STRUCTURAL PECULIARITIES OF ACID-FAST BACTERIAL LIPIDS[1]

R. J. ANDERSON

Department of Chemistry, Yale University, New Haven, Connecticut

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I. Introduction

The lipids contained in certain acid-fast bacteria have been under investigation in this laboratory during the past several years. This work has been carried on in coöperation with the Medical Research Committee of the National Tuberculosis Association, with the object of determining first of all the nature of biologically active compounds and their relation to the pathology of tuberculosis in man and animals. Incidentally, it has been our privilege to study the chemical composition of these compounds and to determine the various cleavage products which are liberated on saponification or hydrolysis of the several complex fractions that have been encountered.

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The lipids elaborated by higher plants and animals are relatively simple in chemical structure. For instance, the fats or glycerides are esters of fatty acids with glycerol. The phosphatides are more complex and are represented by lecithin, cephalin, and sphingomyelin. The waxes occurring in plants and animals vary in composition, but by definition they are esters of higher alcohols with higher fatty acids. The nature of the alcohols and the acids may vary, and often the waxes contain mixtures of several such component esters.

The lipids occurring in acid-fast bacteria are built on quite different plans from those found in plants and animals and their composition is more complex. Up to the present time we have examined the lipids contained in the human tubercle bacillus (1), the bovine (18) and avian types (17) of tubercle bacilli, the so-called leprosy bacillus (41), and the non-pathogenic timothy grass bacillus (26). The different strains of bacilli were grown under identical conditions on the Long synthetic medium (31), which has the following composition: asparagine, 5 g.; ammonium citrate, 5 g.; monopotassium phosphate, 3 g.; sodium carbonate, 3 g.; sodium chloride, 2 g.; magnesium sulfate, 1 g.; iron ammonium citrate, 0.05 g.; glycerol, 50 g.; water, 1000 cc.

The cultivation of the bacteria under uniform conditions on the same synthetic medium was regarded as of prime importance in the comparative studies on the composition of the acid-fast bacteria included in these investigations. The different chemical compounds contained in the bacteria would therefore reflect differences in metabolic activity, depending upon inherent variation in the life processes of the bacterial cells. The results of our investigations have demonstrated that every strain produces lipids peculiar to itself and that no two are alike, although in many respects their properties are similar.

It must be admitted that none of these studies is complete, but we believe that a general outline is evident and that the results that we have obtained can be reproduced, but certain reservations are necessary. It has been found (27), for instance, that five different strains of the human tubercle bacillus which were cultivated under identical conditions showed great variation in chemical constants and in the proportions of the various compounds. However, they all contained the same characteristic components although in varying amounts. It is probable, therefore, that every batch of bacteria that is grown will show some variation in the amount and kind of chemical compounds that are elaborated.

II. The Acetone-soluble Fats of Acid-fast Bacteria

The acetone-soluble fats of acid-fast bacteria are mixtures of neutral fat and large amounts of free fatty acids. In addition to ordinary fatty acids

such as palmitic, stearic, oleic, linoleic, etc., the bacterial fats contain certain new and specific fatty acids. The fat of the human tubercle bacillus differs in its component fatty acids from other members of the acid-fast group, in that it contains a series of new and previously unknown acids. Two of these acids,—namely, tuberculostearic acid (8) and phthioic acid (7),—have been obtained in pure form. Tuberculostearic acid (37) has the formula $C_{19}H_{38}O_2$ and its constitution is expressed as 10-methylstearic acid, $CH_3(CH_2)_7CH(CH_3)(CH_2)_8COOH$. It is optically inactive. It melts at 10–11°C. and is an oil at ordinary temperature. Phthioic acid corresponds in composition to a saturated hexacosanoic acid, $C_{26}H_{52}O_2$. It is a thick oil at ordinary temperature and melts at about 20°C. It is dextrorotatory; $[\alpha]_D^{20^\circ} = +12.6^\circ$. The chemical constitution of phthioic acid has not yet been established, but the acid undoubtedly contains a branched chain (38). Other levorotatory acids of unusual constitution are present in the fat, but they have not been obtained in pure form (7).

The fat of the human tubercle bacillus does not contain glycerides. The water-soluble component of this fat was finally identified as the crystalline disaccharide trehalose (13). In analyzing the fats of the avian and bovine tubercle bacilli (23) and of the timothy grass bacillus (34), glycerol could not be found nor could the water-soluble compounds be identified. When these analyses were made, we were under the impression that the fats were glycerides, and attempts were made to purify the water-soluble constituents by distillation *in vacuo*. During the distillation the materials decomposed and carbonized. In no case did any glycerol distill over. Whether these fats contain trehalose will remain a problem for future investigation. However, the fat of the so-called leprosy bacillus does contain trehalose (14).

While the presence of trehalose has been demonstrated by actual isolation in crystalline form only in the fats of the human tubercle bacillus and in the leprosy bacillus, it is not impossible that the same carbohydrate functions as the alcoholic component in the fats of the other acid-fast bacilli. It is a curious fact that organisms growing on a medium in which glycerol is the chief source of carbon synthesize a complex compound such as the disaccharide trehalose, with which fatty acids are combined to form the neutral fat.

