THE MYCOBACTERIAL CELL WALL

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ABSTRACT

The Mycobacterial cell wall contains a peptidoglycan–glycolipid complex and various more or less defined glycolipid fractions. The detailed chemical structure of the peptidoglycan and the glycolipid (a mycolate of an arabinogalactan) is discussed. *Figure 4* shows a tentative formula for a 'monomer' of the cell wall and *Figure 6* depicts a possible 'decamer.'

Then the chemistry of three glycolipids which seem closely associated with the cell wall is reviewed: (1) Wax D of human strains is a mucopeptide-containing mycolate of arabinogalactan and is probably derived from the cell wall by enzymatic degradation as shown in *Figure 6*; (2) Cord factor is a dimycolate of trehalose (formula **22**); (3) A newly defined sulpholipid is a 2-sulphate of a tetra-acyl trehalose (formula **26**).

Other possible components of the cell wall are mentioned, such as the mycosides, a glucan and lipopeptides. Mass spectrometry has been a very helpful tool for defining the chemistry of all these compounds.

Finally the biological activities of mycobacterial cell walls and their components are considered, such as: specific immunization against tuberculosis, adjuvant activity and stimulation of non-specific resistance to infections.

In this lecture, I shall try to summarize our present knowledge of the chemistry of a very complex and most fascinating natural macromolecule: the cell wall of Mycobacteria and related organisms.

'Very complex' because we shall see that it contains lipids, peptides and carbohydrates.

'Fascinating' because there is a great deal of novel structural chemistry involved and new biosynthetic pathways to explore, and because many of the pathogenic effects of tubercle bacilli and related organisms are due to constituents of the cell wall.

We shall be concerned not only with the insoluble macromolecular, rigid cell wall and its covalent chemical structure, but also with a series of soluble lipid compounds which seem to be located in or on the outer part of the cell wall, such as wax D, cord factor and sulpholipids.

We shall start with a chemical study of the various structures, and shall see that mass spectrometry has been a most helpful tool for bringing precision and order into this most complex field, and we shall end this lecture with a rapid survey of the biological properties of mycobacterial cell walls.

Animal experiments show indeed that preparations derived from mycobacterial cell walls have interesting properties concerning adjuvant activity,

and the stimulation of non-specific resistance, which can be used not only against bacterial and viral infections, but also against certain types of leukaemia and cancer.

I. CHEMICAL STRUCTURE OF THE MYCOBACTERIAL CELL WALL: A PEPTIDOGLYCAN GLYCOLIPID COMPLEX

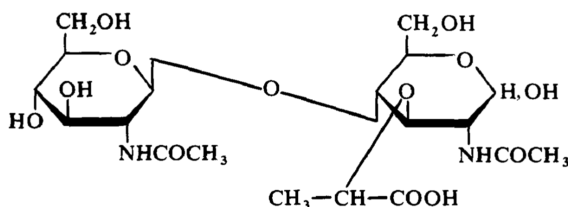
The two major components of the mycobacterial cell wall are: a peptidoglycan and a glycolipid (Kotani *et al.*[1], Takeya *et al.*[2], Misaki *et al.*[3], Kanetsuna[4]).

(1) The peptidoglycan (mucopeptide or murein)†

Here we can distinguish the glycan backbone consisting of a repeating disaccharide unit and a peptide moiety.

(a) *The disaccharide unit*

The classical work of Ghuysen[5,6], Jeanloz[7], Park[8], Salton[9] and Strominger[10] has shown that the cell wall of all bacteria analysed so far contains a peptidoglycan having a glycan backbone of repeating disaccharide units of structure **1**, where *N*-acetyl-D-glucosamine is linked in β1 → 4 to *N*-acetyl-D-muramic acid. The only variations found so far were that *N*-acetyl muramic acid is 6-*O*-acetylated in *Staphylococcus aureus* cell wall[11] and that muramic acid forms a lactame in certain bacterial spores[12].



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The disaccharide unit of the mycobacterial cell wall had not yet been studied in detail; when Drs J. F. Petit and A. Adam at Orsay first isolated it from *M. smegmatis* by the usual enzymatic procedure[6] it was found to have *Rf* values differing from those of the classical disaccharide (**1**). It did contain *N*-acetyl-glucosamine and muramic acid, so it was concluded that the difference was most probably in a substituent of the muramic acid moiety[13].

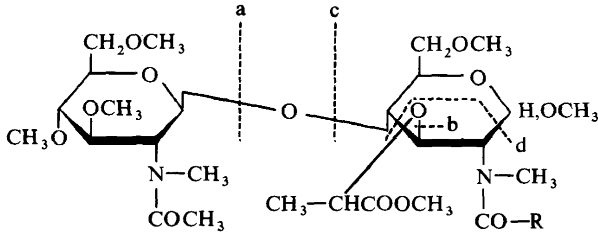
Mass spectrometry of the permethylated disaccharide of *M. smegmatis* in comparison with the permethylated derivative of **1** solved the problem[14].

The mass spectra of the two permethylated disaccharides (**2**) are very similar (*Figure 1*) and show some peaks at identical positions. Others are at 30 mass units higher, in the case of the *M. smegmatis* disaccharide.

The fragmentation (*a*) of the glycosidic linkage of **2** gives, in both cases, an oxonium ion **3** at *m*/*e* 260 confirming the terminal position of *N*-acetyl

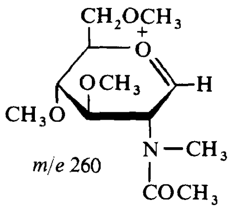
† The terms peptidoglycan, mucopeptide and murein are synonyms.

glucosamine in both disaccharides. Fragmentation (*b*) gives an intense peak at M-103 in both cases, confirming the presence of the lactyl side chain. Fragmentation (*c*) gives peaks at *m*/*e* 332 or at 362.



2 R = CH_3 (*M. lysodeikticus*)
R = CH_2OCH_3 (*M. smegmatis*)

An important cleavage (*d*) leads to fragments **4** containing carbons C-2 and C-3 of the muramic acid moiety with their substituents; *m*/*e* 201 originates from the known structure (**1**) whereas *m*/*e* 231 shows that in the disaccharide from *M. smegmatis* carbon 2 has 30 mass units (i.e. one OMe group) more.



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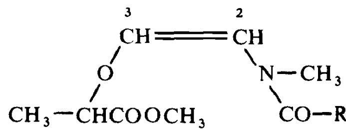
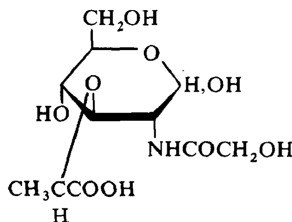


4 R = CH_3 : *m*/*e* 201 (*M. lysodeikticus*)
R = CH_2OCH_3 : *m*/*e* 231 (*M. smegmatis*)

As mass spectrometry after perdeuteriomethylation proved that no methoxyl group was present in the original disaccharide, it was concluded that C-2 of muramic acid has a *N*-glycolyl group instead of an *N*-acetyl group.

Glycolic acid was then identified chemically and *N*-glycolyl muramic acid **5** was synthesized[15] and found to be identical by *Rf* values and mass spectrometry after permethylation with a sample isolated from *M. smegmatis* disaccharide by enzymatic hydrolysis with *Helix pomatia* gut juice[14].



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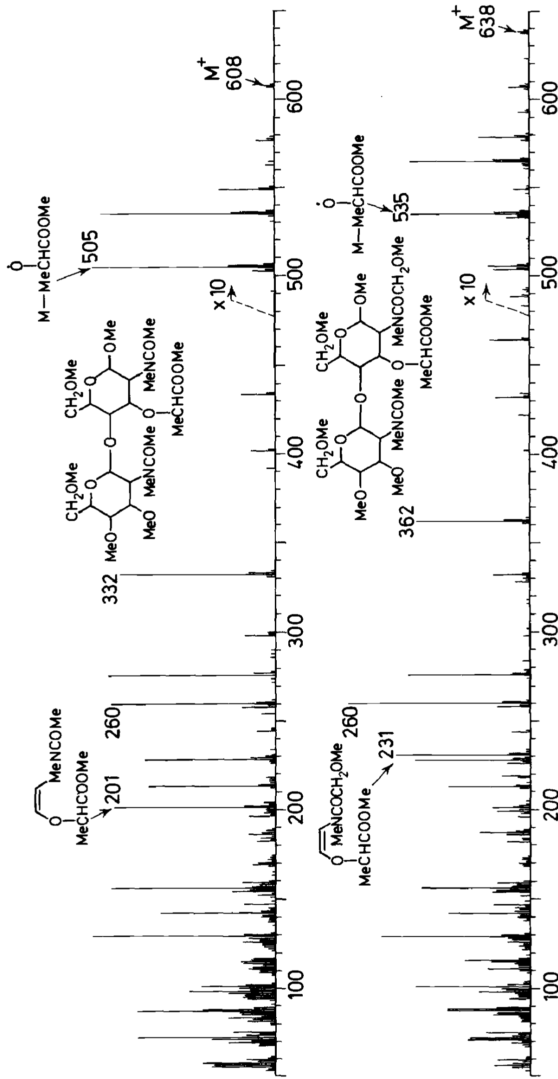
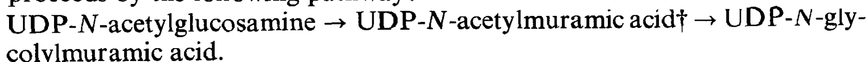


Figure 1. Mass spectrum of permethylated cell wall disaccharide **2**: above: *M. lysodeiktikus*: below: *M. smegmatis* (Adam *et al.*[14]).

