STRUCTURE OF STREPTOCOCCAL CELL WALLS. V*

PHOSPHATE ESTERS IN THE WALLS OF GROUP A *Streptococcus pyogenes*.

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Walls and certain wall constituents of *S. pyogenes* contain 0.6% of organically bound phosphorus. We are reporting the isolation and characterization of D,L-glycerol-1-phosphate, glyceryl-(1'⟶1)rhamnoside-2'-(or 3'-)phosphate and muramic acid-6-phosphate from such walls. It is suggested that the phosphate compounds are involved in the linkage uniting the peptidoglycan and the C-polysaccharide.

METHODS AND MATERIALS

Cell walls were prepared as described earlier (Heymann et al. 1963). Rhamnose was determined according to Dische and Shettles (1948); Mr. J. J. Boltralik demonstrated that the procedure gives reliable results when performed in 1/5 the usual size. The Morgan-Elson and Elson-Morgan reactions were done as described (Heymann et al. 1964). Phosphate was determined by a micro-version of the Martin-Doty extraction procedure (1949), in which 0.5 μg of P gave an absorbancy of 0.3 (final volume 800 μl, light path 10 mm). Periodate oxidations for measuring -CHOH-CH_2OH were performed according to Hanahan and Olley (1958). D-Glycerophosphate was measured with the aid of D-glycerophosphate dehydrogenase. Incubations with alkaline phosphatase (250 μg/ml) were done in M/100 Tris or ammonium carbonate buffer

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pH 9.4 at 37° overnight. Whenever applicable, the digests were desalted by passage over Dowex 50 H^+, or Amberlite MB-3.

Chromatographic solvents employed are:

A. Ethanol: 1 M ammonium acetate pH 3.8; 7:3

B. Pyridine: n-butanol: acetic acid: water: 40:60:3:30

C. Ethanol: 1 M ammonium acetate pH 7.5; 7:3

D. t-Butyl alcohol: water: sat. picric acid; 80:20:4

E. N-Propanol: ammonia: water; 6:3:1

F. Butanol: acetic acid: water, 5:1:4

G. Methanol: ammonia: .002 M Versene; 60:10:30 at 4°

Spots were detected with alkaline silver reagent (Trevelyan et al. 1950), with periodate-silver spray (Richardson and Hough 1962) with ninhydrin, and with Hanes-Isherwood spray and ultra-violet irradiation (Axelrod and Bandurski 1951).

RESULTS AND DISCUSSION

The distribution of phosphorus among cell walls and several wall degradation products appears in Table I.

TABLE I

No.	Material	%P	Reference to origin of material
1	Walls, Group A, T_{14}	0.65	(a)
2	C-polysaccharide, (formamide)	0.7	(b)
3	C-polysaccharide (formamide)	1.1	(a)
4	C-polysaccharide-peptidoglycan complex	1.05	(c)
5	C-polysaccharide (TCA-extracted)	0.36	(d)
6	Peptidoglycan lysozyme lysate (G-25 excluded portion)	.05-.1	(e)
7	Peptidoglycan after TCA (TCA=trichloroacetic acid)	0.18	(d)

References: (a) Heymann et al. 1963; (b) Zittle and Harris 1942; (c) Munoz et al. 1966; (d) unpublished; (e) Heymann et al. 1964.

None of the materials in Table I gave any free phosphate when treated with phosphomonoesterase, whether or not they had been pretreated with venom "diesterase". After gentle hydrolysis (2N HCl, 1-2 hrs., 100°) most of the organic phosphorus was in the form of monoester, and some inorganic phosphorus also appeared.

After drastic hydrolysis for amino acid analysis of a subfraction of complex #4, Table I, an unknown amino acid emerged from a 0.9 x 60 cm. column of BioRad A-4 resin (50°, citrate pH 3.25) well before aspartic acid. After gentle hydrolysis of complex #4, Table I, or of trypsin-treated walls and fractionation of the acid-free hydrolysate on a short column of Dowex 50 by elution with water there appeared, seriatim, rhamnose, ninhydrin - negative phosphate esters, and the aforementioned unknown amino acid that had been weakly retained. The last-named substance contained phosphate which was completely released by phosphatase; it gave an Elson-Morgan color with λ_{max} at 510 mμ. The hexosamine moiety was indistinguishable and inseparable from muramic acid on chromatography in solvent B, and on electrophoresis for 40 min. in 2 N HOAc at 3500 V. The ratio of P: hexosamine was 1.1 : 1. In solvent A muramic acid-6-phosphate migrates with R_f=0.45; $R_{l\text{-glycerophosphate}}$= 0.72. Assignment of the phosphate to position 6 rests upon the great acid stability of the ester linkage and upon the production of glycolaldehyde phosphate by periodate. The muramic acid phosphate (1.8 μmoles) was reduced with $NaBH_4$ until reducing power had been abolished. After deionization and removal of boric acid (Dowex 50, methanol) the material was oxidized with 0.01 M $NaIO_4$ until the rate of consumption dropped off, and the excess reagent was destroyed with glycerol. The volume was reduced to one fourth and aliquots were spotted for paper chromatography in solvents A and G. The unknown solution gave rise to a phosphate-containing, strongly reducing spot moving like authentic glycolaldehyde phosphate; the markers, l-glycerophosphate and glucose-6-phosphate, migrate in the vicinity of glycolaldehyde phosphate but exhibit no or only weak reducing power towards the silver reagent em-