III. The Phosphatides of Acid-fast Bacteria

The phosphatides prepared from acid-fast bacteria resemble ordinary phosphatides in solubility (2, 19, 20, 22, 32). They are easily soluble in ether, and they are precipitated from ether solution with acetone. They form colloidal solutions in water. In other respects, however, the bacterial phosphatides differ entirely from the usual phosphatides of plant or animal

origin. The bacterial phosphatides contain from 2.6 to 3.5 per cent of phosphorus and very small amounts of nitrogen. The phosphorus is present in organic combination, partly as glycerophosphoric acid and partly as a phosphorylated polysaccharide or glycoside (12). The nature of the nitrogen compound has not been established, but a part of the nitrogen is present as ammonia. In no case could either choline or aminoethyl alcohol be found.

All of the bacterial phosphatides that we have examined yield on hydrolysis from 33 to 40 per cent of water-soluble compounds. The water-soluble compounds on complete hydrolysis with dilute sulfuric acid yield a small amount of inorganic phosphoric acid and a larger quantity of organic phosphoric acids which are similar in composition to glycerophosphoric acid. In addition to the phosphoric acids mentioned above, the hydrolyzed solutions contain mannose (16), inositol (6), and a hexose which on treatment with phenylhydrazine gives typical glucosazone (21). The water-soluble hydrolysis products of the bacterial phosphatides are therefore very similar.

In the examination of the component fatty acids we found that every phosphatide differed. The saturated fatty acids were represented chiefly by palmitic acid and the unsaturated acid was chiefly oleic acid, because on catalytic reduction stearic acid was always obtained. However, an unsaturated C_{16} acid was present in some cases, particularly in the acids from the phosphatide of the leprosy bacillus. In addition to these common fatty acids, every phosphatide contained representatives of saturated branched-chain acids of higher molecular weight which were oils at room temperature. The liquid saturated acids obtained from the human tubercle bacillus were dextrorotatory, while the liquid saturated acids from the other acid-fast bacteria were optically inactive.

IV. The Waxes of Acid-fast Bacteria

The so-called waxes of the acid-fast bacteria are quantitatively the most important of the ether-soluble constituents of these organisms, and from a chemical point of view they are exceedingly interesting compounds. The term "wax" for these substances is perhaps a misnomer, since they are mainly esters of optically active hydroxy acids with carbohydrates; nevertheless we have retained this term because of previous usage.

The waxes discussed in this section were obtained by extraction with chloroform after the bacteria had been exhaustively extracted with a mixture of alcohol and ether. They were purified by precipitation from ether or chloroform solution by addition of acetone or methyl alcohol until white amorphous powders were obtained. All the waxes that we have analyzed differed in composition with each strain of bacilli and were apparently

mixtures consisting of the above-mentioned esters of hydroxy acids with carbohydrates together with small amounts of true waxes; sometimes glycerides were also present. The presence of true waxes was indicated by the fact that all the waxes so far examined contained higher secondary alcohols. The waxes of the avian tubercle bacillus, the timothy bacillus, and the leprosy bacillus contained the two alcohols *d*-2-eicosanol and *d*-2-octadecanol. The wax of the human and bovine tubercle bacilli contained a different alcohol,—phthiocerol.

The polysaccharide components of the waxes are unusually interesting. The wax of the human tubercle bacillus contains a specific polysaccharide which has not been found in the waxes of the other acid-fast bacteria. On the other hand, the waxes of the avian tubercle bacillus and the timothy bacillus contain the disaccharide trehalose, while the only water-soluble component of the leprosy bacillus wax was glycerol.

A. WAXES FROM THE HUMAN TUBERCLE BACILLUS

Three wax fractions obtained from the human tubercle bacillus have been analyzed. The crude wax obtained by chloroform extraction, as mentioned above, gave on purification two fractions,—(*1*) the purified wax (3) and (*2*) the soft wax (4); fraction 3 was obtained from the mother liquors in the purification of the phosphatide (42). The composition of these fractions is described below.

1. The purified wax

This fraction was a white amorphous powder which melted with decomposition at 200–205°C. It contained 0.41 per cent of phosphorus and 0.77 per cent of nitrogen. The substance is extremely stable toward acids and may be refluxed with aqueous acid or heated in an autoclave at 120°C. for several hours without undergoing any noticeable hydrolysis. However, when it is refluxed with acidified alcohol it is gradually hydrolyzed, with liberation of ether-soluble compounds and water-soluble carbohydrate. While the wax is very resistant to hydrolysis by acid, it is very sensitive to alkali. If the wax is dissolved in benzene and an alcoholic solution of potassium hydroxide is added to the solution at room temperature, a gelatinous precipitate separates almost immediately. The precipitate consists of a complex water-soluble polysaccharide, whereas the ether-soluble constituents remain in the benzene solution.