A preliminary survey[16] of various Actinomycetales has shown that *N*-glycolyl muramic acid (**5**) is present in all Mycobacteria examined as well as in *Nocardia Kirovani*[17] (and probably also *Micromonospora*[18]), but is absent from *Corynebacteria* and *Streptomyces*. Its presence can thus be used as a taxonomic criterion.

We had suggested[14] that the *biosynthesis* of *N*-glycolyl muramic acid (**5**) proceeds by the following pathway:


UDP-*N*-acetylglucosamine → UDP-*N*-acetylmuramic acid† → UDP-*N*-glycolylmuramic acid.

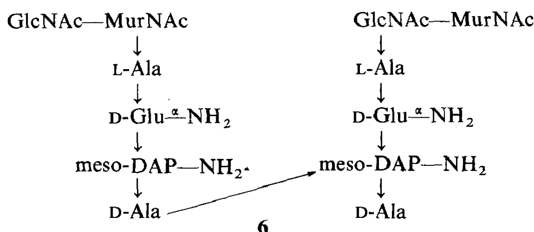
Petit *et al.*[19] have recently obtained experimental evidence in favour of this hypothesis by isolating UDP-*N*-glycolyl-muramyl (Ala, Glu, DAP) from a culture of *M. phlei* grown in the presence of cycloserine, an antibiotic known to inhibit cell wall biosynthesis and leading to the accumulation of UDP-*N*-acylmuramyl derivatives in the cells of sensitive bacteria[20]. A similar experiment has been reported quite recently by Takayama *et al.*[21].

Mycobacteria have thus 'invented' an oxygenase which introduces a hydroxyl specifically into the *N*-acetyl group of muramic acid. A close analogy exists in higher animals: Schoop *et al.*[22] have shown that in pig liver *N*-acetyl-neuraminic acid is oxidized to *N*-glycolyl-neuraminic acid.

(b) *The peptide moiety*

It was known, mainly from the papers of Work[23] and Cummins[24,25], that the cell wall of Mycobacteria and Corynebacteria contains D- and L-alanine, D-glutamic acid and meso-2,6-diaminopimelic acid (DAP).

More recently, Kato *et al.*[26] elaborated structure **6** for the mucopeptide of the cell wall of *C. diphtheriae*, which is the same as that of the cell wall of *E. coli* and *B. megaterium*, i.e. L-Ala-γ-D-Glu-L-meso-DAP‡-D-Ala (Van Heijenoort *et al.*[27]) except that the carboxyl groups of Glu and DAP are amidated.

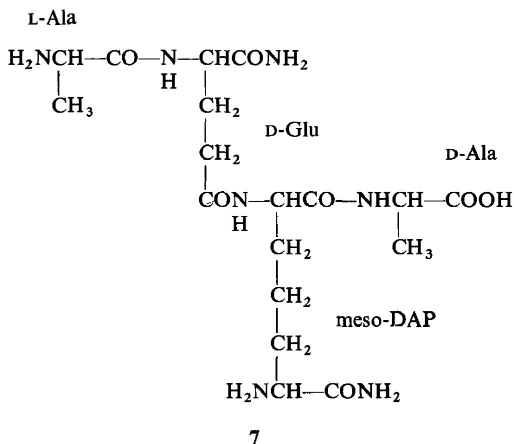


Taxonomic considerations and recent work of Migliore and Jollès[29] on wax D (see below) were in favour of an analogous structure for the peptide moiety of mycobacterial cell walls.

The tetrapeptide of *M. smegmatis* cell wall was isolated by Mrs J. Wietzerbin-Falszpan and Dr J. F. Petit by enzymatic hydrolysis and extensively purified; chemical analysis confirmed that a γ-glutamyl-linkage was present and that the N-terminal Ala was L, the C-terminal Ala was

† Mycobacteria do contain small quantities of *N*-acetyl muramic acid[13].
‡ DAP: 2,6-diamino pimelic acid.

D and that DAP was meso; mass spectrometry of the N-acetylated and permethylated tetrapeptide, performed by Dr B. C. Das, then confirmed structure 7[31], in particular the sequence of the amino acids, the presence of two amide groups and their location, as well as the linkage of L-Ala-γ-D-Glu in α of the carboxyl of meso-DAP carrying D-Ala.



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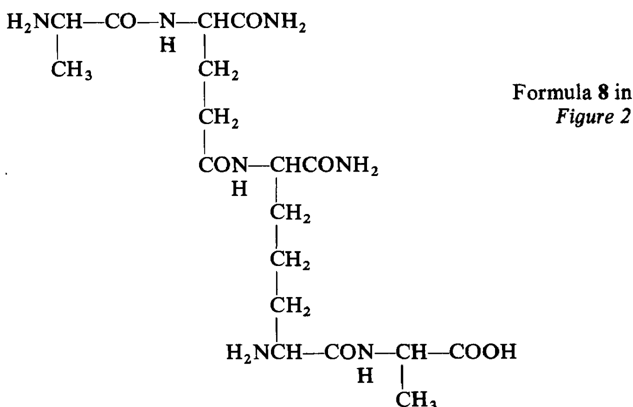
In the mass spectrum of **8** (*Figure 2*) derived from **7** by N-acetylation and permethylation by the technique developed in our laboratory for the sequence determination of amino acid residues in peptides[32–36] the molecular ion is at m/e 683. A peak at m/e 484 is due to fragmentation a, thus confirming structure **7** and excluding the isomeric structure **9**. Sequence peaks are at m/e 128, 298 and 567.



Formula **8** in *Figure 2*

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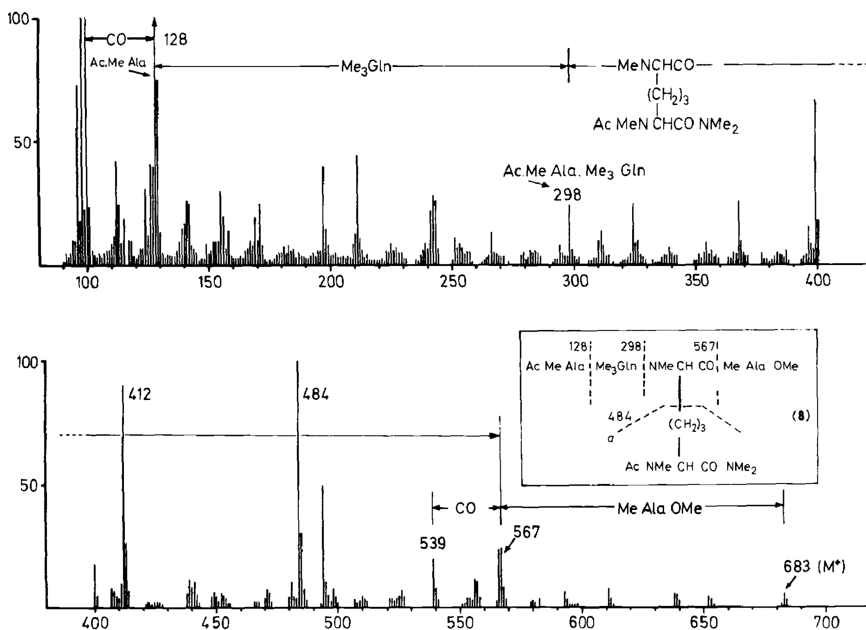
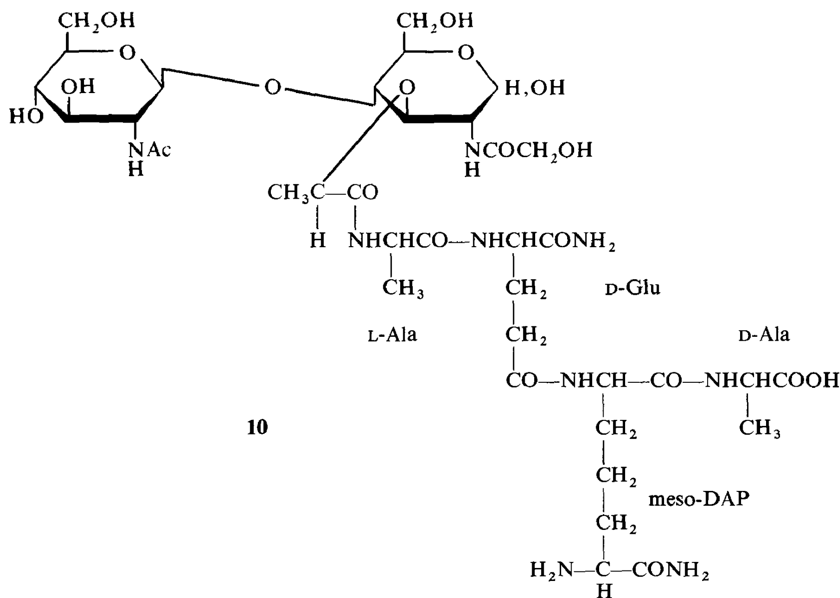


Figure 2. Mass spectrum of the *N*-acetylated and permethylated tetrapeptide diamide of *M. smegmatis* cell walls (Wietzerbin–Falszpan *et al.*[31]).



