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The Cellular Polysaccharide of a Type II non-capsulated *Pneumococcus*

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SUMMARY: A purified cellular polysaccharide was obtained from a non-capsulated pneumococcus (*Streptococcus pneumoniae*, strain R19, derived from a Type II organism). The main purification steps were peptic, tryptic and ribonuclease digestions, removal of protein by the Sevag technique and ethanol precipitation of the polysaccharide. Ribonuclease digestion was an essential step in the purification procedure. The constituent sugars of the isolated polysaccharide were galactose, mannose and glucosamine. The acetyl content of the polysaccharide indicated that the glucosamine was fully acetylated on the N position and that some O-acetyl was also present. The phosphorus content was not entirely explicable in terms of the known ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contamination. Residual amino acid was consistently present which may have been due to bound amino acids or to protein contamination.

A study of the uridine pyrophosphoglycosyl compounds of the pneumococcus (Smith, Mills & Harper, 1957*a*) showed the presence of uridine pyrophosphoglucose (UPPglucose) and uridine pyrophosphoglucuronic acid (UPPglucuronic acid) in *Streptococcus pneumoniae*, strain R19, derived from a Type II organism, and in a capsulated *S. pneumoniae*, Type III, strain A66. The uridine pyrophosphoglycosyl compound, uridine pyrophosphogalacturonic acid (UPPgalacturonic acid), has also been isolated from a capsulated *S. pneumoniae* Type I (Smith, Mills & Harper, 1957*b*). It was suggested by the above authors that such uridine derivatives function as sugar-transferring agents in the synthesis of the capsular polysaccharides of *S. pneumoniae*. Uridine pyrophosphoacetylglucosamine (UPPacetylglucosamine) has also been isolated in considerable amounts from all the above pneumococcal types, and it has been proposed (Smith *et al.* 1957*a*) that this compound is functional in synthesis of the cellular polysaccharide of pneumococci. It was therefore considered essential to investigate the structure of this polysaccharide to determine whether or not acetylglucosamine is a constituent of this material.

Information about composition of the cellular polysaccharide of pneumococci is scant. In 1930 Enders, utilizing an autolysate from a Type I pneumococcus, obtained an immune rabbit serum by repeated injections of the autolysate; when the serum was tested against the type-specific polysaccharide no precipitate was formed, but with an autolysate from smooth Type I pneumococcus the serum gave a definite precipitin reaction. An effect analogous to that described by Enders was reported by Sabin (1931), by Wadsworth &

Brown (1931) and by Ward (1932). Wadsworth & Brown (1933) separated carbohydrate fractions from pneumococci types I, II and III, and also from an atypical strain. The preparations were essentially different from the type-specific substance and were designated as cellular carbohydrate. Micro-analysis of the cellular carbohydrates yielded phosphorus values ranging from 0.43 to 6.54 % and total nitrogen values ranging from 3.03 to 8.92 %; amino-N was also shown to be present. Wadsworth, Crowe & Smith (1934) studied the absorption spectra of cellular polysaccharide preparations at different stages of purification and found that selective absorption in the ultraviolet region was characteristic of the pure substance.

Tillett & Francis (1930) isolated from a non-type-specific R strain of pneumococcus a substance called fraction C; this fraction was chemically distinct from the type-specific capsular carbohydrate and exhibited no type specificity. Tillett, Goebel & Avery (1930) studied the newly isolated fraction which was shown to contain 5.07 % nitrogen, yielded 30 % of reducing sugars on hydrolysis and contained no amino-N. The fraction exhibited a broad species coverage but no type specificity; the conclusion was that fraction C was a common constituent of all pneumococci. Heidelberger & Kendall (1931) further separated pneumococcal cellular polysaccharide into an immunologically inactive fraction and an immunologically active fraction identical with the C fraction. The serologically inactive fraction differed from fraction C in yielding on hydrolysis crystals with the optical rotation of glucosamine. The most comprehensive review of these findings is that of White (1938). The present paper describes the isolation of a polysaccharide from an extreme rough non-capsulated *Streptococcus pneumoniae* derived from a Type II organism.

METHODS

The organism used was a non-capsulated *Streptococcus pneumoniae* Type II, strain R19, kindly supplied by Dr R. D. Hotchkiss (Rockefeller Institute for Medical Research, New York, U.S.A.). Large-scale cultivation in 3.7 % (w/v) Bacto brain heart infusion medium at pH 7.4 and harvesting of the organisms in the Sharples Supercentrifuge were carried out as described by Smith *et al.* (1957a).

Bacto brain heart infusion (solid), *pepsin* and *trypsin* were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

Crystalline ribonuclease was obtained from Armour Laboratories, U.S.A.

Total nitrogen was determined by the micro-Kjeldahl method, using the Markham distillation unit (Ma & Zuazaga, 1942).

