Wax D Fraction of an Unclassified 
*Mycobacterium* Strain

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Wax D prepared from the P-6 strain of the scotochromogenic species of *Mycobacterium scrofulaceum* constituted 0.3% of the dry bacilli. In the acid hydrolysates, alanine, glutamic acid, glycine, and mesodiaminopimelic acid were found as amino acid constituents. Mannose, galactose, and glucosamine were detected by paper chromatography. However, arabinose could not be detected. The quantity of hexosamine was 0.2 to 0.3%.

Wax D was found to be adjuvant-active, as revealed by a positive corneal reaction. The antigenicity of wax D of P-6 was shown by the agglutination reaction between the suspension of wax D and the anti-wax D antiserum. Periodate oxidation of the hydrosoluble portion reduced the inhibitory effect of the hydrosoluble portion on agglutination of the suspension of wax D. The light fraction of wax D had a peptide portion consisting of alanine, glutamic acid, glycine, and diaminopimelic acid and was found to be adjuvant-active in an amount of 1,000 µg.

Mycobacterial glycolipids and peptidoglycolipids have been investigated in relation to their biological activities (5, 21). In particular, the elucidation of the complete structure of wax D has been a challenging problem to biochemists. Wax D from human tubercle bacilli, bovine tubercle bacilli (3), and from a very few other species of mycobacteria has been investigated (16, 20, 25). No report has appeared concerning the wax D composition of what are probably the most common mycobacteria, the slow-growing scotochromogens. This is a study of the biochemical and biological properties of wax D from a disease-related strain, P-6, representative of the species *Mycobacterium scrofulaceum*.

MATERIALS AND METHODS

Wax D was prepared from a 5-week-old culture of P-6 (31) in modified Sauton medium as described by Aebi et al. (1). (A scotochromogen was isolated from a patient and sent to us by E. H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah. It was niacin-negative, catalase-positive, unable to bind the dye neutral red, and showed no cord formation. Modified Sauton medium (1 liter) consists of 5.0 g of sodium glutamate, 0.5 g of MgSO4·7 H2O, 1.0 g of sodium citrate, 1.0 g of K2HPO4, 0.05 g of ferric ammonium citrate, 50 ml of glycerol, 3.0 g of vitamin-free acid-hydrated casein, and 950 ml of distilled water. When complete removal of yellow-colored substances was difficult, extraction with a 1:1 mixture of ether and ethyl alcohol was repeated seven to eight times, but no further.

Pentose was determined by the use of orcinol (13), and hexose was determined by the use of anthrone as described by Roe (30). Hexosamine was measured by the method of Elson and Morgan (10). Nitrogen was determined by the micro-Kjeldahl method and phosphorus by the method of Bartlett (8). Ninhydrin-positive substance was measured by the method of Yemm and Cocking (40). The ratio of individual sugar constituents in the hydrolysate (2 N HCl, 110 C, 2 hr) of wax D was determined by the method of Leloir (22).

Chromatography. For paper chromatography, Toyo filter paper No. 51 was used throughout the experiment.

Amino acids. A sample of each fraction was hydrolyzed with 6 N HCl for 24 hr at 110 C in a sealed tube. After removal of the ether-soluble fraction, the hydrolysate was evaporated and chromatographed in an ascending pattern in solvent systems of n-butanol-formic acid-water (112:23:15, v/v) and n-butanol-acetic acid-water (6:2:2, v/v) for 10 hr at room temperature. Detection was made with ninhydrin.

Sugars. A hydrolysate (2 N HCl, 2 hr, 110 C) of each fraction was extracted with ether, and the remaining aqueous layer was dried and chromatographed in a descending pattern in a solvent system of ethyl acetate-acetic acid-formic acid-water (9:1.5:0.5:2, v/v) for 40 hr at room temperature. Detection was made with the Terveleyan reagent (37).
Hexosamine. A hydrolysate (2 N HCl, 4 hr, 110 C) treated as above was chromatographed in a descending pattern with a solvent system of ethyl acetate–pyridine–n-butanol–butyric acid–water (10:10:5:1:5, v/v) for 40 hr at room temperature (27). Detection was made with the Tревелан reagent.

A qualitative test for muramic acid was made on a hydrolysate of wax D (6 N HCl, 18 hr, 110 C), as described by Barker and Summerson (7).