The same diamidated tetrapeptide **7** has been isolated from BCG, *M. phlei* and *C. fermentans* and identified by mass spectrometry[31].

Similarly the structure of a diamidated tripeptide Ala-Glu-DAP was established. Mass spectrometry has been particularly useful for locating the amide groups.

We can therefore propose structure **10** for the peptidoglycan monomer of Mycobacteria: in the polymer, the C-terminal carboxyl of D-Ala is linked to one of the amino groups of meso-DAP of another glycan strand (as in **6**).

As the tetrapeptide **7** was isolated by the action of the *Myxobacter* Al_1 enzyme which does not seem to hydrolyse D-Ala-meso-DAP bonds, it is probable that peptide **7** is found as such in the cell wall, i.e. not crosslinked to other peptide chains; it might be formed by the action of autolytic enzymes necessary for regulation of cell growth.

The only stereochemical detail not yet known in structure **7** is which of the asymmetric centres of meso-DAP is linked to Glu and D-Ala (the carboxyl of the other being amidated). By analogy with the *E. coli* peptidoglycan[27] the first is expected to be L the second D, thus giving the alternating stereochemistry L, D, L, D which is probably of biological significance (J. M. Ghuysen, personal communication).

(2) The glycolipid moiety

The mucopeptide of the mycobacterial cell wall is linked to a glycolipid containing *mycolic acids* esterified to an *arabinogalactan*.

(a) *The mycolic acids*

Mycolic acids are 'monstrous mycobacterial molecules' discovered by R. J. Anderson[37] during his classical systematic investigation of the chemistry of the lipids of Mycobacteria; the overall formula $C_{88}H_{176}O_4$ proposed by Anderson is not far from reality; precise molecular formulae and structures could, however, be elaborated only recently by Etémadi[38–40] by using mass spectrometry.

With J. Asselineau, to whom we owe the first important developments in mycolic acid chemistry[41,42], we had defined mycolic acids as 'α-branched β-hydroxy acids'[43]. Three principal categories are now known:

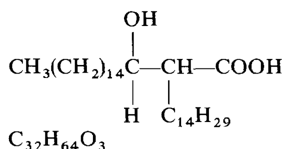
(i) the corynomycolic acids ranging from C_{28} to C_{40}, found mostly in Corynebacteria.

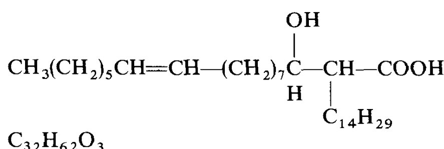
(ii) the nocardic (or nocardomycolic) acids, ranging from C_{40} to C_{60} produced by strains of Nocardia.

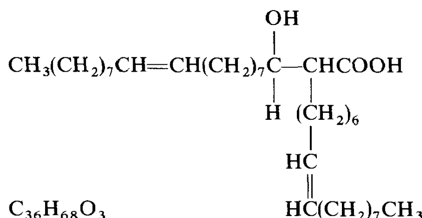
(iii) the mycobacterial mycolic acids, ranging from C_{60} to C_{90}.

(i) *The corynomycolic acids*—The first to be discovered[44] and the most widely distributed in nature is corynomycolic acid $C_{32}H_{64}O_3$ **11**, which has also been found recently in *Nocardia asteroides*[45] esterified to trehalose and in *M. smegmatis*, esterified to glucose[45a]; the mono-unsaturated corynomycolenic acid $C_{32}H_{62}O_3$ **12**[46] has also been found in *N. asteroides* esterified to trehalose[45]. More recently 'corynomycoladienoic' acids having one unsaturation in each of the chains have been found by Welby-Gieusse *et al.*[47] in *C. hofmanii*, by Ioneda *et al.*[45] in *N. asteroides* and by Okazaki *et al.*[48] in

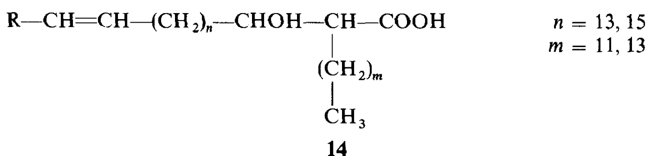
Brevibacterium thiovaginalis†. The most important of these, $C_{36}H_{62}O_3$ has structure **13**[47,48].



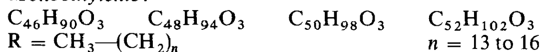




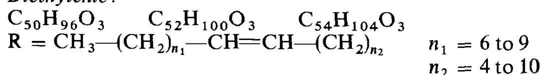
(ii) *The nocardic acids*—These acids were discovered by Michel *et al.*[49] in *N. asteroides*; the precise structure of some of these was later established by mass spectrometry[50]. More recently, nocardic acids (also called nocardo-mycolic acids[42]) ranging from C_{40} to C_{60} have been isolated from various strains of Nocardia; saturated, mono-, di- and tri-unsaturated species have been identified[51]. In all of these the α-chain is saturated. Bordet and Michel[51] have proposed structure **14** for three series of nocardic acids found in *N. asteroides*; see also formulae **19** and **20**.



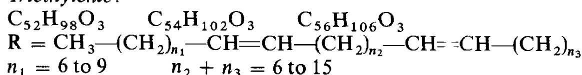
(a) *Monoethylenic*:



(b) *Diethylenic*:



(c) *Triethylenic*:



† An organism apparently unrelated to Mycobacteria.

Table 1. Structures of some mycolic acids of Mycobacteria[61]

Mycolic acid	Strain	Formula[a]	References
"C_{60}-mycolic acid" $CH_3{-}(CH_2)_{17}{-}CH{=}CH{-}(CH_2)_{17}{-}CH(OH){-}CH(C_{22}H_{45}){-}COOH$	*M. smegmatis*	$C_{62}H_{122}O_3$	51a
α-Smegmamycolic acid $CH_3{-}(CH_2)_{17}{-}CH{=}CH{-}(CH_2)_{13}{-}CH{=}CH{-}CH(CH_3){-}(CH_2)_{17}{-}CH(OH){-}CH(C_{22}H_{45}){-}COOH$	*M. smegmatis*	$C_{77}H_{154}O_3$	38
α-Kansamycolic acid $CH_3{-}(CH_2)_{17}{-}CH{-}CH{-}(CH_2)_{14}{-}CH{-}CH{-}(CH_2)_{17}{-}CH(OH){-}CH(C_{22}H_{45}){-}COOH$ (each CH–CH pair bridged by CH_2)	*M. kansasii*	$C_{80}H_{156}O_3$	40

