

# CHEMISTRY AND BIOCHEMISTRY OF SOME BIOLOGICALLY ACTIVE BACTERIAL LIPIDS

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## INTRODUCTION

Work, which was begun in the author's laboratory in 1948, on the chemistry of various lipid fractions of Mycobacteria and other acid-fast micro-organisms, has led to the identification and elucidation of the chemistry of a number of compounds having unusual structures and interesting biological activities. The principal compounds studied are branched-chain high molecular weight fatty acids, glycolipids and peptido-glycolipids. Some information on biosynthetic mechanisms in these series has also been obtained.

The infection of a healthy animal or human being by tubercle bacilli gives rise to several pathological manifestations, such as: (i) formation of tubercles; (ii) establishment of a delayed type of hypersensitivity; (iii) loss of weight, haemorrhages, and death, due to the presence of a toxic factor; (iv) increased production of antibodies; (v) development of resistance to a second infection.

We shall see that all of these pathological phenomena are due to particular lipid fractions of the tubercle bacillus. Besides these, we shall consider also a new category of glycolipids which are typical for certain strains of Mycobacteria (see *Table I*).

*Table I.* Correlation of biological activity and chemical structure

<i>Pathological phenomenon</i>	<i>Chemical structure of the corresponding lipid</i>
Formation of tubercles Delayed hypersensitivity Loss of weight, haemorrhages, death Increased production of antibodies	Branched-chain fatty acids Esters of mycolic acids with carbohydrates Cord factor (6,6'-dimycoloyl-trehalose) Wax D of human strains (a peptido-glycolipid)
Resistance to infection	Phosphoglycolipids (?)
Specific pathogenicity (?)	Mycoside A and B (glycolipids) Mycoside C (a peptido-glycolipid)

## TUBERCLE FORMATION

### Branched-chain fatty acids

Branched-chain fatty acids and their derivatives seem to be the principal compounds producing the characteristic tubercles observed in tuberculosis.

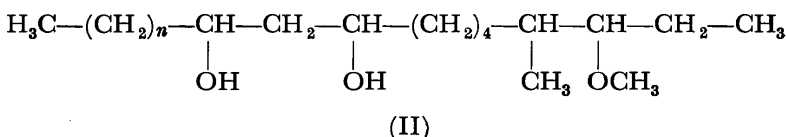
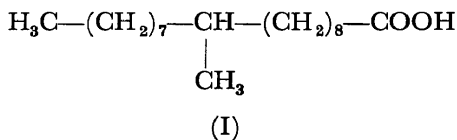
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The excellent work of Anderson<sup>1</sup>, published between 1926 and 1946, has shown that Mycobacteria contain several branched-chain fatty acids. One can distinguish two large groups: (i) compounds having only methyl branchings; (ii) compounds with longer branched chains.

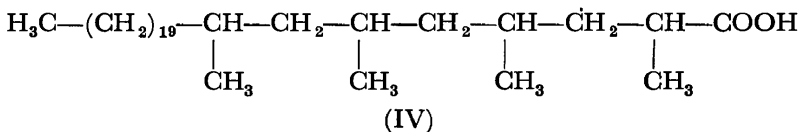
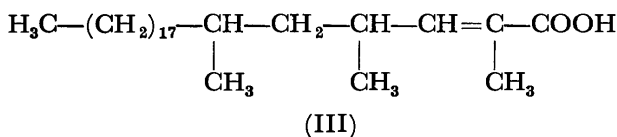
*Compounds with methyl side-chains*

In this group, we find compounds having only one methyl side-chain, such as tuberculostearic acid (I) and phthiocerol (II)<sup>2</sup>, and those having several methyl branchings, such as phthienoic acid (III)<sup>3</sup> and mycocerosic acid (IV)<sup>4</sup>.



(a)  $n = 20$

(b)  $n = 22$



It seems that compounds having only one methyl branching, such as (I) and (II), are devoid of biological activity, whereas those with three or more methyl branchings, such as (III) and (IV), are potent producers of tubercles (see the review by Asselineau<sup>5</sup>).

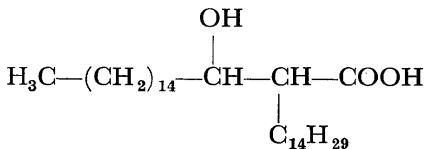
The structural determination of phthienoic and mycocerosic acids is due to Polgar, Cason, J. and C. Asselineau, and S. and E. Stenhagen, and cannot be described in detail here (for recent reviews, see refs. 6 and 7).

Two different mechanisms of biosynthesis can be considered for the fatty acids with methyl side-chains: the "Birch mechanism"<sup>8</sup>, consisting of the fixation of a methyl group (from methionine) onto a polyacetic acid chain\*

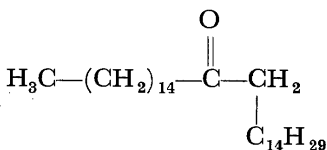
\* Hofman and Liu<sup>9</sup> have shown that the cyclopropane ring of lactobacillic acid is formed *in vivo* by the fixation of a one carbon unit derived from formate onto *cis*-vaccenic acid; the fixation of methyl groups on aliphatic chains by this mechanism does not seem to have been observed yet.



has been isolated from the lipids of *C. diphtheriae* and *C. ovis*. Its structure (VI) has been established by degradation<sup>19</sup> and confirmed by synthesis<sup>23</sup>.



