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Pneumococcal C-Substance, a Ribitol Teichoic Acid Containing Choline Phosphate

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1. Pneumococcal C-substance was isolated from the non-capsulated *Pneumococcus* 1–192R, A.T.C.C. 12213, by extraction with trichloroacetic acid solution followed by chromatography on DEAE-cellulose (HCO_3^- form). 2. The polymer contains 7·0% of phosphorus and 6·0% of nitrogen and is composed of phosphate, *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyldiaminotrideoxyhexose, ribitol and choline in the molecular proportions 2:1:1:1:1:1. 3. After acid hydrolysis, D-galactosamine hydrochloride and galactosamine 6-phosphate were isolated in crystalline form and crystalline derivatives of D-glucose and anhydroribitol were obtained. A product of partial acid hydrolysis was provisionally characterized as 6′-*O*-phosphoryl-[*O*-β-D-galactosaminyl-(1′→6)-D-glucose]. 4. C-substance contains free amino groups accessible to attack by 1-fluoro-2,4-dinitrobenzene and nitrous acid. 5. Choline phosphate and ribitol phosphate are units in the polymer. 6. Treatment with hot alkali gave a fragment comprising phosphate, D-galactosamine, D-glucose, diaminotrideoxyhexose and ribitol in the molecular proportions 2:1:1:1:1. 7. After selective *N*-acetylation, the fragment contained one of its phosphate groups as a phosphomonoester and one as a phosphodiester, shown by potentiometric titration and by treatment with a phosphomonoesterase. 8. C-substance from seven other strains of *Pneumococcus* possesses a structure common to that described for the strain 1–192R. 9. Capsular materials from 26 different strains of *Pneumococcus* were analysed for suspected contamination by C-substance. In 19 cases the presence of C-substance with the normal structure was demonstrated, and in the remaining seven cases the contaminating C-substance was probably similarly constituted. 10. F-substance was isolated and the associated fatty acid material analysed.

A somatic species-specific 'fraction C' from pneumococci (Tillett & Francis, 1930) was shown to contain nitrogen and carbohydrate, to be destroyed by nitrous acid (Tillett, Goebel & Avery, 1930) and to contain phosphorus (Heidelberger & Kendall, 1931). The first detailed structural investigation of C-substance, prepared from a rough type 1 organism (Goebel, Shedlovsky, Lavin & Adams, 1943), substantiated earlier conclusions and showed that it contained hexosamine and darkened when heated with mineral acid. Recent studies on material from a rough type 2 organism, R36a, led to an assignment of the structure of C-substance as a polymer comprising a repeating unit of β-*N*-acetyl-D-galactosamine 1-phosphate joined through its phosphate to the 6-hydroxyl group of the neighbouring amino sugar (Gotschlich & Liu, 1967). In a preliminary communication we have criticized this proposal (Brundish & Baddiley, 1967) on the grounds that it does not explain the observed fragmentation of the polymer by nitrous acid or by periodate. We also demonstrated the presence of ribitol phosphate, and of the diaminotrideoxyhexose described by Distler, Kaufman & Roseman (1966) as incorporated into C-substance from its UDP derivative; choline, described as a pneumococcal cell-wall component by Tomasz (1967), was also found in our C-substance.

As this polymer contains ribitol phosphate and sugar residues, and occurs in the wall of the organism, it is now classified as a teichoic acid. In this paper the composition of highly purified C-substance from a type 1 organism is described. The material gives significantly higher nitrogen and phosphorus values than did any previously studied preparations. Although it is not yet possible to derive a complete structure for this polymer, the present work is reported in view of the increasing

interest in C-substance and in the recently demonstrated significance of choline in the walls of pneumococci (Tomasz, 1968).

EXPERIMENTAL AND RESULTS

Materials

Pneumococcus 1–192R, A.T.C.C. 12213, was grown as described by Brundish, Shaw & Baddiley (1965). After the harvested cells had been washed once with 0·9% NaCl solution, they were dried by successive homogenization with ethanol and acetone and kept as an acetone-dried powder. DEAE-cellulose was purchased from H. Reeve Angel and Co. Ltd. (London, E.C. 4). Glucostat reagent was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Calf intestinal phosphomonoesterase from Sigma Chemical Co. (St Louis, Mo., U.S.A.) was used as a 1% solution in $(NH_4)_2CO_3$ buffer, pH 10.

Analytical methods

Phosphate was determined by the method of Chen, Toribara & Warner (1956), total nitrogen by the method of Sloane-Stanley (1967), hexosamine by the method of Rondle & Morgan (1955), choline by the method of Marenzi & Cardini (1943) and glucose (before and after acid hydrolysis) by the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and (after hydrolysis) by the Glucostat reagent. Periodate was measured spectrophotometrically by the procedure of Dixon & Lipkin (1954) and formaldehyde by the method of Hanahan & Olley (1958).

Paper chromatography

Whatman no. 1 paper, previously washed with 2 N-acetic acid and then water, was used with the following solvent systems: *A*, propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951).

Compounds were detected by the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949), the alkaline $AgNO_3$ reagents for sugars (Trevelyan, Procter & Harrison, 1950) and non-reducing glycosides (Brundish *et al.* 1965), the ninhydrin reagent for amino compounds (Consden & Gordon, 1948) and the $K_4Fe(CN)_6$–$CoCl_2$ reagents for choline and choline phosphate (Heyndrickx, 1953).