Methoxylated mycolic acid	*M. tuberculosis* var. *hominis*, strain Test	$C_{85}H_{168}O_4$	52

```
                        OCH3                                OH
                         |                                  |
CH3—(CH2)17—CH—CH—(CH2)10—CH— CH—(CH2)17—CH—CH—COOH
             |                  \  /                            |
            CH3                 CH2                          C24H49
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β-Mycolic acid	*M. tuberculosis* var. *hominis*, strain Test	$C_{87}H_{160}O_4$	39

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                         O                                  OH
                        ||                                  |
CH3—(CH2)17—CH—C—[C17H34]—CH—CH—(CH2)19—CH—CH—COOH
             |                   \  /                           |
            CH3                  CH2                         C24H49
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Dicarboxylic mycolic acid	*M. phlei*	$C_{56}H_{108}O_5$	53

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                                           OH
                                           |
HOOC—(CH2)14—CH—CH=CH—(CH2)16—CH—CH—COOH
              |                                |
             CH3                            C22H45
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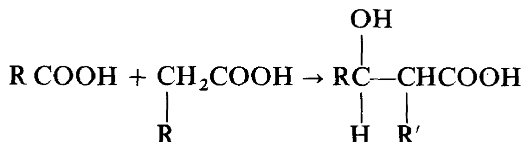
[a] All mycolic acids are mixtures of homologues. The molecular formulae and the structures given in this table are those of the principal member of the homologous series. See also Minnikin and Polgar, ref. 53a and Asselineau *et al.*, ref. 53b.

(iii) *Mycolic acids of Mycobacteria*—We shall not describe in detail the chemistry of these compounds which range from C_{60} to C_{90}; some typical structures are shown in *Table 1* and in formulae **16**, **17** and **25** (for reviews see refs 39, 42).

It is satisfying to learn from a recent paper of C. Asselineau *et al.*[54] that the stereochemistry of six mycolic acids examined, including a corynomycolic acid, a nocardomycolic acid and four from Mycobacteria, is the same: 2R, 3R.

The *biosynthesis* of mycolic acids has been studied by Gastambide[55] and Etémadi[56, 57] in our laboratory; it proceeds by condensation of one long chain molecule in α of another one.



Quite recently Winder *et al.*[58] have reported that isoniazid inhibits mycolic acid biosynthesis.

The methyl branches or methylene groups of the cyclopropane rings of the long mycolic acid chains are introduced by C-methylation, methionine being the donor of the C_1 unit[56, 57, 59].

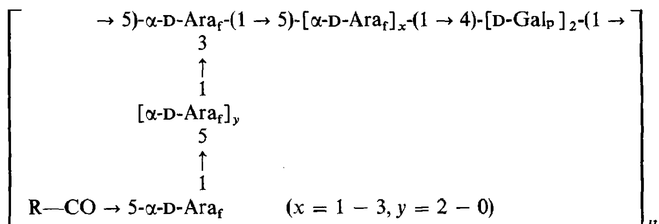
Tuberculostearic acid (10-methyl stearic acid), the methyl substituent of which arises by C-methylation of oleic acid, is widely distributed amongst Mycobacteria, Nocardia and Streptomyces[60]; the C-alkylation reactions leading to branched chain mycolic acids seem, however, to be restricted to Mycobacteria.

Mycolic acids are mostly found in Nature esterified with carbohydrates: with arabinose in the cell wall and in wax D, and with trehalose in 'cord factor' as mentioned below.

(For a review on mycobacterial glycolipids see ref. 61).

(b) *The arabinoglactan*

The arabinogalactan has been studied by Misaki and Yukawa[62], Azuma *et al.*[63] and Vilkas *et al.*[64, 65]; it possesses a branched structure and consists mainly of 1 → 5 linked D-arabinofuranose units and 1 → 4 linked D-galactopyranose units, the two sugars being in approximate molecular proportions of 5 to 2; some of the arabinose units form non-reducing terminal ends.



15 D-Ara$_f$ = D-Arabinofuranose, D-Gal$_p$ = D-Galactopyranose
R—CO = Mycolic acid residue

The tentative formula **15** (as quoted by Azuma *et al.*[63]) was proposed by Misaki and Yukawa[62]; this formula is in agreement with recent work of Amar-Nacasch and Vilkas[65].

(c) *The structure of the glycolipid*†

As shown in formula **15** mycolic acid is linked through its carboxyl to the 5-OH of one of the D-arabinofuranose molecules; this was first stated by Azuma and Yamamura[66] who isolated mycolates of arabinofuranose and of an arabinobiose from 'bound lipids' of a human strain (Aoyama B).

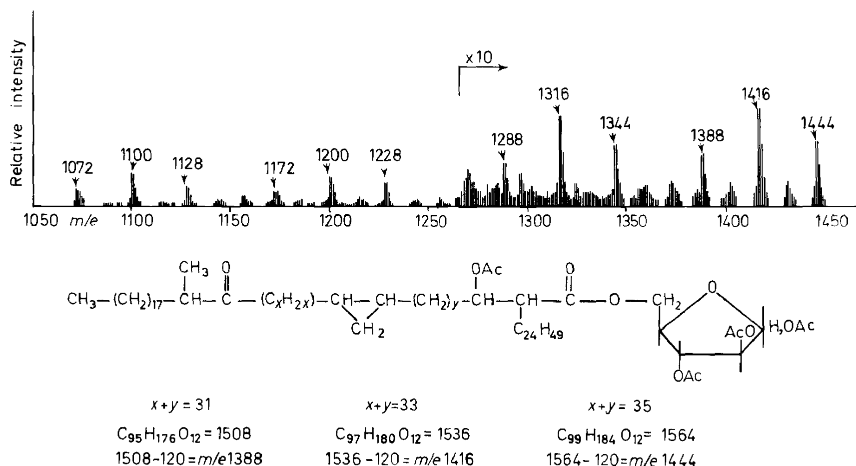
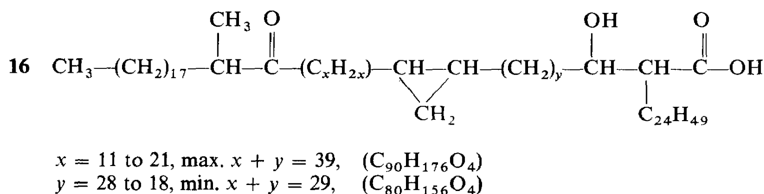


Figure 3. Partial mass spectrum of peracetylated arabinose mycolates isolated from the 'bound lipids' of the BCG strain (Acharya *et al.*[67]).

The structure of the mycolates of arabinofuranose has been studied by Acharya *et al.*[67] by mass spectrometry of the peracetylated compounds isolated from the cell walls of the BCG strain and of *M. kansasii* (*Figure 3*).

In the higher mass region one finds two series of peaks differing by 100 mass units due to the presence of acyl residues corresponding to the mycolic

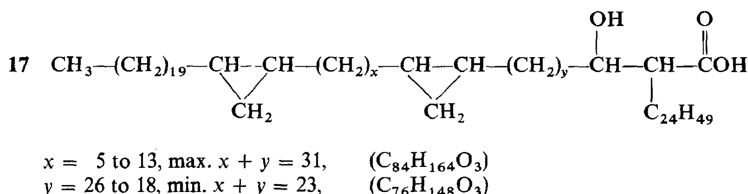


$x = 11$ to 21, max. $x + y = 39$, ($C_{90}H_{176}O_4$)
$y = 28$ to 18, min. $x + y = 29$, ($C_{80}H_{156}O_4$)

acids (**16**) and (**17**) the structure of which had been established previously by mass spectrometry of the methyl esters. The peaks corresponding to the acyl of (**16**) are at *m/e* 1444, 1416 and 1388 and arise apparently by the loss of two molecules of acetic acid (120 m.u.) from the peracetylated compound

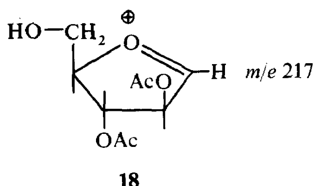
† Some authors use the name lipopolysaccharide.

shown in *Figure 3*. The ions corresponding to the acyl of (**17**) are at *m/e* 1344, 1316 and 1288.



The anhydro-acyl ions (R—CH=C($C_{24}H_{49}$)—C=O) corresponding to (**16**) give (after loss of one H) peaks at *m/e* 1228, 1200 and 1172; the analogous ions derived from the acyl of (**17**) are at *m/e* 1128, 1100 and 1072.

A small peak at *m/e* 217 (not shown in *Figure 3*) can be due to the oxonium ion (**18**).



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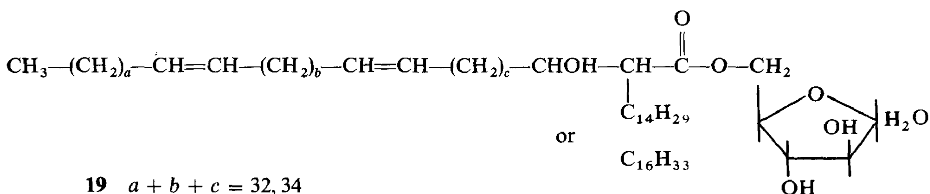
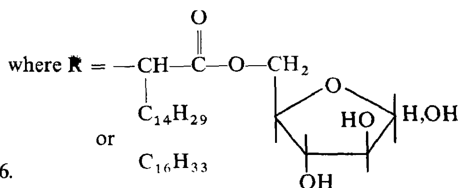
Analogous preparations of arabinose or arabinobiose mycolates have been isolated recently by Kanetsuna *et al.*[68] and Vilkas *et al.*[64, 65].

It seems safe to conclude that in mycobacterial cell walls, as well as in wax D (see below), the lipid moiety consists exclusively of mycolic acids and that they are esterified to the 5-OH of D-arabinofuranose.

The cell wall of *Nocardia* strains has a quite analogous structure where the mycobacterial mycolic acids are replaced by nocardic acids; Lanéelle and Asselineau[69] have quite recently identified arabinose nocardates in 'bound lipids' of *N. brasiliensis*; mass spectrometry of the permethylated glycolipids has shown them to be arabinose esters of nocardic acids $C_{56}H_{106}O_3$, $C_{56}H_{108}O_3$; $C_{58}H_{110}O_3$ and $C_{60}H_{114}O_3$. Structures **19** and **20** have been proposed by Lanéelle and Asselineau[69] for the arabinose nocardates.



In one strain of *Nocardia asteroides* it was found by Ioneda *et al.*[45] that only the cell wall contained nocardic acids (C_{50} to C_{58}), whereas the free lipids contained corynomycolic acids (C_{28} to C_{36}).



