The Occurrence of Muramic Acid in Wax D Preparations of Mycobacteria

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SUMMARY

Acid hydrolysates of wax D preparations from human and bovine strains of *Mycobacterium tuberculosis* were examined by paper chromatography. The amino acids detected in human wax D preparations were alanine, glutamic acid and *meso*- α,ϵ -diaminopimelic acid. In general, amino acids were not found in bovine wax D preparations. Muramic acid was found in all wax D preparations from human strains of mycobacteria but was not detected in wax D preparations from bovine strains. The presence of this typical cell-wall component, as well as the foregoing amino acids, suggests a close relationship between wax D and the cell-wall mucocomplex. The role of muramic acid in the structure of wax D of human strains of mycobacteria is discussed.

INTRODUCTION

The chemical analogy between the water-soluble moiety of wax D and the cell wall of mycobacteria has been suggested (White, Bernstock, Johns & Lederer, 1958; Asselineau & Lederer, 1960; Lederer, 1961*a, b*). The present work was undertaken to determine whether the wax D fraction of mycobacteria represents part of their cell-wall mucocomplex. Following the classical studies on the extraction of lipids from tubercle bacilli by Anderson (1927, 1929) and Anderson & Roberts (1930*a, b*), Aebi, Asselineau & Lederer (1953) isolated wax D of tubercle bacilli from the chloroform-soluble waxes. Wax D prepared from bovine or saprophytic strains of mycobacteria consists in general of mycolic acids esterified to a polysaccharide containing arabinose, galactose and mannose, but without a peptide (Lederer, 1960; Jollès, Samour & Lederer, 1962). Asselineau, Buc, Jollès & Lederer (1958) and Jollès *et al.* (1962) showed that a water-soluble peptido-polysaccharide of Wax D preparations from human strains of mycobacteria contained a hepta-peptide composed of three amino acids (alanine, glutamic acid, α,ϵ -diaminopimelic acid), hexosamines (glucosamine and a smaller amount of galactosamine), and sugars (arabinose, galactose, mannose). Muramic acid was not found although it was 'not excluded that a little muramic acid may be present'.

Muramic acid is an important specific component of the bacterial cell-wall mucopeptide. Salton (1960) showed that the function of muramic acid is to link peptides to other sugars or sugar residues through an amide bond at the carboxyl group of muramic acid. Since wax D preparations from human strains of mycobacteria possess a peptide, hexosamines and a polysaccharide, the demonstration of muramic acid would be of interest in establishing a relation between the wax D

of mycobacteria and their cell walls. The main purpose of the present work was to determine whether muramic acid was present in wax D preparations from mycobacteria.

METHODS

Strains of mycobacteria used. Strain 'C' *Mycobacterium tuberculosis* var. *hominis* was obtained from the Central Veterinary Laboratories, Weybridge. The strains used in the preparation of the wax fractions were *M. tuberculosis* strains Brévannes, Canetti, Test, BCG and Marmorek.

Chemical fractions of mycobacteria. All chemical fractions of mycobacteria were prepared by Professor E. Lederer (Laboratoire de Chimie biologique, Paris) as follows. Mycobacteria were cultivated for 4 weeks on Sauton medium, filtered off, washed with distilled water and extracted several times at room temperature with 5 vol. of a mixture of ethanol + ether (1 + 1, by vol.). After 2 weeks the organisms were filtered off and re-extracted several times with chloroform. The pooled chloroform extracts were extracted with boiling acetone, the insoluble fraction remaining being crude wax D. This was purified by refluxing 2 g. crude wax with 50 ml. acetone for 3 hr., the solvent was filtered off while still hot; after six such extractions the acetone dissolved no more substance. Preparations thus obtained are called purified wax D. Such preparations were obtained from three human strains and two bovine strains of mycobacteria.

Determination of the number of bacilli in wax D preparations and in known weights of organisms. Amounts of dried bacilli of *M. tuberculosis* strain 'C' corresponding to the weights used for hydrolysis were prepared as a suspension in 0.5% Tween 80 and spread evenly over a 1.5 cm. square marked out on a microscope slide. Each film was dried and stained by the Ziehl-Neelsen method. The bacilli in twenty random fields (area 0.0132 sq. mm.) were counted and the number of bacilli per weight of whole organisms hydrolysed was calculated. The same procedure was carried out with 1 mg. samples of the wax D preparations but the suspensions were prepared in chloroform.

Hydrolysis of wax D preparations and whole organisms. Samples were hydrolysed in sealed tubes with 1 ml. 6 N-HCl at 105° for 18 hr. The hydrolysates were filtered to remove insoluble humins, evaporated to dryness over P₂O₅ and KOH in an Edwards centrifugal freeze-drying apparatus and finally resuspended in 0.25 ml. distilled water.

