A Polymer of *N*-Acetylglucosamine 1-Phosphate in the Wall of *Staphylococcus lactis* 2102

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1. Walls of *Staphylococcus lactis* 2102 contain about 40% of a phosphorylated polysaccharide, which was isolated by extraction with cold trichloroacetic acid, with dilute NaOH, and also by digestion with a *Flavobacterium* peptidase. 2. The purified polymer contained equimolar proportions of *N*-acetylglucosamine and phosphate as its sole constituents and was readily hydrolysed under gentle acidic conditions to *N*-acetylglucosamine 6-phosphate. 3. Studies on the intact polymer showed that it is linear and that adjacent acetamido sugar units are joined by phosphodiester bonds between their 1- and 6-positions, the glycosidic linkages having the α-configuration. This polymer is thus the simplest of the known microbial wall polymers possessing sugar 1-phosphate linkages. 4. Alkali degradation of the extracted polymer proceeds predominantly in a stepwise manner from the reducing end, but evidence was obtained for the direct hydrolysis of some of the inter-unit phosphodiester groups.

Polysaccharides and teichoic acids containing glycosyl 1-phosphate residues occur in the walls of certain yeasts (Wickerham & Burton, 1962; Slodki, 1966) and bacteria (Archibald *et al.*, 1968*a*), and the structures and properties of several of these polymers have been described (Archibald *et al.*, 1968*b*, 1971; Archibald & Heptinstall, 1971; Partridge *et al.*, 1971). The simplest so far discovered is that present in walls of *Staphylococcus lactis* 2102 (Archibald *et al.*, 1968*a*). This polymer consists of linear chains containing repeating units of *N*-acetylglucosamine phosphate as the sole component. The structure of this material has been established and some of its chemical properties are described.

Experimental and Results

Materials

Flavobacterium L11 enzyme was a gift from Dr. S. Kotani, Department of Bacteriology, Nara Medical College, Japan. Calf intestinal phosphomonoesterase bovine pancreatic trypsin (type III) and D-glucosamine 6-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. DEAE-cellulose and CM-cellulose were purchased from H. Reeve Angel and Co. Ltd., London E.C.4, U.K., and Bio-Gel P-2 was from Calbiochem Ltd., London W.1, U.K.

Paper chromatography

Paper chromatography was carried out on Whatman no. 1 and no. 4 papers. For preparative work

Whatman 3MM paper was washed with 1 M-HCl and aq. 0.02% disodium EDTA before use. Development was effected by descending chromatography at room temperature in the following solvent systems: (A) propan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (6:3:1, by vol.); (B) butan-1-ol–pyridine–water (6:4:3, by vol.); (C) butan-1-ol–ethanol–water–aq. NH₃ (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase); (D) butan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (5:1:2, by vol.); (E) butan-1-ol–ethanol (9:1, v/v); (F) propan-2-ol–water (4:1, v/v).

Compounds were detected by the following spray reagents: (1) periodate–Schiff for glycols (Baddiley *et al.*, 1956); (2) molybdate for phosphate esters (Hanes & Isherwood, 1949); (3) aniline phthalate for reducing sugars (Partridge, 1949); (4) alkaline AgNO₃ for reducing compounds (Trevelyan *et al.*, 1950); (5) ninhydrin for amino compounds (Consden & Gordon, 1948); (6) Rydon & Smith (1952) reagent for acetamido compounds; (7) Frerejacque (1955) reagent for compounds containing *O*-acyl linkages.

Analytical methods

Both total phosphate and P_i were determined by the method of Chen *et al.* (1956), reducing sugars by the method of Park & Johnson (1949), amino compounds by the method of Rosen (1957), hexosamines by the method of Ghuysen *et al.* (1966), glycerol by the method of Hanahan & Olley (1958), glycolaldehyde by the method of Dische & Borenfreund (1949) and periodate by the method of Aspinall & Ferrier (1957).

Growth of Staphylococcus lactis 2102 and isolation of cell walls

A culture of *Staph. lactis* 2102 was obtained from Dr. H. R. Perkins (National Institute for Medical Research, Mill Hill); the organism has the same characteristics as those of an Oxford laboratory strain (White & Woods, 1965) and is coagulase-negative, does not ferment mannitol, produces acid and not acetoin from glucose and forms gold-coloured colonies on solid media. Cells were grown in 10-litre batches under forced aeration for 4 or 16h at 37°C, as described by Davison (1968), in liquid medium containing Oxoid Nutrient Broth no. 2 (2.5%), Difco Yeast Extract (0.5%), KH_2PO_4 (0.5%), glucose (1%) and trisodium citrate (0.1%). Polyethylene glycol 2000 (2ml) was used as antifoam. Cells were harvested in a Sharples refrigerated centrifuge after 4h incubation, giving 20g wet wt. (approx. 4g dry wt.) of cells. Some batches were harvested after incubation for 16h, when the yield was 35g wet wt. Cells were disrupted and walls were isolated as described by Davison (1968); approx. 3g of freeze-dried walls were obtained from 120g wet wt. of cells. Walls from 4h cultures contained 2.9% of P; this value varied slightly in different preparations, but the walls from the 16h cultures all contained more P (approx. 4%) than those from the 4h cultures. Qualitatively, however, all wall samples contained the same components and walls from the 4h cultures were used for the studies described below. The wall samples did not undergo autolysis on suspension in various buffers at 37°C in the pH range 3–10. Walls were further purified as follows: walls (3.9g) were homogenized in 0.1M-sodium phosphate buffer, pH 7.8 (200ml), trypsin (0.2g) was added and the suspension was stirred at 37°C for 16h. During this time the E_{555} of the suspension decreased by only 5%. Walls were recovered by centrifugation and washed with 0.09% NaCl (3×100ml) and water (6×100ml) and then freeze-dried (yield 3.3g). A sample of these walls (3g) was homogenized in water (50ml) and the suspension was cooled in ice. A solution (50ml) of aq. 80% (w/v) phenol was added and the suspension was stirred at 0°C for 30min. The walls were recovered by centrifugation (15000g, 10min); three layers were formed and the white cell walls, which formed the middle layer, were isolated, washed free of phenol with water (8×100ml) and freeze-dried. These walls contained 2.7% P.