Isolation of C-substance

Dried cells (65g.) were stirred at 4° for 24hr. with 10% (w/v) trichloroacetic acid solution (450ml.). The resulting suspension was centrifuged in the cold for 20min. at 3000*g*. The sediment was treated five times in a similar manner with portions (250ml.) of trichloroacetic acid. The extracts were separately clarified by passage through a pad of Celite, cold ethanol (500ml.) was added to each and the mixtures were kept at 4° for 24hr. The precipitates were collected by centrifugation at 3000*g* for 20min. The supernatant solutions were mixed with cold acetone (1·5l.) and the mixtures were kept at 4° for 24hr. As much of the supernatants as possible was decanted and the precipitates were collected by centrifugation at 3000*g* for 20min. All precipitates were washed by suspending first in cold acetone (3 × 100ml.) and finally in ether (3 × 50ml.). They were dried over NaOH pellets *in vacuo* and dissolved in cold water (25ml.). Insoluble material was removed by centrifugation at 2000*g* for 5min. and the supernatants and precipitate washings were combined and freeze-dried. The extracted material (ethanol and acetone precipitates) weighed 3·83g.

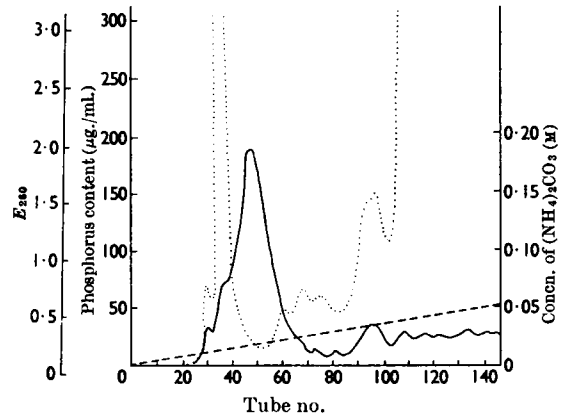


Fig. 1. Fractionation of the trichloroacetic acid extracts on a column of DEAE-cellulose (HCO_3^- form) by elution with a linear concentration gradient of $(NH_4)_2CO_3$. Details are given in the text. ——, Phosphorus content (μg./ml.); ·····, E_{260}; – – –, concn. of $(NH_4)_2CO_3$ (M).

Hydrolysis (2 N-HCl for 3hr. at 100°) of portions (1mg.) of the precipitates, followed by paper chromatography in solvent *A*, showed ribitol phosphates and anhydroribitol in the hydrolysates of the acetone precipitates but negligible amounts of these in the ethanol precipitates.

The acetone precipitates (2·63g.) were dissolved in water (30ml.) and titrated with 0·05 M-Cetavlon solution in portions (10ml.); the precipitates were removed by centrifugation. After three such additions precipitation was complete. The supernatant solution was freeze-dried and the precipitates were dried over P_2O_5 *in vacuo*. Samples (1mg.) of each fraction were examined by acid hydrolysis and paper chromatography as described above. The precipitates contained nucleic acid only and were rejected. Anhydroribitol was produced from the supernatant fraction, but the presence of material that absorbed ultraviolet light indicated that the removal of nucleic acid was incomplete. This fraction (1·90g.) was dissolved in water (100ml.) and applied to a column (28cm. × 2·5cm.) of DEAE-cellulose (HCO_3^- form). The column was washed with water (1l.) and material was eluted with a linear concentration gradient (0–0·15 M) of $(NH_4)_2CO_3$ (total volume 6l.); fractions (15ml.) were collected automatically at a flow rate of 5ml./min. Nucleic acid was measured by the extinction at 260nm. and samples (0·1ml.) were analysed for total phosphate. The elution pattern is given in Fig. 1. Fractions 40–62 were combined and dried by rotary evaporation at

25°; evaporation from water was repeated several times to remove $(NH_4)_2CO_3$. The recovered material (1·03g.) was rechromatographed as described above to remove the small amount of RNA and yielded 982mg. of pure C-substance. After rotary evaporation, the material was dried *in vacuo* over NaOH pellets for 24hr.; it formed a colourless glass that was stable to air and moisture.

Samples of C-substance were prepared by this method from cellular debris of types 2 R36a and SV–1R3, obtained from organisms that had been disrupted for the preparation of enzymes; about 30mg. of material was obtained in each case.

Examination of C-substance

Elemental analysis of a sample of the polymer, converted into the free acid form by passage of a solution through a column of Dowex 50 (H^+ form) resin, gave: P, 7·0; N, 6·0%; it had $[\alpha]_D$ +74±5° (*c* 1·0 in water). The infrared spectrum of the free acid form of the polymer showed only two distinct bands, at 1653 and 1562cm.$^{-1}$, characteristic of an amide; the polymer had no ultraviolet spectrum.

The serological behaviour of the preparation was examined by the agar double-diffusion technique of Ouchterlony, with horse antiserum prepared against C-substance derived from type 7 *Pneumococcus*. The preparation gave a single heavy precipitation band at a concentration of 25μg./ml., identical with that given by authentic C-substance provided by Dr Rachel Brown.

Acid hydrolysis. A sample of polymer (2mg.) was hydrolysed in 2N-HCl at 100° for 3hr. and examined by paper chromatography in the solvents *A* and *B*. By comparison with authentic materials, galactosamine, glucose, choline, choline phosphate, inorganic phosphate, anhydroribitol and the isomeric ribitol phosphates were identified. In addition, compounds with R_F 0·26 and 0·10 in solvent *A* were observed with the periodate–Schiff and acid molybdate reagents; both compounds gave with the periodate–Schiff reagents a brown colour that slowly turned blue.

The hydrolysis was repeated with 400mg. of polymer. Charred material was removed by filtration with charcoal, and the hydrolysis products were separated by ascending paper chromatography in solvent *A*. Material was applied as bands (15cm.) to six sheets of Whatman 3MM paper previously washed twice in solvent *A*. The following were eluted with water and characterized as described.

(*a*) DL-1,4-Anhydroribitol (44mg.) (R_F 0·75), characterized as its tri-*O*-benzoate, m.p. 144° unchanged by mixture with an authentic sample. The infrared spectra (KBr disks) of the natural and synthetic anhydroribitol derivatives were identical.