Chromatography. Amino acids were separated by two-dimensional chromatography on Whatman No. 1 paper (10 × 10 in.) in Smith 10 in. Universal chromatants (Shandon Scientific Co. Ltd., London). Two solvent systems were used: (a) butan-1-ol + acetic acid + water (120 + 30 + 50, by vol.) ascending, followed by water-saturated phenol in NH₃ atmosphere ascending (Smith, 1960); (b) the phenol + water solvent ascending followed by lutidine + water (65 + 35, by vol.) either descending or ascending (Cummins & Harris, 1956). Each solvent was run for 17 hr. The chromatograms were dried and the spots located by dipping in ninhydrin in acetone (0.1%, w/v).

Stereoisomers of α,ϵ -diaminopimelic acid (DAP). These were identified by chromatography on Whatman No. 1 paper (45 × 15 cm.) with methanol + water + 10 N-HCl + pyridine (80 + 17.5 + 2.5 + 10, by vol.) ascending. The spots were located by

dipping in ninhydrin in acetone (0.1%, w/v) and heating at 100° for 2 min. (Rhuland, Work, Denman & Hoare, 1955; Hoare & Work, 1957). DAP spots were characteristically olive-green at first, fading to a permanent yellow colour, in contrast to the purple colour of the other amino acids.

Hexosamines. These were identified by one-dimensional descending chromatography on Whatman No. 3 MM paper (57 × 23 cm.) or Schleicher 2043b paper. The solvent used was a modification of the one described by Bourillon & Michon (1959), butan-1-ol + pyridine + water containing 0.4% glacial acetic acid (60 + 35 + 25, by vol.), and was allowed to descend for 36 hr, after which the chromatograms were dried and the hexosamines located with ninhydrin or with the modified Elson & Morgan reaction of Partridge (1948). With the latter method, glucosamine and galactosamine appeared as pink spots and muramic acid as a salmon-pink spot.

RESULTS

The amino acids and hexosamines detected in the wax fractions are shown in Table 1. The hydrolysates of waxes from human strains of mycobacteria contained alanine, glutamic acid and α,ϵ -diaminopimelic acid as major components, with traces of aspartic acid, serine or glycine in some cases. Amino acids were not detected in the hydrolysates prepared from wax fractions of bovine strains of mycobacteria, with the exception of specimen WL 44, the wax D fraction with a high melting point of strain Marmorek, which showed weak spots for alanine, glutamic acid, aspartic acid and glycine. Chromatograms of whole bacilli of *Mycobacterium tuberculosis* strain 'C' revealed thirteen amino acids, with an increase in the intensity of spots proportional to the weight of bacilli hydrolysed. DAP was not present in the same proportion as either alanine or glutamic acid because the former is present only in the cell wall, while the other amino acids are also cytoplasmic components. In all cases where DAP was detected the chromatographic spot corresponded with that of the DD- or *meso*- forms, but was assumed to be *meso*-DAP after the findings of Work (1951). Bacterial counts showed that 85×10^5 whole bacilli were required to give detectable DAP on a chromatogram.

Glucosamine, galactosamine and muramic acid were located on the one-dimensional chromatograms by Partridge's method. Muramic acid was found in eleven wax D preparations from human strains of mycobacteria. Hexosamines were not found in the wax D fractions of bovine strains with the exception of WL 44, the wax D preparation from strain Marmorek, in which glucosamine and galactosamine were detected. During the preparation of this paper it was learned that Professor E. Lederer and Dr P. Jollès (personal communication) had also found muramic acid in wax D preparations by column chromatography.

Since most wax D preparations are contaminated with small amounts of whole mycobacteria, the possibility that this impurity accounted for the presence of muramic acid was considered. This seemed unlikely since the experiments with whole *M. tuberculosis* organisms showed that 26×10^9 bacilli had to be hydrolysed to yield a faint spot for muramic acid on a chromatogram, and at this concentration strong spots for valine, leucine, isoleucine, threonine and lysine were also apparent. In the human mycobacteria wax D preparations muramic acid was detected without the spots for these latter amino acids. Secondly, bacterial counts showed that too

Table 1. *Amino acid and hexosamine composition of wax D preparations from mycobacteria*

