The Characterization of Pneumococcal C-Polysaccharide as a Ribitol Teichoic Acid

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(Received 11 August 1967)

Pneumococcal C-polysaccharide was first described by Tillett, Goebel & Avery (1930), but was not studied in detail until Goebel, Shedlovsky, Lavin & Adams (1943) showed that it is a polymer containing phosphate and hexosamine but that little, if any, amino acids were present. Their analyses (P, 4.47; N, 5.91%), obtained on material prepared from a rough type 1 organism, agreed closely with analyses obtained by other workers on different occasions. They also noted that solutions of the C-polysaccharide darkened when heated with mineral acid and that nitrous acid destroyed the serological activity of the polymer, one-sixth to one-fifth of the total nitrogen being present in the free amino form; hexosamine accounted for 35% of the total nitrogen in the preparation. Recently it has been suggested that C-polysaccharide from a rough type 2 organism, R36a, is a polymer with a repeating unit composed of β -N-acetyl-D-galactosamine 1-phosphate joined through its phosphate to the 6-hydroxyl group of the neighbouring sugar unit (Gotschlich & Liu, 1967). The work now described indicates that the material also contains ribitol phosphate residues, the 2-acetamido-4-amino-2,4,6-trideoxyhexose reported to be incorporated into C-polysaccharide from its uridine nucleotide (Distler, Kaufman & Roseman, 1966), together with smaller amounts of D-glucose and choline. Polymer of the same composition and indistinguishable structure was obtained from three strains of rough type 1 organisms [1-192R, A.T.C.C. 12213; SV-1R3 and 8a (the strain 190 of Wadsworth & Sickles, 1927)], from the rough type 2 organism R36a, and from capsulated type 7 and type 34.

Freeze-dried whole organisms (type 1-192R, A.T.C.C. 12213) were treated with 10% (w/v) trichloroacetic acid solution at 4° and some RNA was removed from the extract by precipitation with 2 vol. of ethanol. The teichoic acid was precipitated together with some RNA by the addition of 2 vol. of acetone. The polymer was purified by chromatography on DEAE-cellulose (HCO_3^- form) resin with a linear gradient of ammonium carbonate. The polymer was eluted immediately after a weakly bound RNA fraction that contained amino acids and was probably aminoacyl RNA; further purification was effected by rechromatography. The product usually contained 0.5–1.5% of RNA.

A sample of the polymer (Found: P, 4.3; N, 5.5%) contained phosphorus, D-galactosamine, D-glucose and nitrogen in the proportions 1.00:0.79:0.18:2.85. Chromatography on Whatman no. 4 paper in solvent system A [propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.)] (Hanes & Isherwood, 1949), and on ion-exchange columns, revealed the presence of ribitol phosphates, anhydriitol and galactosamine 6-phosphate in the hydrolysis mixture after treatment with 4N-HCl for 3 hr. at 100°.

Treatment of the polymer with fluorodinitrobenzene (Ingram & Salton, 1957) yielded a DNP derivative that, after ion-exchange chromatography, still contained phosphorus and glucose in the ratio 1.00:0.18, indicating that the glucose is an integral part of the polymer. Acid hydrolysis of the DNP-polymer yielded a DNP-amino sugar with the electrophoretic properties described for the DNP derivative of the 2-aminotrideoxyhexose reported by Distler *et al.* (1966). The DNP-polymer, which was still highly active in serological assay, was stable to nitrous acid, whereas the original polymer was destroyed, giving three fragments as indicated by paper chromatography in solvent A. Nitrous acid immediately destroyed the serological activity of the polymer (cf. Goebel *et al.* 1943). The serological behaviour of samples was tested by the agar double-diffusion technique of Ouchterlony against horse antiserum prepared against a C-polysaccharide fraction derived from type 7 *Pneumococcus*. Heavy single precipitation bands were observed at concentrations of 25 $\mu\text{g./ml.}$