(*b*) A mixture (56mg.) of glucose and galactosamine (R_F 0·60). The material was dissolved in water (1ml.) and applied to a column (3ml.) of Dowex 50 (H^+ form) resin. Glucose (21mg.) was obtained by elution with water (10ml.) and galactosamine (33mg.) by elution with 2N-HCl (10ml.). The glucose was characterized as its *p*-nitroanilide (Weygand, Perkow & Kuhner, 1951), m.p. 182°, mixed m.p. with the authentic *p*-nitroanilide of D-glucose 183°. The infrared spectra of the natural and synthetic glucose derivatives were identical. Galactosamine crystallized as its hydrochloride from methanol–water (3:1, v/v) (0·5ml.) after the addition of ether to turbidity; it had $[\alpha]_D$ +93±2° (*c* 0·4 in water) and its infrared spectrum was identical with that of authentic D-galactosamine hydrochloride.

(*c*) Isomeric ribitol phosphates (49mg.) (R_F 0·30–0·33). The material was dissolved in water (2ml.) and treated with phosphomonoesterase solution (3ml.) at 37° for 16hr. On examination of the solution by paper chromatography in solvent *A* ribitol was observed. By heavily loading the paper chromatogram a trace of anhydroribitol was detected.

(*d*) Material (9mg.) (R_F 0·26) that gave ribitol and galactosamine on treatment of a sample (0·5mg.) with phosphomonoesterase solution. The mixture was applied to a column (1ml.) of Dowex 50 (H^+ form) resin and this was washed with water (5ml.) to remove the ribitol phosphates. Development with 0·2N-HCl (5ml.), followed by evaporation of the eluate, gave 4·1mg. of material. The residue was dissolved in water (0·4ml.), kept in a deep-frozen state and then thawed; crystals were recovered by centrifugation. The infrared spectrum of a small sample of the solid was not distinct but was similar to that of galactosamine 6-phosphate, synthesized by the method of Distler, Merrick & Roseman (1958), and the compound had the same paper-chromatographic mobility and gave the same blue colour with the periodate–Schiff reagents.

(*e*) Material (76mg.) (R_F 0·10) that was shown by paper chromatography to contain galactosamine 6-phosphate and inorganic phosphate as contaminants. The mixture was dissolved in water (3ml.) and applied to a column (15cm.× 1cm.) of DEAE-cellulose (HCO_3^- form); material was eluted with a linear concentration gradient (0–0·3M) of $(NH_4)_2CO_3$ (total volume 500ml.). Fractions (4ml.) were collected automatically at a flow rate of 0·5ml./min. and samples (0·2ml.) were analysed for phosphate. Fractions 18–50 gave material (17mg.) that contained galactosamine 6-phosphate as impurity. The preparation was applied as bands (10cm.) to two Whatman no. 1 papers, previously washed twice with solvent *A*, for 16hr. After development in solvent *A*, the chromatographically homogeneous material (5·1mg.) with R_F 0·10 was eluted with water; it had $[\alpha]_D$+27·9±0·3° (*c* 0·4 in water). A sample (1·5mg.) was examined on a Technicon AutoAnalyzer; it behaved as the 'peak B' of Liu & Gotschlich (1963). A further sample (1·5mg.) was heated in 3N-HCl at 100° for 16hr. and the hydrolysate was divided. One part was examined by paper chromatography in solvents *A* and *B*, with the periodate–Schiff and $AgNO_3$ reagents respectively; glucose, galactosamine and galactosamine 6-phosphate were detected, together with a large amount of the starting material. These findings were confirmed by autoanalysis of the second part of the hydrolysate. A sample (1·5mg.) of the original material in water (0·1ml.) was treated with a mixture of 33% (v/v) acetic acid solution and 5% (w/v) $NaNO_2$ solution (1:1, v/v) (0·1ml.) (Williamson & Zamenhof, 1963) at room temperature for 10min. The mixture was passed through a column (0·5ml.) of Dowex 50 (H^+ form) resin and the eluate was evaporated to dryness. A sample (1mg.) of synthetic galactosamine 6-phosphate was treated similarly with nitrous acid. The reaction mixture was examined by paper chromatography in solvents *A* and *B*, with the periodate–Schiff and $AgNO_3$ reagents respectively. The compound yielded glucose and the deamination products of galactosamine 6-phosphate.

Quantitative analysis. A solution of the polymer (2mg.) in water (10ml.) was analysed for total phosphate (0·5ml.) and total hexose (1·0ml.); the molecular ratio phosphate:hexose was 2·00:0·74.

Samples (2mg.) of polymer were heated at 100° with

4N-HCl for various times and each was adjusted to a final volume of 10ml. at pH6. The solutions were analysed for total phosphate (0·2ml.), D-glucose (1·0ml.) and galactosamine (1·0ml.). Glucose was maximal after 60min. and galactosamine after 20min. The polymer contained the molecular proportions phosphate:D-glucose:galactosamine 2·00:0·74:0·94.

A sample of polymer (10mg.) was heated at 100° with 6N-HCl for 10hr. and the solution was adjusted to a final volume of 10ml. at pH5. Portions were analysed for total phosphate (0·1ml.) and choline (0·5ml.). The polymer gave the molecular ratio phosphate:choline 2·00:0·9±0·1.

Action of 1-fluoro-2,4-dinitrobenzene. A sample (20mg.) of polymer and $NaHCO_3$ (10mg.) in water (2ml.) was shaken in the dark for 5hr. at room temperature with 5% (v/v) 1-fluoro-2,4-dinitrobenzene in ethanol (4ml.). The mixture was transferred to a dialysis sac with water (10ml.) and dialysed against water (5×8l.) during 48hr. The resulting solution was extracted with ether (3×30ml.) and the aqueous layer was evaporated to dryness *in vacuo*. The amount of DNP substituent, calculated from the extinction at 360nm., was related to the phosphate content; the molecular ratio DNP substituent:phosphate was 0·4:2.