Glucosamine was estimated by the method of Rondle & Morgan (1955); *acetyl* groups were determined by a micro-modification of the method of Heidelberger, Kendall & Scherp (1936), which allowed determinations of the order of 0.2–1.0 mg. acetyl; *DNA-deoxyribose* estimations were carried out by the method of Ceriotti (1955) as modified by Paul (1956) and *micro-phosphorus* estimations by the method of Griswold, Humoller & McIntyre (1951). Determination of *total reducing sugar* was carried out by the method of Hagedorn &

Jensen (1923). *Paper chromatography* of sugars was carried out in the butanol/acetic acid/water solvent and the butanol/ethanol/water solvent of Partridge (1948) on Whatman no. 1 paper; the method of Consden & Stanier (1952) was employed for *ionophoresis of sugars* as borate complexes on Whatman 3MM paper strips; the aniline hydrogen phthalate reagent of Partridge (1949) was used for the development of the sugar spots. *Amino acid chromatography* was carried out on Whatman no. 1 paper using the two-dimensional butanol/acetic acid/water and phenol/ammonia system (Partridge, 1948). *Purine base and pyrimidine nucleotide chromatography* was carried out in the isopropanol/HCl solvent of Wyatt (1951).

RESULTS

Purification of the polysaccharide

The centrifuged organisms from 8 l. culture medium were suspended in 500 ml. distilled water, the pH value adjusted to pH 1–2 with dilute HCl and 1 g. pepsin added; digestion was allowed to proceed for 24 hr. at 37°. At the end of this period the pH value was adjusted to 9 with 2N-KOH, 2 g. trypsin added and digestion continued for a further 2 days at 37°. Tryptic activity was destroyed by neutralizing the digest and bringing it rapidly to the boil; the digest was then cooled rapidly to 30°–37°.

The pH value was readjusted to 7.8 with dilute KOH and 100 mg. crystalline ribonuclease added, followed by incubation at 37° for 3 hr. The pH value was again readjusted to 9 and a further tryptic digestion made by adding 1 g. trypsin and a 24 hr. incubation period at 37°.

At the end of this period the solution was filtered, 10 g. sodium acetate, 1 ml. acetic acid, 20 ml. chloroform and 4 ml. *n*-butanol added for every 100 ml. solution and protein removed exhaustively by the method of Sevag (1934).

The polysaccharide was precipitated from solution by the addition of 5 vol. ethanol, centrifuged and washed with 95 % (v/v) ethanol in water followed by an ether washing; the material so obtained was dried *in vacuo*. The dried product was dissolved in 50 ml. water and dialysed for 2 days at 0° against several changes of distilled water. The insoluble residue obtained on dialysis was discarded and the polysaccharide was reprecipitated from solution by the addition of sodium acetate (10 g./100 ml.), acetic acid (1 ml./100 ml.) and 5 vol. ethanol. The centrifuged polysaccharide was washed with 95 % (v/v) ethanol in water followed by an ether washing and was then dried *in vacuo*; yield 50 mg.

Analysis and properties of the polysaccharide

Constituent sugars obtained by acid hydrolysis. Five mg. of the isolated polysaccharide were hydrolysed with 1 ml. N-H₂SO₄ at 110° for 18 hr. and the hydrolysate neutralized with solid BaCO₃. The precipitated BaSO₄ was removed by centrifugation, washed with 1 ml. water, recentrifuged, the supernatant fluids pooled and dried *in vacuo*. The dried material was dissolved in 500 µl. distilled water, any further trace of BaSO₄ removed by centrifugation,

and 50 μ l. samples were then taken for paper chromatography and ionophoresis. As will be seen from Table 1, the constituent sugars of the isolated polysaccharide obtained by hydrolysis were galactose, mannose and glucosamine; galactose was the major component.

Table 1. *Constituent sugars of the cellular polysaccharide from Streptococcus pneumoniae Type II, strain R19*

R_{glucose} values by chromatography in two solvents and ionophoresis in borate buffer at pH 8.6.

	Butanol/acetic acid/water	Butanol/ethanol/ water	Ionophoresis
Polysaccharide hydrolysate	0.65	0.70	0.53
	0.85	0.90	0.82
	1.26	1.24	0.91
Glucosamine	0.66	0.72	0.52
Mannose	1.27	1.27	0.81
Galactose	0.84	0.89	0.92

Trace amounts of ribose (probably from RNA contamination) were identified in both chromatographic solvents.

Glucosamine content. Fig. 1 shows the liberation of glucosamine from the polysaccharide during hydrolysis at 100° with 3N-HCl as measured by the method of Randle & Morgan (1955); 3N-HCl gave optimal liberation of glucosamine from the polysaccharide. Glucosamine standards were run simultaneously.

Fig. 1. Liberation of glucosamine as measured by the method of Randle & Morgan (1955) from the polysaccharide during hydrolysis at 100° with 3N-HCl.