Examination of cell walls

Chromatographic examination of samples of wall that had been hydrolysed in 2M-HCl at 100°C for 3h showed that in addition to amino acids and amino sugars derived from the peptidoglycan a large amount of a reducing phosphate, subsequently identified as

D-glucosamine 6-phosphate, was present. Hydrolysis of walls in 0.1M-HCl for 1h at 100°C gave a large amount of N-acetyl-D-glucosamine 6-phosphate. No ribitol or glycerol or their phosphates were detected in either of these hydrolysates showing that, although rich in phosphate, the walls did not contain teichoic acid. Samples of wall (2mg) and the methyl ester of alanine (1mg) were separately treated at room temperature with (a) 2M-aq. NH_3 – methanol (1:1, v/v) (0.4ml) and (b) aq. NH_3 (sp.gr. 0.88)–methanol (1:1, v/v) (0.4ml). After incubation for 5h wall debris was removed by centrifugation (30000g, 25min) and the supernatant solutions were examined chromatographically in solvent (A). Alanine methyl ester gave alanine and alanine amide under both conditions; these products were not obtained from the wall samples, and ester-linked alanine is therefore not present in the wall.

Isolation of the polysaccharide

(a) *By extraction into trichloroacetic acid.* Walls (500mg) were suspended in cold aq. 5% (w/v) trichloroacetic acid (25ml) and the suspension was stirred for 27h at 4°C. Wall debris was removed by centrifugation (20000g, 25min) and the supernatant solution was poured into cold acetone (100ml) and left overnight at 4°C. The resulting precipitate was recovered by centrifugation (20000g, 25min), washed with acetone and diethyl ether and then dissolved in water (5ml). A small amount of insoluble material was removed by centrifugation (20000g, 25min) and the solution was freeze-dried to give a hygroscopic white solid (110mg). Under these conditions extraction was incomplete; prolonged extraction resulted in substantial hydrolysis of the phosphorylated polysaccharide.

(b) *By extraction with NaOH.* Walls (250mg) were homogenized in 0.5M-NaOH (20ml) and stirred at room temperature for 4h. Wall debris was removed by centrifugation and the supernatant solution was neutralized with 0.5M-HCl and then deionized by dialysis against water and freeze-dried to give a hygroscopic white solid (90mg). Recovery of polysaccharide was almost quantitative by this procedure, although the preparation contained small amounts of amino acids (cf. Archibald *et al.*, 1969), which were not present in acid-extracted preparations.

Examination of the polymer

Polysaccharide obtained by both the above extraction procedures contained 9.2% of P (by wt.) and had $[\alpha]_D^{25}$ +105° (2%, w/v, in water). After hydrolysis in 0.1M-HCl at 100°C for 15min, enzymic dephosphorylation gave 100% P_i and the ratio of reducing sugars, determined by the method of Park & Johnson (1949), with N-acetylglucosamine as standard, to phosphate was 1:1.06.

Determination of O- and N-acetyl groups

Polysaccharide (1 mg) isolated by extraction with trichloroacetic acid was examined chromatographically by using reagent (7) against a standard of penta-O-acetylglucose. No reaction was obtained with the polysaccharide, indicating the absence of O-acetyl residues. A further sample (9.4 mg) was hydrolysed in 2M-HCl (1 ml) at 100°C for 3 h in a sealed tube. The hydrolysate was adjusted to pH 8 with 2M-NaOH and then evaporated to dryness under diminished pressure. The resulting solid was dissolved in water containing an internal standard of dioxan (2.5 ml/litre) and adjusted to pH 3 with 0.1 M-HCl. Samples were chromatographed on a polyethylene glycol succinate column at 170°C and N_2 pressure 9.8 N/cm² (1 kg/cm²) by using a Pye model 24 gas–liquid chromatograph. The ratio of acetic acid to phosphate in the polymer was 1.06:1.

Acid hydrolysis

Polysaccharide (3 mg) was heated in 2M-HCl (0.2 ml) at 100°C for 3 h. After removal of acid by evaporation *in vacuo* over KOH pellets, examination of the products showed the presence of a small amount of material having the chromatographic properties of glucosamine, the major product had the properties of glucosamine phosphate, and a small amount of material having the mobility of a diphosphate was also present. Similar hydrolysis in 0.1 M-HCl gave products having the properties of N-acetylglucosamine and its monophosphate. No trace of a component which could be the N-acetyl derivative of the presumed diphosphate was observed, and this suggested that its presence in the 2M-HCl hydrolysate might be an artifact, a conclusion substantiated by the observation that treatment of a pure sample of glucosamine 6-phosphate with 2M-HCl as described above gave a component having the same chromatographic properties as those of the presumed diphosphate.

Isolation and characterization of glucosamine

Polysaccharide (7 mg) was heated at 100°C for 3 h in 2M-HCl. After removal of acid and incubation with phosphomonoesterase, ions were removed on columns (20 mm × 10 mm) of Dowex 50 (H^+ form) and Dowex 1 (CO_3^{2-} form). Samples of the eluate were then subjected, as described by Archibald *et al.* (1968*b*), to degradation with ninhydrin, when arabinose was formed, and with HNO_2 followed by reduction with $NaBH_4$, when the product was 2,5-anhydromannitol. These results showed that the amino sugar was either glucosamine or mannosamine; these sugars are separated on chromato-

graphy in solvent (F), which showed that the sugar in the polysaccharide was glucosamine.