Ref. no. of wax sample*	Source and nature of wax D preparation	Wt. wax hydrolysed (mg.)	No. bacilli/ mg. substance	Amino acids†						Hexosamines		
				Alanine	Glutamic	DL-DAP	Aspartic	Serine	Glycine	Muramic acid	Glucosamine	Galactosamine
W. 6	Human strains of mycobacteria	5.0	34 × 10 ³	+	+	+	+	+	+	+	+	+
WL 8	Canetti	3.5	17 × 10 ³	+	+	+	+	+	+	+	+	+
WL 13	Brévannes intermediate fraction	2.1	—	+	+	+	+	+	+	+	+	+
WL 14	Canetti	10.0	68 × 10 ³	+	+	+	+	+	+	+	+	+
WL 16	Test m.p. 217–221°	8.7	17 × 10 ³	+	+	+	+	+	+	+	+	+
WL 19	Test m.p. 220°	8.0	51 × 10 ³	+	+	+	+	+	+	+	+	+
WL 20	Test	6.4	34 × 10 ³	+	+	+	+	+	+	+	+	+
WL 45	Test; chromatographed	2.4	—	+	+	+	+	+	+	+	+	+
WL 52	Canetti	7.1	136 × 10 ³	+	+	+	+	+	+	+	+	+
WL 53	Brévannes; chromatographed	3.6	—	+	+	+	+	+	+	+	+	+
WLT	Lipopolysaccharide strain Test	10.9	None seen	+	+	+	+	+	+	+	+	+
	Bovine strains of mycobacteria											
WL 7	B.C.G.	6.0	—	—	—	—	—	—	—	—	—	—
WL 18	Marmorek; m.p. 47–49°	5.3	170 × 10 ³	—	—	—	—	—	—	—	—	—
WL 21	BCG; m.p. 160–165°	5.0	119 × 10 ³	—	—	—	—	—	—	—	—	—
WL 44	Marmorek; m.p. 212°	5.0	—	+	+	—	+	—	+	—	+	—
WL 60	Marmorek	2.0	68 × 10 ³	—	—	—	—	—	—	—	—	—

* White/Lederer reference numbers; these conform with biological results published elsewhere (White *et al.* 1958).

† Valine, leucine, proline, arginine, not detected.

few bacilli were present in the wax D fractions to yield a detectable spot for muramic acid, and although the contamination of bovine wax D preparations with bacilli was as great as that for human wax D preparations, neither amino acids nor hexosamines were detected.

DISCUSSION

The presence of muramic acid in wax D preparations from human mycobacterial strains as an integral component suggests a close relation with the cell wall of the organism. Analyses of mycobacterial cell walls (Cummins & Harris, 1958) have shown the presence of amino acids (alanine, glutamic acid, *meso*-DAP), hexosamines (glucosamine, muramic acid) and sugars (arabinose, galactose). Thus the demonstration of muramic acid together with alanine, glutamic acid and *meso*-DAP in wax D preparations supports the hypothesis that these waxes are integral components of the cell-wall mucocomplex. Further support for this hypothesis comes from the finding (Jollès *et al.* 1962; White, Jollès, Samour & Lederer, 1964) that the molecular proportions of these amino acids in wax D preparations are within the ranges described for the cell walls of mycobacteria (Belknap, Camien & Dunn, 1961).

Fig. 1. Incorporation of muramic acid into the hypothetical structure of wax D of human strains of mycobacteria.

Asselineau *et al.* (1958) proposed a structure for the wax D of human strains of *Mycobacterium tuberculosis* in which hexosamines were established as the intermediates between the heptapeptide and the polysaccharide. Jollès, Cros & Lederer (1960) postulated that the heptapeptide and polysaccharide were linked by a bond between a carboxyl group of *meso*-DAP and galactosamine, and a glycosidic linkage between galactosamine and arabinose. Asselineau *et al.* (1958) showed that there were no free amino groups in the heptapeptide. However the structure of Jollès *et al.* (1962) incorporating galactosamine as the intermediate hexosamine, would presumably leave two free amino groups. It is possible that the role of muramic acid as a structural component of wax D could be to link the heptapeptide to sugars or sugar residues as it does in the cell wall. If there are no free amino groups it is conceivable that muramic acid is linked to each molecule of *meso*-DAP and a possible structure for wax D of human strains of *M. tuberculosis* might be as shown in Fig. 1.

The relationship of the wax D of bovine mycobacterial strains to the cell-wall mucocomplex requires further explanation. Recently it has been suggested that the cell-wall mucocomplex may be composed of a mixture of polymers of relatively

high and different molecular weights (Rogers, 1963). The wax D of mycobacteria may also consist of a mixture of different sized polymers. On the basis of the structure proposed above the polymer building-blocks would be: (A) muramic acid + heptapeptide, (B) *n*-acetylgalactosamine + *n*-acetylglucosamine, and (C) polysaccharide + mycolic acid. On the basis of the results of the composition of the waxes, wax D of human strains of *M. tuberculosis* would contain the building-blocks A, B and C linked together. The high melting point fraction of the bovine wax D preparation, WL 44, may contain polymer building-blocks B and C linked together and the wax D of bovine strains of *M. tuberculosis* could be polymer building-block C.

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