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Fractionation of wax D, a peptidoglycolipid of *Mycobacterium tuberculosis*

Wax D is a macromolecular glycolipid extractable from *Mycobacterium tuberculosis*[1]. WHITE *et al.*[2] found that wax D of human type *M. tuberculosis* which contains amino acids has a characteristic biological activity, an adjuvant effect, while wax D from the other strains of *M. tuberculosis* lacking amino acid has no such activity. Very recently, JOLLÈS, SAMOUR AND LEDERER[3] reported further fractionation of wax D of human type *M. tuberculosis* by ultracentrifugation into a supernatant fraction designated as Ds (6–10 %) which is free from amino acids, and five other fractions, all of which are sedimentable and contain meso-DAP, Glu, Ala and sometimes Gly.

In the present study, chromatography of wax D on a column of silicic acid or magnesium trisilicate repeatedly proved unsatisfactory. But in the course of chemical modifications of wax D, it was found that acetylated wax D (AD) of human type *M. tuberculosis* ($H_{37}Ra$) could be easily separated by chromatography and thus the heterogeneity of it was confirmed. Further findings were: (1) fractions free from amino acids amounted to 30 %; (2) another 30 % covered fractions containing Glu, Ala and DAP; (3) a fraction of 14 % contained at least six amino acids including two amino acids which have not hitherto been recognized in wax D.

Crude wax D was kindly supplied by Dr. LEDERER of the University of Paris. It was further purified by exhaustive extraction with boiling acetone (15 times, 10 h for each extraction) until the amount of a soluble material was negligible. 2 g of

Abbreviation: DAP, diaminopimelic acid.

wax D thus purified was dissolved in a mixture of 30 ml of dried pyridine and 20 ml of acetic anhydride and the reaction mixture was kept at 28° (bath temperature). After 6 h, infrared spectra showed that acetylation was complete. The reaction mixture was kept for further 6 h and then 250 ml of alcohol was added. The resulting precipitate was washed thoroughly in alcohol and dried. 1.8 g of wax D thus acetylated was dissolved in 100 ml of hexane and the insoluble fraction was treated 6 times in the same manner until no soluble material was obtained. This was designated as hexane-insoluble AD. The hexane-soluble fraction (1.354 g) was put on a column of silicic acid (50 g) and Hyflosuper cel (50 g), and was then separated into six further fractions designated as AD1 (eluted by benzene, yield 6%, m.p. 40°), AD2 (eluted by chloroform–methanol (99:1, v/v) yield 11%, m.p. 63–65°), AD3 (eluted by chloroform–methanol (98:2, v/v), yield 10%, m.p. 170–225°), AD4 (eluted by chloroform–methanol (98:2, v/v) after AD3, yield 9%, m.p. 225°), AD5 (eluted by chloroform–methanol (9:1, v/v), yield 13%, m.p. 250–260°) and AD6 (eluted by chloroform–methanol (8:2, v/v) yield 22%, m.p. 260–270°).