(VI)



(VII)

The presence of free palmitic acid and of palmitone in the lipids of *C. diphtheriae*<sup>25</sup>, as well as previous work on the mycolic acids of Mycobacteria, suggested that corynomycolic acid is formed *in vivo* by the condensation of two molecules of palmitic acid (probably as palmitoyl-coenzyme A) and reduction of the intermediate  $\beta$ -ketoester. Palmitone (VII) could be formed through saponification and decarboxylation of the  $\beta$ -ketoester.

Recent experiments of Gastambide-Odier<sup>24</sup> with 1-<sup>14</sup>C-palmitic acid have confirmed this hypothesis, and have shown that corynomycolic acid is, in fact, formed *in vivo* by the condensation of two molecules of palmitic acid.

Cultures of diphtheria bacilli (*C. diphtheriae*, strain Parke-Williams 8) were grown on Loiseau-Philippe medium containing 10  $\mu\text{C}$  of the potassium salt of 1-<sup>14</sup>C-palmitic acid (specific activity  $(43.1 \pm 6.0) \times 10^7$  counts  $\text{min}^{-1}$   $\text{mmole}^{-1}$ ). After 12 days at 37°, the cells were centrifuged, washed, and extracted with alcohol-ether (1:1).

Corynomycolic acid was isolated in a pure state by chromatography on magnesium silicate-celite, esterification, distillation under reduced pressure, and finally chromatography of the methyl ester on alumina; after several subsequent stages of purification, 0.4 mg of pure methyl corynomycolate (m.p. 59–62.5° and specific activity  $44.7 \times 10^8$  counts  $\text{min}^{-1}$   $\text{mmole}^{-1}$ ) were isolated. This represents about 3 per cent of the total radioactivity added with the potassium palmitate.

If corynomycolic acid is synthesized *in vivo* from two molecules of palmitic acid, then, in these experiments with carboxyl-labelled palmitic acid, only the C-1 and C-3 atoms should be labelled, and each should contain half of the radioactivity. This was proved to be the case experimentally, the reactions involved being represented schematically as follows:



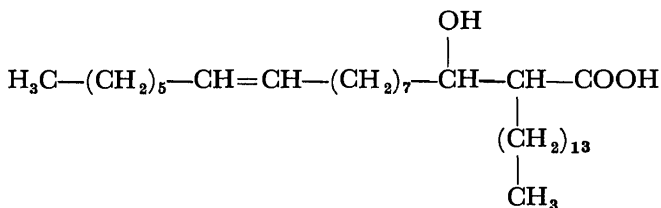
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acid (X) having the same specific activity ( $(12.5 \pm 3.4) \times 10^4$ ) as the palmitone; decarboxylation of this palmitic acid then gave again an inactive amine (XII) and barium carbonate having the same radioactivity as the initial sample of palmitone (VII).

This proves that, in the corynomycolic acid formed *in vivo* in the presence of 1- $^{14}\text{C}$ -palmitic acid, only the C-1 and C-3 atoms are radioactive; this can only be explained by the condensation of two intact molecules of palmitic acid; otherwise, if the palmitic acid had undergone an initial degradation, radioactivity would have been "smeared out" over the whole molecule.

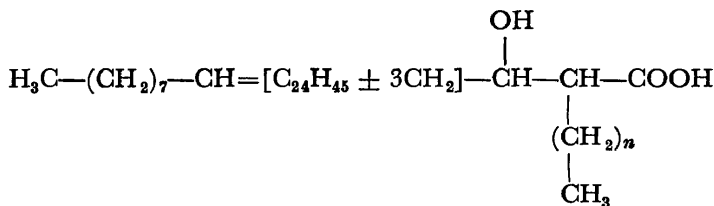
It can be surmised that one of the molecules of palmitoyl-CoA has to be carboxylated to tetradecylmalonyl-CoA. The detailed mechanism of the condensation reaction has not yet been studied.

Corynomycolenic acid (XIII), the second  $\text{C}_{32}$ -mycolic acid isolated from *C. diphtheriae*<sup>20</sup>, is obviously formed from one molecule of palmitoleic acid,  $\text{CH}_3-(\text{CH}_2)_5-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$  (which also exists in large quantities in the free state in the diphtheria bacillus), and one molecule of palmitic acid. Here, an intermediary  $\beta$ -keto-ester could give a ketone,  $\Delta^7$ -palmitenone,  $\text{CH}_3-(\text{CH}_2)_5-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COC}_{15}\text{H}_{31}$ , on saponification; this has also been isolated from the lipids of *C. diphtheriae*<sup>25</sup>.