ployed. Paper electrophoresis (60 V/cm, 25 mA, 25 min. in 0.14 M NaOAc buffer pH 4.5 containing 0.65 g per liter of ethylenediaminetetraacetic acid) likewise supported the conclusion that glycolaldehyde phosphate had been formed. Muramic acid phosphate was first described by Agren and Verdier (1958); it was encountered by Liu and Gotschlich (1963) in pneumococcal polysaccharide; it is involved in the linkage between peptidoglycan and the atypical teichoic acid of Staphylococcus lactis (Button et al., 1966), and has been conjectured upon as having a similar role in L. casei (Hall and Knox, 1965). In these cases, 6-substitution had not been proved specifically, but it appears likely that in all instances the 6-ester was the compound involved.

The non-nitrogenous phosphate fraction alluded to above was adsorbed in 0.001 N ammonia on a column of Dowex-1 chloride. Elution with 0.025 N HCl yielded three well-separated fractions, I, II and III. Of these, III appeared homogeneous on chromatography in solvents C, D, E and F, and migrated like l-glycerophosphate in all of them. Phosphatase liberated all of the phosphorus and a compound with the R_F of glycerol (0.59, solvent F). The ester reacted with l-D-glycerophosphate dehydrogenase (Boehringer) and hydrazine (Boltralik and Knoll 1960) to the extent of 49.7% and thus is D,L-l-glycerophosphate. In agreement with this conclusion, the compound in fraction III gave 1.04 mole of formaldehyde per atom of P; after dephosphorylation the ratio was 2.1 : 1.

Fraction II contained glycerol, rhamnose and phosphomonoester. The product from phosphatase action exhibited no reducing groups (Park-Johnson, $NaBH_4$-treatment) but gave formaldehyde on periodate oxidation. After hydrolysis the amount of formaldehyde doubled, relative to rhamnose. However, the ratios of the constituents of fraction II were not integer numbers, and only after tedious purification on 1 x 100 cm. columns of Sephadex G10 and G15 did a compound result, which furnished data in agreement with the formulation as glyceryl (1'$\longrightarrow$1)-rhamnoside-2'-(or 3'-)

phosphate. Pertinent data appear in Table II.

TABLE II

Glyceryl (1'⟶1)-rhamnoside-2'-(or 3'-)phosphate

Solvent	C	D	E	F
R_P:	1.61			
$R_{1\text{-glycero-P}}$:	1.09	1.06	1.01	0.53
R_F:				0.06

Rhamnose: Phosphate = 1.02:1

Glyceryl (1'⟶1)-rhamnoside

$R_{Rhamnose}$	0.92
$R_{Glycerol}$	0.78
R_F	0.43

Rhamnose: Formaldehyde = 1 : 0.97

Rhamnose: Formaldehyde, after hydrolysis = 1 : 2.3

Since the characterized phosphate esters here described arise as products of acid hydrolysis, the possibility of phosphoryl migrations cannot be ruled out, and the conclusions are not necessarily applicable to the phosphate esters as they exist in native walls.

Fraction I off the Dowex-1 chloride column was complex, giving a streak in most solvents; glycerol, rhamnose and phosphate are among its constituents.

The data in Table I in conjunction with the characterization of phosphates just presented suggest a hypothesis: C-polysaccharide is linked to cell wall peptidoglycan by phosphate-containing bridges made up of one or more units, the organic moiety of which is glycerol or possibly glyceryl rhamnoside. A reasonable inference is that these bridges terminate in muramic acid-6-phosphate units that are part of the peptidoglycan. The frequency and length of such bridges cannot be determined without more quantitative data than are now available, nor can one exclude the possi-

bility that phosphate ester groupings occur in the interior of the rhamnose polysaccharide portion of the cell walls. To date, we have not yet been able to remove any phosphate compounds from the walls or wall complexes without at the same time effecting profound degradation. Finally, phosphate substitution of certain muramic acid residues in the peptidoglycan offers an explanation for resistance of a portion of the glycan linkages to the several muralytic enzymes studied, e.e. lysozyme, phage muralysin, and Streptomyces albus F_1-muramidase.

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