The polysaccharide of the purified wax. The polysaccharide (36) on purification is obtained as a white amorphous powder which is somewhat hygroscopic. The substance contains phosphorus and nitrogen, and it gives a precipitin reaction with immune horse serum in dilutions up to 1:1,000,000. On hydrolysis with dilute acids, the polysaccharide yields

reducing sugars amounting to about 57 per cent, calculated as glucose. The reducing sugars were separated as hydrazones and identified as mannose, *d*-arabinose, and galactose, while a trace of glucosamine was isolated as the hydrochloride. A trace of inositol was also found, but the bulk of the non-reducing portion of the polysaccharide molecule could not be identified.

The amounts of reducing sugars obtained from the polysaccharide are shown in table 1.

The occurrence of *d*-arabinose in the polysaccharide is a matter of interest, because this pentose is very seldom found in nature, and is an indication of the peculiar metabolic activity of the tubercle bacillus. Heidelberger and Menzel (29) isolated *d*-arabinose among the products of hydrolysis of the specific polysaccharide from the tubercle bacillus and in our work we have found the same sugar, not only in the wax polysaccharides but in the polysaccharide associated with the firmly bound lipids. It is apparent, therefore, that *d*-arabinose is the principal reducing sugar obtained on hydrolysis of all the polysaccharide fractions of the tubercle bacillus that give precipitin reactions with immune serum.

TABLE 1

Reducing sugars from wax polysaccharide

	per cent
Mannose	2.1
d-Arabinose	35.9
Galactose	17.5

The ether-soluble constituents of the purified wax. The ether-soluble compounds obtained after saponification of the purified wax consisted principally of mycolic acid (40), a small amount of lower fatty acids, and the alcohol phthiocerol (39). These components are very firmly combined and are liberated only on prolonged saponification.

Mycolic acid. The principal fatty acid contained in the purified wax was named mycolic acid; it amounted to about 56 per cent of the wax. Mycolic acid has a very high molecular weight; its probable formula is $C_{88}H_{176}O_4$. It contains one hydroxyl group and one methoxyl group to one carboxyl group. Mycolic acid melts at 54–56°C. and has a low dextrorotation, $[\alpha]_D = +1.8°$. It is a saturated compound, since it absorbs no iodine. However, when the acid is treated in chloroform solution with an excess of bromine, varying amounts of bromine are absorbed, apparently by substitution, since hydrobromic acid is liberated. Mycolic acid is acid-fast. It is the only substance that we have found in the tubercle bacillus that possesses the property of acid-fastness, and it is probably the compound which is responsible for the acid-fastness of the bacilli.

When mycolic acid is heated under reduced pressure to a temperature above 280°C., it decomposes with the liberation of normal hexacosanoic acid, $C_{26}H_{52}O_2$, which distills off, leaving a practically colorless, non-volatile, unsaturated residue (5).

When mycolic acid is demethylated by heating with hydriodic acid, and the resulting monoiodonormycolic acid is reduced, hydroxynormycolic acid is formed (30). This new acid on pyrolysis is split in the same manner as mycolic acid. On treatment with more concentrated hydriodic acid, it is possible to replace both the methoxyl and hydroxyl groups with iodine. The diiodonormycolic acid thus formed gives normycolic acid, $C_{87}H_{174}O_2$, on reduction. When normycolic acid is subjected to pyrolysis, it is decarboxylated and no hexacosanoic acid is obtained. It is evident, therefore, that the liberation of *n*-hexacosanoic acid on pyrolysis depends upon the presence of the hydroxyl group in mycolic acid.

2. The soft wax

The fraction designated soft wax (4) was obtained on the evaporation of the mother liquors from the purified wax. After saponification, glycerol was the only water-soluble compound that could be found. The ether-soluble constituents consisted of mycolic acid, certain lower fatty acids, and the alcohol phthiocerol. Judging by these cleavage products the substance was a mixture of glycerides and some true waxes.

3. The wax contained in the alcohol–ether extract

The lipids which are extracted from the tubercle bacillus with alcohol–ether contain, in addition to acetone-soluble fat and phosphatide, a small amount of wax-like material (42). The latter represents a mixture of glycerides, esters of fatty acids with a carbohydrate, and true waxes. Two water-soluble compounds were obtained on saponification,—namely, glycerol and a carbohydrate. The carbohydrate on hydrolysis with dilute acid yielded only mannose and inositol, and thus has a very different composition from the specific polysaccharide contained in the "purified wax."

The fatty acids consisted mainly of mycolic acid, but notable quantities of other fatty acids were present,—namely, *n*-hexacosanoic acid, tuberculostearic acid, phthioic acid, and a levorotatory acid corresponding to the formula $C_{31}H_{62}O_2$. An appreciable quantity of the alcohol phthiocerol was also present.

Phthiocerol. The alcohol phthiocerol was first isolated from the purified wax of the human tubercle bacillus by Stodola and Anderson (39). It has since been found as a constituent of all the wax fractions of the human tubercle bacillus. It also occurs in the wax of the bovine tubercle bacillus,

but it has not been found in any of the other acid-fast bacteria. Phthiocerol crystallizes from ethyl acetate in rosettes of prismatic needles, m.p. 73–74°C., $[\alpha]_D$ in chloroform = −4.8°. Its composition corresponds to the formula $C_{35}H_{72}O_3$, or possibly $C_{36}H_{74}O_3$. It contains two hydroxyl groups and one methoxyl group. The chemical constitution of phthiocerol has not yet been established.