(XIII)

The nocardic acids,  $\text{C}_{50}\text{H}_{98}\text{O}_3 \pm 3\text{CH}_2$ , have been isolated recently from the pathogenic Actinomycete *Nocardia asteroides*, and the partial structure (XIV) established (Michel *et al.*<sup>21</sup>); they are probably synthesized *in vivo* by the condensation of three molecules of long chain ( $\text{C}_{16}$ ?) acids in a similar way.



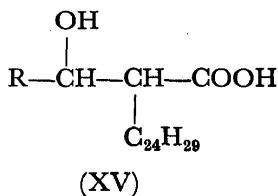
(XIV)

(a)  $n = 11$

(b)  $n = 13$

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Mycolic acids are typical constituents of Mycobacteria, and were discovered in 1938 by Lesuk and Anderson<sup>26</sup>. Detailed studies of their chemical structure, pursued in our laboratory principally by J. Asselineau, have yielded the following results: mycolic acids of human and bovine strains of *M. tuberculosis* have the approximate formula  $C_{38}H_{176}O_4 \pm 5 CH_2$  and the general structure (XV):

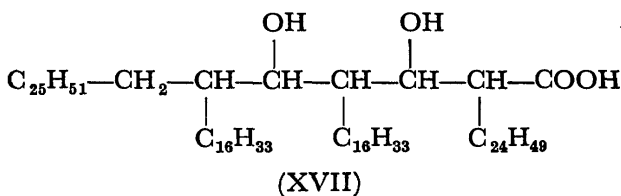
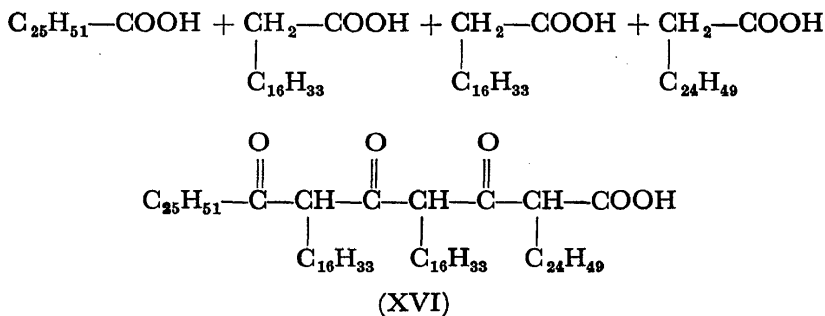


where R is a radical containing about 60 carbon atoms with one oxygen function (OH, or  $OCH_3$  or a carbonyl) and three chains.

Extensive degradative evidence has shown that in some of these mycolic acids the second oxygen function is either on C-5 or further away from the carboxyl, and that C-4 or probably C-4 and C-6 carry side-chains<sup>6, 22</sup>.

As to the length of the various side chains, it is known that at least one of these (besides that at C- $\alpha$ ) has 24 to 26 carbon atoms, and that the others are shorter ( $C_{16}$  to  $C_{18}$ ).

All experimental evidence is in agreement with formulae (XVII) and (XVIII), which could result from the condensation of four molecules of long-chain fatty acids ( $2 \times 26 + 2 \times 18 = 88$ ) by the mechanism which we have proven for corynomycolic acid. An appropriate reduction of the intermediate (quite hypothetical) triketo-compound (XVI) gives the two types of mycolic acids (XVII) and (XVIII). These structures are in agreement with all experimental findings<sup>27</sup>.







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normal fatty acid synthesis stops at  $C_{26}$ , branched acids with several methyl groups have 21 to 34 carbon atoms, whereas mycolic acids have 32 to 88 carbon atoms.

Mycolic acids, as well as their esters with carbohydrates, are potent producers of tubercles *in vivo*<sup>5</sup>; we shall see that glycolipids and peptidoglycolipids containing 50–88 per cent mycolic acid have other interesting biological activities.

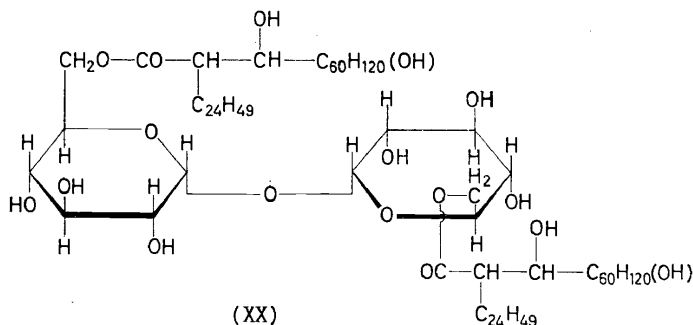
### DELAYED TYPE OF HYPERSENSITIVITY

The injection of tuberculin, a protein (or mixture of proteins) secreted by the tubercle bacillus into the culture filtrate, is not sufficient to produce the typical "tuberculin hypersensitivity". Raffel<sup>31</sup> has shown that one can elicit this hypersensitivity by simultaneous injection of tuberculin and a purified wax fraction. It was later found that all esters of mycolic acid with carbohydrates are active<sup>32</sup>.

