The Action of Nitrous Acid on C-Teichoic Acid (C-Substance) from the Walls of *Diplococcus pneumoniae*

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1. C-teichoic acid (C-substance) from the walls of *Diplococcus pneumoniae* contained free amino groups accessible to attack by nitrous acid. Treatment with nitrous acid, followed by reduction with borohydride and hydrolysis with acid, gave ribitol, glucitol and their respective phosphates. 2. Hydrolysis of the polymer with alkali followed by treatment of products with nitrous acid yielded glucose. 3. When alkali hydrolysis was followed by treatment with a phosphomonoesterase, nitrous acid degradation of C-substance yielded glucose and a disaccharide identified as 2-O-(N-acetylgalactosaminy)-D-ribitol. 4. A partial structure for C-teichoic acid was deduced in which the order of the constituent residues and the position of phosphodiester linkages were established.

There is a continuing and growing interest in C-substance, a species-specific antigen of *Diplococcus pneumoniae* (Tillett *et al.*, 1930). In addition to its importance as a pneumococcal group-specific antigen it is found that minor modifications in its structure cause marked changes in autolytic behaviour and division of the cells (Tomasz, 1967). C-substance gives a precipitate with a protein (C-reactive protein) that appears in the serum of man and rabbits during acute infections and inflammatory processes of various origins (MacLeod & Avery, 1941; McCarty, 1947); this and other studies on immunoglobulins that react with C-substance support the view that a knowledge of the structure of this antigen could assist our understanding of antigen-antibody interactions. Such knowledge would also be helpful in studies on the mechanism of the immunological responses of lymphocytes where reactions of these cells to pneumococcal type III polysaccharide (a common technique in the study of antibody production by lymphocytes) may be complicated by the presence of very small amounts of the much more reactive C-polysaccharide (J. H. Humphrey, personal communication).

It has been shown (Brundish & Baddiley, 1968) that C-teichoic acid (C-substance) is a cell-wall polymer with a repeating structure comprising the residues ribitol phosphate, N-acetylgalactosamine, glucose, N-acetyldiaminotriideoxyhexose and choline phosphate. The presence of the diamino sugar and the high nitrogen content suggested that the polymer would be degraded into smaller fragments with nitrous acid. Early work (Tillett *et al.*, 1930) has shown this to be the case. A preliminary study (Brundish & Baddiley, 1968) of the action of nitrous acid on C-teichoic acid showed that the molecule was readily fragmented and three products were detected. Each product contained phosphate and yielded glucose,

galactosamine and anhydrosorbitol on hydrolysis with acid.

This paper reports a study of the nature of the products of reaction of C-teichoic acid with nitrous acid and the implication of these findings on the structure of the polymer itself.

Experimental and Results

Materials

Pneumococcal C-teichoic acid was isolated by the procedures described by Brundish & Baddiley (1968). Calf intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Paper chromatography

Paper chromatography was carried out on Whatman no. 1 paper by using 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 *et al.*, 1951). Compounds were detected by the periodate–Schiff reagents for α -glycols (Baddiley *et al.*, 1956), the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949), the alkaline AgNO_3 reagents for glycols (Trevelyan *et al.*, 1950) and for non-reducing glycosides (Brundish *et al.*, 1965), the aniline phthalate reagent for reducing sugars (Partridge, 1949) the $\text{K}_4\text{Fe}(\text{CN})_6\text{--CoCl}_2$ reagents for choline and choline phosphate (Heyndrickx, 1953) and the Morgan–Elson reagent for N-acetylhexosamines (Partridge, 1948).

Examination of C-teichoic acid

Action of nitrous acid on C-teichoic acid, followed by borohydride reduction and acid hydrolysis. C-teichoic acid (15 mg) was treated with 20 mM-HCl (0.6 ml) and NaNO_2 (150 mg) in water (5 ml) and the

