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DEMONSTRATION THAT THE GALACTOSYL AND ARABINOSYL RESIDUES IN THE CELL-WALL ARABINOGALACTAN OF *Mycobacterium leprae* AND *Mycobacterium tuberculosis* ARE FURANOID

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ABSTRACT

By a complex process involving methylation, partial hydrolysis with acid, reduction with sodium borodeuteride, ethylation, further hydrolysis and reduction, and subsequent capillary gas–liquid chromatography–mass spectrometry of the derived alditol acetates, it was established that the arabinogalactans of *Mycobacterium leprae* and *Mycobacterium tuberculosis* contain arabinofuranosyl and galactofuranosyl residues exclusively. Thus, the covalently bound, highly immunogenic arabinogalactan of mycobacteria, and presumably of other actinomycetes, is highly unusual, in that all of the glycosyl residues are in the furanoid form. Furthermore, by establishing that the galactofuranosyl residues are either 5-, 6-, or 5,6-linked, their linkage pattern was established, and the literature is corrected on this point.

INTRODUCTION

The distinguishing feature of actinomycetes and coryneform bacteria, such as most members of the *Corynebacterium, Mycobacterium, Nocardia, Rhodococcus,* and "aurantiaca" taxa is a chemotype IV cell-wall marked by major quantities of *meso*-diaminopimelic acid and arabinogalactan[1,2]. Arabinogalactan, covalently linked to both peptidoglycan and the high-molecular-weight, branched mycolic acids, constitutes ~35% of the cell-wall mass of most mycobacteria, and it may be the single most dominant immunogen[3,4]. Although the polysaccharide has been the subject of intensive research by several generations of workers[5–7], a few Japanese researchers are credited with the majority of the present information on its definition. Misaki *et al.*[8–10] clearly demonstrated that the polymer is composed of D-arabinose and D-galactose, and excluded the possibility, raised in earlier work, that glucose, mannose, and amino sugars might be present. They also showed that the arabinosyl residues were predominantly 5-linked arabinofuranose, and that the

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galactosyl residues are either 4-linked galactopyranose or 5-linked galactofuranose. It was initially presumed by these workers[8] that the galactosyl residues are all in the 4-linked galactopyranosyl form, a misbelief still perpetuated in the secondary literature. However, from partially cleaved arabinogalactan of *Mycobacterium tuberculosis*, Vilkas *et al.*[11] isolated the disaccharide, Gal*f*-(1→6)-Gal, which served to demonstrate the presence of at least some galactofuranosyl and 6-linked galactosyl residues in the arabinogalactan. Because 6-linked galactosyl residues were not evident among the methylation products described by Misaki *et al.*[10], and as the question of the ring form in which the galactosyl residues appear had not been resolved, we have addressed this fundamental question in a study of the arabinogalactans from the most notable of the mycobacterial pathogens, namely, *M. tuberculosis* and *Mycobacterium leprae*.

RESULTS AND DISCUSSION

*The presence of 6-linked Gal*f *in the arabinogalactan.* — Cell walls, or a cell-wall-containing insoluble fraction, were prepared from *M. tuberculosis* and *M. leprae* as described in the Experimental section. Amino acid and amino sugar analysis showed the expected peptidoglycan components[4]: glucosamine, muramic acid, alanine (glycine in the case of *M. leprae*[12]), and diaminopimelic acid (results not shown). The glycosyl composition of the cell walls of both *M. tuberculosis* and *M. leprae* was dominated by the presence of arabinosyl and galactosyl residues, as expected (see Table I).