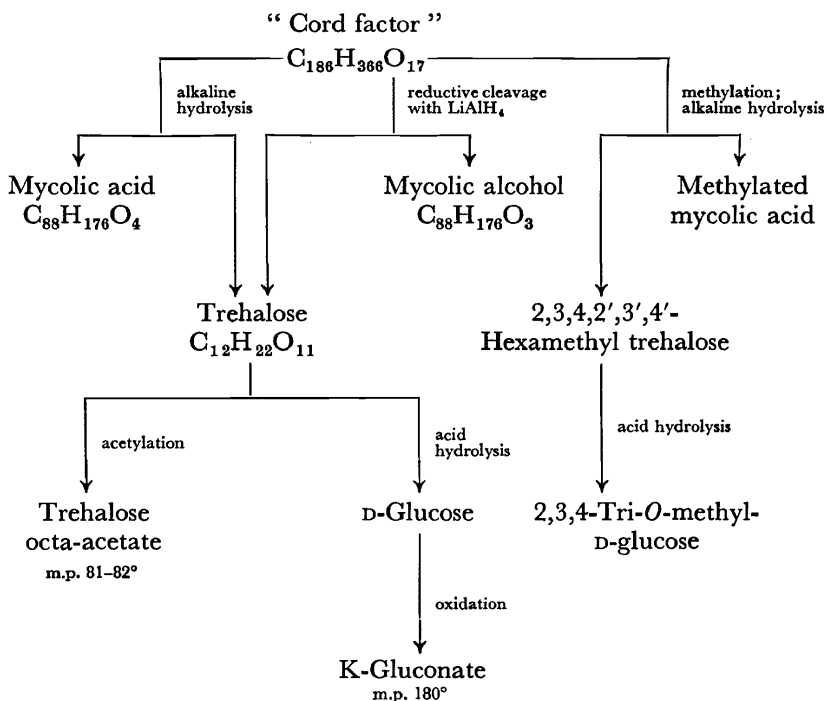
Amongst the lipids produced by tubercle bacilli, a particular wax fraction, called wax D, is also active in this respect. We shall say more about this fraction later.

### THE TOXIC FACTOR

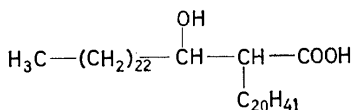
The toxic factor has been called "cord factor", because it is found in "cord forming" organisms, i.e. virulent and attenuated strains which grow in the culture medium in "serpentine cords"<sup>33</sup>. One intravenous injection of  $20\mu\text{g}$  of natural cord factor kills mice within 10–20 days; haemorrhages in the lung are the principal lesions observed.



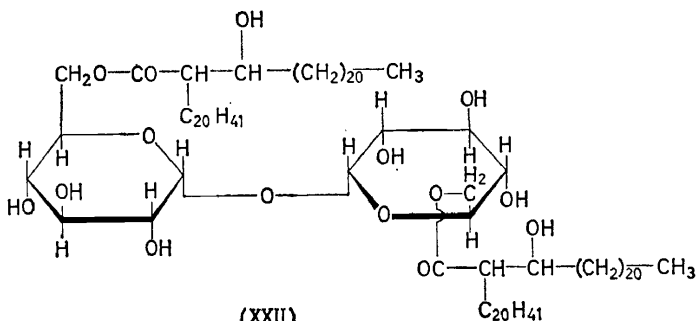
Cord factor has been isolated in a pure state after repeated chromatographic purifications on magnesium silicate and silicic acid, and its structure as the 6,6'-dimycolate of trehalose (XX) ( $C_{186}H_{366}O_{17} \pm 10 CH_2$ ) established by degradation<sup>34</sup>, and by synthesis<sup>35, 36</sup>, starting from trehalose and natural mycolic acids.



Simple, synthetic mycolic acids, such as (XXI) obtained by condensation of two molecules of methyl docosanoate, have also been esterified with trehalose and have yielded "small cord factors", *e.g.* (XXII) ( $C_{100}H_{194}O_{15}$ )<sup>36</sup> which show approximately the same biological activity as natural cord factor.