mixture was heated at 50°C in a sealed tube for 2 h. The solution was passed through a column (12 ml) of Dowex 50 (NH₄⁺ form) resin eluted with water and the eluate (120 ml) was evaporated *in vacuo* to 25 ml, heated on a boiling-water bath for 10 min and evaporated to dryness *in vacuo*. A sample of the residue was chromatographed in solvent A. Three products, all containing phosphate, with *R_F* 0.09, 0.17 and 0.23 were detected. The remainder of the residue was dissolved in water (4 ml), NaBH₄ (70 mg) was added and the solution kept for 12 h at 21°C. A few drops of acetic acid were added and the solution was passed through a small column (6 ml) of Dowex 50 (H⁺ form) resin and eluted with water (60 ml). The eluate was evaporated to dryness *in vacuo* and boric acid was removed by distillation (three times) with methanol. The residue was hydrolysed in 2 M-HCl (1.5 ml) at 100°C for 3 h, the acid was removed by evaporation *in vacuo* over KOH and part of the hydrolysate was chromatographed in solvents A and B. The products were detected as follows: periodate-Schiff reagents for ribitol, a hexitol (presumably glucitol), anhydribose, two anhydrohexitols with *R_{glucose}* 1.37 and 1.63 in solvent B, ribitol phosphates and hexitol phosphates; molybdate reagent for ribitol phosphates, hexitol phosphates and P₁; alkaline AgNO₃ for ribitol, a hexitol, glucose and galactosamine; aniline phthalate for glucose and galactosamine; K₄Fe(CN)₆-CoCl₂ reagents for choline.

The remainder of the hydrolysate was chromatographed as a band in solvent A and the products were detected with the periodate-Schiff reagents and the molybdate reagent. The phosphates were eluted from the paper with water. The eluates were evaporated to dryness *in vacuo* and the residues dissolved in water (0.5 ml). Each was mixed with intestinal phosphomonoesterase (0.5 mg) and (NH₄)₂CO₃ (2 mg), and kept for 12 h at 37°C. The solution was evaporated to dryness *in vacuo*. The residue was dissolved in water and examined in solvent B. Ribitol and a hexitol were detected with the periodate-Schiff reagents and alkaline AgNO₃.

Action of nitrous acid on the products of alkali hydrolysis of C-teichoic acid. C-teichoic acid (4 mg) was dissolved in 1 M-NaOH solution (0.5 ml) and the solution was heated at 100°C in a sealed tube for 1 h. The hydrolysate was passed through a small column (2 ml) of Dowex 50 (NH₄⁺ form) resin and material was eluted with water. The eluate was evaporated *in vacuo* to a small volume and freeze-dried. The material (3 mg) was treated with 20 mM-HCl (0.1 ml) and NaNO₂ (30 mg) in water (1 ml) and the mixture was heated at 50°C in a sealed tube for 2 h. The solution was passed through a column (2.5 ml) of Dowex 50 (NH₄⁺ form) resin eluted with water and the eluate (25 ml) heated on a boiling-water bath for 10 min and

evaporated to dryness *in vacuo*. The residue was examined by chromatography in solvents A and B. Glucose and several unidentified phosphorus-containing products were detected.

Alkali hydrolysis of C-teichoic acid followed by treatment with phosphomonoesterase. C-teichoic acid (100 mg) was dissolved in 1 M-NaOH (9 ml) and heated at 100°C in a sealed tube for 1 h. The hydrolysate was passed through a column (20 ml) of Dowex 50 (NH₄⁺ form) resin and was eluted with water. The eluate (200 ml) was evaporated *in vacuo* to a small volume and then freeze-dried. The residue was dissolved in water (14 ml) and treated with (NH₄)₂CO₃ (40 mg) and phosphomonoesterase (9 mg). A few drops of toluene were added and the solution was kept at 37°C for 18 h. The mixture was then evaporated to dryness *in vacuo*. The residue was dissolved in water (5–10 ml) and re-evaporated to dryness. This procedure was repeated several times to remove (NH₄)₂CO₃. The residue was chromatographed in solvent A for 18 h as a band on Whatman 3MM paper twice prewashed in solvent A. Two groups of products with mobilities *R_{Glycerol 1-phosphate}* 0.4 (19 mg) and 0.8 (35 mg), containing phosphorus, were detected with the periodate-Schiff reagents (purple colours which developed rapidly) and the molybdate spray, and were eluted with water. The group of products of mobility *R_{Glycerol 1-phosphate}* 0.8 are subsequently referred to as group A products and the products of *R_{Glycerol 1-phosphate}* 0.4 as group B.

In another experiment, after treatment of the alkali-hydrolysis products (10 mg) with phosphomonoesterase, part of the residue was chromatographed in solvent A on Whatman no. 1 paper (previously washed in solvent A) for 30 h. A third group of products (low yield, of *R_{Glycerol 1-phosphate}* 0.9) was detected which rapidly gave a purple colour with the periodate-Schiff reagents and showed no reaction with the molybdate spray. The remaining phosphatase-treated products were chromatographed as a band in solvent A and the phosphorus-free material was eluted with water. The eluate was evaporated to dryness *in vacuo* and the residue hydrolysed in 2 M-HCl for 3 h at 100°C. The acid was removed *in vacuo* over KOH and the hydrolysate was examined by chromatography in solvents A and B. The products identified were galactosamine, glucose, ribitol and anhydribose.