The cell-wall preparations were per-*O*-methylated and a portion of each product was hydrolyzed, the sugars reduced, and the alditols acetylated. The resulting acetates were analyzed by capillary-column gas–liquid chromatography (g.l.c.) and gas–liquid chromatography–mass spectrometry (g.l.c.-m.s.). The results (see Table II) were consistent with those reported earlier by Misaki *et al.*[10], except that a major new component, 1,4,6-tri-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-

TABLE I

GLYCOSYL COMPOSITION OF *M. tuberculosis* AND *M. leprae* CELL WALLS

Glycosyl residue	*Mole %*	
	M. tuberculosis	M. leprae
Rhamnosyl	0.6	1.7
Ribosyl	0.7	ND[a]
Arabinosyl	53	79
Mannosyl	3	1
Galactosyl	41	19
Glucosyl	1	ND

[a]ND = none detected.

TABLE II

GLYCOSYL-LINKAGE ANALYSIS OF *M. tuberculosis* AND *M. leprae* CELL WALLS

Glycosyl residue	O-*Acetyl position*	O-*Methyl position*	*Linkage deduced*	*Mole %*	
				M. tuberculosis	M. leprae
Ara	1,4	2,3,5	t-Ara*f*	12	13
Ara	1,2,4	3,5	2-Ara*f*	10	12
Ara	1,4,5	2,3	5-Ara*f* *or* 4-Ara*p*	31	41
Ara	1,3,4,5	2	3,5-Ara*f* *or* 3,4-Ara*p*	11	11
Man	1,5	2,3,4,6	t-Man*p*	1	0.4
Gal	1,4	2,3,5,6	t-Gal*f*	1	1
Gal	1,4,5	2,3,6	5-Gal*f* *or* 4-Gal*p*	21	11
Gal	1,4,6	2,3,5	6-Gal*f*	10	5
Gal	1,4,5,6	2,3	5,6-Gal*f* *or* 4,6-Gal*p*	2	4

methyl-galactitol (*m/z* 117, 118, 159, 162, and 233) was observed. This product was probably overlooked in earlier analyses, simply because the packed g.l.c. columns of earlier times did not have the resolving capacity of present-day capillary columns. The presence of this product confirmed the report of Vilkas *et al.*[11] of 6-linked galactosyl residues in the arabinogalactan of mycobacteria, and further demonstrated that such galactosyl residues are in the furanose form.

Strategy for determination of ring form. — Conventional methylation analysis (*cf.*, Table II) can establish the ring form of a glycosyl residue only if a methyl group appears on O-4 in the case of sugars in the pyranose form, or on O-5 in the case of sugars in the furanose form. After *O*-methylation, the glycosyl residues of the methylated arabinogalactan cannot contain a methyl group on O-4 or O-5. Thus, in order to identify the ring form of the glycosyl residues, the *O*-methylated arabinogalactan was subjected to partial hydrolysis with acid, followed by reduction with sodium borodeuteride, *O*-ethylation, complete hydrolysis, further reduction with sodium borodeuteride, and, finally, acetylation[13]. The rationale for this series of reactions is presented in Fig. 1. For a glycosyl residue whose ring form is to be determined, cleavage at its glycosidic bond (C-1; pathways b and d in Fig. 1) or at its aglycon bond (C-4 for pyranosyl residues or C-5 for furanosyl residues; pathways a and c in Fig. 1) converts it into a new type of glycosyl or glycose residue whose ring form is decipherable by the elaborate series of analyses described previously[13]. Accordingly, a 4-linked Gal*p* residue cleaved at C-1 (but not at C-4) is converted into 4-*O*-acetyl-1-deuterio-1,5-di-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol, as illustrated in pathway b in Fig. 1, and a 5-linked Gal*f* residue, when cleaved at C-1 but not at C-5) is converted into 5-*O*-acetyl-1-deuterio-1,4-di-*O*-ethyl-2,3,6-tri-*O*-methyl-galactitol (pathway d, Fig. 1). In a similar fashion, cleavage of a 4-linked Gal*p* at