B. THE WAX OF THE BOVINE TUBERCLE BACILLUS

The crude chloroform-soluble wax of the bovine tubercle bacillus, which was isolated by Anderson and Roberts (18), amounted to 8.5 per cent of the dried bacteria. The wax was purified and analyzed by Cason and Anderson (25). The purified wax was a white amorphous powder, having the following properties: m.p. (unsharp), 47–54°C.; $[\alpha]_D$ in benzene, + 15.5°; iodine number, 3.2. It contained 0.30 per cent of phosphorus and a trace of nitrogen. In properties and in chemical composition the wax showed an interesting similarity to the wax fraction described in the preceding paragraph, which had been obtained from the alcohol–ether extract of the human tubercle bacillus (42).

The purified wax yielded the following cleavage products on saponification: glycerol, 1.3 per cent; carbohydrate, 9.0 per cent; crude bovine mycolic acid, 61.0 per cent; lower fatty acids, 19.4 per cent; and unsaponifiable matter, 5.3 per cent.

The carbohydrate was an amorphous white powder which contained 2.2 per cent of phosphorus and a trace of nitrogen. On hydrolysis it gave mannose, inositol, and inositolmonophosphoric acid.

The purified bovine mycolic acid was very similar in properties to the mycolic acid obtained from the wax fractions of the human tubercle bacillus. It melted at 56–58°C.; $[\alpha]_D$ in chloroform = +2.70; molecular weight by titration, 1219. It contained one hydroxyl group and one methoxyl group to one carboxyl group. The bovine mycolic acid, when subjected to pyrolysis at a temperature of 250–300°C., decomposed in the same manner as mycolic acid. The volatile acid that distilled off was identified as *n*-hexacosanoic acid ($C_{26}H_{52}O_2$; m.p., 87–88°C.; molecular weight by titration, 399).

The examination of the lower fatty acids revealed some new interesting acids. The mixed acids were first separated by means of the lead salt–ether procedure into solid and liquid acids. The solid acids were converted into methyl esters, and the esters were fractionated by distillation *in vacuo*. The first ester fraction proved to be methyl palmitate, m.p. 27–28°C., which on saponification gave pure palmitic acid (m.p., 62–63°C.; molecular weight by titration, 252). The next ester fractions were apparently mixtures, but the highest boiling fraction gave on saponifica-

tion an acid which, after repeated recrystallization from acetone, had a constant melting point of 76–77°C. The composition (C, 78.20 per cent; H, 13.12 per cent) and molecular weight (368) are in agreement with the calculated values of a tetracosanoic acid, $C_{24}H_{48}O_2$. However, the acid differed in crystal form from the normal higher fatty acids. It crystallized from acetone in bulky diamond-shaped crystals and from methyl alcohol in boat-shaped needles. The low melting point and unusual crystal form would indicate that the acid possessed a branched-chain structure.

The acids obtained from the ether-soluble lead salts formed a semi-solid mass and had an iodine number of 5. The mixture was subjected to catalytic reduction, after which the lead salt–ether procedure was repeated. A small amount of solid reduced acid was obtained from the ether-insoluble lead salt, but the substance was a mixture from which no pure acid could be isolated. The ether-soluble lead salts yielded a mixture of acids that was converted into methyl esters. The esters on fractionation *in vacuo* gave two principal fractions.

Fraction I was an optically inactive colorless oil, b.p. 112–114°C. at 0.006 mm. After saponification the free acid was obtained as a colorless crystalline mass, m.p. 29–30°C. The analysis gave: C, 75.93 per cent; H, 12.73 per cent; molecular weight by titration, 284.5. These values agree with the calculated composition of stearic acid. The 2,4,6-tribromoanilide was prepared and recrystallized from methyl alcohol, yielding snow-white needles that melted at 96–96.5°C. This new acid is apparently a branched-chain isomer of stearic acid and an analog of tuberculostearic acid.

Fraction II distilled at 172–175°C. at 0.003 mm. and melted at 22–24°C.; $[\alpha]_D = -5.3°$. After the ester had been saponified, the free acid was obtained as a colorless wax-like solid, having the following properties: m.p., 33–34°C.; $[\alpha]_D$, $-3.98°$; molecular weight by titration, 430. This levorotatory acid was apparently a mixture, and no pure tribromoanilide could be obtained from it.