The DNP-polymer was examined serologically by the Ouchterlony technique as described above. It was comparable in activity with the original polymer. The DNP-polymer was stable to nitrous acid when tested as described above. Attempts to recover the DNP derivative of the diaminotrideoxyhexose from the DNP-polymer by acid hydrolysis as described by Distler *et al.* (1966) were not successful.

Colour tests for the diaminotrideoxy sugar. A sample (4mg.) of polymer in water (0·2ml.) was heated at 100° for 5min. with Bial's reagent (0·2ml.) (Heyworth & Bacon, 1957). A faint red colour with an absorption maximum at 405nm. was observed.

A sample (4mg.) of polymer in water (0·2ml.) was heated at 100° for 5min. with Ehrlich's reagent (0·2ml.) (Heyworth & Bacon, 1957). A faint red colour with an absorption maximum at 570nm. was observed.

Action of nitrous acid. A sample (1mg.) of the polymer in water (0·1ml.) was treated with $NaNO_2$ and acetic acid as described above. After treatment with Dowex 50 (H^+ form) resin, the reaction mixture was examined by paper chromatography in solvent *A*; three products, all containing phosphate, with R_F 0·08, 0·14 and 0·22 were detected. The reaction was repeated on a larger scale (5mg. of polymer) and the three products were isolated by preparative paper chromatography in solvent *A*. Acid hydrolysis and paper chromatography revealed the presence of glucose, galactosamine and anhydroribitol in the hydrolysates of all three fragments.

A sample of the mixture of products was examined serologically by the Ouchterlony technique, after treatment with Dowex 50 (H^+ form) resin and repeated evaporation *in vacuo* to remove all traces of nitrous acid. The products were serologically inactive at a concentration corresponding to 500μg. of original polymer/ml.

Action of 10mM-periodate. A sample of polymer (5mg.) was added to 20mM-$NaIO_4$ solution (2·5ml.) and the mixture was diluted to 5ml. with water. The mixture was kept at room temperature in the dark and samples (0·1ml.) were withdrawn at intervals, diluted to 10ml. with water and analysed for periodate. Oxidation was complete at 72hr. Samples (0·1ml.) of the oxidation solution were analysed for phosphate and others (0·5ml.) for formaldehyde. The molecular proportions phosphate:periodate reduced:formaldehyde formed were 2·00:3·24:0·04.

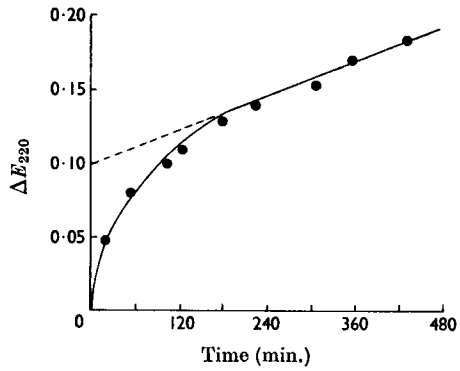


Fig. 2. Periodate oxidation of C-substance. Oxidation with 1mM-$NaIO_4$ was carried out as described in the text.

Oxidation with periodate and reduction with borohydride. A sample (2ml.) of the oxidation solution from the experiment described above was treated with $NaBH_4$ (10mg.) at 4°. After 16hr. the excess of borohydride was destroyed by the addition of Dowex 50 (H^+ form) resin. The resin was removed by filtration and the filtrate was evaporated to dryness. Boric acid was removed by evaporation with three successive portions (1ml.) of methanol on a steam bath. The residue was dissolved in 2N-HCl (0·4ml.) and heated at 100° for 3hr. Examination of a portion (one-fifth of the total) of the hydrolysate by paper chromatography in solvents *A* and *B* revealed the presence of galactosamine and glycerol together with phosphates. The remaining portion of the hydrolysate was applied as a band (5cm.) to a sheet of Whatman no. 1 paper and run for 16hr. in solvent *B*. Phosphates did not move from the base line; they were recovered by elution with water and treated with an equal volume of phosphomonoesterase solution at 37° for 16hr. Paper chromatography revealed the formation of ethylene glycol, glycerol, galactosamine and an unidentified compound (R_{Glc} 0·30 in solvent *B*) giving a purple colour rapidly with the periodate–Schiff reagents. This behaviour is characteristic of a 1-substituted glycerol or similar compound capable of yielding formaldehyde on oxidation with periodate (Roberts, Buchanan & Baddiley, 1963). Insufficient of this material was available for further study.

Action of 1mM-periodate. A sample (5mg.) of the polymer was added to 20mM-$NaIO_4$ solution (2·5ml.) and the mixture was diluted to 50ml. with water and kept at room temperature in the dark. Portions (1ml.) were withdrawn, diluted to 10ml. with water and analysed for periodate at intervals of 20min. during 4hr. and then at intervals of 60min. during a further 4hr. The amount of periodate reduced rapidly, denoted by the intercept in the rate curve (Fig. 2), was related to the amount of phosphorus; the molecular ratio phosphate:periodate reduced rapidly was 2:1.

Action of periodate followed by NN-*dimethylhydrazine.* A sample (5mg.) of polymer was allowed to react with $NaIO_4$

(5mg.) in water (0·2ml.) for 3 days in the dark at room temperature. One drop of ethylene glycol was added and, after 5min., a freshly prepared 1% solution of *NN*-dimethylhydrazine in dilute acetic acid (1ml.) at pH6 was added (modification of the procedure of Barry, 1943). The mixture was kept at 37° for 16hr. and extracted with several portions (1ml. each) of chloroform. The mixture was applied as a band (6cm.) to a Whatman no. 1 paper and the chromatogram was run in solvent *A*; authentic choline phosphate was run as a standard. Material was eluted from the appropriate area of the chromatogram with water. The eluate was run again on a paper chromatogram in solvent *A*. The $K_4Fe(CN)_6$–$CoCl_2$ reagents revealed choline phosphate.