The crude mycolic acid fraction was prepared by the method of Stodola et al. (33). To 100 mg of wax D in 10 ml of benzene was added 8 ml of 5% methanolic KOH. The resulting mixture was heated at 85 C for 80 hr under reflux. The saponified material was dried and taken up in water. After acidification of the aqueous phase, it was repeatedly extracted with ether. The ether-extract was dried in a current of CO2 gas. The above fraction of mycolic acid was loaded on a column of alumina prepared as described by J. Asselineau (Ph.D. Thesis, Univ. of Paris, 1950). Elution was effected with benzene, ether, ether-acetic acid (95:5), and ether-acetic acid (90:10), successively. These four fractions were analyzed by thin-layer chromatography on silica gel plates (20 by 20 cm) impregnated with 2.5% acetic acid in ether in a solvent system of ether-heptane-acetic acid-water (110:110:20, v/v) (9). Detection was made with 1.5% iodine in methanol. The titration equivalents of the two fractions eluted with ether-acetic acid (95:5) and ether-acetic acid (90:10), respectively, were determined with 0.01 N methanolic KOH.

A stereosomer of diaminopimelic acid was identified by treating the hydrolysate of wax D (6 N HCl, 24 hr, 110 C) with acetone powder of Escherichia coli as a source of diaminopimelic acid (DAP)-decarboxylase (14, 29). The resulting enzymatic digest was deproteinized with ethanol alcohol and was analyzed by ascending paper chromatography in a solvent system of methanol-water-10 N HCl-pyridine (80:17.5:2.5:10, v/v).

Acetylation of wax D was carried out as described by Tanaka and Kitagawa (35), and the water-soluble portion of wax D was prepared by the method of J. Asselineau (Ph.D. Thesis, Univ. of Paris, 1950). Ultracentrifugal analysis of wax D was carried out by the method of Jolles et al. (15). Wax D (809 mg) was suspended in 100 ml of ether, and the insoluble residue (designated as DE-152 mg) was removed by centrifugation for 20 min at 3,000 rev/min. The remaining ether-soluble fraction was subjected to ultracentrifugation at 50,000 × g for different periods of time (15, 35, 70, and 150 min), and the fractions obtained were designated by the method of Jolles et al. (Ds, Dp15, Dp35, Dp70, and Dp150 (15)).

The cell wall fraction was prepared as described by K. Fukushi (unpublished data). A suspension of P-6 (50 mg/ml) in 0.25 M sucrose was disrupted in a Ribi Sorvall refrigerated cell fractionator and was then centrifuged at 10,000 × g for 30 min. The residue was suspended in 0.25 M sucrose and centrifuged at 3,000 rev/min to remove unbroken cells and cell debris. The cell wall fraction in the resulting supernatant fluid was negatively stained with phosphotungstic acid and was observed with a Hitachi HU-11 B electron microscope at 75 kv of accelerating voltage.

Experiments on biological properties. The adjuvant activity was tested by means of oral and parenteral injection tests. Guinea pigs weighing about 250 g received an injection into the hind footpad of 0.2 ml of a 1:1 mixture of Dracekle no. 6, Arlacel A, and 0.15 M NaCl containing 2 mg of thrice crystallized ovalbumin and varying amounts of each fraction to be tested (wax D, 1.10, 100 μg; acetylated wax D-10, 100 μg; Ds-100, 500 and 1,000 μg; cell wall fraction-10, 100 μg).

At 3 weeks after the above sensitizing injection, a 0.4% solution of ovalbumin in 0.15 M NaCl was injected intracorneally with a Mantoux needle, as described by White et al. (38). The cornea was observed for turbidity and accompanying reactions after 24 hr.

A quantitative precipitation reaction was made as described by Kabat and Mayer (18). Sera were taken 4 weeks after the sensitizing injection. Protein was determined by the method of Lowry et al. (23). An anti-wax D serum was prepared by seven intravenous injections in rabbits of 3 mg of wax D suspended in 0.3 ml of methyl myristate at intervals of 4 days. Blood was taken 5 days after the last injection.

The anti-P-6 serum was prepared by two intramuscular injections in rabbits of 1 ml of a 50 mg/ml suspension in Dracekle no. 6 of wax D at intervals of 10 days. Blood was taken 1 month after the second injection. To determine agglutination reaction, a suspension of wax D was prepared as described by Choucrour et al. (12). The procedure for determining the agglutination reactions to the above antigens was similar to those described by Tanaka et al. (36); 0.1 ml of the suspension of wax D received an additional 0.5 ml each of the twofold serial dilutions with TME buffer (34) of the antisera. The results were read after incubation at 37 C for 2 hr and at 4 C for 3 days. Passive cutaneous anaphylaxis tests were carried out as described by Ovary (28). A 0.1-ml amount each of the 10-fold, 100-fold and 200-fold dilutions with 0.15 M NaCl of the anti-P-6 and anti-wax D sera, respectively, were injected intracutaneously at different sites of the abdomen of guinea pigs weighing 300 to 400 g.