Acid hydrolysis of group A and B products. Samples of both groups of products were hydrolysed with 2 M-HCl for 3 h at 100°C and the hydrolysate was examined by paper chromatography in solvents A and B. Both groups yielded galactosamine, glucose, ribitol, anhydribose, choline and choline phosphate. Other unidentified products were detected.

Nitrous acid degradation of group A and B products. Group A and group B products (3 mg samples) were

each dissolved in 20mM-HCl (0.1ml) and treated with NaNO₂ (30mg) in water (1ml). Each mixture was heated at 50°C for 2h and then passed through a column (2.5ml) of Dowex 50 (NH₄⁺ form) resin and eluted with water. The eluates (25ml) were heated at 100°C on a water bath for 10min and evaporated to dryness *in vacuo*. The residues were examined by chromatography in solvent B and the major products in each case were glucose and a disaccharide identified as 2-*O*-(*N*-acetylgalactosaminyl)ribitol. Less disaccharide was present in the hydrolysate of the group B products.

The disaccharide (5mg) was isolated from group A products (12mg) by elution from paper chromatograms.

Characterization of N-acetylgalactosaminylribitol

Chromatographic properties. The disaccharide gave a purple colour rapidly with the periodate-Schiff reagents and gave an intense reaction with alkaline AgNO₃ only when the chromatogram was steamed; this is typical of a glycoside (Brundish *et al.*, 1965). It had a mobility $R_{\text{Galactose}}$ 0.58 in solvent B.

Acid hydrolysis. The disaccharide (1mg) was hydrolysed in 2M-HCl (0.1ml) at 100°C for 3h and the hydrolysate was examined by paper chromatography in solvent B. Galactosamine and ribitol were detected.

Partial acid hydrolysis. The disaccharide (2mg) was dissolved in formic acid (66%, v/v; 0.2ml) and heated at 100°C for 35min in a sealed tube. The hydrolysate was evaporated to dryness *in vacuo*, the residue was dissolved in a few drops of dilute aq. NH₃ and again evaporated *in vacuo*. The residue was examined by chromatography in solvent B. Ribitol and *N*-acetylgalactosamine were detected with the periodate-Schiff reagents, the latter appearing as a slowly developing yellow spot. *N*-Acetylgalactosamine was also detected with the Morgan-Elson reagent. A trace of galactosamine was present as shown by the alkaline AgNO₃ reagents. Visual examination indicated that the products were present in about equimolar amounts.

Periodate oxidation followed by borohydride reduction and acid hydrolysis. The disaccharide (2mg) was treated with 0.1M-NaIO₄ (0.35ml) and the mixture kept at 21°C for 3 days. The solution was then treated with NaBH₄ (10mg) and kept for 12h. The excess of borohydride was decomposed with a few drops of acetic acid, the solution was passed through a column (1ml) of Dowex 50 (H⁺ form) resin and material was eluted with water (10ml). The eluate was evaporated to dryness *in vacuo* and then with methanol (three times) on a hot plate. The residue was hydrolysed with 2M-HCl (0.2ml)

at 100°C for 3h. Glycerol was the only product detected. No ethylene glycol was present.

Discussion

Pneumococcal C-substance (C-teichoic acid) occurs in the wall of the organism and contains in its structure ribitol phosphate, galactosamine, glucose, 2-acetamido-4-amino-2,4,6-trideoxyhexose and choline phosphate (Brundish & Baddiley, 1968). A repeating unit was proposed comprising galactosamine, glucose, the diamino sugar and ribitol linked through phosphodiester bridges, with choline phosphate occurring as appendages to the main chain.