Action of alkali. A sample (5mg.) of polymer was heated at 100° for 16hr. with 2N-NaOH solution (0·2ml.). The hydrolysate was passed through a column (1ml.) of Dowex 50 (NH_4^+ form) resin; the eluate was evaporated to dryness and a sample (1mg.) was examined by paper chromatography in solvent *A* (descending development for 16hr.). A single spot with $R_{\alpha GP}$ ($R_{glycerol\ 1\text{-}phosphate}$) 0·09 was observed. A further portion (1mg.) was heated with 4N-HCl at 100° for 6hr. and examined by paper chromatography in solvent *A* and with the Technicon AutoAnalyzer. Ribitol phosphates and galactosamine 6-phosphate were observed amongst the hydrolysis products. The remaining part of the hydrolysate was treated with phosphomonoesterase solution (3ml.) at 37° for 16hr. and samples (0·1ml.) were analysed for inorganic and total phosphate; 31% of the total phosphate was converted into inorganic phosphate by the enzyme. The dephosphorylation solution was evaporated to dryness several times to remove $(NH_4)_2CO_3$ and it was examined by paper chromatography in solvent *A* (periodate–Schiff and modified $AgNO_3$ reagents). Dephosphorylation was incomplete but, in addition to starting material, a non-reducing glycoside, $R_{\alpha GP}$ 0·28, that reacted with the $AgNO_3$ reagents only on steaming the paper chromatogram was observed.

The experiment was repeated with 10mg. of polymer and the products with $R_{\alpha GP}$ 0·09 and 0·28 were separated by preparative paper chromatography. The materials (about 3mg. of each) were separately *N*-acetylated in water (0·5ml.) containing methanol (0·05ml.) by the addition of acetic anhydride (0·03ml.) at 0°. The solutions were kept for 90min. at pH7 by the addition of 0·1N-NaOH (Roseman, Distler, Moffatt & Khorana, 1961). Excess of acetic anhydride was destroyed by heating at 70° on a steam bath and the solutions were passed through columns (1ml.) of Dowex 50 (H^+ form) resin. The eluates were evaporated to dryness and examined by paper chromatography in solvent *A*. The material with original $R_{\alpha GP}$ 0·09 gave material with $R_{\alpha GP}$ 0·27, and the material with original $R_{\alpha GP}$ 0·28 gave a compound with $R_{\alpha GP}$ 0·60. The *N*-acetylated product, $R_{\alpha GP}$ 0·27, was completely converted by phosphomonoesterase into the product with $R_{\alpha GP}$ 0·60, as judged by paper chromatography.

Analysis of the N-acetylated alkali-hydrolysis product. A sample (100mg.) of polymer was heated at 100° for 3hr. with 2N-NaOH (3ml.) in a polypropylene tube. The reaction mixture was passed through a column (15ml.) of Dowex 50 (NH_4^+ form) resin and the eluate was evaporated to dryness. The material was dissolved in water (5ml.) containing methanol (0·5ml.) and selectively *N*-acetylated and worked up as described above. The resulting material was purified by preparative paper chromatography on four sheets of Whatman no. 1 paper with solvent *A* (descending development for 48hr.). The product (12mg.) was eluted from the appropriate area with water. On prolonged development (120hr.) this was separated into two groups of two closely running compounds.

The material was dissolved in CO_2-free water (1ml.) and passed through a column (0·5ml.) of Dowex 50 (H^+ form) resin previously washed with CO_2-free water. The acid form of the product was eluted with CO_2-free water (5ml.) and the eluate was titrated with 0·1N-NaOH by using an Agla micro-syringe and a Beckman pH-meter. Alkali was added to 10μl. portions and the curve $\Delta pH/\Delta v$ against v (the total volume of alkali added) was plotted. Two maxima were observed in the ratio 2·3:1·0, corresponding to primary and secondary acidic groups respectively.

The titrated solution was made to 25ml. with NH_3-free water, and samples (0·2ml.) were analysed for nitrogen and phosphate; the ratio nitrogen:phosphorus was 3·4:2·00. Samples (1·0ml.) were analysed for glucose (as total hexose). The molecular ratio glucose:phosphate was 0·74:2·00. Further samples (0·2ml.) were incubated with phosphomonoesterase solution (3·8ml.) at 37° for 16hr. and the inorganic phosphate was determined; 52% of the total phosphate was converted into inorganic phosphate.

The remainder of the solution of isomeric phosphates was evaporated to yield 10·7mg. of material; it was dissolved in water (1ml.). A sample (0·1ml.) of this solution was heated with 4N-HCl (0·4ml.) at 100° for 30min. and adjusted to 10ml. at pH6; the molecular ratio galactosamine:phosphate in the hydrolysate was 1·3:2·00. A further sample (0·1ml.) was heated with 6N-HCl (0·5ml.) at 100° for 10hr. and products were examined by paper chromatography in solvent *A* with the $K_4Fe(CN)_6$–$CoCl_2$ reagents; no choline or its phosphate was detected, whereas a strong spot corresponding to choline was obtained from the hydrolysis of a corresponding amount (1mg.) of the original polymer. A similar hydrolysate was evaporated to dryness and examined by paper chromatography in solvent *B*; galactosamine, glucose and anhydroribitol were detected.