Isolation and characterization of N-acetyl-D-glucosamine 6-phosphate

Polysaccharide (500 mg) was hydrolysed in 0.1 M-HCl (10 ml) at 100°C for 15 min, diluted to 100 ml with water and then applied to a column (200 mm × 18 mm) of DEAE-cellulose which had previously been equilibrated against pyridine–acetate buffer, pH 5.5 (prepared by the addition of pyridine to 0.5 M-acetic acid), and then water. The column was eluted with a linear gradient (600 ml) of 0–0.5 M-pyridine–acetate buffer, pH 5.5, and fractions (5 ml) were collected and analysed for phosphate. All of the phosphate was eluted in a single peak of P_i between 0.1 and 0.2 M-pyridine–acetate buffer. Buffer was removed by repeated rotary evaporation at 30°C and the final solution was freeze-dried. A sample (70 μg of P) of the purified phosphate was dissolved in water (2 ml) and mixed with $NaBH_4$ (20 mg). Reduction was allowed to proceed at room temperature for 5 h and then the solution was acidified with acetic acid and borate was removed by distillation with methanol. The product was dissolved in water (1 ml), 0.2 M-sodium metaperiodate (1 ml) was added and oxidation was allowed to proceed in the dark at room temperature. Samples (0.05 ml) were removed at intervals, diluted to 50 ml with water and their E_{223} read. Oxidation was complete after 27 h when 1.94 mol.prop. of periodate had been reduced per mol of phosphate. Aqueous $Ba(OH)_2$ was added to the oxidation mixture until the solution was just alkaline to phenolphthalein, the insoluble barium salts were filtered off and Ba^{2+} ions were removed by passing the filtrate through a column (50 mm × 9 mm) of Dowex 50 (Na^+ form). The eluate was chromatographed on a column (90 mm × 9 mm) of Dowex 1 (Cl^- form; 2% cross-linked; 100–200 mesh) which was successively eluted with water (100 ml), 0.025 M-HCl (250 ml) and 0.05 M-HCl (250 ml). Fractions (5 ml) were collected and samples were analysed for phosphate and aldehyde (Dische & Borenfreund, 1949). A single peak of aldehyde containing all of the phosphate in the sample was eluted in 0.05 M-HCl. Fractions constituting this peak were combined, neutralized with 0.1 M-NaOH and reduced with $NaBH_4$ (5 mg). The reduction was terminated and borate removed as described above, and Na^+ ions were removed on a column (50 mm × 9 mm) of Dowex 50 (H^+ form). A sample (7 μmol of P) was incubated with phosphomonoesterase, which converted all of the phosphate into P_i. The products were deionized on columns (20 mm × 9 mm) of Dowex 50 (H^+ form) and Dowex 1 (OH^- form). Chromatographic examination (solvent A) showed only one product (demonstrated by spray reagent 1) which

had the same properties as ethylene glycol ($R_{glycerol}$ 1.23). The original phosphate was therefore N-acetyl-glucosamine 6-phosphate.

A sample (10mg) of the N-acetylglucosamine 6-phosphate was dissolved in water, adjusted to pH2 and then passed through a column (50mm×9mm) of Dowex 50 (H$^+$ form) to remove any traces of glucosamine phosphate. The optical rotation of the resulting solution was $[\alpha]_D^{25}$ +26° (c 2% in 0.5M-sodium acetate). N-Acetyl-D-glucosamine 6-phosphate has been reported (Distler et al., 1958) to have $[\alpha]_D^{25}$ +29.5° (c 8% in 0.5M-sodium acetate).

Oxidation of the polysaccharide with sodium meta-periodate

Preliminary studies showed that oxidation at room temperature in unbuffered sodium periodate led to overoxidation and liberation of iodine, a consequence, presumably, of the instability of the products of oxidation. The following conditions were therefore employed. Polysaccharide (10mg) was dissolved in water (2ml), passed through a column (40mm× 10mm) of Dowex 50 (Na$^+$ form) and freeze-dried. A sample (7.8mg) was dissolved in 0.1M-sodium acetate buffer, pH5.8 (0.5ml), and 0.1M-sodium periodate (0.5ml) was added. Oxidation was allowed to proceed in the dark at 4°C, and samples (0.05ml) were removed at intervals, diluted to 50ml with
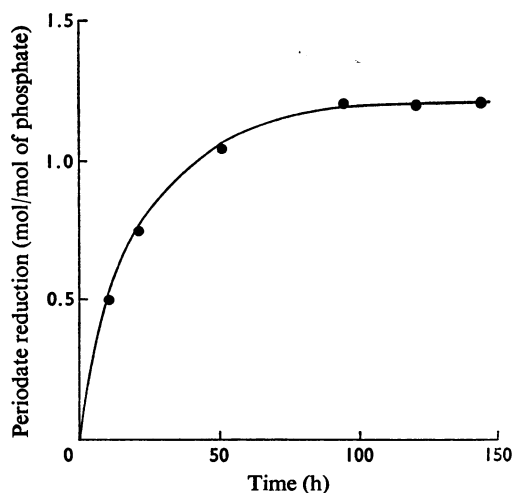

Fig. 1. *Periodate oxidation of the polysaccharide*

Polysaccharide was oxidized at 4°C in a twofold excess of 0.025M-sodium periodate. At intervals samples were removed, diluted in water and the periodate uptake was determined spectrophoto-metrically as described in the text.