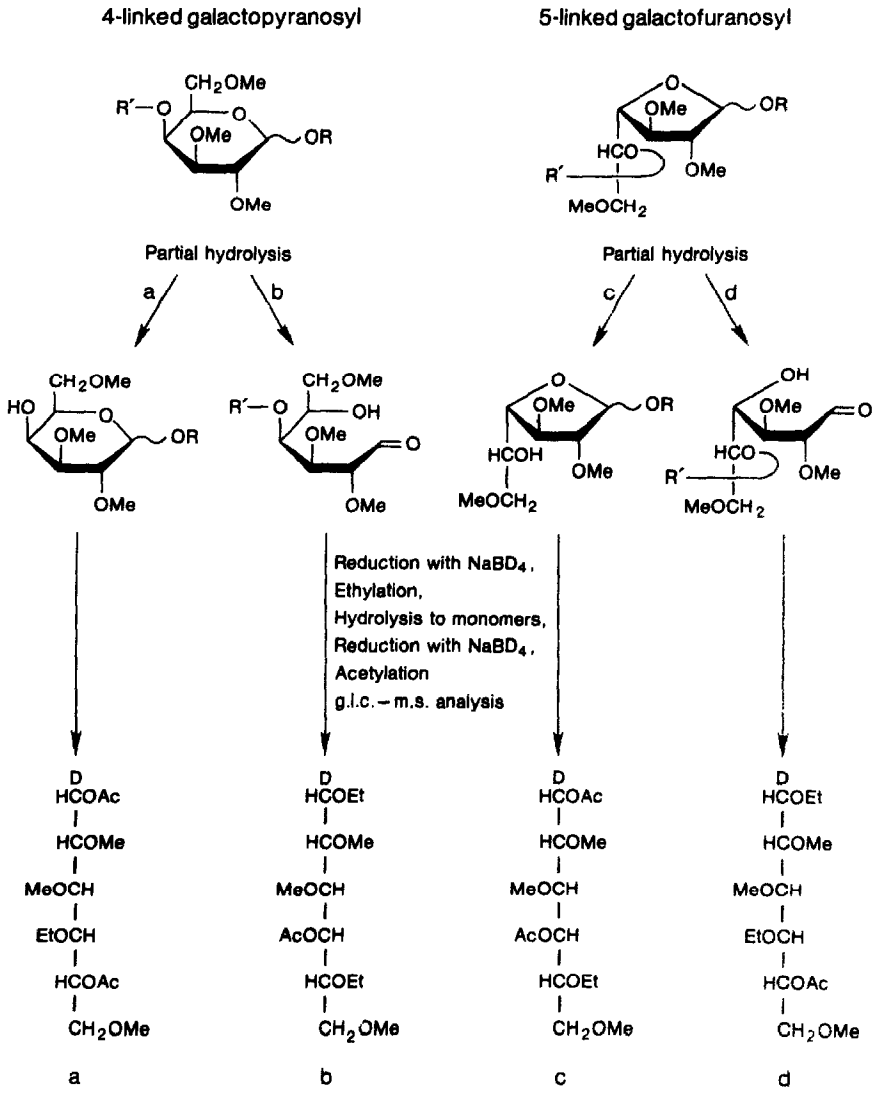

Fig. 1. A scheme for distinguishing 4-linked galactopyranosyl from 5-linked galactofuranosyl residues. The formation of the four different, partially *O*-acetylated, partially *O*-ethylated, partially *O*-methylated alditols, two resulting from pyranosyl residues (a and b) and two resulting from furanosyl residues (c and d), is illustrated. The four different partially *O*-alkylated alditol acetates may be distinguished by g.l.c.–m.s. (see Fig. 3).

C-4 leads to 1,5-di-*O*-acetyl-1-deuterio-4-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol (pathway a, Fig. 1), and cleavage at C-5 of a 5-linked Gal*f* leads to 1,4-di-*O*-acetyl-1-deuterio-5-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol (pathway c, Fig. 1). These four different, partially *O*-acetylated, partially *O*-ethylated, partially *O*-methylated alditols are readily distinguished from one another by mass spectrometry[13,14].