N-acetyl and O-acetyl evaluations. Total acetyl was determined by steam distillation of acetic acid from the polysaccharide after hydrolysis with *p*-toluenesulphonic acid. The method of Heidelberger *et al.* (1936) was modified by using a micro-distillation unit with subsequent titration of acetic acid with the Conway burette (1947). The results are recorded in Table 2. It will be observed that the acetyl content of the polysaccharide was greater than can be accounted for by *N*-acetylation of the glucosamine present; hence the following experiment was carried out to determine *O*-acetyl groups by means of their lability to alkali (Smith & Zwartouw, 1956). Six mg. polysaccharide in 1 ml. 2% (w/v) KOH were allowed to stand at room temperature for 30 min.; 10 ml. cold ethanol was added and the precipitated polysaccharide washed twice with ethanol (5 ml.). The acetyl content of the recovered polysaccharide is shown in Table 2, the results indicating that approximately 50% of the total acetyl in the polysaccharide was *N*-acetyl. The values correspond to complete acetylation of the glucosamine found in the polysaccharide.

Table 2. *Analysis of the cellular polysaccharide from Streptococcus pneumoniae Type II, strain R19*

	%
Total reducing sugar (calculated as glucose)	60
Glucosamine	20
Galactose + mannose (calculated)	45
Total acetyl	10.0
<i>N</i> -acetyl	5.3
Total nitrogen	4.8
Total phosphorus	1.3
RNA-phosphorus	0.7
DNA-phosphorus	0.05

Total reducing sugar. Hydrolysis of the polysaccharide with 3*N*-HCl at 100° for 3 hr. provided optimal conditions for liberation of reducing groups. Such hydrolysates were dried *in vacuo*, and total reducing sugar estimated by the method of Hagedorn & Jensen (1923). A value of 60% (calculated as glucose) was obtained; allowing for the fact that glucosamine has only 75% of the reducing power of glucose in the method used, this value would correspond to a combined galactose and mannose value of 45%.

DNA-deoxyribose estimations indicated that the polysaccharide contains 0.5% (w/v) DNA.

Ribonucleic acid content. In view of the negligible contamination of the polysaccharide with DNA, RNA contamination of the isolated material was assessed by the ultraviolet absorption spectrum, making the necessary correction for protein contamination (assuming that all the nitrogen unaccounted for by glucosamine, etc., was present as protein). When digestion with crystalline ribonuclease was omitted during the purification procedure, the polysaccharide showed 70–75% contamination with RNA; hydrolysis and chromatography confirmed the presence of adenine, guanine, uridine-5'-monophosphate and cytidine-5'-monophosphate. Inclusion of the ribo-

nuclease digestion procedure decreased contamination with RNA to the order of 7%.

Total phosphorus. Micro-phosphorus determinations showed that the isolated polysaccharide contained 1.3% phosphorus. On the basis of 7% contamination with RNA and 0.5% contamination with DNA, the phosphorus unaccounted for by either DNA or RNA is of the order of 0.55%.

Total nitrogen. The total nitrogen present in the isolated polysaccharide was 4.8%; allowing for the nitrogen contributed by glucosamine (1.37%), RNA (0.7%) and DNA (0.05%), the residual nitrogen value is 2.68%.

Amino acid analysis. Two mg. of polysaccharide were hydrolysed with 1 ml. 6N-HCl in a sealed tube for 18 hr. at 110°, evaporated to dryness *in vacuo*, the residue dissolved in 100 μ l. distilled water and chromatographed two dimensionally in butanol + acetic acid + water followed by phenol + ammonia. The following amino acids were detected: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, leucine, iso-leucine, valine, methionine, proline.

DISCUSSION

Analysis of the cellular polysaccharide from *Streptococcus pneumoniae*, Type II, strain R19, showed the constituent sugars to be galactose, mannose and acetylglucosamine. The presence of acetylglucosamine lends support to the proposal (Smith *et al.* 1957*a*) that the UPPacetylglucosamine present in *S. pneumoniae*, Types II and III, may be functional as a glycosyl donor in synthesis of the cellular polysaccharide. An extension of this investigation to the cellular polysaccharides of *S. pneumoniae*, Types I and III, has now shown that an identical sugar pattern exists for each type.

Galactose appears to be the major constituent of the sugars obtained on acid hydrolysis of the polysaccharide; this is of interest in view of the fact that the UPPglucose isolated from *Streptococcus pneumoniae*, Type II, strain R19, has been found to be a mixture of UPPglucose and UPPgalactose (unpublished observation).

During the isolation of the cellular polysaccharide it was found that RNA was a major contaminant unless a ribonuclease digestion were included in the purification procedure. This may account for the observation of Wadsworth *et al.* (1934) that selective absorption in the ultraviolet region was characteristic of the 'pure' substance. The different phosphorus values of previous preparations (0.43–6.54%) may also be accounted for by the contaminating RNA. The residual 7% RNA associated with the present preparation can probably be attributed to the residual core of RNA which is not attacked by ribonuclease.

A considerable amount of nitrogen (2.68%) is unaccountable for in terms of glucosamine-N, RNA-N or DNA-N. From the amino acid pattern obtained it seems probable that this nitrogen is associated with protein. Attempts to remove this protein by repeated Sevag treatments and fractional alcohol precipitation of the purified polysaccharide have so far failed, nor have such procedures led to any change in the amino acid pattern.

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