water and their E_{223} read. Oxidation was complete (Fig. 1) when slightly more than 1mol.prop. of periodate was reduced per mol of phosphate. A further sample (7.8mg) of the sodium salt of the polysaccharide was oxidized as described above and then dialysed against water for 16h at 4°C to remove salts. Less than 10% of the phosphate diffused out in this time and the non-diffusible material was freeze-dried and dissolved in water (5ml). A sample (0.69μ-mol of P) was made 1M with respect to NaOH, left at room temperature for 3.5h and then neutralized with 2M-HCl. A second sample (1.38μmol of P) was adjusted to pH11.9 with 0.1M-NaOH and left at room temperature for 1.5h. A third sample (0.69μmol of P) was added to 0.04M-glycine buffer, pH10.3 (1ml), and left at room temperature for 18h. Samples of all three mixtures were analysed for P_i before and after incubation with phosphomonoesterase. The results (Table 1) show that substantial amounts of P_i were liberated under all three conditions. A further sample (4.6mg) of the polysaccharide was oxidized under these conditions and then NaBH$_4$ (5mg) was added followed, after 5h, by a few drops of acetic acid. Borate and salts were removed as described above, and the product was dissolved in 0.1M-HCl (0.3ml) and hydrolysed at 100°C for 5min. The hydrolysate was evaporated to dryness over KOH pellets and dissolved in water (0.3ml). A sample was chromatographed (solvent A) with a glycerol 1-phosphate marker. Reagents (1) and (2) showed the presence of a single phosphate, which had the mobility and colour reactions of glycerol 1-phos-phate. A second sample (0.1ml) was dephosphoryl-ated enzymically and chromatographed (solvent A) against glycerol. Reagent (1) showed a single spot identical in colour and mobility with glycerol. A third sample (0.1ml) was reduced with NaBH$_4$, deionized and chromatographed in solvent (A). Reagent (6) showed the presence of a single spot having the same mobility as a standard of 2-acetamido-2-deoxyglycerol prepared from N-acetyl-D-glucosamine by reduction, oxidation with periodate and further reduction with NaBH$_4$.

A further sample of glycerol monophosphate was prepared as described above from 30.5mg of poly-saccharide, and isolated by preparative chromato-graphy on Whatman 3MM paper. Samples (4.8μmol of P) were analysed for formaldehyde produced on periodate oxidation (Hanahan & Olley, 1958) and 0.98mol.prop. of formaldehyde was produced per mol of phosphate.

Chain length of the polysaccharide

Samples of polysaccharide isolated by extraction with trichloroacetic acid gave 13% P_i on incubation with phosphomonoesterase. Such extraction con-ditions cause hydrolysis of sugar 1-phosphate link-

Table 1. *Action of alkali on periodate-oxidized polysaccharide*

A solution (1.5ml containing 0.69 μmol of P) of oxidized polysaccharide in 1 M-NaOH was incubated at room temperature for 3.5h. A second sample (1ml containing 1.38 μmol of P) of oxidized polysaccharide was adjusted to pH11.9 with 0.1 M-NaOH and incubated at room temperature for 1.5h and a third sample (0.5ml containing 0.69 μmol of P) was mixed with 0.04 M-glycine buffer, pH10.3 (1ml), and incubated at room temperature for 18h. At the end of the respective incubation periods the samples were neutralized with 2 M-HCl and analysed for P_i before and after incubation with phosphomonoesterase.

Treatment	% total phosphate present as P_i at end of incubation	% total phosphate present as P_i after subsequent incubation with phosphomonoesterase
1 M-NaOH	71	84
NaOH, pH11.9	47	92
Glycine buffer, pH10.3	40	86

ages and so should give short chains, the average length of which in the present case is 7–8 units, terminating in a phosphomonoester residue at the non-reducing terminus. Enzymic dephosphorylation of samples that had been extracted by NaOH gave only 2.5% P_i. The mechanism of this extraction procedure is not understood, so that although there is one phosphomonoester residue for every 40 units it is not at present possible to deduce from this any information about chain length. Careful examination of the products of hydrolysis in 0.1 M-HCl showed that although most of the polysaccharide was converted into N-acetylglucosamine 6-phosphate, a small amount of free N-acetylglucosamine was also formed. Biosynthetic considerations (Brooks & Baddiley, 1969) suggest that the polysaccharide should terminate at the non-reducing end in an N-acetylglucosamine unit which has no phosphate group attached to the C-6 hydroxyl group. Mild acid hydrolysis should therefore give one molecule of N-acetylglucosamine per chain, the other units being liberated as N-acetylglucosamine 6-phosphate, so that the chain length is given by the ratio of these two products. This ratio has been determined by using walls and a polysaccharide–peptidoglycan complex isolated after digestion of walls with *Flavobacterium* enzyme preparation. Walls (96mg) isolated from a 4h culture were homogenized in 0.1 M-HCl (10ml) and heated at 100°C for 30min. Wall debris was removed by centrifugation (20000g, 20min), the supernatant solution was adjusted to pH7 with 1 M-NaOH and applied to a column (100mm × 10mm) of Dowex 1 (CO_3^{2-} form), which was then eluted successively with water (50ml) and 0.25 M-$(NH_4)_2CO_3$ (50ml). The neutral products were decreased in volume to 10ml and samples (0.2ml) were assayed for reducing sugar. The ratio of reducing sugar (present as N-acetylglucosamine) to N-acetylglucosamine phosphate was 1:24.4, indicating an average chain length of 25.4 units.

A polysaccharide–glycan complex was isolated by using the *Flavobacterium* L11 enzyme (Kato *et al.*, 1968; Kato & Strominger, 1968). A crude enzyme preparation (750mg) was dissolved in cold 0.01 M-sodium phosphate buffer, pH8 (100ml). After removal of some insoluble material by centrifugation (10000g, 25min) the brown solution was applied to a column (150mm × 18mm) of CM-52 cellulose which had been equilibrated with 0.01 M-sodium phosphate buffer, pH8. The column was washed with this buffer (150ml) and gave a brown-coloured eluate which contained no lytic activity. The column was then eluted stepwise with solutions (50ml) of sodium phosphate buffer, pH8.0, of increasing concentration (0.05, 0.10 and 0.20 M). The eluates were dialysed against water and then tested for lytic activity; this was present only in the fractions eluted in 0.20 M buffer. The non-diffusible material in this eluate was freeze-dried to give 400mg of partially purified enzyme.

