Specific Capsular Polysaccharide of Type 46 Streptococcus pneumoniae (American Type 73)

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The specific capsular polysaccharide of type 46 Streptococcus pneumoniae (American type 73) was isolated in pure form and shown to be a high-molecular-weight, glycosidically linked polymer composed of D-galactose (2 mol), N-acetyl-D-glucosamine (1 mol), N-acetyl-D-galactosamine (1 mol), and N-acetyl-L-fucosamine (1 mol).

Type 46 Streptococcus pneumoniae (American type 73) has been detected among disease isolates in Africa (1, 15), and the inclusion of its specific capsular polysaccharide in polyvalent vaccines for use in certain geographical areas has been proposed (4). Because of this interest, a study of the preparation, purification, and determination of the physical properties and chemical composition of the polysaccharide was undertaken.

MATERIALS AND METHODS

Experimental methods were the same as those described for the analysis of the type 45 S. pneumoniae polysaccharide (7).

Type 46 S. pneumoniae was obtained from R. M. Douglas, of the University of Pennsylvania School of Medicine. The culture was preserved in a lyophilized state in the Merck Institute Rahway Stock Culture Collection. The cells were identified by Gram stain and the Quellung reaction with rabbit typing antiserum to type 46 S. pneumoniae obtained from the Statens Seruminstitut, Copenhagen, Denmark.

The organism was grown in a 14-liter fermenter inoculated with a 1-liter growth from the original seed culture. During the 17-h fermentation, the temperature was maintained at 37°C; the pH was monitored and adjusted to between 6.2 and 7.0 with 10% NaOH.

After fermentation, phenol was added to a final concentration of 1%. The clear centrifuged medium was brought to a 40 to 60% ethanol concentration, and the collected precipitated material was reprecipitated with 2-propanol to a 20 to 30% concentration, the presence of capsular material being verified with antiserum. Protein and nucleic acids were removed by digestion with trypsin, ribonuclease, and deoxyribo-nuclease (Worthington Biochemicals Corp., Freehold, N.J.) followed by dialysis against distilled water. The polysaccharide was collected by precipitation with 2 volumes of 95% ethanol, washed with ethanol and acetone, and dried in vacuo.

RESULTS AND DISCUSSION

Crude type 46 pneumococcal polysaccharide obtained by fractional ethanol precipitation of the growth medium of type 46 S. pneumoniae was a water-soluble white powder having $\alpha_0 = +2.6^\circ$ (c = 0.1 in water) which, after acid hydrolysis, was found to be composed of ribose (<1%), fucose (<3%), galactose, mannose, galactosamine, glucosamine, fucosamine, phosphate, and choline. The complex nature of this material suggested that it was heterogeneous. On immunodiffusion, the material gave a strong precipitin line against type 46 pneumococcal antiserum (Statens Seruminstitut) and against a myeloma protein specific for phosphorylcholine (14).

The crude polysaccharide (220 mg) was fractionated by diethylaminoethyl (DEAE)-Sephaphore CL-6B chromatography in tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.1 M, pH 7.6) as previously described (7) to yield two fractions. One fraction (31%) eluting in the buffer showed no reactivity with the type 46 pneumococcal antiserum. A second fraction (69%) eluting at the beginning of the salt gradient was shown by immunodiffusion to contain type 46 specific polysaccharide.

The fraction containing type 46 polysaccharide was refractionated by DEAE- Sephaphore CL-6B chromatography, using a sodium acetate-acetic acid (0.025 M, pH 6.5) buffer, followed by a 0 to 1 M sodium chloride gradient in the same buffer (Fig. 1). The initial material eluting in the salt gradient did not react with type 46 pneumococcal antiserum, but the remainder of the fractions did. On the other hand, the initial fractions reacted strongly with the myeloma protein (specific for phosphorylcholine), but the latter fraction did not. The presence of components eluting from the column which were reactive with the type 46 pneumococcal antiserum but were unreactive with the myeloma protein provided the first indication that the type 46 polysaccharide did not contain phosphorylcholine. The DEAE-Sephaphore chromatographic separation, although showing the impure nature of the crude polysaccharide, did not appear to
provide a satisfactory method for large-scale purification of the type-specific polysaccharide. Subsequent studies showed that the specific polysaccharide could be obtained by selective precipitation as its insoluble cetyltrimethylammonium salt complex.

