[6] E. P. CLARK, *Semimicro Quantitative Organic Analysis*, Academic Press, New York, 1943, p. 68.
[7] A. ELEK, in *Organic Analyses*, Vol. 1, Interscience Publishers, New York, 1953, p. 89.
[8] E. KLENK AND G. UHLENBRUCK, *Z. Physiol. Chemie*, 307 (1957) 266.
[9] L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604.
[10] W. AYALA, L. V. MOORE AND E. L. HESS, *J. Clin. Invest.*, 30 (1951) 781.
[11] L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
[12] B. L. HORECKER, J. HURWITZ AND A. WEISSBACH, *J. Biol. Chem.*, 218 (1956) 793.
[13] D. A. MCFAYDEN, *J. Biol. Chem.*, 158 (1945) 107.
[14] S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 149.
[15] D. G. COMB AND S. ROSEMAN, *J. Biol. Chem.*, 235 (1960) 2529.

Received November 28th, 1963

Biochim. Biophys. Acta, 83 (1964) 129–132

SC 2331

Alkaline degradation of the phosphomucopolysaccharide from cell walls of *Bacillus megaterium* KM

About 40% of the dry weight of cell walls of *Bacterium megaterium* KM consist of a phosphomucopolysaccharide containing phosphate, glucose and *N*-acetylglucosamine in the molar ratio 1:2:1.3 as well as a polyol[1–3]. Because of its resemblance to the glycerol and ribitol teichoic acids found in other Gram-positive bacterial walls, it has been referred to as X-teichoic acid[3]. According to SALTON AND MILHAUD[4], acid hydrolysates of radioactive *B. megaterium* KM cell walls or of the radioactive component(s) released from them by a phage enzyme, contain a compound of which the chromatographic behaviour is that of anhydroribitol; this observation suggested that the polyol of the phosphomucopolysaccharide complex might be ribitol. Further studies are reported in this paper.

As already described[2,3], *B. megaterium* KM cell walls were lysed through the action of the F_2B enzyme preparation from *Streptomyces albus* G and the phosphomucopolysaccharide was separated from the glycopeptide fragments by paper electrophoresis. It was then further purified by gel filtration on Sephadex G-50, employing water to equilibrate the column and as eluting solvent. Filtration of the phosphomucopolysaccharide complex was almost identical to that of the teichoic acid from *S. aureus* (strain Copenhagen)[5]. The polymer was collected between 11 and 26 ml while bovine serum albumin and NaCl, used as controls to calibrate the column, were collected at about 12 ml and 45 ml, respectively.

The purified phosphomucopolysaccharide preparation had the same chemical composition as the preparations obtained earlier[3]. (It should be noted that the F_2B preparation is a mixture of lytic enzymes. The phosphomucopolysaccharide prepared in this way is devoid of fragments of glycopeptide. If, instead of the F_2B preparation purified enzymes are employed to lyse the cell walls—as the F_1 acetylhexosaminidase (F_1 *N*-acetylmuramide glycanohydrolase) or the 32 enzyme from *S. albus* G or egg white muramidase (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17, formerly known as lysozyme)—, the phosphomucopolysaccharide contains fragments of the glycopeptide, apparently covalently linked. The peptide component is released by muraminyl amidase, an enzyme present in the F_2B complex, which splits the amidic linkage between a *N*-acetylmuramic acid residue and a peptide moiety.) It contained no P_i and less than one phosphomonoester group for 100 total phosphate (as estimated

after incubation with a bacterial alkaline phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1)). However, by treatment with 1 N NaOH at 60°, phosphomonoester groups were progressively liberated and, at completion of the reaction (after 10 h of incubation), they represented about 80% of the total phosphate. (For this experiment the phosphomucopolysaccharide was dissolved in 1 N NaOH and the tube was immersed in a water bath at 60°. Aliquots of 10 μl, corresponding to 40 mμmoles of total phosphate, were removed after various times of incubation. The aliquots were neutralized with 10 μl of 1 N HCl and added to 20 μl of 0.5 M Tris buffer (pH 9.5) and 10 μl of alkaline phosphomonoesterase (0.4 mg/ml, Sigma Chemical Co.). After incubation at 37° for 1 h (which liberated all of the phosphomonoester groups), P_i was measured.) Using paper chromatography (isobutyric acid–conc. NH_4OH–water, 1000:40:560, v/v), the NaOH-degraded products (previously neutralized with isobutyric acid) were separated into two components with R_F 0.0 and 0.32, respectively.

The material with R_F 0.0 contained a small amount of organic phosphate (about 10% of the total phosphate) and, after periodic acid oxidation, it was very reactive with the Schiff spray[1]. This material was eluted from the chromatogram and freeze-dried. It represented about 65% of the dry weight of the original phosphomucopolysaccharide. The reducing power[6] of 1 mg was equivalent to 2.4 mμmoles of glucose. On acid hydrolysis (3 N HCl, 100°, 2 h) it gave rise to two main compounds which were separated by chromatography in the isobutyric acid solvent and then further identified as glucose (R_F 0.39) and glucosamine (R_F 0.62).