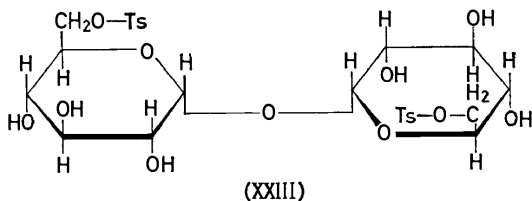
(XXI)



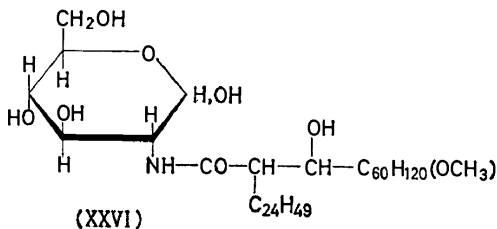
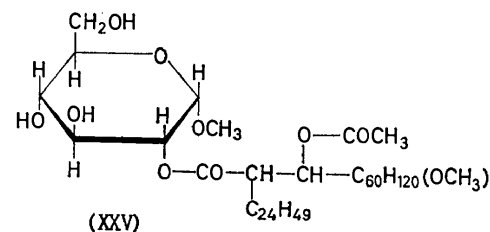
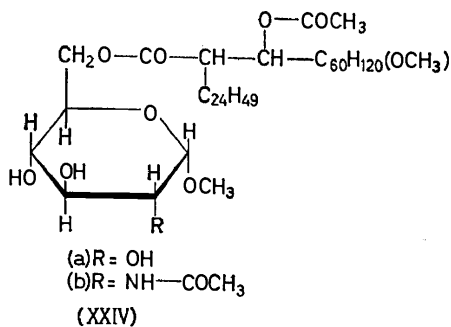
(XXII)

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The most specific method of obtaining 6,6'-diesters of trehalose is to heat 6,6'-ditosyl trehalose (XXIII), with two molecules of the potassium salt of an acid<sup>37</sup>.



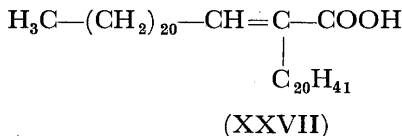
Concerning the relation between chemical structure and cord factor activity, the following preliminary statements have been made<sup>38</sup> on the basis of unpublished experiments by Bloch with compounds prepared in the author's laboratory: 6-mycolates of monoses (D-glucose, D-galactose, D-glucosamine, *e.g.* (XXIVa, b)) are toxic, but to a lesser degree than trehalose esters; the corresponding 2-mycolates (XXV) and mycolamides (XXVI) are not toxic.



The 6,6'-dimycolates of trehalose seem more active than the 6-mono- or 2,6,6'-trimycolates.

Acetylation of the  $\beta$ -hydroxyl of mycolic acid diminishes only slightly the activity of cord factor; fully acetylated cord factor is inactive.

The influence of the structure of the acid esterifying trehalose can be characterized as follows: even behenic (docosanoic) esters of trehalose are active, but doses larger than 0.1 mg are necessary. The 6,6'-diester of trehalose with the synthetic mycolic acid  $C_{44}H_{88}O_3$  (XXI) has about 50 per cent of the activity of natural cord factor. Dehydration of the latter acid gives the unsaturated acid (XXVII):



which, as the 6,6'-diester of trehalose, is inactive at 0.1 mg dose levels.

Concerning the mechanism of action of cord factor, Kato *et al.*<sup>40</sup> have found that injection of cord factor decreases the activity of most DPN-linked dehydrogenases.

For reviews on cord factor, see Lederer<sup>38</sup> and Noll<sup>39</sup>.

## INCREASED PRODUCTION OF ANTIBODIES

Freund's adjuvant, consisting of a water-in-oil emulsion containing killed Mycobacteria in the oil phase and the antigen in the aqueous phase, has been widely used by immunologists for increasing antibody production in animals. Freund<sup>41</sup> has reviewed our knowledge of the mode of action of this type of adjuvant.

After the preliminary experiments of White *et al.*<sup>42</sup> with a "purified wax fraction", we showed with White *et al.*<sup>43</sup> that a particular wax fraction of human strains of *M. tuberculosis* can effectively replace the whole bacilli in Freund's adjuvant mixture.

This wax fraction, which we call *wax D*<sup>5-7</sup>, is extracted from the bacilli with chloroform and can be separated from other wax fractions as a result of its insolubility in boiling acetone.

The wax D fractions of human strains of tubercle bacilli are high-melting dextrorotatory solids which represent 6-8 per cent of the dry weight of virulent strains, but only about 2 per cent of non-virulent strains<sup>44</sup>.

A recent study<sup>45</sup> of the chemical structure of wax D has shown that it is a high molecular weight lipid, and that the composition and molecular weight varies from strain to strain. The best analysed wax D fraction, that of the human virulent strain "Brévannes", has a molecular weight of about 54,000. On saponification, about 50 per cent of mycolic acid  $C_{88}H_{176}O_4$  is obtained (corresponding to approximately 22 molecules) and 50 per cent of a water-soluble portion. This latter is a peptido-mucopolysaccharide and seems to be homogeneous on ultracentrifugation and by immunological reactions.