Pure type 46 polysaccharide (0.23 g) was obtained from the crude polysaccharide preparation (0.5 g) by cetyltrimethylammonium salt complexing in the same way as that described for the purification of type 45 pneumococcal polysaccharide (7). On DEAE-Sepharose CL-6B chromatography in the sodium acetate-acetic acid buffer system, the polysaccharide was eluted in the sodium chloride gradient (Fig. 2) as a peak with consistent relative glycoside composition across the fractions. All further attempts to fractionate the polysaccharide were unsuccessful, and we believe that it represented the pure capsular polysaccharide elaborated by type 46 S. pneumoniae. The DEAE-Sepharose adsorption and insoluble cetyltrimethylammonium salt formation by the polysaccharide were probably due to the presence of a low proportion of unsubstituted phosphate groups.

The pure type 46 polysaccharide had \[ \alpha_\text{n} = +1.5^\circ \] (c = 1.5 in water), and on Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gel filtration in 0.2 M ammonium acetate it eluted as a sharp peak having \( K_d = 0.04 \). The infrared spectrum of the polysaccharide showed strong absorption at 1,650 cm\(^{-1}\) (C=O of NHCOCH\(_3\)) and no other bands in the carbonyl absorption region, indicating the absence of uronic acid and O-acetyl groups. Quantitative colorimetric analyses also showed the absence of uronic acid (3) and O-acetyl groups (8). The proton magnetic resonance spectrum of the polysaccharide (95°C in D\(_2\)O) showed signals at \( \delta 1.25 \) (m, 3H; CH\(_3\) of fucosamine), 1.99, 2.06, and 2.17 (9H, CH\(_3\) of three NAc groups of 2-acetamido-2-deoxyglycoses) and 5.3 (\( J_{1,2} = 2 \) Hz) and 5.5 ppm (\( J_{1,2} = 2 \) Hz). The \( ^{13}\)C nuclear magnetic resonance spectrum (25.16 MHz in D\(_2\)O at 81°C) showed signals at \( \delta 17.36 \) (CH\(_3\) of fucosamine), 23.98 (3C, NHCOCH\(_3\) of 2-acetamido-2-deoxyglycoses), 50.27, 51.31, and 57.26 (CHNHCOCH\(_3\), 3C), 63.10 (CH\(_2\)OH), 176.51, 175.80, and 175.33 (NHCOCH\(_3\), 3C).

The type 46 polysaccharide (60 mg) was hydrolyzed with 3 N hydrochloric acid (7 ml) at 100°C for 8 h, and the solution was concentrated below 35°C. The residue (54 mg) was passed down a column of Rexyn 101 (H\(^+\)) ion-exchange resin (5 ml), and the eluate and water washings were concentrated to a syrup (9 mg) which on paper chromatography gave a single spot corresponding in color reaction and mobility to galactose. A portion of this fraction was completely oxidized by \( \beta \)-galactose oxidase (2), and a second portion, after reduction (NaBH\(_4\)) and acetylation (13), afforded galactitol hexaacetate which on gas-liquid partition chromatography (GLC) gave a single peak corresponding in retention time to an authentic derivative. The remainder of the fraction gave \( \beta \)-galactose having melting point and mixture melting point of 165°C and \[ \alpha_\text{n} = +69^\circ \] (c = 0.24 in water).