When C-teichoic acid was subjected to degradation with nitrous acid, serological activity was destroyed and the polymer was degraded to small fragments. It was therefore proposed (Brundish & Baddiley, 1968) that nitrous acid acted on the 4-amino-4-deoxyhexose of the polysaccharide in a manner analogous to the deamination of glycosides of 2-amino-2-deoxyhexoses with subsequent glycosidic cleavage or fragmentation. Work (Al-Radhi *et al.*, 1970; Ng Ying Kin *et al.*, 1971; Ng Ying Kin & Williams, 1971) on the nitrous acid deamination of 4-amino-4-deoxypyranose amines has clarified the pathways by which these amino sugars are degraded. In particular the studies have shown that degradation of 4-amino-4-deoxy sugars in which the amino group is equatorial can proceed not only with loss of the aglycone but also with removal of an alkyl or glycosyl group at C-3. The degradation pattern of the diamino sugar in C-polysaccharide and the products of hydrolysis of C-substance with alkali, discussed below, are consistent with these observations. A study of the sugar residues and substituents in the fragments from nitrous acid treatment enabled the order of these residues in the repeating structure to be established.

When C-substance was treated with nitrous acid and the products were reduced with borohydride and hydrolysed with acid, ribitol, a hexitol with the properties of glucitol and their respective phosphates were produced. The other components of the hydrolysate were galactosamine, glucose, anhydro-ribitol, anhydrohexitols, choline and unidentified phosphates. Nitrous acid degradation of the products of alkali hydrolysis of the polymer yielded glucose. These results lead to partial structure (I) for C-teichoic acid in which phosphodiester linkages join ribitol of one unit to position 6 of glucose in a neighbouring unit. The position of substitution and configuration of the ribitol residue is discussed by Brundish & Baddiley (1968).

During the course of alkali hydrolysis of C-teichoic acid, intermediate five-membered cyclic phosphate formation occurred involving the 2-hydroxyl group of D-ribitol. Isomeric phosphomonoesters were thus

obtained with phosphate groups residing on ribitol. Treatment of these products with nitrous acid resulted in degradation of the diamino sugar units and subsequent formation of glucose. In the polymer itself degradation of the diamino sugar, although mono-*N*-acetylated, still occurred with the formation of reducing glucose residues linked at position 6 by the phosphodiester bridge (evidence for the position of the phosphate substituent is discussed below). Borohydride reduction of the degradation products and hydrolysis with acid yielded ribitol, glucitol and their respective isomeric phosphates. Hydrolysis of the phosphodiester linkages was random owing to the presence of vicinal hydroxyl groups in both ribitol and glucitol. Anhydro-ribitol and two other products, with the chromatographic properties of anhydroglucitols described by Baddiley *et al.* (1957*a,b*), were also obtained. The presence of a phosphate group on glucitol would facilitate anhydroglucitol formation, thus leading to the relatively high proportion of the two anhydroglucitols in the above hydrolysis products.

In the earlier structural work, the polymer was subjected to the sequence periodate oxidation, borohydride reduction and hydrolysis with acid (glycerol, glycerol phosphates and ethylene glycol phosphate were among the products). These observations, together with the fact that glucosyl residues are destroyed by periodate, are accommodated in partial structure (I).

Under the conditions of alkali hydrolysis used in structural work on the pneumococcal capsular materials containing ribitol phosphate and *N*-acetylhexosamine residues (Rao *et al.*, 1966) complete de-*N*-acetylation does not usually occur. To effect complete de-*N*-acetylation and thereby simplify the pattern of alkali hydrolysis of C-teichoic acid the polymer was previously subjected to a prolonged hydrolysis. Hydrolysis under milder conditions has now been studied and the resulting isomeric phosphates have been treated with phosphomonoesterase. Two groups of products were obtained in

good yield; they were separated by preparative paper chromatography. Each group contained phosphorus, gave a purple colour rapidly with the periodate-Schiff reagents (Baddiley *et al.*, 1956) indicative of the presence of a glycol system oxidizable to formaldehyde, and on hydrolysis with acid yielded ribitol, glucose, galactosamine, anhydroribitol, choline and unidentified phosphates. No ribitol phosphates were detected.

When both groups of products were treated with nitrous acid, glucose and a non-reducing disaccharide were among the products. The disaccharide yielded galactosamine and ribitol on vigorous hydrolysis with acid. Controlled hydrolysis yielded *N*-acetylgalactosamine and ribitol. The disaccharide is therefore an isomer of *N*-acetylgalactosaminylribitol. When subjected to the sequence periodate oxidation, borohydride reduction and hydrolysis with acid, glycerol was the only polyol detected. This must originate from C-4, C-5 and C-6 of the galactosamine and three carbon atoms of the ribitol; consequently the ribitol is substituted at position 2 or 3. The colour reaction of the disaccharide with the periodate-Schiff reagents confirmed substitution at position 2: it rapidly gave a purple colour, and the absence of yellow, characteristic of a malondialdehyde derivative which would be obtained from ribitol linked at position 3 (Hardy *et al.*, 1963; Hardy & Buchanan, 1963), excluded 3-substitution. On biosynthetic grounds it is concluded that teichoic acids containing ribitol phosphate residues are derivatives of D-ribitol 5-phosphate: in this disaccharide the sugar residue must occupy the 2 position on D-ribitol.