The unsaponifiable matter of the bovine bacillus wax consisted mainly of a crystalline higher alcohol (24) which separated from ethyl acetate in aggregates of colorless prismatic crystals, m.p. 73–74°C., $[\alpha]_D$ in chloroform $= -4.06°$. It contained two hydroxyl groups and one methoxyl group, and its composition corresponded to the formula $C_{35}H_{72}O_3$ or $C_{36}H_{74}O_3$. The above alcohol was identical in composition, crystal form, and properties with the alcohol phthiocerol, which had previously been isolated from the human tubercle bacillus wax. It is an interesting fact, and one that appears to have some significance, that the waxes of the human and bovine tubercle bacilli contain the alcohol phthiocerol, because this alcohol has not been found in the other acid-fast bacteria. Moreover, the

bovine mycolic acid resembles mycolic acid from the human tubercle bacillus and both yield *n*-hexacosanoic acid on pyrolysis. These similarities in chemical constituents of the waxes of the human and bovine tubercle bacilli indicate such a close relation between the two organisms that it sets them apart from other members of the acid-fast groups of bacteria.

The cleavage products of the bovine bacillus wax indicate that the substance was a mixture containing glycerides, fatty acid esters of a special phosphorus-containing carbohydrate, and fatty acid esters of the alcohol phthiocerol. The fatty acids were a complex mixture in which only one ordinary acid,—namely, palmitic acid,—was present. All of the other acids were new and specific compounds. The bovine mycolic acid resembled the mycolic acid of the human tubercle bacillus in properties, but its molecular weight was lower and undoubtedly the two acids differ in chemical constitution, although both gave *n*-hexacosanoic acid on pyrolysis. The wax contained also at least three new fatty acids of lower molecular weight and of unknown constitution: (*a*) a solid tetracosanoic acid, $C_{24}H_{48}O_2$, m.p. 76–77°C., which probably possessed a branched-chain structure; (*b*) a new branched-chain acid, $C_{18}H_{36}O_2$, m.p. 29–30°C., which was optically inactive and the lead salt of which was soluble in ether, (*c*) a mixture of higher levorotatory acids whose lead salts were also easily soluble in ether.

C. THE TIMOTHY BACILLUS WAX

The chloroform-soluble wax was purified by precipitation from ether solution by addition of acetone until a nearly white powder was obtained. The purified wax, as reported by Pangborn and Anderson (33), had the following properties: m.p., 45°C.; ash, 1.7 per cent; phosphorus, 0.29 per cent; nitrogen, 0.41 per cent; iodine number, 20.5; saponification number, 66.9; $[\alpha]_D$ in chloroform, +15.1°. After saponification the following cleavage products were isolated: glycerol, 3.0 per cent; carbohydrate, 4.6 per cent; mixed fatty acids, 68.0 per cent; neutral material, 13.0 per cent. The nature of the phosphorus and nitrogen components could not be determined.

The glycerol was identified by preparing the crystalline tribenzoyl derivative, which melted at 75–76°C. The carbohydrate fraction could not be crystallized directly, probably owing to small quantities of impurity, but after acetylation a crystalline acetyl derivative was obtained which was identified as trehalose octaacetate. This was the first time that the disaccharide trehalose had been found as a constituent of a bacterial wax.

In the examination of the fatty acids none of the ordinary acids could be found. The acids were apparently unsaturated, optically active hydroxy acids of high molecular weight, but since they did not crystallize but separated as amorphous white powders the degree of purification attained

was uncertain. What appeared to be the purest fraction corresponded in composition to an acid having the formula $C_{70}H_{138}O_6$. It was probably a dibasic acid, because the molecular weight found by titration was 518 but the Rast method gave a value of 1000. On acetylation only one hydroxyl group could be demonstrated. No methoxyl could be found. The acid melted at 56–57°C.; $[\alpha]_D$ in chloroform = +6.1°; iodine number = 15.2.

The unsaponifiable or neutral material was found to contain two new interesting crystalline secondary alcohols, *d*-2-eicosanol and *d*-2-octadecanol, which were optically active. They were the first higher alcohols to be isolated and identified as constituents of acid-fast bacterial waxes and whose chemical composition and constitution could be established.

The composition of the alcohols indicated the formulas $C_{20}H_{42}O$ and $C_{18}H_{38}O$, and on oxidation with chromic acid the corresponding ketones

TABLE 2

Properties of the two new alcohols (and their derivatives) isolated from the timothy bacillus wax

ALCOHOLS AND DERIVATIVES	MELTING POINT	$[\alpha]_D$ IN $CHCl_3$	KETONES AND DERIVATIVES	MELTING POINT
	°C.			°C.
d-2-Eicosanol	62–63	+4.2°	2-Eicosanone	58–59
Acetyl derivative	35–37	+1.5°	Semicarbazone	128
Benzoyl derivative	39–40		Oxime	73–74
Phenylurethan	78–78.5			
d-2-Octadecanol	56	+5.7°	2-Octadecanone	52
Phenylurethan	72–73	+7.9°	Semicarbazone	127.5

were obtained in crystalline form and in nearly quantitative yield. The ketones were identified as 2-eicosanone, $CH_3(CH_2)_{17}COCH_3$, and 2-octadecanone, $CH_3(CH_2)_{15}COCH_3$. Accordingly, the alcohols possessed the following constitutions: *d*-2-eicosanol, $CH_3(CH_2)_{17}CHOHCH_3$; *d*-2-octadecanol, $CH_3(CH_2)_{15}CHOHCH_3$.