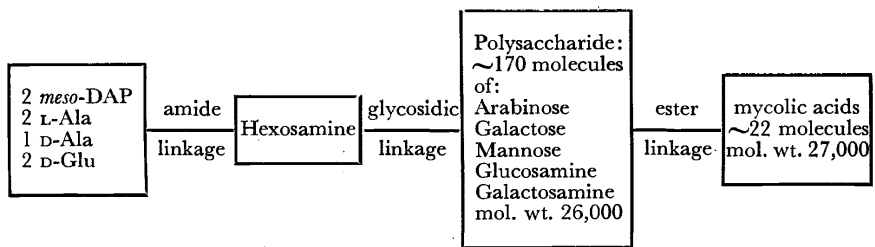
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The carbohydrate moiety of this peptido-mucopolysaccharide contains the following sugars: D-arabinose, D-mannose, D-galactose, glucosamine and galactosamine\*; traces of muramic acid are present, probably as an impurity.

The peptide moiety of wax D of human strains is of particular interest, because the analogous wax D fractions of bovine and saprophytic strains, which are nitrogen-free glycolipids, are inactive as adjuvants; thus, the peptide moiety seems to play a definite rôle in the adjuvant activity of wax D<sup>43</sup>.

It had already been found several years ago that wax D fractions of all human strains investigated contain the same three amino-acids: *meso*-diaminopimelic acid, glutamic acid and alanine<sup>6</sup>.

The particular wax D of the strain "Brevannes" which was studied in detail contains two molecules of *meso*-diaminopimelic acid (DAP), two molecules of D-glutamic acid (Glu) and three molecules of alanine (Ala), one of which is D. The presence of several amino-acids of "unnatural" configuration is quite remarkable. A tentative structure for wax D of the human virulent strain "Brevannes" of *M. tuberculosis* has been proposed<sup>45</sup> as follows:



Quite recently, it has been shown that these seven molecules of amino-acids form a heptapeptide, and the sequence of amino-acids in this heptapeptide has been established as: *meso*-DAP.D-Ala.D-Glu.D-Glu.L-Ala.*meso*-DAP.L-Ala<sup>47</sup>.

From the products of the partial acid hydrolysis of the water-soluble part of wax D, a compound was isolated containing one molecule of *meso*-DAP, one of galactosamine and one of arabinose. Galactosamine was found to be linked to a carboxyl-group of *meso*-DAP. This shows that the heptapeptide is linked by a carboxyl from one of its *meso*-DAP molecules to galactosamine, which is itself joined by a glycosidic linkage with arabinose, one of the components of the polysaccharide moiety. A second linkage probably exists between one carboxyl-group of D-glutamic acid and arabinose<sup>47</sup>.

It should be mentioned that delipidated bacterial residues of human, bovine, avian and saprophytic strains are also active adjuvants; this can be explained by the fact that these residues contain essentially cell-walls. It is

\* The optical configuration of these amino-sugars has not yet been determined; however, Haworth *et al.*<sup>46</sup> have found D-glucosamine in a "lipid-bound" polysaccharide of *M. tuberculosis* closely related to the polysaccharide of wax D.

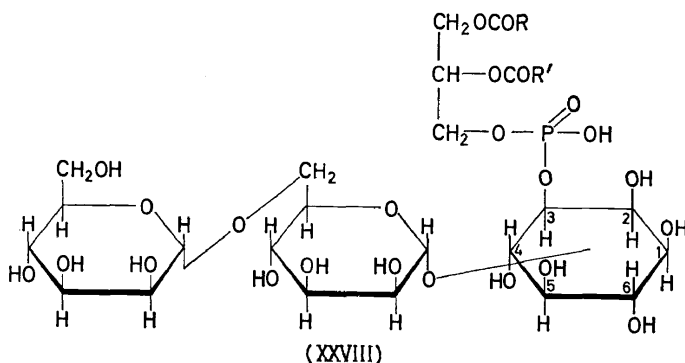
known that mycobacterial cell-walls are mainly composed of three amino-acids (alanine, glutamic acid, and  $\alpha, \epsilon$ -diaminopimelic acid) linked to a polysaccharide containing arabinose, mannose, galactose and muramic acid<sup>48</sup>. They thus have an overall composition very similar to that of the water-soluble part of wax D of human strains.

The adjuvant activity of wax D of the human strain of *M. tuberculosis* is probably due to its general chemical analogy with the cell-wall, and one might consider the cellular reactions which result in the increase of antibody production as a general reaction of the tissues of the higher organism to contact with mycobacterial cell-walls.

### DEVELOPMENT OF RESISTANCE TO INFECTION

Boquet and Nègre<sup>49, 50</sup> of the Pasteur Institute, Paris, have shown long ago that methanol extraction of acetone-defatted tubercle bacilli yields an extract which possesses immunizing properties. They had called this crude fraction "antigène méthylique", and Macheboeuf *et al.*<sup>51</sup> had shown that phospholipids are the main antigenic components of this extract.

More recently, Weiss and Dubos<sup>52</sup> have confirmed the findings of Boquet and Nègre<sup>49</sup>, and have studied the preparation of active extracts and some biological aspects of immunization.



In view of the interesting biological properties of the crude phospholipid fraction, we have undertaken a more detailed study of its constituents.