Walls (989mg), which had been purified by digestion with trypsin and extraction with phenol, were homogenized in 0.01 M-sodium phosphate buffer, pH8 (50ml), and added to a solution (35ml) of partially purified enzyme (400mg) in the same buffer. Samples (0.5ml) were removed at intervals, mixed with water (2.5ml) and their E_{500} read. After incubation for 24h at 37°C the extinction of the suspension had decreased to 12% of its original value. After removal of insoluble material the digest was dialysed against water and the non-diffusible fraction was freeze-dried (yield 1.01g). Samples of this material were analysed and found to contain a range of amino acids presumably derived from enzyme present in the preparation. This was removed by ion-exchange chromatography on DEAE-cellulose (acetate form) by elution with a gradient of 0.05–1 M-sodium acetate buffer, pH5.2. Most of the protein was eluted in buffer of low concentration and all of the phosphate was eluted in a single peak in 0.6 M

buffer. Tubes containing this phosphate were combined, dialysed against water and freeze-dried to give a teichoic acid–peptidoglycan complex (300 mg). Hydrolysis of this material in 0.1 M-HCl at 100°C for 15 min gave N-acetylglucosamine and N-acetylglucosamine 6-phosphate in the ratio 1:21, corresponding to an apparent chain length of 22.

Action of alkali on the polysaccharide

Initial studies showed that treatment of polysaccharide with 1 M-NaOH at 100°C produced substantial charring and gave P_i, glucometasaccharinic acid phosphate [identified, after enzymic dephosphorylation, as described by Archibald & Buchanan (1969)] and residual organic phosphates, which contained unhydrolysed phosphodiester bonds. Polysaccharide isolated by extraction into trichloroacetic acid gave similar results to that isolated by alkali and the degradation was studied further to examine the origin of the resistant phosphodiesters. Samples (0.2 ml) of a solution of polysaccharide (6 mg) in water (1.2 ml) were added to small Pyrex test tubes containing 1 M-NaOH (0.2 ml). The tubes were then evacuated, sealed and heated at 100°C. At intervals tubes were cooled and the contents were neutralized by passage through columns (20 mm × 10 mm) of Dowex 50 (NH_4^+ form) and freeze-dried. Analysis of these samples showed that production of P_i approached a limit of 20–25% after 2–3 h (Fig. 2). A larger sample (94 mg) was similarly heated for 3 h at 100°C in 1 M-NaOH (7 ml), then desalted and freeze-dried. In this case 22.8% of the phosphate was P_i. The residual glucosamine was determined after enzymic dephosphorylation of a sample which had been hydrolysed in 2 M-HCl at 100°C for 3 h, and the glucosamine/phosphate ratio was 0.52:1, showing that 50% of the glucosamine originally present was destroyed on treatment with alkali. Incubation of the alkali hydrolysate with phosphomonoesterase gave 63% P_i, showing that 37% of the original diesters remained intact. These phosphodiester groups were hydrolysed on further treatment of the initial hydrolysate in 1 M-NaOH for 3 h at 100°C, after which enzymic dephosphorylation gave 81% P_i. However, after incubation with phosphomonoesterase, further alkali hydrolysis of the initial hydrolysate left only 4% of the initial phosphodiester groups intact. Thus hydrolysis of the polysaccharide at 100°C results in a rapid initial breakdown of 63% of the phosphodiester groups. On subsequent hydrolysis for 3 h a total of 81% of the phosphodiester groups are hydrolysed, but prior incubation with phosphomonoesterase leads to hydrolysis of 96% of the phosphodiester groups. Thus enzymic removal of the phosphomonoester groups renders the remaining phosphodiester groups more susceptible to hydrolysis in alkali. The nature of the remaining phospho-
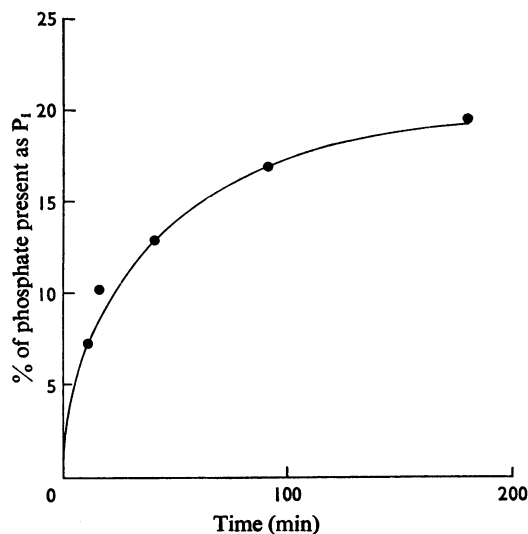

Fig. 2. *Formation of P_i on treatment of the polysaccharide in 1 M-NaOH at 100°C*

Sealed evacuated tubes containing a solution (0.4 ml) of polysaccharide (1 mg) in 1 M-NaOH were kept at 100°C for various times. The contents were then neutralized and analysed for P_i as described in the text.

diester groups was examined by isolation of the appropriate fraction of the alkali hydrolysate. Fractions rich in phosphodiester residues were obtained by fractionation on columns of DEAE-cellulose and Bio-Gel P-2. Both fractions had similar properties, but Bio-Gel P-2 gave better resolution and this is described here. A sample (2.4 mg of P) of the polysaccharide, which had been hydrolysed for 3 h at 100°C in 1 M-NaOH as described above, was applied in aqueous solution to a column (560 mm × 18 mm) of Bio-Gel P-2 which had been equilibrated in water. The column was eluted with water (120 ml) followed by 0.15 M-pyridine–acetate buffer, pH 5.2 (100 ml). A single peak (peak 1) of phosphate (peak vol. 85 ml) was eluted with water. This accounted for 31% of the phosphate applied to the column, and all of this phosphate was organic. The remaining phosphate (peak 2), 37% of which was P_i, was eluted with 0.15 M-pyridine–acetate buffer. Samples (each containing 7 μg of P) of the above fractions and of the unfractionated alkali hydrolysate were subjected to electrophoresis on Whatman no. 1 paper in 0.2 M-pyridine–acetate buffer, pH 4.9, for 1.5 h at a potential gradient of 21.5 V/cm. Reagent (2) showed that three components were present in the unfractionated hydrolysate: two of these ran as discrete spots at 10 and 16 cm from the origin and the third migrated as a band at 5–9 cm from the origin.