20 $a + b + c + d = 32, 34, 36.$

(3) The glycolipid–peptidoglycan complex

The nature of the linkage of the glycolipid to the peptidoglycan is not yet quite clear. Liu and Gotschlich[70] have shown that muramic acid-6 phosphate exists in *M. butyricum* and subsequent authors have confirmed the presence of muramyl phosphate in all Mycobacteria examined[28, 29, 71]. This suggests that there might be a phosphodiester linkage of the glycan backbone to the arabinogalactan but there is as yet no clear experimental proof. Considering the probability of a phosphodiester bridge we can propose for the glycolipid peptidoglycan 'complex' of the mycobacterial cell wall the structure shown in *Figure 4*; Kanetsuna[71] has obtained some evidence for another, glycosidic,

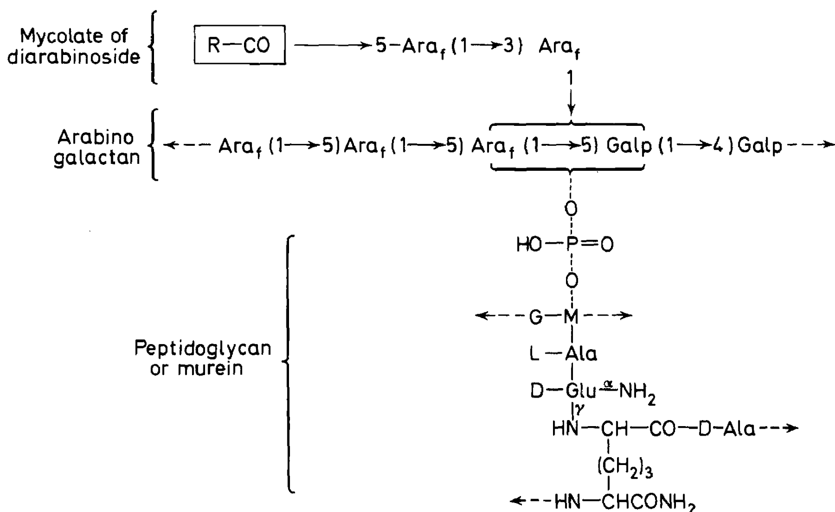


Figure 4. Tentative structure of the 'monomer' of the cell wall of Mycobacteria.

linkage existing between the glycolipid and possibly glucosamine of the mucopeptide, and has proposed the structure shown in *Figure 5*.

The molecular weight of a 'monomer' such as that shown in *Figure 4* is about 3200: Misaki *et al.*[3] have found that the molecular weight of the arabinogalactan of the BCG strain is approximately 30000. Considering that only about one out of eight to ten molecules of muramic acid is phosphorylated one can propose the (very hypothetical) scheme of a 'decamer' shown in *Figure 6* (the arrows indicate a possible mode of formation of wax D, by enzymatic hydrolysis, as explained later.

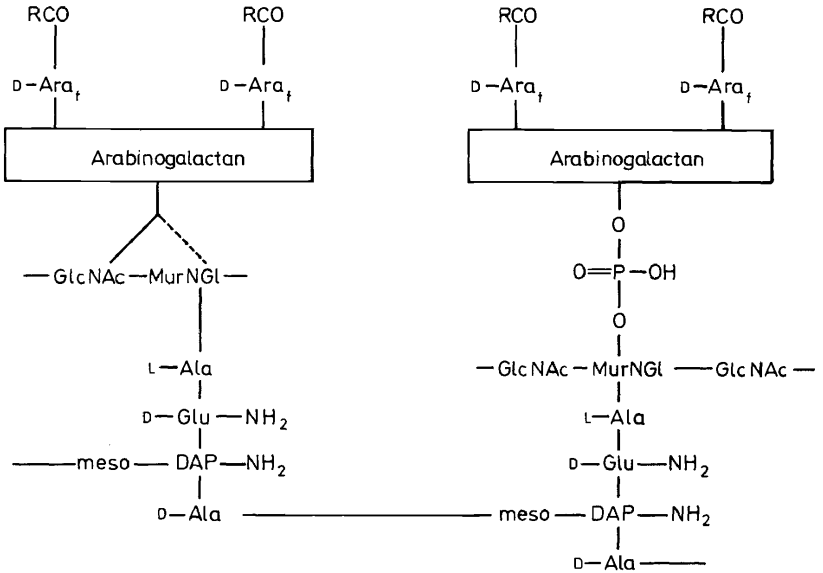


Figure 5. Tentative structure of a mycolic acid–arabinogalactan–mucopeptide complex of mycobacterial cell wall. RCO, mycolic acid residue; D-ara$_f$ denotes D-arabinofuranose; GlcNAc denotes *N*-acetylglucosamine; MurNGl is *N*-glycolylmuramic acid (Kanetsuna[71]).

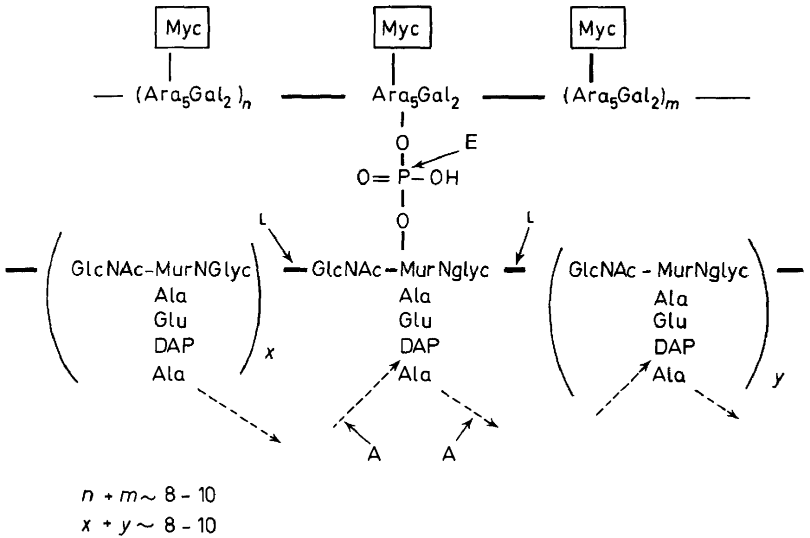


Figure 6. Tentative proposal for the structure of a 'decamer' of the cell wall of Mycobacteria. (Myc: indicates a molecule of mycolic acid esterified to arabinose).

II. GLYCOLIPIDS ASSOCIATED WITH THE CELL WALL

Under this heading we shall consider the following categories of compounds: wax D, cord factor and sulpholipids.

(1) Wax D

Wax D is the acetone insoluble fraction of a chloroform extract of Mycobacteria previously defatted by exhaustive treatment with alcohol--ether[42].

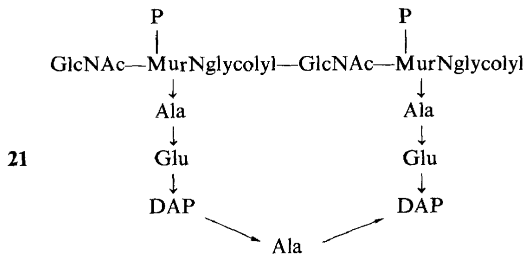
Many attempts have been made to purify wax D, but no homogeneous preparation has so far been obtained. Phospholipids and cord factor are frequent contaminants. The presence of mannose reported previously in various wax D preparations was due to contamination by phospholipids (Vilkas *et al.*[75]).

Wax D preparations of 'non-human strains' of Mycobacteria are nitrogen-free glycolipids: mycolates of an arabinogalactan.

Wax D preparations of human strains of *M. tuberculosis* and of *M. kansasii* are peptidoglycolipids, mycolates of an arabinogalactan linked to the typical mucopeptide (Jollès *et al.*[29,73,74]; Vilkas *et al.*[72,75]) (see also ref. 61). These latter wax D preparations have a most interesting immunological adjuvant activity (see below).

Azuma (personal communication) has shown the immunological identity of the arabinogalactan of cell walls and of wax D and parallel studies of the arabinogalactan of cell wall and of wax D of human virulent strains by Vilkas *et al.*[72] have confirmed the close analogy of their structures.

More recently Migliore and Jollès[30] have isolated the mucopeptide of wax D of a human strain and have proposed structure **21** showing the close analogy with the mucopeptide of the cell wall.



Two carboxyl groups of the heptapeptide are amidated.

Bound wax D

Anderson[37] had already found that part of the lipids of Mycobacteria can only be extracted after hydrolysis of the insoluble bacterial residue with 0.1 N HCl; these 'bound lipids' are chemically very similar to wax D preparations of 'non-human' strains (i.e. they are nitrogen-free mycolates of arabinogalactan).

Kotani *et al.*[76] and Kanetsuna[71] have isolated a 'bound wax D' from the cell wall of Mycobacteria by treatment with lysozyme and *Streptomyces* enzymes. These preparations contain a mycolate of arabinogalactan linked

to the mucopeptide and are thus closely similar to the mucopeptide containing wax D preparations extractable by chloroform from human strains of *M. tuberculosis* and from *M. kansasii.*

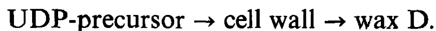
The so-called 'bound lipids', liberated by weak acid hydrolysis, are thus apparently none other than the arabinogalactan–mycolates of the cell wall, whereas the 'bound wax D' liberated by enzymatic hydrolysis can be considered to be a sort of 'monomer' of the cell wall.

The biogenesis of wax D

The chloroform-soluble wax D fractions are probably either oligomers or parts of oligomers of the cell wall not having been used for polymerization, or autolysis products, liberated from the cell wall by the action of autolytic enzymes hydrolysing the polymer at various linkages and thus producing a complex mixture of analogues[71].

The arrows in *Figure 6* show how such wax D fractions could be formed; the action of a phosphodiesterase (E) would yield a liposoluble arabinogalactan–mycolate (wax D of 'non-human' strains) whereas the action of lysozyme (L) and a D-alanine-endopeptidase (A) would yield mucopeptide containing wax D fractions such as are found in human strains and in *M. kansasii.*

David *et al.*[77] have quite recently reported that the synthesis of wax D in *M. tuberculosis* is inhibited by cycloserine; this finding is in favour of a common biosynthetic pathway for cell walls and wax D and in agreement with the sequence:



UDP-precursor → cell wall → wax D.

Wax D of Nocardia strains

Quite recently Lanéelle and Asselineau[69] have isolated from *N. brasiliensis* a wax D fraction, hydrolysis of which gives nocardic acids, galactose and arabinose as well as Ala, Glu and DAP.

(2) Cord factor

Cord factor is a toxic glycolipid discovered by Bloch[78] in petrol ether extracts of virulent, cord-forming Mycobacteria. (For reviews on the structure, synthesis and biological activity, see refs. 79, 80.)

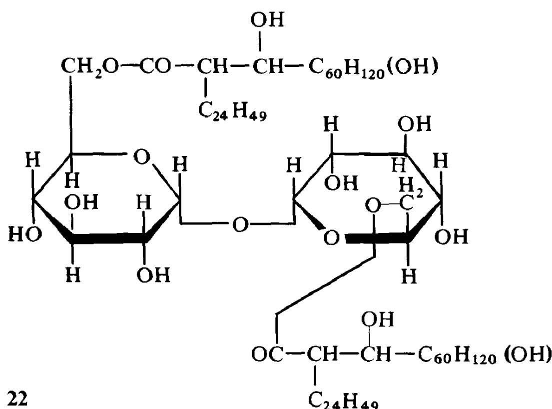
Noll *et al.*[81] have shown that cord factor is a 6,6′-dimycolate of trehalose and, at a time when precise structures for mycolic acids were not yet known, formula (**22**) was proposed.

Since then a whole series of natural diesters of trehalose have been isolated from bacterial lipids; let us mention them by increasing molecular weight of the acyl radical they contain.

In *M. fortuitum* Vilkas *et al.*[82,83] have found a dipalmitate of trehalose and a diester of trehalose; the mass spectrum of the latter showed unambiguously that the trehalose molecule was unsymmetrically substituted, both acyl radicals (mostly palmitic and tuberculostearic acids) being on the same glucose moiety.