C-substance from other strains of Pneumococcus

Preparations (2mg.) from *Pneumococcus* type 1 nos. 5 and 8a, type 2 no. 3, type 3 no. 2 and type 7 prepared by the procedures described in the earlier literature, and from types 2 R36a and SV–1R3 prepared by the procedure described above, were examined by acid hydrolysis followed by paper chromatography as described above. Products characteristic of the C-substance from type 1–192R, i.e. anhydroribitol, ribitol phosphates, galactosamine and glucose, were observed in all cases. Autoanalysis of acid hydrolysates (2mg. of polymer heated in 2N-HCl for 16hr. at 100°) for amino compounds revealed in all cases, except those of type 2 R36a and SV–1R3, the amino compounds characteristic of pneumococcal cell-wall peptidoglycan, i.e. glucosamine, muramic acid, muramic acid phosphate, alanine, glutamic acid and lysine (cf. Gotschlich & Liu, 1967). Galactosamine 6-phosphate was present in the hydrolysates from all preparations. The preparation from type 1 no. 5 was shown by the Ouchterlony technique to contain about 25% of C-substance, and a second serologically active component was present; autoanalysis indicated that the preparation was heavily contaminated with protein in

Table 1. *Analysis of impure pneumococcal capsular polysaccharides for C-substance*

Samples were hydrolysed with alkali and enzymically dephosphorylated. The partially dephosphorylated product was recovered and hydrolysed in acid. Details are given in the text.

	Type no. given by R. Brown	Preparation no.	Current Danish type no.
Preparations giving the glycoside with $R_{\alpha GP}$ 0·28 that gave glucose, galactosamine and anhydroribitol on acid hydrolysis	5	10a	5
	6a	4b	6A
	7A	2a	7A
	7B	1a	7B
	10	5c	10
	15B	2c	15B
	16	2c	16
	19A	1	19A
	21	2c	21
	26	1b	38
	32	3	32
	33	1b	9A
	34	1	34
	35A	2	35A
	37	1	37
	40	1	33A
39254 related to	6	1b	—
3814 related to	11	1a	—
39458 related to	19	1a	—
Preparations as above but giving galactose as an additional product of acid hydrolysis	10A	1b	10A
	18A	2	18A
	29	2a	29
	30	1a	33B
	30	—	33B
	35	1	35
	36	1	36

which proline, valine, leucine, tyrosine, phenylalanine and isoleucine were prominent components.

Samples (5mg.) of the preparations were heated at 100° for 16hr. with 2N-NaOH (0·3ml.). The hydrolysates were passed through columns (1·5ml.) of Dowex 50 (NH_4^+ form) resin and the eluates were applied as bands (6cm.) to Whatman no. 1 papers. These were developed (descending) for 16hr. in solvent *A* and the product with $R_{\alpha GP}$ 0·09 was eluted with water. The resulting solutions were treated at 37° for 16hr. with equal volumes of phosphomonoesterase solution and, after removal of $(NH_4)_2CO_3$ by repeated evaporation, the residues were applied as bands (5cm.) to Whatman no. 1 papers. The chromatograms were developed (descending) for 48hr. in solvent *A* and material with $R_{\alpha GP}$ 0·28 was recovered by elution with water. The solutions were heated with equal volumes of conc. HCl for 16hr. at 100° and products were examined by paper chromatography in solvent *B*. All samples gave the dephosphorylation product with $R_{\alpha GP}$ 0·28 that gave glucose, galactosamine and anhydroribitol on acid hydrolysis.

Capsular polysaccharide preparations contaminated with C-substance. Samples (15mg.) of 26 impure capsular polysaccharides were examined by the sequence of operations described above involving hydrolysis with alkali followed by enzymic dephosphorylation, recovery of the partly dephosphorylated product and acid hydrolysis of this to glucose, galactosamine and anhydroribitol. Of the samples 19 (Table 1) contained C-substance with the properties described in this paper. The remaining seven samples yielded galactose in the final acid hydrolysis, in addition to glucose, galactosamine and anhydroribitol.

Table 2. *Percentage composition of fatty acid methyl ester mixture from pneumococcal F-substance*

The chromatography of methyl esters is described by Brundish *et al.* (1965). The esters are designated *x*:*y*, where *x* is the no. of carbon atoms/fatty acid mol. and *y* is the no. of double bonds/fatty acid mol.

Methyl ester	Composition (moles/100moles)
12:0	10
Hydroxy acid (?)*	12
14:0	13
14:1	6
16:0	25
16:1	10
18:0	16
18:1	8

* Peaks the relative retention times of which were changed after trimethylsilylation of the fatty acid methyl ester mixture.

F-substance

F-substance was prepared as described by Goebel *et al.* (1943); 350mg. of material yielded 20mg. of fatty acids by the method described by these authors. The fatty acids were converted into their methyl esters (Hornstein, Alford, Elliott & Crowe, 1960) and analysed as described by Brundish *et al.* (1965); the results are given in Table 2. The mixture of fatty acid methyl esters was re-examined after the addition of Pearce Tri-Sil reagent.

Paper-chromatographic analysis of the water-soluble products of acid hydrolysis of F-substance indicated that these were the same as those from C-substance.

DISCUSSION

Pneumococcal C-substance occurs in the wall of the organism (unpublished work by M. McCarty, quoted by Liu & Gotschlich, 1963); it has been prepared here for the first time in a pure state, free of components of wall peptidoglycan and with a significantly higher optical rotation and phosphorus and nitrogen content than previously reported. The isolation and purification procedures, described in detail in the previous section, comprise the following steps: material extracted from defatted organisms with cold 10% trichloroacetic acid solution was treated with Cetavlon solution to remove a part of the RNA; ion-exchange chromatography on DEAE-cellulose (HCO_3^- form) gave a product containing about 5% of RNA; further

chromatography yielded pure material with high serological activity containing no amino acids or material that absorbed ultraviolet light. Although Goebel *et al.* (1943) reported that C-substance contained no amino acids, later analyses (Liu & Gotschlich, 1963; Gotschlich & Liu, 1967) showed that the preparations contained the components of wall peptidoglycan. A range of samples prepared by earlier procedures was provided by Dr Rachel Brown and Dr Jean M. Tyler; they were shown to contain the components of peptidoglycan. Our preparations were free of these impurities, probably owing to the method of extraction, which involves treatment with trichloroacetic acid. The work described here shows the C-substance is a polymer containing ribitol phosphate and sugar residues and, as it is a wall component, it must be regarded as a teichoic acid. Wall teichoic acids are usually attached to peptidoglycan through a terminal phosphodiester linkage (cf. Button, Archibald & Baddiley, 1966), and the process of extraction with dilute trichloroacetic acid causes the release of teichoic acid free from other wall components.