Peak 2 contained only the two faster-moving components and peak 1 contained only the material moving as a band between 5 and 9 cm from the origin. Peak 2 was composed principally of P_i and glucometasaccharinic acid phosphate, whereas 74% of the phosphate in peak 1 was present as phospho-diester. Peak 1 was examined further to obtain information on the nature of the alkali-resistant phosphodiester groups. Acid hydrolysis gave P_i, glucosamine 6-phosphate and glucosamine as the sole products. A sample (70 μg of P) was hydrolysed in 1 M-HCl (0.5 ml) at 100°C for 3 h and then, after removal of acid, applied to a column (20 mm × 8 mm) of Dowex 1 (CO_3^{2-} form). Neutral products were eluted with water (20 ml) and phosphates were eluted with 0.25 M-$(NH_4)_2CO_3$ (20 ml). The neutral products contained 0.20 μmol of glucosamine and the phosphate fraction contained 2.20 μmol of phosphate, 19% of which were present as P_i. A further sample of peak 1 material (70 μg of P) was incubated with phosphomonoesterase, when 26% P_i was formed.

Discussion

Walls of *Staphylococcus lactis* 2102 contain 30–40% by weight of a phosphorylated polysaccharide consisting of linear chains of N-acetylglucosamine 1-phosphate in which units are held together by phosphodiester bridges between the 1- and 6-positions of adjacent units. The only other major component of the wall is peptidoglycan, which is similar in composition (Archibald *et al.*, 1969) to that of several other staphylococci. Only small amounts of 'protein' amino acids are present in isolated walls.

The polysaccharide is extracted on treatment of walls with cold 5% trichloroacetic acid, but this causes considerable hydrolysis of the sugar 1-phosphate linkages, and most of the present studies were carried out on samples that had been extracted into dilute NaOH as described by Archibald *et al.* (1969). This gives almost quantitative yields of polysaccharide, the low phosphomonoester content (2.5%) of which indicates that little or no degradation has taken place.

Isolated polysaccharide is completely hydrolysed on treatment with 0.1 M-HCl at 100°C for 15 min, when the sole products are N-acetylglucosamine 6-phosphate and a small amount of N-acetylglucosamine; quantitative analysis showed that after enzymic dephosphorylation N-acetylglucosamine and phosphate were present in equimolar proportions. Hydrolysis in 2 M-HCl for 3 h at 100°C gave glucosamine and glucosamine monophosphate; enzymic dephosphorylation gave glucosamine as the sole sugar component, and this was characterized chromatographically and by degradation with ninhydrin to arabinose and with HNO_2 followed by reduction with $NaBH_4$ to 2,5-anhydromannitol. The N-acetylglucos-

amine phosphate was identified as the 6-phosphate by reduction with $NaBH_4$ followed by periodate oxidation, when 2 mol.prop. of periodate was reduced per mol of phosphate. Reduction of the products gave ethylene glycol phosphate as the sole phosphate, showing the position of substitution in the N-acetylglucosamine phosphate. The complete hydrolysis of the polymer under gentle acid conditions indicates the presence of sugar 1-phosphate linkages. Since acid-catalysed migration of the phosphate group is unlikely to be substantial under these conditions the nature of the product indicates that the polymer consists of repeating units of N-acetylglucosamine 1-phosphate attached by phosphodiester linkages between the 1- and 6-positions of adjacent units. Since the sole products of hydrolysis are N-acetylglucosamine 6-phosphate and a little N-acetylglucosamine it follows that the polymer is a linear molecule, since any branch-point would give rise to a diphosphate equivalent to at least one-half the molecular proportions of free N-acetylglucosamine produced on hydrolysis: this would have been readily detected in the present study. It is noteworthy, though of no structural significance, that small amounts of a product having the chromatographic properties of a diphosphate were observed in studies when the polymer was hydrolysed in 2 M-HCl. No such component was present after gentle acid hydrolysis, and our subsequent observation that treatment of a pure sample of glucosamine 6-phosphate with 2 M-HCl gave the same apparent diphosphate shows that this is an artifact caused by either transphosphorylation or transglycosylation reactions occurring either during heating in the acid or during removal of acid under diminished pressure.

Although the behaviour of other sugar 1-phosphate polymers (Archibald *et al.*, 1968a; Archibald & Heptinstall, 1971) shows that phosphate-group migration does not occur during gentle acid hydrolysis it was desirable to confirm the position of the phosphodiester linkages by other procedures. On oxidation with a twofold excess of periodate under carefully controlled conditions 1 mol.prop. of periodate was reduced per mol of phosphate. This is consistent only with 1→6 phosphodiester linkages. The lability of the oxidized polysaccharide in alkali, resulting in extensive depolymerization and elimination of P_i, is also evidence for the structure shown in Scheme 1, P_i being formed by β-elimination reactions at C-1 owing to the aldehyde group at C-3 and at C-6 owing to the aldehyde group at C-4. After reduction of the product of oxidation with $NaBH_4$, acid hydrolysis gave glycerol 1-phosphate; further reduction of the hydrolysate gave 2-acetamido-2-deoxyglycerol. These results confirm the structure shown in Scheme 1 and the optical rotation of the polysaccharide, $[\alpha]_D^{25}$ +105°, shows that the N-acetylglucosaminyl 1-phosphate linkages have the α anomeric configuration.

