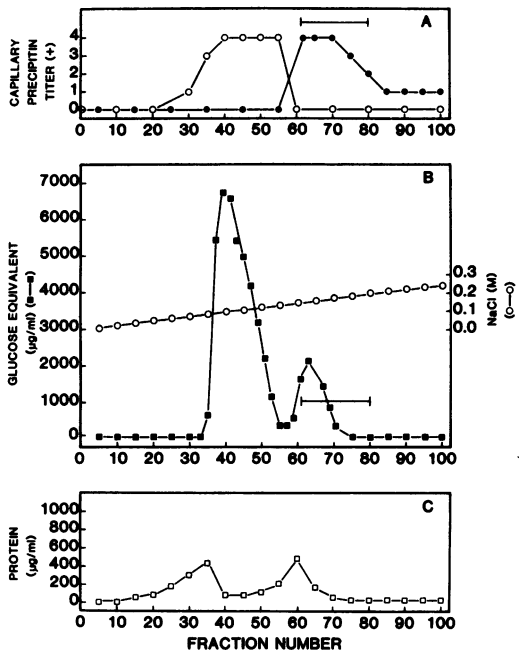

FIG. 3. Isolation of type Ia polysaccharide from culture supernatant by DEAE-Sephacel chromatography. A 20-liter culture of strain DS/1204/78 was incubated at 37°C. After 6 h, at about the end of the exponential growth phase, the culture was neutralized to pH 7.0 with NaOH, and incubation was continued for 24 h. Bacteria were removed by filtration, and the extracellular culture fluid was concentrated, dialyzed, and heat inactivated. This concentrate (500 ml in 0.01 M Tris, pH 7.0) was then bound to DEAE-Sephacel (100 ml) equilibrated with the same buffer. The resin was poured into a column that was washed with 0.05 M NaCl in 0.01 M Tris (pH 7.0). Bound components were eluted with a shallow, linear sodium chloride gradient in a total volume of 1,200 ml of 0.01 M Tris (pH 7.0), and 8-ml fractions were collected. Symbols are as in Fig. 1.

(type II) and quadruplicate (type Ia) analyses. For example, the mean sialic acid content of the type Ia polysaccharide was 35.6 ± 2.5% (four separate determinations). Also shown in Table 1 is the chemical composition of the previously (10) purified type III polysaccharide. According to the thiobarbituric acid assay, sialic acid constituted 20.2, 33.2 ± 0.5, and 28.9% of the dry weight of the type II, type Ia, and type III polysaccharides, respectively. These thiobarbituric acid values yielded molar ratios of sialic acid to glucosamine of 1.0, 1.24, and 1.1, respectively. The purified type II and type Ia polysaccharide preparations did not contain detectable amounts of rhamnose (a major constituent of group B-specific polysaccharide), phosphorus, or nucleic acids. The component sugars accounted for 104 and 96.3% of their dry weights, respectively.

Serological properties of the purified type II and type Ia polysaccharides. In capillary precipitins, immunoelectrophoresis (Fig. 5), and counter-immunoelectrophoresis, the purified type polysaccharides reacted only with their homologous antisera and failed to react with antiserum specific for the group B polysaccharide. In immunoelectrophoresis, both polysaccharide antigens migrated toward the anode (Fig. 5).

Molecular weight estimations. Based on the compositions given in Table 1 and on reducing end group determinations, the average molecular weights of the type II, type Ia, and the previously isolated type III polysaccharides were estimated at 97 × 10^3, 94 × 10^3, and 91 × 10^3, respectively. Figure 6 shows the gel filtration profiles of these three type polysaccharides and of the culture supernatant-derived group B-specific polysaccharide. After calibration of the

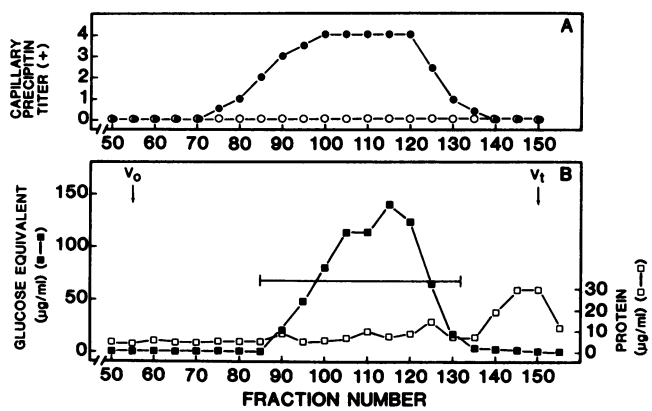

FIG. 4. Sepharose 4B filtration of type Ia polysaccharide. The pronase and trypsin-treated type Ia preparation (fractions 61 to 80, Fig. 3) was applied to and eluted from a Sepharose 4B column (5 by 80 cm) in 0.01 M Tris (pH 7.3) containing 0.6 M NaCl. Fractions of 10.5 ml were collected. V_o (577 ml) and V_t (1575 ml) were determined with blue dextran 2000 and glucose, respectively. Symbols are as in Fig. 1.