The properties of the alcohols and ketones and their derivatives are shown in table 2.

Considering the nature of the saponification products obtained, it seems evident that the timothy bacillus wax was composed of a complex mixture of solid glycerides, fatty acid esters of the disaccharide trehalose, and esters of *d*-2-eicosanol and *d*-2-octadecanol. The only previously known components were glycerol and trehalose.

D. THE AVIAN TUBERCLE BACILLUS WAX

The chloroform-soluble wax from the avian tubercle bacillus isolated by Anderson and Roberts (17) was purified and studied by Reeves and Anderson (35). The crude wax was purified by precipitation from ether

and chloroform by addition of methyl alcohol, and two purified fractions were obtained as white amorphous powders. The properties of the two fractions and their saponification products are given in table 3.

The main differences between the two fractions were in the magnitude of the optical rotation and in the percentage of carbohydrate. Fraction I had the highest rotation and the highest per cent of carbohydrate. Glycerol was absent from both fractions.

The fatty acids consisted mainly of optically active hydroxy acids of high molecular weight, which will be mentioned more fully below. The small amount of lower fatty acids evidently represented a mixture from which no pure acid could be isolated. The ordinary straight-chain fatty acids were entirely absent.

TABLE 3

Properties of the purified wax of the avian tubercle bacillus and products formed upon its saponification

	FRACTION I	FRACTION II
Properties of the wax:		
Melting point, °C	54–55	53–55
$[\alpha]_D$ in chloroform	+38.6°	+17.7°
Iodine number	4.5	8.7
Products formed upon saponification of the wax:		
Carbohydrate, per cent	12.3	5.6
Hydroxy acids, per cent	81.0	84.8
Lower fatty acids, per cent	2.4	6.8
Unsaponifiable matter, per cent	9.9	9.0

1. Identification of trehalose

The carbohydrate component remained as an insoluble mass in the reaction flask after the wax had been saponified with alcoholic potassium hydroxide. It was dissolved in water, and a slight precipitate which appeared on acidification with acetic acid was removed by filtration. The addition of lead acetate gave a slight precipitate, which was removed by filtration and discarded. The carbohydrate contained in the filtrate was precipitated with basic lead acetate and ammonia. The lead salt was filtered off, washed with dilute ammonia, suspended in water and decomposed with hydrogen sulfide. After removing the lead sulfide, the filtrate was concentrated to a thick syrup, and the latter was dehydrated by grinding in a mortar under absolute alcohol until a fine white powder was produced. The substance gave no color reactions for pentose, and it did not reduce Fehling's solution until it had been boiled for some time with dilute acid. The acetyl derivative was prepared and on recrystalliza-

tion from methyl alcohol was obtained as colorless prismatic needles, m.p. 97–98°C., $[\alpha]_D$ in chloroform = +163.7°. The acetic acid liberated on saponification was 70.6 per cent. The properties identify the substance as trehalose octaacetate. Trehalose itself was isolated, after the acetyl derivative had been saponified, and was crystallized from 80 per cent alcohol. Large colorless prismatic crystals were obtained, m.p. 98°C., $[\alpha]_D$ in water = +178°. The crystals lost 9.85 per cent in weight on drying, corresponding to two molecules of water of crystallization. The properties of the sugar were in complete agreement with those of the disaccharide trehalose.

2. The unsaponifiable matter

The unsaponifiable matter consisted mainly of *d*-2-eicosanol, but a small amount of *d*-2-octadecanol was also found. The two alcohols were identical in composition and properties with the alcohols first isolated from the timothy bacillus wax, and on oxidation with chromic acid the corresponding ketones, 2-eicosanone and 2-octadecanone, were obtained and identified.

3. The hydroxy acids

The hydroxy acids did not crystallize but separated as colorless fine globular particles. Attempts to purify the acids or their acetyl derivatives were not successful, but the results indicated that a mixture of acids was present. Fractionation of the potassium salts of the mixed acids resulted in the isolation of two acids that differed in composition and properties. The potassium salts were insoluble in alcohol but easily soluble in ligroin. However, on extraction of the ligroin solution with methyl alcohol a portion of the potassium salt went into the alcoholic layer. By repeating the process several times with each fraction, it was possible to obtain two acids whose properties did not change on further purification. The salt contained in the alcoholic solution gave an acid having a molecular weight of about 500. This acid was later designated as avian α-mycolic acid (9). The ligroin solution contained the salt of an acid which has a molecular weight of about 1300 and was called avian β-mycolic acid (9). The acids had low iodine numbers but when treated with an excess of bromine they gave bromo derivatives through substitution with liberation of hydrobromic acid. Both acids were acid-fast.

The properties of the acids and of some derivatives are given in table 4.

The avian mycolic acids when subjected to pyrolysis under reduced pressure were split in the same manner as mycolic acid, yielding volatile crystalline acids and leaving nearly colorless non-volatile residues. Avian α-mycolic acid on heating to 210°C. gave a volatile crystalline acid in a

yield of 25.4 per cent. This acid crystallized in branching feathery forms and was probably a branched-chain pentacosanoic acid, $C_{25}H_{50}O_2$.