It was already known from the work of Anderson<sup>1</sup> that mycobacterial phospholipids contain inositol and mannose. We have found with Mrs Vilkas that a phosphatidyl-inositol-dimannoside is the principal component of these phospholipids, and we have proposed for this compound formula (XXVIII), in which inositol is linked to the C-1 or C-3 atom of the *meso*-inositol, and in which the exact position of the 6-*O*- $\alpha$ -D-mannopyranosido- $\alpha$ -D-mannopyranose is not yet known<sup>53</sup>. The fatty acid molecules linked to glycerol are normal and methyl-branched C<sub>16</sub> to C<sub>19</sub>-fatty acids.

The phosphatidyl-inositol-dimannoside is accompanied by other phospholipids, amongst which a phosphatidyl-pentamannoside<sup>54, 55</sup> and a phosphatidyl-pentagluside<sup>56</sup> are most remarkable.

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In the course of recent immunization experiments conducted in collaboration with Bloch, Vilkas obtained fractions of phospholipids which produce (in 20  $\mu\text{g}$  doses) a distinct increase in survival time of mice; the main constituent of these high-melting, strongly dextrorotatory fractions seems to be a phosphatidyl-inosito-trimannoside\*.

### THE MYCOSIDES, TYPE-SPECIFIC GLYCOLIPIDS

Smith *et al.*<sup>57, 58</sup>, using the combined techniques of chromatography and infra-red spectroscopy, were able to show that some lipid compounds are limited in distribution to a single species of micro-organism; their presence or absence may thus serve to distinguish between various species of Mycobacteria.

The distribution of these substances in the various species of Mycobacteria is as follows<sup>58</sup>: (i) phthiocerol dimycocerosate is present in the lipids of 28 out of the 30 human strains studied; (ii) a compound, first called  $G_B$ , is present in all 7 bovine strains studied; (iii) a compound, first called  $G_A$ , is present in all 17 photochromogenic strains studied; (iv) a compound, called Jav, is present in 11 out of 13 avian strains, and also in 3 out of 6 non-photochromogenic strains.

As it was then shown<sup>59</sup> that compounds  $G_A$ ,  $G_B$  and Jav are glycolipids, containing characteristic *O*-methylated 6-deoxyhexoses in glycosidic linkage, it was suggested that these compounds be called "mycosides"; a mycoside is defined as a "type specific glycolipid of mycobacterial origin"<sup>60</sup>.

*Mycoside A* (the former  $G_A$ ) is a nearly colourless solid, melting at  $105^\circ$ ,  $[\alpha]_D = -37^\circ$ ; it contains three different *O*-methylated 6-deoxyhexoses, which have been identified as 2-*O*-methylfucose, 2-*O*-methylrhamnose and 2,4-di-*O*-methylrhamnose<sup>59</sup>. The lipid moiety of mycoside A is a di- or trimycocerosate of an aromatic alcohol with a typical ultra-violet light absorption spectrum (maxima at 222, 274 and 278  $m\mu$  in hexane).

*Mycoside B* (the former  $G_B$ ) is a colourless wax melting at  $25^\circ$ ,  $[\alpha]_D = -22^\circ$ ; it has approximately the same ultra-violet light absorption maxima as mycoside A; the probable molecular formula is  $C_{82}H_{152}O_{10}$ ; mycoside B contains only one sugar, identified by MacLennan *et al.*<sup>59</sup> as 2-*O*-methylrhamnose.

The lipid moiety of mycoside B has the formula  $C_{75}H_{140}O_6$  and is a diester of two molecules of a branched-chain acid fraction of mean molecular composition  $C_{22}H_{44}O_2$ , with a methoxylated phenolic triol  $C_{31}H_{56}O_4$ . In mycoside B, the deoxyhexose is linked glycosidically to the one phenolic hydroxyl-group of the lipid moiety (Demarteau-Ginsburg and Lederer, unpublished experiments).

*Mycoside C* differs from the other mycosides by its nitrogen content, which is due to the presence of several amino-acids; mycoside C is a peptidoglycolipid; as a matter of fact, it is a mixture of closely-related compounds which can be separated by chromatography on silicic acid; three of the fractions thus obtained have been investigated in more detail (see Table 2)<sup>61</sup>.

The peptide portion of these three fractions contains three different amino-acids linked in a pentapeptide: one molecule of *D*-phenylalanine

\* More recently, the immunizing activity of this fraction has been rather irregular.