From *Corynebacterium diphtheriae* Ioneda *et al.*[84] have isolated a toxic glycolipid containing trehalose and two molecules of C_{32} acids, coryno-

mycolic (**11**) and corynomycolenic (**12**) acid. Here again trehalose is esterified in the 6,6′-position, as shown by permethylation studies.



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C. hofmanii contains a trehalose diester of the C_{36} corynomycoladienoic acid (**13**)[47].

Quite recently we became interested in the structure of cord factor of *Nocardia* strains, which had not yet been examined. We had expected to find diesters of nocardic acids and in fact from *N. rhodochrous* a trehalose diester was isolated containing nocardic acids ranging from C_{40} to C_{46}, but in a strain of *N. asteroides* the acyl groups of the isolated cord factor were a mixture of corynomycolic acids, corynomycolenic and corynomycoladienoic acids ranging from C_{28} to C_{36} [45].

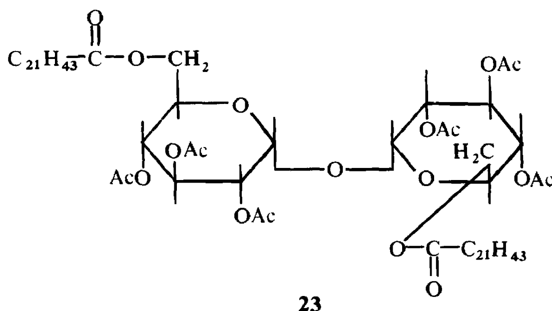
Thus this glycolipid was structurally closely related to the one produced by Corynebacteria. We then wondered whether this particular strain did produce nocardic acids; a detailed mass spectrometric analysis showed that in the free lipids only corynomycolic acids were found, whereas saponification of the cell wall gave nocardic acids ranging from C_{50} to C_{56}. This shows that nocardic acids are specifically used for the glycolipid of the cell wall[45] (in agreement with observations of Bordet *et al.*[85]).

Mass spectrometry of trehalose diesters

Adam *et al.*[86] have studied the mass spectrometry of some synthetic 6,6′-diacyl-trehaloses prepared for biological experiments[87]; it was intended to check the structure of these and analogous natural compounds and to see how far mass spectrometry could be used in the high molecular weight region.

The peracetylated synthetic 6,6′-dieicosanoyl-trehalose **23** gave a molecular ion at m/e 1238; the cleavage of the glycosidic bond leads to an oxonium ion at m/e 611.

Mass spectrometry of the peracetylated cord factor of *C. diphtheriae* showed that three different molecular species were present; a trehalose diester having two saturated C_{32} acyl radicals, another having two unsaturated C_{32} acyl radicals and a third species having one saturated and one unsaturated acyl radical (molecular ions at m/e 1630, 1632 and 1634[88]).



The synthetic 2,3,4,2′,3′,4′-hexaacetyl-6,6′-di(α-eicosanyl-β-acetoxytetracosanoyl)trehalose[87] (**24**) (*Figure 7*) showed no molecular ion, but peaks (M-60) at *m/e* 1910, (M-120) at *m/e* 1850 and (M-180) at *m/e* 1790 as well as an oxonium ion at *m/e* 977 which loses 60 m.u. to give a strong peak at *m/e* 917; the acyl ion (*m/e* 689) loses 60 m.u. to give a strong peak at *m/e* 629.

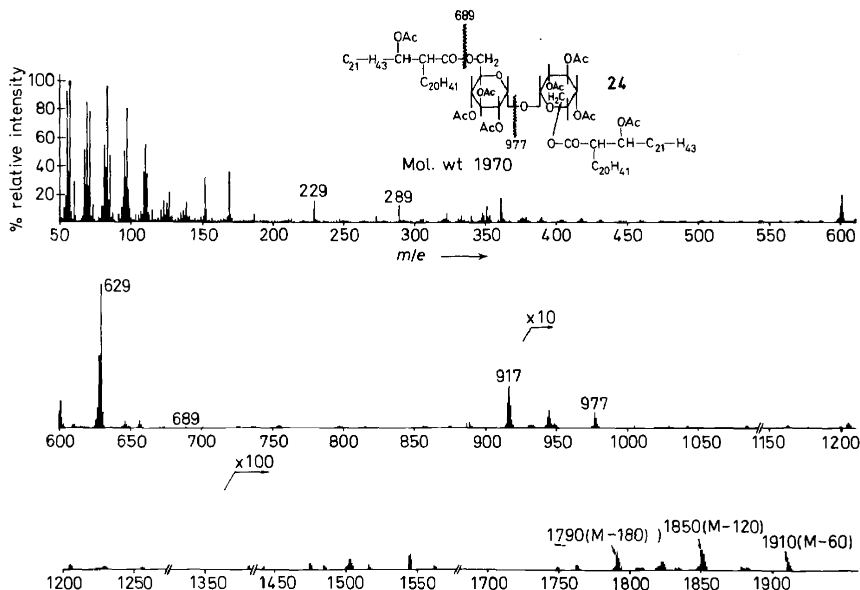


Figure 7. Mass spectrum of a peracetylated synthetic 6,6′-trehalose diester (Adam *et al.*[86]).

Before analysing the cord factor of a human strain of *M. tuberculosis* Adam *et al.*[86] prepared the methyl ester of the mycolic acids it contained and analysed them by mass spectrometry. A series of homologous mycolic acids was found, ranging from $C_{78}H_{154}O_4$ to $C_{90}H_{178}O_4$ and containing one methyl, one methoxyl group and one cyclopropane ring on the long chain, a structure which seems typical for mycolic acids of human strains of *M. tuberculosis* (see *Table 1*).

The peracetylated cord factor gave a mass spectrum (*Figure 8*) showing no molecular ions (which would have been at *m/e* 2776 to *m/e* 3000) but a

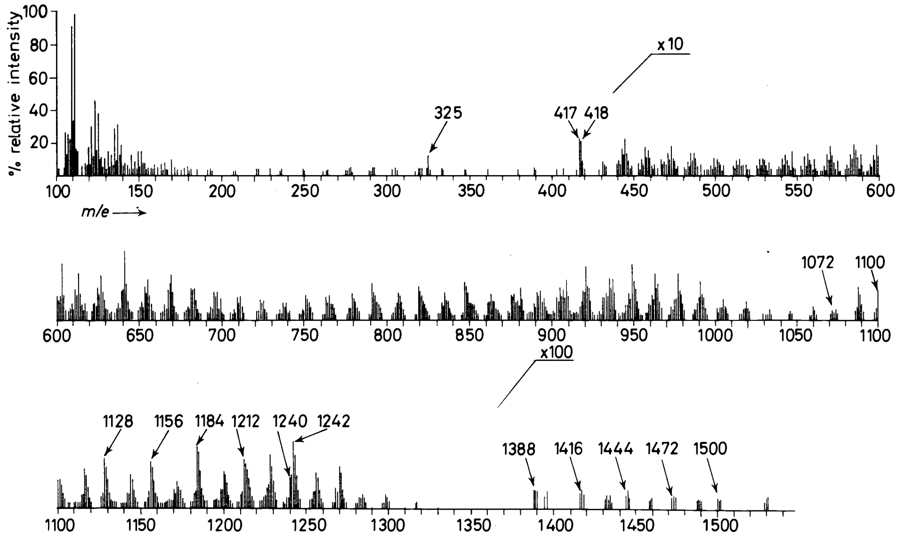
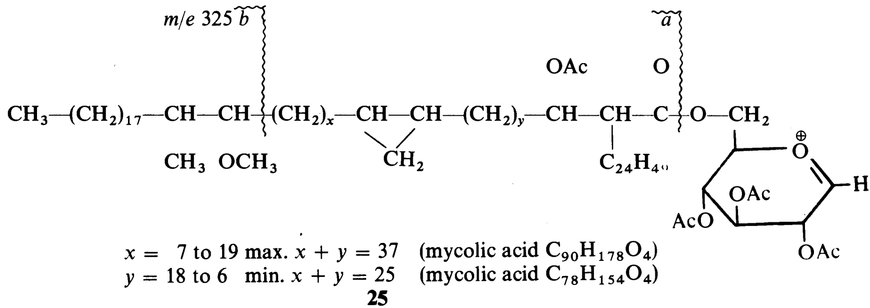


Figure 8. Mass spectrum of the peracetylated cord factor of a human strain of *M. tuberculosis* (Adam *et al.*[86]).

series of homologous peaks at *m/e* 1388, 1416, 1444, 1472 and 1500, corresponding to oxonium ions **25** (after loss of one H†). The peaks at *m/e* 1072 to 1240 are due to the corresponding acyl ions of the homologous series of mycolic acids (fragmentation a), after loss of one molecule of acetic acid; the peak at *m/e* 325 corresponds to $C_{22}H_{45}O$ (fragmentation b); the identification of peaks at *m/e* 1530, 1242, 417 and 418 is described in the original paper[86].



Some comments on the biological properties of cord factor

In the first few years after the discovery of cord factor, its toxic properties were mainly studied; experiments attempting to produce immunity with cord factor or 'anticord factors' had failed; more recently, however, Bekierkunst *et al.*[89] have shown that, under certain experimental conditions

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† Trehalose diesters of cyclopropane containing mycolic acids give 'oxonium ions' with even mass numbers, whereas other diesters (synthetic, as well as those of *M. butyricum*, which do not contain cyclopropane rings) give normal oxonium ions with uneven mass numbers[86].

(in the presence of mineral oil), injections of 10 to 20 μg of cord factor into mice produce granulomas in the lung and local immunity.

Kato[90] has studied the action *in vivo* and *in vitro* of cord factor on mitochondria and has found that it causes the structural disintegration of the mitochondrial membrane and a decrease in mitochondrial respiration and phosphorylation.

BCG (and other mycobacteria) increase the susceptibility of experimental animals to endotoxins of gram-negative bacteria; from the experiments of Suter[91] it seems that cord factor alone has this (rather undesirable) property; bacteria, previously defatted with acetone, have lost this property (when injected in saline; unpublished experiments with L. Chedid and A. Lamensans).

Trehalose diesters have been found more recently also in micro-organisms not related to Mycobacteria, thus Okazaki *et al.*[48] have found a trehalose diester of the C_{36}-corynomycoladienoic acid **13** in *Brevibacterium thiovaginalis* and trehalose diesters of partially undefined structure have been found recently by Suzuki *et al.*[92] in bacteria grown exclusively on paraffin as carbon source (*Arthrobacter paraffineus* and others). These authors write: 'the trehalose lipids displayed a significant activity as a surface active agent when added to mixtures of *n*-paraffin and aqueous solution. This suggests that this lipid may play a possible role in *n*-paraffin utilization by this micro-organism'. They also mention the analogy with hydrocarbon-utilizing yeasts (*Torulopsis*) in which the occurrence of sophorose lipids has been reported; the latter participate in dispersion of *n*-paraffin and the aqueous solution of nutrients.

It thus seems that the detergent properties of trehalose diesters are used by bacteria for absorption of lipids.

(3) Sulpholipids

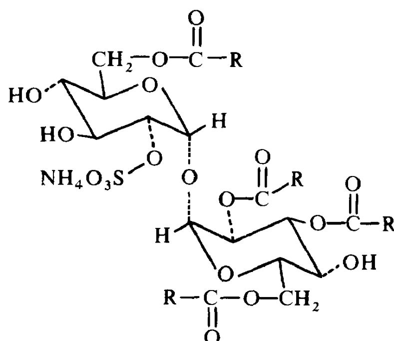