Several authors have claimed that C-substance contains material that absorbs ultraviolet light (Wadsworth, Crowe & Smith, 1934; Goebel *et al.* 1943: Hornung & Berenson, 1963). During the ion-exchange chromatography of our material a fraction that absorbed ultraviolet light was observed with similar chromatographic behaviour to that of C-substance (Fig. 1). This fraction contained a few amino acids and, as it was eluted from the column before the main body of the nucleic acid, it seems likely that it is aminoacyl-RNA and that it accounts for the absorption of ultraviolet light by the earlier preparations.

From 65g. of dry, defatted cells nearly 1g. of pure C-substance with high serological activity was isolated. Previous analyses of C-substance gave optical rotations of about 50° and phosphorus and nitrogen values of about 4·5 and 5·6% respectively. Material prepared by the new procedure had $[\alpha]_D + 74 \pm 5°$ with 7·0% of phosphorus and 6·0% of nitrogen. After acid hydrolysis D-galactosamine hydrochloride and galactosamine 6-phosphate were isolated in crystalline form. In addition, anhydroribitol was characterized as its crystalline tri-*O*-benzoate and D-glucose as its crystalline *p*-nitroanilide. Other components of the hydrolysis mixture were recognized by their behaviour on paper chromatograms as choline, choline phosphate, inorganic phosphate, the isomeric ribitol phosphates and a disaccharide phosphate corresponding to the 'peak B' of Liu & Gotschlich (1963). This disaccharide phosphate gave glucose and the deamination products of galactosamine 6-phosphate on treatment with nitrous acid and yielded galactosamine, galactosamine 6-phosphate and glucose on vigorous acid hydrolysis; the β-configuration was assigned to the glycosidic linkage from the optical rotation of the compound. As C-substance has a fairly high positive rotation, it is possible that the other glycosidic linkages in the polymer have the α-configuration. The isomeric ribitol phosphates yielded ribitol and a trace of anhydroribitol on treatment with intestinal phosphomonoesterase; the anhydroribitol presumably originates from anhydroribitol phosphate.

Quantitative analysis of the polymer gave the proportions phosphate:nitrogen:D-glucose:total hexose:galactosamine:choline 1·00:1·90:0·37:0·37:0·47:0·45 ± 0·05, or about 2:4:1:1:1:1. Additional evidence discussed below supports the presence of two phosphate groups in the repeating unit. A polymer comprising a repeating unit with this composition and containing one unit of ribitol would require 6·7% of phosphorus and 6·0% of nitrogen, in good agreement with the experimentally determined values. The somewhat low value for glucose may be due to the extensive charring that occurs on hydrolysis of the polymer with acid. One nitrogen presumably represents galactosamine and one represents choline. The remainder is believed to be contained in 1 mol.prop. of the 2-acetamido-4-amino-2,4,6-trideoxyhexose demonstrated to be a component of C-substance by Distler *et al.* (1966). Although the polymer possesses free amino groups, i.e. it forms a DNP derivative, and is decomposed into serologically inactive small fragments by nitrous acid, a DNP-diaminotrideoxyhexose has not yet been clearly identified among the products of hydrolysis of the DNP derivative with acid, although impure preparations of the polymer gave a substance with the correct paper-electrophoretic mobility (Brundish & Baddiley, 1967). Supporting evidence for the inclusion of the acid-labile diamino sugar in the polymer comes from its high nitrogen content, the extensive charring in acid and the faint positive Bial and Ehrlich tests; Distler *et al.* (1966) obtained a 'direct Ehrlich' reaction from the diamino sugar in glycosidic linkage. No information is available on the action of nitrous acid on 4-amino-4-deoxyhexoses, but the degradation of C-substance to small fragments by this reagent indicates that glycosidic cleavage or fragmentation of the sugar must occur in a manner analogous to the degradation of glycosides of 2-amino-2-deoxyhexoses.

As the products of acid hydrolysis of the polymer include galactosamine phosphate, choline phosphate and those typical of the acid degradation of a derivative of ribitol phosphate, it follows that the two phosphate groups in the repeating unit must be attached to galactosamine, choline and ribitol. It is improbable that choline phosphate could be in the main polymer chain, but the rapid destruction of this chain by nitrous acid and by dilute periodate

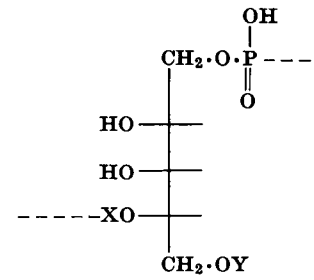
solution, with destruction of the diaminohexose and ribitol residues respectively, indicates that these two components are a part of the chain. The rapid and complete oxidation of ribitol residues with the reduction of 1 mol.prop. of periodate shows that an unsubstituted glycol group occurs on ribitol (Fig. 2); the glycol must be in the main polymer chain in view of the fragmentation caused by the oxidation and because no formaldehyde is produced. This glycol grouping must occur adjacent to a terminal phosphate on ribitol to account for the production of ethylene glycol phosphate in the sequence periodate oxidation–borohydride reduction–acid hydrolysis (Smith degradation).

In all ribitol teichoic acids and related compounds where the structure of ribitol phosphate residues is known the configuration is L-ribitol 1-phosphate, corresponding to the configuration in the biological precursor, CDP-ribitol. It is likely then that the residue in C-substance has this configuration, and the glycol grouping must occupy positions 2 and 3 in the L-ribitol 1-phosphate. Position 4 must be substituted, as only 1 mol.prop. of periodate is reduced rapidly.

When the diamino sugar residues have been destroyed with nitrous acid, a variety of procedures fails to yield recognizable simple glycosides of ribitol, and this suggests that the diamino sugar residue is directly attached to ribitol but that the attachment of the other sugar residues is indirect. A tentative partial structure consistent with these observations is given in (I), but the present evidence does not clearly establish the relative positions of the substituents X and Y.