TABLE 1. Composition of purified type II, Ia, and III polysaccharides[a]

Component	Type II polysaccharide			Type Ia polysaccharide			Type III polysaccharide		
	% of dry wt	μmol/mg	Molar ratio[b]	% of dry wt	μmol/mg	Molar ratio	% of dry wt	μmol/mg	Molar ratio
Galactose	37.9	2.10	3.3	30.2	1.68	2.0	32.6	1.81	2.3
Glucose	25.6	1.42	2.3	12.5	0.69	0.8	15.7	0.87	1.1
Glucosamine[c]	14.0	0.63	(1.0)	18.2	0.83	(1.0)	17.8	0.80	(1.0)
Neuraminic acid[c]	26.3	0.85	1.35	35.4	1.14	1.4	30.0	0.97	1.2
Sum	103.8		96.3				96.1		

[a] Determined by gas liquid chromatography.
[b] Molar ratios based on glucosamine = 1.0.
[c] As the *N*-acetyl derivatives.

column with Dextran T70, the apparent average molecular weights of the type Ia, type III, and group B polysaccharides were estimated to be 100×10^3, 80×10^3, and 20×10^3, respectively. The type II polysaccharide eluted near the void volume and outside of the Dextran T70 calibrated range of the column. Thus, we were unable to obtain a precise estimate of its molecular weight by this method and can only state that the type II polysaccharide may have an average molecular weight of over 17×10^4.

DISCUSSION

The data presented show that strains of type II and type Ia, group B streptococci, secrete into the culture medium substantial amounts (4 to 8.5 mg/liter) of high-molecular-weight polysaccharides that carry their respective type-specific immunodeterminants, in a manner similar to that previously described for type III strains (10, 17). Secretion of these polysaccharides into a medium containing only low-molecular-weight, completely dialyzable constituents permitted their rapid removal from cells and culture medium, without exposure to chemical or even physical stresses. In fact, because of the possible modification of polysaccharides by endogenous enzyme activities, such as the neuraminidase known to be present in some strains of group B streptococci (32, 33), the concentrated supernatants were exposed to 60°C for 30 min. Thus, we have obtained these antigens in a state that should be very similar to, or identical with, the original bacterial products. This contention is substantiated by the high content (percentage of dry weight) of neuraminic acid present in each of

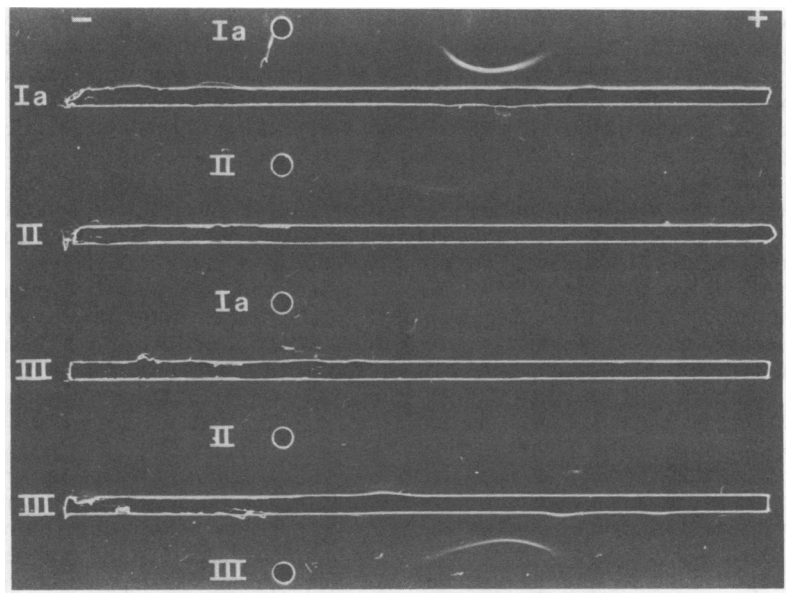

FIG. 5. Immunoelectrophoresis of purified type Ia, II, and III polysaccharides. The type III antigen was prepared as described previously (10). Troughs contained the indicated type-specific antisera (Ia, II, or III) prepared in rabbits to formalinized whole cell vaccines.