In 1959 Middlebrook *et al.*[93] had described the isolation of an anionic sulphur-containing lipid from human virulent strains of *M. tuberculosis*. Preliminary data suggested that the cytochemical neutral-red fixing activity of viable, cord-forming virulent tubercle bacilli was attributable to the sulpholipid; subsequently Gangadharam *et al.*[94] found a correlation between levels of sulpholipid elaborated by twelve different strains and their order of infectivity for the guinea-pig, thus suggesting a possible role of this lipid in the pathogenesis of tuberculosis.

Quite recently, Goren[95] has described the isolation of a mixture of sulpholipids of the virulent human strain $H_{37}Rv$ and has unravelled the principal features of the structure of one of these (**26**).

Trehalose is esterified by four acyl radicals and one molecule of sulphuric acid, the latter being on the 2-OH of one glucose moiety which carries one fatty acyl group on the 6-OH. The other glucose moiety carries three acyl groups, on the 2, 3 and 6 positions.

Goren[95] had shown that at least three of the four acyl radicals of the sulpholipid are different (acids A, B and C); acid B had been identified with palmitic acid.

A closer examination of the structure of the acyl radicals by mass spectrometry[96] led to the identification of A and C. Acid C is a mixture of



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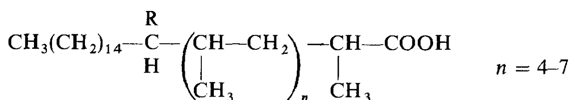
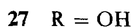
homologous acids representing a new type of hydroxy acids (**27**) showing a repeating sequence of methyl groups. Mass spectrometry shows the presence of acids having 31, 34, 37 and 40 carbon atoms.

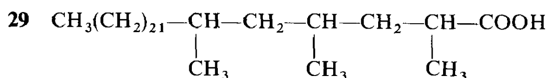
After elimination of the hydroxyl group of acid C a compound was obtained the methyl ester of which was identical with the one derived from acid A which thus has structure **28**, resembling closely the structure of the mycocerosic acids (for instance) (**29**); the latter, however, are laevorotatory whereas acids A and C are dextrorotatory and have thus the same stereochemistry as the dextrorotatory phtienoic acids (for instance) (**30**).

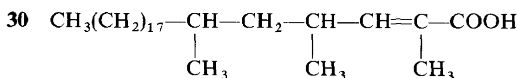


$n = 4\text{–}7$



27 R = OH
28 R = H

29 

30 

The biosynthesis of acids A and C by condensation of palmitoyl CoA with five to eight successive propionate units is most likely, in view of the presence of the homologous series of C_{31}, C_{34}, C_{37} and C_{40} acids and in view of previous studies of Gastambide-Odier[97] in our laboratory showing the incorporation of propionic acid into the mycocerosic acids **29** (as predicted by Polgar and Robinson[98]).

III. OTHER POSSIBLE COMPONENTS OF THE MYCOBACTERIAL CELL WALL

We may now ask: have we mentioned all the components of the mycobacterial cell wall? The answer is: probably not. Some other categories of molecules should probably be added: the *mycosides*, a *glucan*, and a *lipopeptide* (or lipoprotein). Teichoic acids have not yet been found in Mycobacteria.

The mycosides are 'type specific glycolipids of mycobacterial origin'[98a], discovered by Smith, Randall and McLennan[99, 100]. Nothing seems to be known about their biological properties, but Fregnan *et al.*[101] have observed distinct differences of the surface structure of colonies of mycoside-containing and mycoside-free strains; it thus seems that mycosides are also located on the surface of the cells.

We have reviewed their chemistry in recent years[61, 102]; no significant advance seems to have been made since.

A glucan has been described by several authors and is possibly located on or in the cell wall[62, 65].

The lipopeptide: several 'non-mucopeptide' amino acids have been identified in hydrolysates of cell walls. These can be isolated in a separate fraction after acetolysis or action of Myxobacter AL1 enzyme and seem to be part of a lipopeptide (or peptidolipid) which is possibly linked to the cell wall in a way analogous to that described by Braun and Sieglin[103] for a murein–lipoprotein of *E. coli* cell wall (Petit *et al.*, Vilkas *et al.*, unpublished).

It is difficult, for the moment, to reconcile the probably rather simplified picture we have given of the mycobacterial cell wall with the results of an electron microscope investigation by Imaeda *et al.*[104] who distinguish 'an outermost layer composed of lipopolysaccharides containing glucose, galactose and arabinose, a middle layer being a lipopolysaccharide–lipid–protein–complex and an innermost layer containing the lipopolysaccharide–mucopeptide complex which consists of fibril-containing mycolic acid–polysaccharide embedded in a membraneous arabinogalactan–mucopeptide layer'. See also Winder and Rooney[105].

IV. BIOLOGICAL ACTIVITIES OF MYCOBACTERIAL CELL WALLS

The study of the chemistry of bacterial cell walls is actively pursued in many laboratories and is amply justified by its intrinsic importance. Mycobacterial cell walls have, however, an additional interest: they produce in experimental animals a series of biological events which lead to increased production of antibodies and, in general, an increase of the resistance to infections not only by Mycobacteria, but also by agents quite unrelated to Mycobacteria.

These biological effects are an additional stimulus for our studies and open interesting possibilities for clinical applications; they are summarized below.

(1) Specific immunization against tuberculosis

Anacker *et al.*[106] have described, in a series of papers, the immunization against tuberculous infection of experimental animals with oil-treated

mycobacterial cell walls. More recently, however, they have shown[107] that their cell wall preparations can be inactivated by organic solvent extraction and by alkali or lipase treatment; these inactivated preparations combined with a wax D fraction were again highly protective.

It is not known how specific is the immunization described by Anacker *et al.*[106, 107].

(2) Adjuvant activity

Freund's adjuvant[108] (mycobacterial cells in a water in oil emulsion containing the antigen in the water phase) is well known to immunologists[109, 110]. The mucopeptide containing wax D fractions of human strains and of *M. kansasii* can replace the whole mycobacteria in Freund's adjuvant[111, 112]. This observation allowed the study of the specificity of adjuvant action; it could thus be concluded that the mucopeptide as well as the long chain mycolic acids are necessary. Structures such as those shown in *Figure 4* or *Figure 5* seem to be the minimal requirement for adjuvant activity.

For a recent review on the mechanism of adjuvant action see Paraf[113].

(3) Stimulation of non-specific resistance to infections

Antibacterial action

Dubos *et al.*[114, 115] have shown that injection of intact mycobacterial cells into mice can produce immunity to heterologous infections.

Howard *et al.*[116] have reported that, in mice, BCG produces a stimulatory effect on the phagocytic activity of the reticuloendothelial system and thus increases resistance to infection with *Salmonella enteridis.*