Scheme 1. *Alkaline degradation of the phosphorylated polysaccharide of Staphylococcus lactis* 2102

Abbreviation: Ac, acetyl.

After lysis of walls by incubation with the *Flavobacterium* peptidase, fractionation of the products gave a polysaccharide–peptidoglycan complex which on gentle acid hydrolysis gave N-acetylglucosamine and N-acetylglucosamine 6-phosphate in the ratio 1:21. On the reasonable assumption that all of the N-acetylglucosamine is derived from the non-reducing terminal end of the polysaccharide chain this indicates that the average chain length of the polysaccharide is 22 repeating units.

On treatment with 1 M-NaOH at 100°C the polysaccharide decomposed with charring and produced P_i, glucometasaccharinic acid phosphates and residual unhydrolysed organic phosphates. The interunit phosphodiester linkages shown in Scheme 1 should be stable in alkali, since they are not flanked by unsubstituted hydroxyl groups which would permit hydrolysis through intermediate five-membered cyclic phosphates. Degradation would therefore be expected

to proceed in a stepwise manner from the reducing end of the polysaccharide, principally by its conversion into saccharinic acid on to which the phosphodiester group can cyclize so giving saccharinic acid phosphate and exposing the reducing group of the next residue (Scheme 1). Alternatively the reducing N-acetylglucosamine residue could undergo a reverse aldol condensation. Such reactions have been observed to occur on alkaline degradation of glucose 6-phosphate (Degani & Halmann, 1968). This would leave glyceraldehyde attached to the end of the polysaccharide chain. This could then form a cyclic phosphate as before and expose the reducing group of the next unit, but alternatively it could undergo a β-elimination reaction leaving a phosphomonoester residue attached to the reducing group of the next sugar molecule. The molecule should then be stable in alkali because of the absence of free reducing groups. The alkali-resistant phosphodiester

groups should therefore be present in chains which terminate in sugar 1-phosphate units. In addition to these processes glyceraldehyde phosphate, formed as above, would further decompose in alkali giving P_i, and any open-chain terminal reducing sugar could undergo cyclic phosphate formation before saccharinic acid formation or reverse aldol condensation. A variety of products would therefore be formed, including substantial proportions of glucometasaccharinic acid phosphate and P_i, as were indeed found, and a certain amount of unhydrolysed phosphodiester groups, all of which should be present in chains which terminate in sugar 1-phosphate units (Scheme 1).

Formation of P_i approached a limit of about 22% after treatment of the polysaccharide in 1 M-NaOH for 3 h at 100°C. During this time approx. 50% of the glucosamine was destroyed, but 37% of the original phosphodiester groups remained intact. These phosphodiester groups were slowly hydrolysed on further treatment with 1 M-NaOH at 100°C, but prior enzymic removal of the phosphomonoester groups led to rapid hydrolysis of almost all of the residual phosphodiesters on subsequent treatment with alkali. Thus the phosphodiesters remaining after degradation of the polysaccharide in alkali are stabilized by the presence of certain phosphomonoester groups. That these are, as forecast above, sugar 1-phosphomonoester groups was shown by the detection of such groups in the isolated resistant material. A sample of this material was isolated by chromatography on Bio-Gel and had the same electrophoretic mobility as one of the three components detected by electrophoresis of the degradation mixture. Hydrolysis in 1 M-HCl gave glucosamine phosphate, glucosamine and P_i in the mol.prop. 1.78:0.20:0.42. No saccharinic acid or saccharinic acid phosphate was produced. Under such hydrolytic conditions P_i can be formed only by hydrolysis of sugar 1-phosphomonoester groups, and its formation shows that 19% of the phosphate in the resistant fraction is present in this form. Since, as shown above, enzymic removal of the phosphomonoester groups renders the phosphodiester groups much more susceptible to degradation on further treatment with alkali, it is reasonable to conclude that the sugar 1-phosphate monoester groups are responsible for the resistance of the diester groups and that the latter are present in chains of glucosamine phosphate of average length 5.6 units. One might expect these chains to result from stepwise degradation from the reducing end of the polysaccharide until an elimination reaction leaves a phosphomonoester group attached to the reducing end. In this case each of the derived chains should terminate at the non-reducing end in a glucosamine residue, and so acid hydrolysis should give equivalent proportions of glucosamine and P_i representing each end of the chain. However, the observed molar ratio of glucos-

amine to P_i was 1:2.1, so that there are only one-half as many non-reducing terminal glucosamine residues as reducing terminal glucosamine 1-phosphate residues. The most likely explanation of this is that the non-reducing terminal glucosamine residue of about one-half of the chains bears a phosphomonoester group. This is consistent with the observation that incubation with phosphomonoesterase gives 26% P_i, showing that some 7% of the phosphate is present as monoester other than the 19% present at the reducing terminal end of the chain. The alkaliresistant fraction thus consists of a mixture of short chains of glucosamine phosphate, all of which terminate in glucosamine 1-phosphate, and approximately one-half of which have glucosamine and the rest glucosamine phosphate at the non-reducing terminals. Such a mixture could arise through hydrolysis of a proportion of the interchain *N*-acetylglucosaminyl 1-phosphate linkages, possibly by a mechanism involving 4:6-cyclic ester formations. Cyclic esters of this type are known and the stable cyclohexylamine salt of methyl α-D-glucoside 4:6-cyclic phosphate has been synthesized (Baddiley *et al.*, 1954). Little is known, however, about the formation of 4:6-cyclic phosphates in alkaline solution, though the present results show fairly clearly that in some way a proportion of the inter-unit phosphodiesters undergo hydrolysis so as to leave the phosphate group at the newly formed non-reducing terminus. Thus the alkali-resistant fraction does undergo further hydrolysis on continued treatment with alkali, though such hydrolysis is slower than the degradation that takes place after enzymic removal of the reducing terminal phosphomonoester residues when only 11% as opposed to 54% of the originally resistant phosphodiesters remain intact after further treatment in alkali for 3 h. Although the mechanism of this hydrolysis is uncertain, these results show that lability of such polymers to alkali cannot be taken to imply that the original polymer contains either free reducing groups or free hydroxyl groups adjacent to the phosphodiester groups.