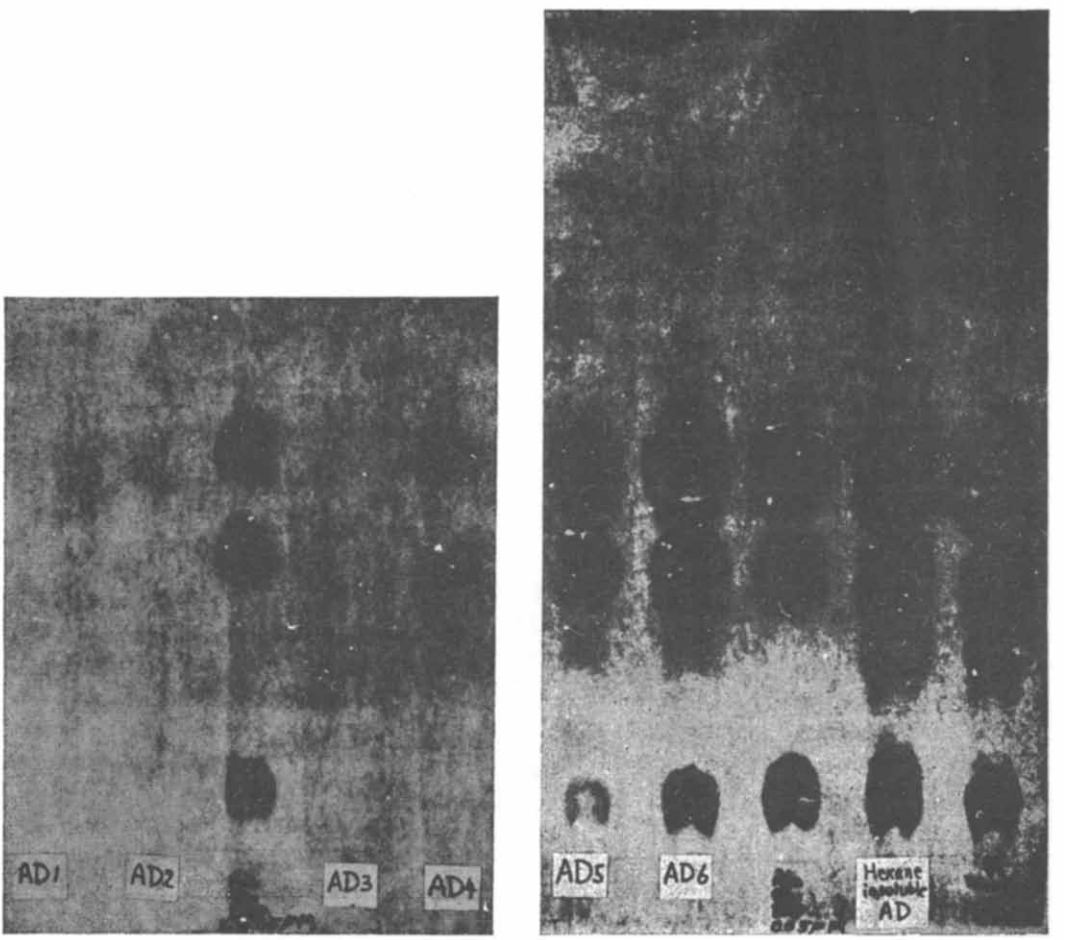

Fig. 1. Paper chromatograms of acid hydrolysates (6 N HCl, 110°, 20 h) of each AD fraction (5 mg). Toyo Roshi No. 50, *n*-butanol–formic acid–water (112:23:15, v/v). Spots were revealed by ninhydrin.

Infrared spectra of AD1, AD2, AD3, and AD4 indicated no absorptions corresponding to the peptide bond, while those of AD5, AD6 and hexane insoluble AD revealed the presence of it at 1670 cm^{-1} and 1540 cm^{-1}. Paper chromatography confirmed the above results (Fig 1).

The paper chromatograms also revealed that the hexane-insoluble AD and AD6 contained a greater amount of amino acids than AD5, a result which accords to that of JOLLÈS *et al.* However, there exists a difference in amount of the fraction lacking amino acids between our findings and those of JOLLÈS *et al.* The reason for this might be that one of the AD fractions lacking amino acids in our experiments corresponds to the Ds fraction of JOLLÈS *et al.* and the other AD fractions lacking amino acids may correspond to those which are sedimentable by ultracentrifugation, thus diluting to some extent the amino acid content of the sedimented fractions.

Cleavage of the amide bond between the peptide and the polysaccharide in wax D by acetylation is not conceivable under the present conditions. The change in weight ratio of the fractions by acetylation should be small, because all fractions are similar, chloroform-soluble, lipids consisting most of mycolic acids and polysaccharides. It is interesting that the paper chromatogram of the acid hydrolysate of the hexane-insoluble AD showed at least 6 ninhydrin-positive spots corresponding to Leu, Phe, Ala, Glu, Gly and DAP, while those of AD5 and AD6 showed only clear spots corresponding to Ala, Glu and DAP (Fig. 1). This finding suggests, firstly, that no correlation exists between Gly and such amino acids as Ala, Glu and DAP which are characteristic of wax D; this might explain the data of JOLLÈS *et al.*, that the fractions separated by ultracentrifugation "sometimes" contain Gly. But secondly, the possibility cannot be excluded that there is contamination by a very small amount of protein occurring in wax D obtained by a usual method. Whether such contamination by a minute amount of protein is possible or not in this chloroform-soluble wax fraction seems important from the view point of the antigenicity of wax D itself, which is a related problem[4,5]. Further investigations are under way along this line, as well as on the chemical properties and biological activities of each AD fraction.

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