The avian β-mycolic acid when heated under the same conditions to a temperature of 280°C. gave a volatile acid in a yield of 21 per cent. This acid crystallized in thin colorless plates similar to the usual crystal form of the higher normal fatty acids and its composition corresponded to tetracosanoic acid, $C_{24}H_{48}O_2$.

TABLE 4

Properties of the avian wax acids

	AVIAN α-MYCOLIC ACID	AVIAN β-MYCOLIC ACID
Melting point, °C	69–70	60–61
$[\alpha]_D$ in chloroform	+5.6°	+5.5°
Analysis, per cent	C, 78.99; H, 12.78	C, 82.46; H, 13.49
Molecular weight by titration	501–520	1280–1300
Acetyl derivative, m.p., °C	54–55	48–57
Methyl ester, m.p., °C	54–55	49–50
Active hydrogen of ester, per cent	0.92	0.82
Bromo derivative, m.p., °C	47–49	43–49
Bromo derivative, per cent bromine	22.4	22.9
Iodine number, Hanus	6.5	5.5

TABLE 5

Properties of the volatile acids obtained by pyrolysis of the avian mycolic acids

PROPERTIES	AVIAN α-MYCOLIC ACID	AVIAN β-MYCOLIC ACID
Melting point, °C	78–79	83
Carbon, per cent	78.67	78.30
Hydrogen, per cent	13.23	13.05
Molecular weight by titration	380	368
Formula	$C_{25}H_{50}O_2$	$C_{24}H_{48}O_2$

The properties of the acids obtained on pyrolysis of the avian mycolic acids are given in table 5. The acids produced by pyrolysis were undoubtedly homogeneous, because the melting points of the top fractions were identical with those of the acids recovered from the mother liquors.

Attention is called to the fact that there is an interesting similarity in the components of the wax of the avian tubercle bacillus and of the timothy bacillus wax. From both waxes the disaccharide trehalose was obtained and they also contained the same higher alcohols,—namely,

d-2-eicosanol and *d*-2-octadecanol. The hydroxy acids also possessed similar properties but they were not identical. Neither wax contained any of the ordinary straight-chain normal fatty acids.

E. LEPROSIN

In the purification of the phosphatide of the so-called leprosy bacillus as described by Uyei and Anderson (41), a considerable amount of wax-like material was recovered from the mother liquors. This material, which was called leprosin, was purified and its composition was studied by Anderson, Crowder, Newman, and Stodola (11). Purified leprosin was a white amorphous powder, having the following properties: m.p., 50–51°C.; $[\alpha]_D$ in chloroform, +4.0°; iodine number, 5. Analysis showed that it contained only carbon, hydrogen, and oxygen.

The following products were obtained on saponification of leprosin: glycerol, 6.0 per cent; mixed fatty acids, 93.0 per cent; and unsaponifiable matter, 7.0 per cent. The only water-soluble constituent that could be found was glycerol, and it was identified by means of the tribenzoyl derivative.

The fatty acids were composed of a complex mixture in which at least six different acids were present. The ordinary normal fatty acids were represented by myristic, palmitic, stearic, and tetracosanoic acids. There was also present a notable amount of an acid which appeared to be a tricosanoic acid.

The most interesting component fatty acid was a new optically active hydroxy acid named leprosinic acid, which amounted to about 20 per cent of leprosin. The properties of leprosinic acid were similar to those of other hydroxy acids which we have found in the bacterial waxes, and it also was acid-fast. It melted at 62–63°C.; $[\alpha]_D$ in chloroform = +4.0°; iodine number = 6. The exact formula could not be established, but the simplest formula calculated from the analytical values was $C_{44}H_{88}O_3$. It is probable, however, that leprosinic acid is a dibasic acid, and the above formula should therefore be doubled.

The unsaponifiable matter consisted largely of the secondary alcohol *d*-2-eicosanol, but a small amount of *d*-2-octadecanol was also present.

It is evident from the cleavage products obtained on saponification that leprosin is a mixture of solid glycerides and esters of fatty acids with the two higher alcohols mentioned above. Leprosin is the only wax-like substance obtained from acid-fast bacteria—except the soft wax of the human tubercle bacillus—that we have examined that was free from carbohydrate. It is noteworthy that leprosin contained such a large proportion of normal fatty acids, from myristic to tetracosanoic acid. The phosphatide and the acetone-soluble fat of the leprosy bacillus like-

wise contained a large proportion of normal fatty acids,—acids which occur to but a small extent in the lipid fractions of the other acid-fast bacteria. However, the presence of the hydroxy acid, leprosinic acid, and the secondary alcohols *d*-2-eicosanol and *d*-2-octadecanol indicates a family resemblance of the leprosy bacillus to some other members of the acid-fast group of bacteria, notably to the timothy grass bacillus and to the avian tubercle bacillus.