The aminoglycoses liberated from the polysaccharide and retained by the ion-exchange resin were recovered by elution of the column with 2 N hydrochloric acid. The mixture of aminoglycoses (38 mg) was fractionated by preparative paper chromatography using pyridine-ethyl acetate-water (2:5:5, vol/vol; top layer) as the mobile phase. The aminoglycoses were lo-
icated by ninhydrin, and the excised paper strips were eluted with 0.1 N hydrochloric acid to yield three products. Mobility \( R_{GN} \) was determined relative to d-glucosamine hydrochloride. The glycosylate with the slowest mobility on paper chromatography had \( R_{GN} = 0.78 \), corresponding to d-galactosamine hydrochloride, and \( \alpha_1 = +77^\circ \) (c = 0.34 in water). On reduction (NaBH\(_4\)) and acetylation, it gave 2-acetamido-2-deoxy-d-galactitol pentaacetate, which on GLC (17) gave a single peak corresponding in retention time to authentic material. The second aminoglycoside on paper chromatography gave a single spot with \( R_{GN} = 1.00 \) and had \( \alpha_1 = +71^\circ \) (c = 0.3 in water). On reduction and acetylation, the glycosylate afforded 2-acetamido-2-deoxy-d-glucitol pentaacetate, which on GLC gave a single peak corresponding in retention time to an authentic derivative. The aminoglycosylate with the fastest chromatographic mobility had \( R_{GN} = 1.28 \), corresponding to fucosamine hydrochloride, and \( \alpha_1 = -60^\circ \) (c = 0.2 in water); on reduction and acetylation, it yielded 2-acetamido-2-deoxy-L-fucitol tetracetate, which on GLC gave a single peak with the same retention time as an authentic sample. GLC analysis of the trimethylsilylated derivatives of the derived N-acetylated aminoglycosides (16) gave peaks having the same retention values as the derived corresponding trimethylsilylated derivatives of N-acetyl-d-glucosamine, N-acetyl-d-galactosamine, and N-acetyl-L-fucosamine. Ion-exchange chromatography (18) of the aminoglycosylate hydrochloride derivatives confirmed the above identifications.

Quantitative analysis of the type 46 polysaccharide was done by GLC analysis of the derived alditol acetate derivatives obtained after sealed-tube hydrolyses at 100°C (13, 17). Direct hydrolysis (3 N H\(_2\)SO\(_4\); 14 h) and colorimetric analysis (9) indicated a maximum galactose content of 25%; however, prior treatment of the de-N-acetylated polysaccharide with nitrous acid (6) followed by the usual acid hydrolysis showed a maximum indicated galactose content of 32.5%. It appears that, because of the high aminoglycosylate content of the polysaccharide, it is difficult to achieve full release of galactose under the usual hydrolytic conditions. Aminoglycosides were determined by GLC analysis of their derived 2-acetamido-2-deoxyglucitol acetate derivatives (17) and by ion-exchange chromatography (18) after hydrolysis with 4 N HCl for 16 h (Table 1).

The composition data considered in conjunction with the spectroscopic data indicate that the type 46 polysaccharide is a high-molecular-weight polymer composed of d-galactose (2 mol), N-acetyl-d-galactosamine (1 mol), N-acetyl-d-glucosamine (1 mol), and N-acetyl-L-fucosamine (1 mol). It is interesting to note that the specific capsular polysaccharide of type 45 S. pneumoniae (7) is composed of the same four glycosyl components as the type 46 polysaccharide together with L-rhamnose, which is not present in the type 46 polysaccharide.

Despite the similarities in the glycosyl compositions of the type 45 and 46 polysaccharides, no cross-reactivity between them and their specific typing antisera was observed. Structural studies of the type 46 polysaccharide are being done, but methylation and hydrolytic methods have been difficult because of the chemical nature of the polymer. Periodate oxidation of the polysaccharide resulted in the oxidation of the d-galactose and N-acetyl-d-galactosamine components leaving, after Smith-type hydrolysis (11), a high-molecular-weight polymer composed of only N-acetyl-L-fucosamine and N-acetyl-d-glucosamine, which may represent a backbone structure of the molecule.

### Table 1. Percentage composition of the type 46 S. pneumoniae specific capsular polysaccharide obtained pure via its cetyltrimethylammonium salt complex

<table>
<thead>
<tr>
<th>Component</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Galactose</td>
<td>32.5</td>
</tr>
<tr>
<td>N-acetyl-d-galactosamine</td>
<td>17.3</td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine</td>
<td>20.3</td>
</tr>
<tr>
<td>N-acetyl-L-fucosamine</td>
<td>13.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\) Analysis found: C, 38.76%; H, 5.87%; N, 4.23%; ash, 1.5%.

\(\beta\) Calculated as anhydrohexose.

### LITERATURE CITED