Weiss *et al.*[117, 118] have shown that this heterologous immunogenicity of BCG was retained in the insoluble residue obtained after methanol extraction. Fox *et al.*[119] investigated the stimulation of non-specific resistance to infection by crude cell wall preparation from *M. phlei* and Misaki *et al.*[3] showed that a mucopeptide preparation of BCG had a protective effect against staphylococcal infection in mice, which was as high as that of intact BCG cells.

In collaboration with Drs L. Chedid and A. Lamensans at the Pasteur Institute, we have obtained analogous results; various more or less purified preparations of Mycobacterial cell walls are active in stimulating the resistance of mice against infection with *Klebsiella pneumoniae* (*Table 2*).

Antiviral and antitumour action

Gorhé *et al.*[120] have shown that pretreatment of mice with Freund's complete adjuvant or adjuvant-active wax D preparations shortens the delay of production of interferon appearance after an injection of virus and thus has a beneficial effect on viral infections.

Several authors have studied the favourable effects of BCG infections on viral leukaemia (Lemonde *et al.*[121, 122]; Lamensans *et al.*[123]) and on viral tumours (Berman *et al.*[124]).

Halpern *et al.*[125] have shown that in rats BCG inhibits the growth of a transplanted tumour (atypical epithelioma T-8) and the development of

Table 2. Action of BCG and cell wall preparations on infection by *Klebsiella pneumoniae*

Material injected	*Dose* (μg)	*Mean survival time in days*	*Survivors after 30 days/total number of animals*
Saline	—	1.6	0/50
S. enteridis endotoxin	1	5.7	0/10
BCG	100	13.8	2/10
Acetone treated BCG	100	11	1/10
M. smegmatis crude cell walls	100	7.3	0/10
M. tuberculosis $H_{37}Rv$ purified cell walls	100	15.2	0/10
M. kansasii	100	14.2	2/10
Acetone treated *M. kansasii*	100	10.7	1/10
M. kansasii purified cell walls	100	20.4	5/10

The compounds were injected intravenously to mice, 24 h before intravenous infection with 10^5 bacteria [L. Chedid and A. Lamensans, unpublished experiments].

Ehrlich ascites in mice (Biozzi *et al.*[126]). Old *et al.*[127] have reported that BCG infection inhibits the growth of sarcomas induced by methylcholanthren.

Let us quote from a recent paper on the effect of BCG on adenovirus type 12 tumorigenesis in mice (Sjögren and Ankerst[128]). 'The pronounced effect of BCG treatment in the work described here indicates that it is quite feasible to obtain efficient protection against at least some tumours by non-specific stimulation of the immune responsiveness of the hosts at a relatively late stage of the latent period before tumour development.'

This is confirmed by unpublished experiments with Drs L. Chedid and A. Lamensans (Institut Pasteur) which have shown favourable effects of cell wall preparations (obtained by Drs A. Adam and J. F. Petit at Orsay) on the survival time of mice infected with lymphoid leukaemia (*Table 3*) or with Ehrlich ascites (*Table 4*).

Table 3. Action of BCG and cell walls of *M. kansasii* against a lymphoid leukaemia in mice

Materials injected	*Dose* (μg)	*Mean survival time in days*	*Survivors after 60 days/total number of animals*
Saline	—	25.5	0/10
BCG	10	41.4	3/10
	30	49.2	4/9
	100	43.9	3/9
Purified cell walls of *M. kansasii*[a]	10	38.6	1/10
	30	50.6	5/10
	100	53.6	8/10

[a] Prepared by A. Adam and J. F. Petit.
Hybrid $(C_{57}Bl/6 \times AK)F_1$ mice; intraperitoneal injections of BCG or cell walls, 8 days before intraperitoneal inoculation of 10^2 cells [L. Chedid and A. Lamensans, unpublished experiments].

Clinical experiments with successful application of BCG to the treatment of acute lymphoblastic leukaemia have been reported by Mathé *et al.*[129].

It seems rather probable that some, at least, of these effects of whole BCG cells are due to the glycolipid–mucopeptide complex discussed above;

analogous experiments with *C. parvum* containing a 'reticulostimuline' are in agreement with this hypothesis†.

Table 4. Action of BCG, *M. kansasii* and its cell walls on survival of mice inoculated with Ehrlich ascites

Materials injected	*Dose* (μg)	*Mean survival time in days*	*Survivors after 60 days/total number of animals*
Saline	—	21.5	0/67
S. enteridis	10	29.5	1/7
endotoxin	100	29	1/10
BCG (phenol killed)	100	47.7	5/19
	300	49.5	22/46
	1000	47.4	4/9
Acetone extracted BCG	300	40.5	2/8
	1000	52.7	4/7
M. kansasii (phenol killed)	300	41.2	2/19
M. kansasii crude cell walls[a]	300	54.9	7/10
	1000	57.6	8/10

[a] prepared by A. Adam and J. F. Petit.
Hybrid (C57B 1/6 × AK)F_1 mice.
Treatment: intraperitoneal injection, 14 days before intraperitoneal inoculation of 10^5 cells [L. Chedid and A. Lamensans, unpublished experiments].

The task of the chemist—There can be no doubt that whole mycobacterial cells and some of their fractions stimulate the reticuloendothelial system and that this property could find interesting applications in the treatment of human diseases.

Whole mycobacterial cells have, however, several disagreeable side-effects, such as sensitization to tuberculin and to endotoxins, granuloma formation, etc. We may thus ask: would it be possible to isolate mycobacterial fractions having only the desirable effects?

It is too early to give an answer to this question; a first approach consists of defining the minimal chemical structure responsible for the 'desirable effect' (adjuvant action and/or stimulation of the reticuloendothelial system).

This seems to be possible, for the moment, only for adjuvant activity, where it is known that active wax D preparations must contain the mucopeptide and mycolic acids. No data are yet available concerning the structural specificity of the arabinogalactan moiety.

We conclude that structures such as **10** or those shown in *Figures 4* and *5* are necessary for full adjuvant activity. Would a monomer be fully active, or only an oligomer, or a polymer? It is too early to give an answer to this question.

We may then ask if it will be possible to obtain fractions having only adjuvant activity and no 'reticulostimuline' action and vice-versa. Preliminary experiments of L. Chedid and A. Lamensans with preparations obtained by A. Adam and J. F. Petit show that this seems possible.

† Prévot *et al.*[130] have reported that the anaerobic *C. parvum* has a strong 'reticulostimuline' activity; the cell walls of this strain are also active[131]; their mucopeptide contains LL-diaminopimelic acid[132].

The task of the chemist will be to degrade mycobacterial cell walls by gentle, mostly enzymatic methods, to try to isolate the simplest type of molecule having still one or another of the 'desirable activities'; this work must of course be pursued in close contact with competent biologists. At the same time synthetic model compounds will have to be prepared so as to be able to obtain, finally, active compounds by total synthesis.

This may yet take several years of hard work, but the prospects of success seem fair and the goal rewarding.

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