V. The Firmly Bound Lipids

The so-called firmly bound lipid of the acid-fast bacteria is so strongly combined in the cellular structure that it can not be removed by extraction with neutral solvents. The bacterial residues, after exhaustive extraction at room temperature with a mixture of equal parts of alcohol and ether, followed by extraction with chloroform, contain only traces of lipid that is soluble in neutral solvents. However, after the bacterial residues have been treated with a mixture of alcohol and ether containing 1 per cent of hydrochloric acid, they will yield from 8 to 19 per cent of additional lipids on extraction with ether and chloroform. The greater portion of the bound lipids can be obtained as white amorphous powders by precipitation from ether or chloroform solutions by addition of alcohol. The mother liquors yield small quantities of soft salve-like material. The solid products are easily soluble in ether, chloroform, benzene, and ligroin, but nearly insoluble in alcohol or acetone.

So far only the solid purified fractions obtained from the human (15) and avian (10) tubercle bacilli and from the leprosy bacillus (28) have been examined in some detail. While the solid fractions of the firmly bound lipids are easily soluble in ether or chloroform, it has been found that, when such solutions are forced through a Chamberland filter, only a part of the dissolved material will pass through the filter. We have thus obtained filterable and unfilterable fractions. However, solutions of the firmly bound lipid of the leprosy bacillus passed completely through the Chamberland filter.

The results of the analyses of the firmly bound lipids are summarized in table 6. It will be noted that the unfilterable lipids contained twice as much polysaccharide as the filterable fractions.

The cleavage products obtained from the firmly bound lipids on saponification indicate that the substances were principally esters of hydroxy acids with polysaccharides. The filterable bound lipids from the human tubercle bacillus contained, however, a notable quantity of lower fatty acids, among which tuberculostearic acid was identified but from which phthioic acid was absent.

The hydroxy acid from both the unfilterable and the filterable fractions

of the bound lipids of the human tubercle bacillus appeared to be identical with mycolic acid. The alcohol phthiocerol, which occurs in the wax fractions, was entirely absent in the bound lipids. The polysaccharide was similar in composition to the polysaccharide contained in the purified wax (36).

The hydroxy acid contained in the bound lipids of the avian tubercle bacillus differed in properties from avian α- and β-mycolic acids and was called avian γ-mycolic acid. On pyrolysis it decomposed with the liberation of 18 per cent of a crystalline acid that distilled off. The volatile

TABLE 6

Percentage composition of firmly bound lipids

	UNFILTERABLE		FILTERABLE		
	Human	Avian	Human	Avian	Leprosy
Firmly bound lipid	4.7	2.68	7.5	8.16	19.5
Polysaccharide	50.5	31.3	25.5	15.0	40.5
Glycerol	None	None	2.0	None	None
Hydroxy acids	51.0	52.8	41.1	69.0	56.3
Lower fatty acids	4.0	3.8	28.4	3.7	4.3
Neutral material	None	10.6	0.8	8.2	5.5
Percentage composition of the polysaccharides					
Precipitin reaction	1:1,000,000	1:500,000			1:2,000,000
Phosphorus, per cent	0.38	1.04			None
Nitrogen, per cent	0.95	1.81			Trace
Reducing sugar on hydrolysis, per cent	57.0	50.1			50.5
Mannose, per cent	6.6	27.3			None
d-Arabinose, per cent	38.7	19.9			41.4
Galactose, per cent	12.2	3.5			1.0
Inositol	Present	Present			None
Glucosamine	Present	Present			None

acid corresponded in composition to a tetracosanoic acid, $C_{24}H_{48}O_2$, which probably had a branched-chain structure. The neutral material contained the two secondary alcohols *d*-2-eicosanol and *d*-2-octadecanol.

The polysaccharide component gave a precipitin reaction with immune serum. On hydrolysis, reducing sugars were liberated and identified as mannose, *d*-arabinose, and galactose. The composition of the polysaccharide was therefore strikingly different from that of the carbohydrate contained in the wax fraction, which consisted of the disaccharide trehalose.

The hydroxy acid contained in the bound lipids of the leprosy bacillus appeared to be identical in properties with leprosinic acid (11). On pyrol-

ysis it decomposed in the same manner as the mycolic acids. The volatile acid that distilled off was apparently a new branched-chain tetracosanoic acid. The neutral material contained the same alcohols as were present in leprosin,—namely, *d*-2-eicosanol and *d*-2-octadecanol.

The polysaccharide gave a high precipitin reaction with immune serum, but it differed in composition from all the polysaccharides that we have found in the wax fractions of acid-fast bacilli. On hydrolysis, about 50 per cent of reducing sugars were liberated and 41 per cent of the polysaccharide was identified as *d*-arabinose. The presence of some other pentose was indicated, but it could not be identified. The only other reducing sugar that could be isolated was galactose. No mannose could be found.

All of the bacterial polysaccharides that we have studied,—from the purified wax of the human tubercle bacillus as well as from the above-mentioned firmly bound lipids,—give about 50 per cent of reducing sugars on hydrolysis. We have been able to account for practically all of the reducing sugars as mannose, *d*-arabinose, and galactose by actual isolation by means of appropriate hydrazones. Unfortunately it has not been possible to identify the non-reducing portion of the polysaccharides.

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