Amounts (250 and 500 μg) of the hydrosoluble part of wax D in 1.0 ml of a 0.25% solution of Evan's Blue in 0.15 M NaCl were injected by heart puncture. After 15 min, the skin of the abdomen was detached under anesthesia with ether, and the intensity and the extent of the reaction were observed.

Attempts to produce adjuvant arthritis were made by the method of Bonhomme et al. (11), and attempts to produce disseminate encephalitis were made by the method of White et al. (39), using rats of the Donryu strain.

RESULTS

Of the lipid fractions of P-6 extracted as described by Aebi et al. (1), the fraction soluble in a 1:1 mixture of ether and ethyl alcohol constituted 22.1% of the dry P-6; the chloroform-soluble
fraction, 2.9%; the wax B, 0.6%; the wax C, 1.4%; and the wax D, 0.3%.

The melting point of wax D was 40 °C and its \([\alpha_0]^{14} \text{ in chloroform } = +6.7\). Alanine, glutamate, glycine, DAP, and muramic acid were found in the four samples shown in Table 1.

As sugar constituents, only galactose and mannose were found in wax D, Ds, and in the hydrosoluble portion. The mannose to galactose ratio, as determined by the method of Leloir (22), was 6:1. Arabinose was found in the cell wall fraction. However, the presence of arabinose could not be confirmed in the hydrolysate of wax D, Ds, and in the hydrosoluble portion of wax D. Glucosamine was detected in all four samples (Table 1). Galactosamine, on the other hand, was found only in the cell wall fraction. The quantity of the ninhydrin-positive substances and that of hexose were highest in the cell wall fraction. It is to be noted that the quantity of hexosamine was 0.2 to 0.3% for wax D and 0.6 to 0.7% for Ds. The same results were also obtained when alkaline hydrolysis with 5% methanolic KOH for 1 min (17) preceded the acid hydrolysis (2 N HCl, 4 hr, 110 °C).

In the corneal test, a remarkable corneal turbidity accompanied by chemosis was observed in the guinea pigs injected with a mixture of ovalbumin and 100 µg of wax D. The guinea pigs injected with a mixture of ovalbumin with 10 µg of wax D or with 100 µg of acetylated wax D showed weaker reactions (moderate corneal turbidity) than those injected with 100 µg of wax D. In the guinea pigs injected with ovalbumin alone, no corneal turbidity was observed. The cell wall fraction caused moderate corneal turbidity in an amount of 100 µg, and Ds, in an amount of 1,000 µg. However, the results for 500 µg of Ds were indefinite.

The ether-soluble fraction, after saponification by the method of Stodola et al. (33), constituted 72% of wax D. On a thin-layer chromatogram of the fractions eluted from the alumina column, there was a spot with an \(R_f\) value of stearic acid in the fraction eluted with ether. It was also found in the ether-acetic acid (95:5) fraction and in the ether-acetic acid (90:10) fraction. The titration equivalent was 685 for the ether-acetic acid (95:5) fraction and 845 for the ether-acetic acid (90:10) fraction. Infrared spectra of these two fractions are shown in Fig. 1. The ultracentrifugal analysis of 657 mg of the ether-soluble portion of wax D was as follows: Ds, 92% (604 mg); Dp 15, 49.3% (32 mg); Dp 35, 1.2% (8 mg); Dp 70, none; and Dp 150, 20% (13 mg). The quantitative precipitation test demonstrated a marked increase in the anti-ovalbumin level in the sera from animals injected with 1,000 µg of Ds and 100 µg of the cell wall preparation.

The anti-ovalbumin antibody level ranged from 300 to 590 µg of N per ml of serum for 1,000 µg of Ds, and 366 to 435 µg of N per ml of serum for 100 µg of cell wall, as compared to 9 to 73 µg of N per ml of serum in a control group. The results of chemical analysis and animal experiments using Ds were almost identical to those obtained using the cell wall.

By comparison of the paper chromatograms before and after treatment with DAP-decarboxylase by the method of Rhuland et al. (29), it was found that a yellow spot with an \(R_f\) value of DAP disappeared, and a spot with an \(R_f\) value of lysine appeared. Thus, the optical configuration of DAP was identified as the mesoform. The agglutination reaction to a suspension of wax D was observed up to a dilution of 1:32 for anti-P-6 serum and anti-wax D serum, respectively.

This agglutination of the suspension of wax D was inhibited by the addition of 200 µg of the hydrosoluble portion of wax D. On the contrary, agglutination was observed even when 400 µg of the periodate-oxidized hydrosoluble portion was added to the reaction mixture. Thus, it was found that oxidation with periodate caused a loss of the inhibitory effect of the hydrosoluble portion on agglutination of wax D.