The production of glucose by nitrous acid deamination of both groups of products confirms partial structure (I). The other product of fragmentation, 2-*O*-(*N*-acetylgalactosaminyl)-D-ribitol, suggests that the diamino sugar is attached to the disaccharide unit, the characterization of which establishes the position of *N*-acetylgalactosaminyl residues in C-teichoic acid. A partial structure (II) may now be drawn for C-teichoic acid in which *N*-acetylgalactos-

aminyl units are glycosidically linked to position 2 of D-ribitol. In this arrangement of component sugars for C-polysaccharide the pattern of deamination is in agreement with the observations for 4-amino-4-deoxypyranose amines in which the amino group at C-4 is equatorial. Glucose residues bearing phosphate groups, or glucose in the products of alkali hydrolysis of the polysaccharide, which are linked to the hydroxyl at C-3 of the *N*-acetyldiaminohexose, would be removed. Cleavage of the glycosidic linkage and loss of the aglycone is also possible and explains the formation of the disaccharide on deamination of the two groups of products after alkali hydrolysis of the polymer. The sequence of sugar residues in the repeating structure (II) differs from that tentatively suggested in the earlier work (Brundish & Baddiley, 1968). The incorrect conclusion that *N*-acetylgalactosamine occupied the non-reducing terminal position was based on the isolation of a disaccharide apparently comprising galactosamine and reducible glucose. It now seems likely that this was a mixture possibly of galactosaminylribitol and glucose. The earlier periodate-oxidation studies showed that the *N*-acetylgalactosaminyl residues were not destroyed by periodate and were therefore substituted at position 3 or 4. It was previously suggested (Brundish & Baddiley, 1968) that the choline phosphate residues were attached to D-ribitol at position 1. This conclusion was based on the formation of choline phosphate in a modified Barry degradation of the polymer. In view of the presence of units terminating in ribitol after alkali hydrolysis and enzymic dephosphorylation of the polymer, this now seems unlikely. Moreover these degradation products still contain choline. It is more probable that the choline phosphate is attached to the *N*-

acetylgalactosaminyl residues. The isolation of galactosamine phosphate reported by Brundish & Baddiley (1968) and by Liu & Gotschlich (1963) is consistent with this conclusion. The formation of choline phosphate by the modified Barry procedure would be expected if *N*-acetylgalactosamine is attached to position 2 on ribitol and substituted on its 3-hydroxyl group with choline phosphate. After periodate oxidation of ribitol phosphate residues, an α -elimination in the oxidized ribitol followed by a β -elimination in the sugar would lead to the formation of choline phosphate.

As it is possible to produce *N*-acetylgalactosaminyl-ribitol under conditions that do not remove choline phosphate, it appears that not all of the *N*-acetylgalactosaminyl residues in the polymer are substituted with these residues. This was confirmed by prolonged paper chromatography of the products of hydrolysis of the polymer with alkali and phosphatase, when a small amount of a phosphate-free product was isolated. Hydrolysis of this product with acid caused considerable darkening, characteristic of acid-catalysed decomposition of the diamino sugar, and glucose, galactosamine, ribitol and anhydrosorbitol were formed.

The presence of some *N*-acetylgalactosaminyl residues lacking choline phosphate is in agreement with the observation (Higginbotham *et al.*, 1970) that antiserum prepared against C-substance cross-reacts with type 4 pneumococcal capsular polysaccharide from which the pyruvyl substituents have been removed. The cross-reaction has been attributed to multiple residues of *N*-acetylgalactosamine in type 4 substance and thus suggests the presence of a number of such residues lacking substituents in the C-teichoic acid.

From immunological studies it has now been shown (Heidelberger *et al.*, 1972) that phosphorylcholine residues are also antigenic determinants in C-polysaccharide. The homologous precipitin reaction of C-polysaccharide and anti-(C-polysaccharide), as well as of the cross-reaction of depyruvylated type 4 polysaccharide and anti-(C-polysaccharide), is fairly strongly inhibited by phosphorylcholine.

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