The polymer was rapidly degraded to small fragments on treatment with 10 mM-sodium periodate solution. A rate study demonstrated that approx. 1 mol.prop. of periodate was reduced rapidly for each phosphorus atom, completely destroying ribitol without the production of formaldehyde. After reduction of the oxidized polymer with sodium borohydride followed by hydrolysis in acid, ethylene glycol phosphate was one of the products. This indicated that phosphate is attached to a primary hydroxyl group on ribitol, and that an unsubstituted hydroxyl group is adjacent to it. Such a structure is consistent with the view that the polymer originates biologically from the nucleotide cytidine diphosphate ribitol.

Glucose was completely destroyed by the oxidation but galactosamine was largely unaffected, thereby indicating that the galactosamine must be substituted at position 3 or 4.

No oligosaccharides could be produced from the polymer by acid hydrolysis under a variety of conditions, and browning with the concurrent production of ammonia was always observed. Acid released the reducing group of galactosamine very rapidly (cf. Goebel *et al.* 1943). Hydrolysis in 2N-NaOH solution for 6 hr. at 100° gave a phosphate (R_{α} -Glycerol phosphate 0.09 in solvent A) that, although apparently homogeneous when examined by paper chromatography, was presumably a mixture of isomeric phosphomonoesters as it gave both galactosamine phosphate and ribitol phosphate on acid hydrolysis. Treatment of the product with intestinal phosphomonoesterase at pH 9.3 (ammonium carbonate) produced a phosphorus-free glycoside (R_{α} -Glycerol phosphate 0.35 in solvent A) that, like other polyol glycosides, reacted with the alkaline AgNO_3 reagent only on steaming the paper chromatogram (Brundish, Shaw & Baddiley, 1965). The glycoside was hydrolysed by vigorous acid treatment (6N-HCl for 6 hr. at 100°) to yield anhydribose and galactosamine; no smaller ribitol glycoside was observed. This suggests that the acid-labile diaminotrioxohexose is glycosidically linked to ribitol, whereas the rapid release of the reducing function of the galactosamine residue from the original polymer by acid suggests that the *N*-acetylgalactosamine is linked glycosidically to the acid-labile amino sugar. The above evidence is consistent with a structure for C-polysaccharide in which a repeating unit, *N*-acetylgalactosaminyl - *N* - acetyldiaminotrioxohexosylribitol 5-phosphate, is joined through its phosphate group to either position 3 or 4 of galactosamine on an adjacent repeating unit. If 0.2 mol. prop. of D-glucose is included in the repeating unit, the resulting structure requires P, 4.9, and N, 6.6%, which is reasonably near the experimentally determined values. An additional product of acid hydrolysis has been identified by paper chromato-

graphy and from its colour reactions as choline. This confirms an observation by (Tomasz, 1967) that choline is a component of C-polysaccharide and that radioactive choline is incorporated into the polymer.

It would seem that the natural polymer is more complex than the suggested repeating unit might imply. Nevertheless, the phosphorus-free ribitol glycoside has been obtained from all strains of *Pneumococcus* studied in this Laboratory. In particular, the present work is not in agreement with the structure proposed by Gotschlich & Liu (1967), as their structure does not contain either ribitol or the diaminotrioxo sugar, nor would it be degraded to small molecules by treatment with nitrous acid or periodate. As C-polysaccharide contains polyol phosphate and sugar residues, and is located in the walls of the bacteria, it is a teichoic acid. Thus the *Pneumococcus*, like all other Gram-positive bacteria examined, contains at least one teichoic acid in its cellular construction.

We are indebted to Dr J. M. Tyler for gifts of antisera and C-polysaccharide from type 7 *Pneumococcus*, and to Dr Rachel Brown for samples of material from type 1 no. 8a and capsular polysaccharide (which contains a significant amount of C-polysaccharide) from type 34. D. E. B. held a Turner and Newall Fellowship during this work.

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