Passive cutaneous anaphylaxis (a distinct blue spot) was provoked with 500 µg of the hydro-
Fig. 1. (A) Infrared spectrum of the fraction eluted with ether-acetic acid (95:5) in column chromatography on alumina of the ether-soluble fraction after saponification of wax D. (B) Infrared spectrum of the fraction eluted with ether-acetic acid (90:10) in column chromatography on alumina of the ether-soluble fraction after saponification of wax D.

Fig. 2. Electron micrograph of the cell wall fraction of P-6, negatively stained with phosphotungstic acid. × 18,000.
soluble portion of wax D at the sites of injection of a 100-fold dilution of anti-P-6 serum and of a 50-fold dilution of anti-wax D serum. With 250 µg of the hydrolysable portion of wax D, the reaction (a faint blue spot) was weaker than that observed for 500 µg.

Electron microscopic examination of the cell wall fraction revealed that it consists predominantly of pure cell wall and a small quantity of cytoplasmic material in empty envelopes (Fig. 2). The similarity in the amino acid constituents of wax D to the cell wall was confirmed also in P-6, thus extending the findings of other investigators (19, 26).

**DISCUSSION**

Studies on wax D fractions from various mycobacteria have shown that only those with peptide are adjuvant-active (16, 38, 39). Although a tentative structural formula for a human strain (Brévannes) has been presented by Asselineau (3) with a molecular weight estimated to be 30,000 to 54,000, the complete structure of wax D has not been made clear. Because purification is difficult, a variety of methods has been applied for the preparation of a homogeneous wax D fraction.

Direct chromatographic purification, (3, 4), chromatography of acetylated wax D (35), and enzymatic digestion followed by molecular filtration (24) contributed to partial characterization of wax D.

Jollès et al. (15) applied the technique of ultracentrifugal analysis to wax D. White et al. (39) showed that the Ds fraction of wax D in the supernatant fluid after 150 min of centrifugation at 50,000 × g had no amino acids and was devoid of adjuvant activity, in contrast to the heavy Dp fractions containing amino acids. He showed further that the Ds fraction was the major constituent of wax D in M. phlei, M. avium, and in several other mycobacteria, whereas the quantity of the heavy Dp fractions exceeded that of the Ds fraction in human tubercle bacilli.

In our experiment on P-6, although our method of preparation of wax D differed somewhat from that of Jollès et al., wax D was found to have amino acids and was found to be adjuvant-active in an amount of 100 µg, and Ds in an amount of 1,000 µg. Thus, it is possible that Ds from P-6 represents a very small homogeneous unit of adjuvant-active peptidoglycolipid. This hypothesis was also supported by the fact that Ds was isolated with ether from the column of Sephadex LH-20, whereas the elution of phosphorus-containing impurities was retarded to some extent (unpublished observation).

For the full characterization of wax D and Ds fractions of P-6, however, additional studies are required, using more vigorous criteria for homogeneity.

The unsuccessful attempts to produce disseminated encephalitis and adjuvant arthritis under the present experimental conditions may be ascribed to the fact that the proportion of peptide moiety is too small for wax D to give positive results.

The yield of wax D was as low as 0.3% of the dry P-6. Similar results have been reported for an avian strain (25) and a nonphotochromogen (20). However, if one takes into consideration the influence of age on culture (32) and accepts Andrejew's concept of autonutrition (2), the low yield of wax D may not be a definitive character of P-6 because the yield may vary according to the stage of culture. Despite this, the results of our experiment strongly suggest that wax D from P-6 is quite different to that from the human strains, so far as its proportion of Ds, its yield, its melting point, and its amino acid and sugar constituents are concerned.

Arabinose was not found in wax D of P-6 as in the case of M. smegmatis (32). Probably the very small value of pentose, as determined by the orcinol reaction, is due to a nonspecific reaction common to sugars other than pentose.

As to the mycolic acid fraction, the titration equivalents for the two fractions were lower than those reported by Azuma et al. (6).

These lower values probably resulted from the concomitant presence of fatty acids with a shorter carbon chain length, as shown by thin-layer chromatography.

It is noteworthy that wax D from P-6 exhibited a remarkable adjuvant activity in spite of its low hexosamine content, because only wax D with more than 1% of hexosamine has been reported as adjuvant-active (17). Furthermore, the low nitrogen content in Ds, the positive results of the corneal test, and an increase in the antibody level in the animals injected with ovalbumin and Ds strongly suggest that Ds was isolated as a light adjuvant-active peptidoglycolipid from P-6.

**LITERATURE CITED**