The phosphorylated polysaccharide is held in the wall by covalent attachment to peptidoglycan, but the nature of its association and the reasons for its ready extraction into dilute alkali are unknown. Several teichoic acids are similarly extracted into dilute alkali (Hughes & Tanner, 1968; Archibald *et al.*, 1969), but it is noteworthy that teichuronic acid is not removed from walls of *Bacillus licheniformis* under such conditions (Hughes & Tanner, 1968). Hughes (1970) has shown that teichuronic acid is held in the wall of *B. licheniformis* by a sugar 1-phosphate linkage between *N*-acetylgalactosamine and muramic acid. Since this linkage is stable under these conditions and since the inter-unit linkages in the phosphorylated polysaccharide of *Staph. lactis* 2102 are also stable, the linkage of the phosphorylated

polysaccharide to peptidoglycan must involve an alkali-labile bond, and so linkage is presumably not directly through a sugar 1-phosphate bond to mur-amic acid.

The phosphorylated polysaccharide of *Staph. lactis* 2102 is a simple linear polymer. Its biosynthesis is accomplished by membrane-bound enzymes (Brooks & Baddiley, 1969) and proceeds by transfer of *N*-acetylglucosamine 1-phosphate residues from UDP-*N*-acetylglucosamine to a lipid intermediate and thence to the non-reducing terminal end of the growing chain (Hussey *et al.*, 1969). It resembles teichoic acid in being rich in phosphate groups and it may serve a similar role in the uptake of metal ions, although it differs from teichoic acids in not possess-ing D-alanyl ester residues, the presence of which in teichoic acids can substantially influence their Mg^{2+}-binding capacity (Heptinstall *et al.*, 1970).

References

Archibald, A. R. & Buchanan, J. G. (1969) *Carbohyd. Res.* **11**, 558–560

Archibald, A. R. & Heptinstall, S. (1971) *Biochem. J.* **125**, 361–363

Archibald, A. R., Baddiley, J., Button, D., Heptinstall, S. & Stafford, G. H. (1968*a*) *Nature (London)* **219**, 855–856

Archibald, A. R., Baddiley, J. & Button, D. (1968*b*) *Biochem. J.* **110**, 543–557

Archibald, A. R. Coapes, H. E. & Stafford, G. H (1969) *Biochem. J.* **113**, 899–900.

Archibald, A. R., Baddiley, J., Heckels, J. & Heptinstall, S. (1971) *Biochem. J.* **125**, 353–359

Aspinall, G. O. & Ferrier, R. J. (1957) *Chem. Ind. (London)* 1216

Baddiley, J., Buchanan, J. G. & Szabo, J. (1954) *J. Chem. Soc. London* 3826–3832

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956) *J. Chem. Soc. London* 2818–2823

Brooks, D. & Baddiley, J. (1969) *Biochem. J.* **115**, 307–314

Chen, P. S., Toribara, T. V. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758

Consden, R. & Gordon, A. H. (1948) *Nature (London)* **162**, 180–181

Davison, A. L. (1968) *Biochem. J.* **110**, 557–558

Degani, Ch. & Halmann, H. (1968) *J. Amer. Chem. Soc.* **90**, 1313–1317

Dische, Z. & Borenfreund, E. (1949) *J. Biol. Chem.* **180**, 1297–1300

Distler, J. J., Merrick, J. M. & Roseman, S. (1958) *J. Biol. Chem.* **230**, 497–509

Frerejacque, M. (1955) *C. R. Acad. Sci.* **240**, 1804–1805

Ghuysen, J. M., Tipper, D. J. & Strominger, J. L. (1966) *Methods Enzymol.* **8**, 685–699

Hanahan, D. J. & Olley, J. N. (1958) *J. Biol. Chem.* **231**, 813–828

Hanes, C. S. & Isherwood, F. A. (1949) *Nature (London)* **164**, 1107–1112

Heptinstall, S., Archibald, A. R. & Baddiley, J. (1970) *Nature (London)* **225**, 519–521

Hughes, R. C. (1970) *Biochem. J.* **117**, 431–439

Hughes, R. C. & Tanner, P. J. (1968) *Biochem. Biophys. Res. Commun.* **33**, 22–28

Hussey, H., Brooks, D. & Baddiley, J. (1969) *Nature (London)* **221**, 665–666

Kato, K. & Strominger, J. L. (1968) *Biochemistry* **7**, 2754–2761

Kato, K., Hirata, T., Murayama, Y., Suginaka, H. & Kotani, S. (1968) *Biken J.* **11**, 1–12

Park, J. T. & Johnson, M. J. (1949) *J. Biol. Chem.* **181**, 149–151

Partridge, M. D., Davison, A. L. & Baddiley, J. (1971) *Biochem. J.* **121**, 695–700

Partridge, S. M. (1949) *Nature (London)* **164**, 443

Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10–15

Rydon, H. N. & Smith, P. W. G. (1952) *Nature (London)* **169**, 922–923

Slodki, M. E. (1966) *J. Biol. Chem.* **241**, 2700–2706

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445

White, P. J. & Woods, D. D. (1965) *J. Gen. Microbiol.* **40**, 243–253

Wickerham, L. J. & Burton, K. A. (1962) *Bacteriol. Rev.* **26**, 382–397