Table 2. Composition of three different fractions of mycoside C

		Fraction I/17	Fraction II/7	Fraction III/7+8
Melting point		~200°	~200°	195–200°
[α] <sub>D</sub> (in chloroform)		–44°	–34°	–34°
Probable molecular formula		C <sub>78</sub> H <sub>131</sub> N <sub>5</sub> O <sub>28</sub>	C <sub>80</sub> H <sub>137</sub> N <sub>5</sub> O <sub>24</sub>	C <sub>68</sub> H <sub>111</sub> N <sub>5</sub> O <sub>23</sub>
Products of hydrolysis	Hydroxyacid	C <sub>24</sub> H <sub>48</sub> O <sub>3</sub> ±2CH <sub>2</sub>	C <sub>32</sub> H <sub>64</sub> O <sub>3</sub> ±2CH <sub>2</sub>	C <sub>20</sub> H <sub>38</sub> O <sub>3</sub> ±2CH <sub>2</sub>
	Acetic acid (moles)	2	2	2
	Deoxyhexoses* (moles)	4	3	3
	Amino-acids (moles)	1 D-Phe 2 D-allo-Thr 2 D-Ala	1 D-Phe 2 D-allo-Thr 2 D-Ala	1 D-Phe 2 D-allo-Thr 2 D-Ala

\* The deoxyhexoses of these fractions have been identified by MacLennan<sup>63</sup>; they are: 6-deoxytalose, 3-*O*-methyl-6-deoxytalose and 3,4-di-*O*-methylrhamnose.

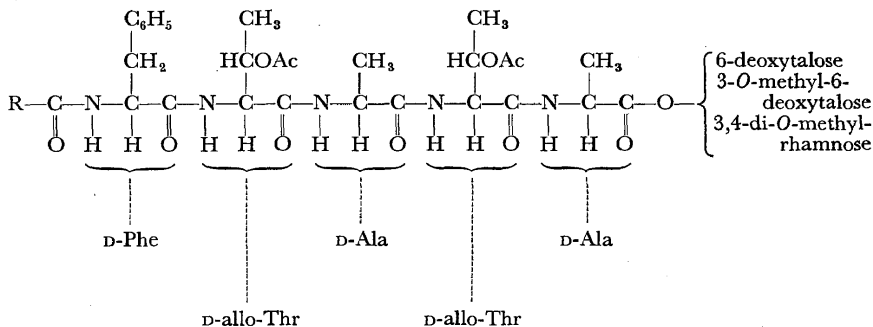
(Phe), two molecules of D-allo-threonine (Thr) and two molecules of D-alanine(Ala); the pentapeptide of fraction III/7 + 8 has the structure:



The unnatural configuration of all the constituent amino-acids, and the presence of D-allo-threonine (which had not previously been isolated from natural sources), are remarkable features<sup>62</sup>.

These mycoside C preparations contain three different 6-deoxyhexoses: 6-deoxytalose, 3-*O*-methyl-6-deoxytalose and 3,4-di-*O*-methylrhamnose (MacLennan<sup>63</sup>). It can be seen from Table 2 that fraction I/17 contains four molecules of deoxyhexoses, whereas the other two fractions contain three molecules.

The lipid moiety of these mycoside C preparations has not yet been fully characterized; it seems to be a mixture of saturated and unsaturated hydroxyacids, the approximate molecular formula of the saturated acids varying from C<sub>20</sub>H<sub>40</sub>O<sub>3</sub> to C<sub>30</sub>H<sub>60</sub>O<sub>3</sub>. Two *O*-acetyl groups are also present in each of these fractions. For fraction III/7 + 8, the tentative structure (XXIX) has been proposed:



R—COOH = a fatty acid, mean mol. wt. 330

(XXIX)



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In this structure, the terminal carboxyl group of D-alanine is not free but esterified with a hydroxyl group of one of the sugar molecules. As the three mycoside C fractions studied all have two molecules of D-allo-threonine and two O-acetyl groups, it is assumed that the acetyl groups are esterified to the hydroxyl groups of allo-threonine.

Amongst the apparently type-specific lipids, we might also mention a sugar-free peptido-lipid isolated from the pathogenic Actinomycete *Nocardia asteroides*<sup>64</sup>; this fraction is a mixture of closely related compounds, m.p. 215–217°;  $[\alpha]_D = +44.5^\circ$ , consisting of 30 per cent of a lipid moiety of approximate molecular formula  $C_{32}H_{64}O_2$ , joined by an amide linkage to an oligopeptide containing the following six amino-acids: threonine, alanine, valine, isoleucine, leucine and proline. Two-thirds of the alanine and isoleucine are D-; here again the presence of D-amino-acids is noteworthy<sup>62</sup>.

### CONCLUSION

In the course of a detailed study of the relations between biological activity and chemical structure of bacterial lipids, we have determined the structure, and mechanism of biosynthesis, of high molecular weight branched-chain hydroxyacids. New types of glycolipids and peptido-glycolipids have been isolated, and their chemical structures have been defined; their interesting biological activities have been characterized.

The simultaneous presence of several lipophilic and hydrophilic groups in the same molecule confers to glyco- and peptido-glycolipids peculiar physicochemical properties (such as emulsification, detergent action, etc. . . .) which might explain many of their biological activities and which open new fields of investigation.

Whereas D-amino acids had previously been known to exist only in antibiotics and in bacterial cell-walls, the work described above shows that they are also frequent constituents of liposoluble bacterial products, such as peptido- and peptido-glycolipids. This might indicate a close biochemical and physiological relationship of these products with cell-wall.

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