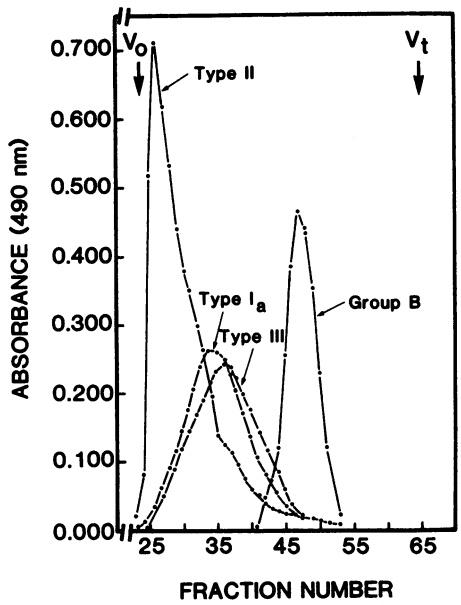

FIG. 6. Molecular weight estimations. The type II, Ia, and III and group B polysaccharides were filtered on a column (1.5 by 51 cm) of Sepharose 6B in 0.01 M Tris-hydrochloride (pH 7.3) containing 0.6 M NaCl. Each polysaccharide was run separately; fractions of 1.2 ml were collected, and profiles were monitored by assaying for neutral hexose. V_o and V_t were determined with blue dextran 2000 and glucose, respectively, and the column was calibrated with dextran T70.

the three polysaccharides (Table 1). For each of our polysaccharides, the molar ratio of neuraminic acid to glucosamine is more than 1:1 (Table 1). These high ratios of neuraminic acid to glucosamine cannot be accommodated in the reported proposed structures of the oligosaccharide repeating units for the type III (23), type Ia (24), or the very recently proposed structure for the type II oligosaccharide repeating unit (25).

The reason(s) for the difference in molar ratios between our type polysaccharides and those of Baker et al. (6) Jennings et al. (23–25) are not entirely clear. However, one or more of the following could contribute to these discrepancies. First, it seems possible that antigen extraction by stirring cells with glass beads for 15 h at 4°C in 0.05 M phosphate (pH 7.4), 0.15 M NaCl, and 0.01 M EDTA, as described by Kasper and co-workers (6, 22–25), could permit the chemical or enzymatic removal of highly labile neuraminic acid residues. As stated above, we not only very rapidly separate culture supernatants from cells in the cold, but we also pasteurize concentrated supernatants as soon as possible. Second, we have carefully examined our products for their purity and freedom from possible contamination with group B-reactive polysaccharide. Our analyses account for at least 96% of the dry weight of the preparations as the component monosaccharides. Jennings et al. do not provide data concerning the purity, quantitative chemical composition (other than molar ratios), or reactivity with group B antibodies of their type II (24) or Ia (25) polysaccharides. The possible presence of group B polysaccharide in their preparations is of some importance, since the group B polysaccharide contains both galactose and glucosamine (36) and its presence could possibly increase the relative amounts of these two monosaccharides in their preparations. Previously, some difficulty was encountered in separating type III and group B reactivity in polysaccharides prepared by extracting cells by mixing with glass beads (28). The type II and Ia polysaccharides isolated and purified from the supernatant culture medium described in this paper do not contain detectable group B antigen and are virtually free of nucleic acid and protein. Although they are not completely homogenous in size, their average molecular weights are described. These molecular weights (and therefore chain lengths) are substantially smaller than the molecular weight of 650,000 or more claimed by Baker and collaborators (6, 28) for their type III polysaccharide. However, their claim was based on gel filtration at low ionic strength (0.01 M Tris-hydrochloride, pH 7.3), conditions that also lead to elution at low K_{av} values for our extracellular type III polysaccharide (10) and for our type II polysaccharide (Fig. 2). The reasonably good agreement of molecular weights of type Ia and type III polysaccharides determined by two independent methods, reducing end groups and gel filtration, increases our level of confidence in the precision of these values.

It is clear from previous studies (15, 16) that both type and group polysaccharides are integral parts of the cell walls of group B streptococci and are covalently linked to the cell wall peptidoglycan. In fact, Doran and Mattingly (16) showed that about 88% of the neuraminic acid present in a culture of a type III group B streptococcus culture was present in the isolated cell wall fraction. Since previous studies showed little evidence of loss of cytoplasmic contents via lysis (17), it seems unlikely that the amounts of type and group B polysaccharides found in the medium are due to gross cellular lysis. However, it is possible that selective hydrolysis of a small number of covalent linkages could cause the release of cell wall polymers from the surface of these streptococci into the culture medium. Evidence that the wall peptidoglycans of other species of streptococci are not lost by turnover (34) argues against this possibility. Alternately, these extracellular polymers could result from the direct secretion of perhaps ex-

cess preassembled precursors that fail to be covalently attached to the cell wall peptidoglycan. Recent data (13, 29) obtained with a different organism, *Bacillus subtilis* subsp. WM, is consistent with this last proposal. In this organism, a portion of the anionic polymers, normally covalently linked to the cell wall peptidoglycan, appears to be directly secreted into the culture medium.

As will be documented elsewhere, these type-specific polysaccharides are nontoxic and are immunogenic in humans.

ACKNOWLEDGMENTS

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