The compound with R_F 0.32, obtained after NaOH degradation of the phosphomucopolysaccharide complex, was also eluted from the chromatogram. It contained at least 60% of the original phosphate and was identified as glycerophosphate. By treatment with the alkaline phosphomonoesterase, 100% of the phosphate was liberated and, by further chromatography in the isobutyric solvent, the polyol (R_F 0.60) was obtained. This R_F is characteristic of glycerol or anhydroribitol while ribitol has an R_F of 0.54. This polyol was eluted from the chromatogram and was specifically oxidized with a bacterial glycerol dehydrogenase (glycerol:NAD oxidoreductase, EC 1.1.1.6) in presence of DPN, identifying it as glycerol. The polyol was not oxidized by the ribitol dehydrogenase (ribitol:NAD oxidoreductase) of *Aerobacter aerogenes*. Finally, the ratio of P_i to glycerol, both released by the action of the alkaline phosphomonoesterase upon the NaOH-treated phosphomucopolysaccharide, was 0.9–1. (The NaOH-degraded phosphomucopolysaccharide was incubated with alkaline phosphomonoesterase. Then aliquots containing 20–80 mμmoles of material (measured as phosphate) were added to 500 μl of a 0.13 M carbonate buffer (pH 10). After obtaining the initial absorbancies, 80 μl of a reagent, containing 1 part of the solution of glycerol dehydrogenase (Worthington Biochemical Co.), 1 part of 1 M $(NH_4)_2SO_4$ and 2 parts of 0.05 M DPN at pH 7, were added. The absorbancies of the solutions were read at 340 mμ at intervals during 30 min, at which time the reaction was complete.)

These findings are consistent with two types of structure. The phosphomucopolysaccharide compound could consist of a polysaccharide of glucose and acetylglucosamine with side chains of glycerophosphate attached to the polysaccharide through the phosphate group. Such a structure would be similar to that of the specific polysaccharide of Type-XVIII Pneumococcus[8]. Alternatively, a polysaccharide of

glucose and acetylglucosamine could be linked in some manner to a second polymer, a glycerol phosphate polymer (*i.e.* a teichoic acid) resembling that of *Lactobacillus casei*[9] (but without esterified D-alanine residues). Further work will be needed to decide between these possibilities.

We are indebted to Dr. H. J. FROMM for a sample of ribitol dehydrogenase of *A. aerogenes*.

Bacteriological Service, University of Liège, Liège (Belgium) and Pharmacology Department, Washington University, St. Louis, Mo. (U.S.A.) J. M. GHUYSEN

[1] J. M. GHUYSEN, *Biochim. Biophys. Acta*, 50 (1961) 413.
[2] J. M. GHUYSEN, M. LEYH-BOUILLE AND L. DIERICKX, *Biochim. Biophys. Acta*, 63 (1962) 286.
[3] J. M. GHUYSEN, M. LEYH-BOUILLE AND L. DIERICKX, *Biochim. Biophys. Acta*, 63 (1962) 297.
[4] M. R. J. SALTON AND G. MILHAUD, *Biochim. Biophys. Acta*, 35 (1959) 254.
[5] J. M. GHUYSEN AND J. L. STROMINGER, in the press.
[6] J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.
[7] H. J. FROMM, *Biochim. Biophys. Acta*, 57 (1962) 369.
[8] S. ESTRADA-PARRA, P. A. REBERS AND M. HEIDELBERGER, *Biochemistry*, 6 (1962) 1175.
[9] M. V. KELEMAN AND J. BADDILEY, *Biochem. J.*, 80 (1961) 246.

Received June 20th, 1963

Biochim. Biophys. Acta, 83 (1964) 132–134

SC 83002

Application of mass spectrometry to methylated monosaccharides identification

The standard procedure for the structural analysis of carbohydrate biopolymers, based on methylation, still remains tedious and wasteful, due to the lack of appropriate methods for the identification of methylated monosaccharides. This is despite the fact that the methylation procedure[1,2], and the methods for the separation of methylated sugars which are produced by hydrolysis[3–6], have been considerably improved during the last few years.

We have recently investigated the pathways of fragmentation of α-methyl-2,3,4,6-tetra-*O*-methyl-D-glucoside (I) after electron impact[7]. The structures of the majority of the fragments produced were established on the basis of the mass spectra of α-methyl[2-*O*-*Me*-$^2H_3$]-2,3,4,6-tetra-*O*-methyl-D-glucoside (II), α-methyl[2,3-*di*-*O*-*Me*-$^2H_3$]-2,3,4,6-tetra-*O*-methyl-D-glucoside (III), α-methyl[4,6-*di*-*O*-*Me*-$^2H_3$]-2,3,4,6-tetra-*O*-methyl-D-glucoside (IV) and α-methyl[6-*O*-*Me*-$^2H_3$]-2,3,4,6-tetra-*O*-methyl-D-glucoside (V). The data obtained make it possible to predict the positions of the major peaks in the mass spectra of all the other possible methyl [*Me*-$^2H_3$]-tetra-*O*-methyl- and [*di*-*Me*-$^2H_3$]-tetra-*O*-methyl-glucosides*. The spectra predicted, together with the spectral data measured for I–V are shown in Table I. These data reveal that each of the compounds exhibits a unique mass spectrum, which can thus establish, unequivocally, the position of the $-C^2H_3$ group.

* Obviously the fragmentation pattern of α-methyl[*tri*-*O*-*Me*-$^2H_3$]-tetra-*O*-methyl-D-glucosides must be essentially similar to that of methyl[*O*-*Me*-$^2H_3$]-tetra-*O*-methyl-D-glucosides.