Among the products of acid hydrolysis was a disaccharide of galactosamine 6-phosphate and glucose; this is believed to be represented by 'peak B' in the studies of Liu & Gotschlich (1963). Although evidence for the structure of this disaccharide phosphate is incomplete, the behaviour of C-substance itself towards periodate is relevant. With an excess of 10mM-periodate the polymer reduces 3·24 mol.prop. of reagent for 2 mol.prop. of phosphate; ribitol and glucose are destroyed but *N*-acetylgalactosamine is largely unaffected. The ribitol accounts for only 1 mol.prop. of oxidant, and so it is likely that the glucose residues reduce 2 mol.prop. of periodate. The disaccharide structure 6′-*O*-phosphoryl-[*O*-β-D-galactosaminyl-(1→6)-D-glucose] would be consistent with these observations, and would account for at least a part of the glycerol obtained by Smith degradation of C-substance.

Much of the choline in C-substance is converted into free choline during acid hydrolysis, despite the known stability of choline phosphate towards acid. This indicates that choline is attached to the polymer through a phosphodiester linkage to a



(I; X = diamino sugar; Y = H or choline phosphate)

hydroxyl group on either a sugar or ribitol, and that during the hydrolysis there is a hydroxyl group adjacent to the phosphodiester. Hydrolysis would occur in part through the usual cyclic phosphate mechanism, giving free choline and leaving the phosphate on sugar or ribitol. It is possible that the adjacent hydroxyl group required for this mechanism to operate is absent from C-substance itself but is revealed only during acid hydrolysis. These suggestions are supported by the modified Barry degradation of the polymer (periodate oxidation followed by *NN*-dimethylhydrazine to effect base-catalysed elimination); during this treatment choline phosphate was formed.

The phosphate at the L-1-position on ribitol is presumably in phosphodiester linkage to the *N*-acetylgalactosamine of a neighbouring residue. However, the formation of galactosamine 6-phosphate during acid hydrolysis of C-substance does not prove that a phosphodiester linkage in the polymer involves a hydroxyl group at position 6 on galactosamine, as acid-catalysed migration of the phosphate to the 6-position could occur. In fact, it is more likely that linkage involves position 3 or 4 in galactosamine in view of the stability of this residue towards periodate.

Hydrolysis of the polymer with alkali gave a mixture of probably four closely related isomers; this is consistent with the view that a phosphodiester group with neighbouring hydroxyl groups on both substituents is hydrolysed through intermediate cyclic phosphates. In support of this, galactosamine phosphate and the decomposition products of a ribitol phosphate residue were produced by acid hydrolysis of the mixture of phosphates. When the mixture of isomeric phosphates produced by the action of alkali was treated with the phosphomonoesterase 31% of its phosphorus was converted into inorganic phosphate, whereas after selective *N*-acetylation of this mixture 52% of the phosphorus was converted into inorganic phosphate; apparently the phosphomonoesterase is inhibited by the basic amino groups in the hydrolysis products, and it is

concluded that these contain one phosphomonoester and one phosphodiester. This was confirmed by potentiometric titration. Analysis of the products of hydrolysis with alkali indicates the proportions phosphate : nitrogen : glucose : galactosamine : choline to be about 2:3:1:1:0. Thus choline has been destroyed by the alkali, but the phosphate to which it was attached presumably remains as a phosphodiester.

Seven samples of C-substance from different sources were examined by hydrolysis with acid and alkali. Preparations that had been isolated by a procedure including extraction with trichloroacetic acid were free of amino acids, whereas others contained the typical components of peptidoglycan. A procedure for the analysis of small amounts (about 1 mg.) of material was developed; it involved hydrolysis with alkali followed by recovery of the product, partial dephosphorylation, recovery of the dephosphorylation product and comparison of this with authentic material. Acid hydrolysis of the mono-dephosphorylated product then gave glucose, galactosamine and anhydroribitol. The seven preparations behaved identically with the preparation from *Pneumococcus* 1–192R, A.T.C.C. 12213.

This procedure was used to study 26 samples of pneumococcal capsular material (15 mg. of each) suspected from serological examination to be contaminated with C-substance. Of these samples 19 gave the repeating unit with the structure of that of *Pneumococcus* 1–192R; the remaining seven yielded a mono-dephosphorylated product, but this gave galactose in addition to glucose, galactosamine and anhydroribitol on acid hydrolysis. The galactose was probably produced from contaminating fragments originating from the capsular polysaccharides, and it is likely that the C-substance in these cases has the normal structure. Ribitol is a component of a number of capsular polysaccharides from pneumococci, and consequently the presence of ribitol derivatives among the products of hydrolysis of capsular polymers with alkali may be expected. Nevertheless, the production of only traces of anhydroribitol by hydrolysis of capsular preparations with acid (cf. Shabarova, Buchanan & Baddiley, 1962) probably indicates contamination with C-substance.

Pneumococcal F-substance was prepared from *Pneumococcus* 1–192R as described by Goebel *et al.* (1943). It gave the typical hydrolysis products normally obtained from C-substance on heating in acid, but also contained 5·7% by weight of fatty acid esters. Goebel *et al.* (1943) reported a fatty acid content of 6·5% by weight and an acid equivalent of 372. We find that the lipid material is a mixture of eight fatty acids, one of which may be a hydroxy acid as its relative retention time on the gas–liquid-chromatographic column was altered after trimethylsilylation of the fatty acid methyl ester mixture (Table 2). The saturated and unsaturated acids are the same as those in the glycolipids (Brundish *et al.* 1965) and phospholipids (Brundish, Shaw & Baddiley, 1967) of this organism, but the hydroxy acid has not been found in these other lipids. The 'average acid equivalent' of the fatty acid mixture was about 245.

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