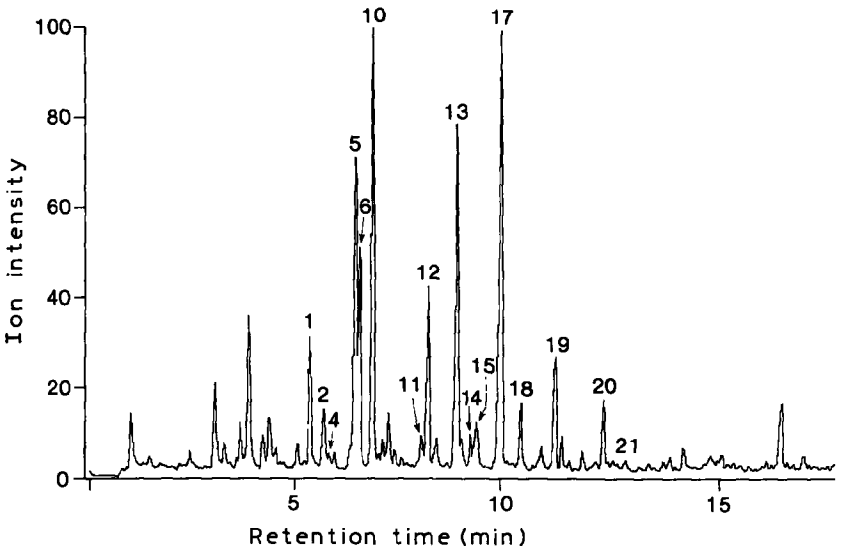

Fig. 2. The total ion chromatography generated during g.l.c.–m.s. analysis of the partially *O*-alkylated alditols prepared from the cell-wall arabinogalactan of *M. tuberculosis*. The compounds identified in Table III and in the text are those contained in the numbered peaks.

Ring form of galactosyl residues in the arabinogalactan. — Methylated cell-wall arabinogalactan from *M. tuberculosis* was partially hydrolyzed by treating it with 2M CF_3CO_2H for 1 h at 90°. The methylated arabinogalactan from *M. leprae* was partially hydrolyzed with 2M CF_3CO_2H for 1 h at 80°. The conditions selected for partial hydrolysis were arrived at as described[14], and resulted in the cleavage of 40 to 60% of the glycosyl linkages, with greater cleavage of the arabinogalactan from *M. tuberculosis* than of that from *M. leprae*. The partial hydrolyzates were reduced with sodium borodeuteride, the products ethylated, the ethers fully hydrolyzed, the sugars reduced with sodium borodeuteride, the alditols acetylated, and the complex mixture of partially alkylated alditol acetates analyzed g.l.c. and g.l.c.–m.s.[13,14]. The total-ion chromatogram resulting from g.l.c.–m.s. analysis of the ensuing complex mixture of partially alkylated alditol acetates derived from *M. tuberculosis* cell-wall arabinogalactan is presented in Fig. 2. The identity of the partially alkylated alditol acetates, the deduced linkage and ring form of their corresponding glycosyl residues, and the mole percent recovered are presented, for the products obtained from both *M. leprae* and *M. tuberculosis*, in (Table III). Of particular note are compounds **11** and **17**, which correspond to those compounds produced by pathways d and c in Fig. 1. The emergence of these products demonstrates the presence of 5-linked Gal*f* residues in the cell-wall arabinogalactan of both *M. leprae* and *M. tuberculosis*. The mass spectrum of compound **17** is presented in Fig. 3, and is compared to that of 1,5-di-*O*-acetyl-1-deuterio-4-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol arising from a known 4-linked Gal*p* residue in the galactan prepared from citrus pectin[15].

TABLE III

IDENTIFICATION OF THE PARTIALLY ALKYLATED ALDITOL ACETATES DERIVED FROM CELL WALLS OF *M. tuberculosis* AND *M. leprae*

Compound number[a]	*Glycosyl residue*	*Position of*			*Linkage position and ring form*[b]	*Position cleaved by partial hydrolysis*[c] *with acid*	*Mole %*	
		O-*Acetyl*	O-*Methyl*	O-*Ethyl*			M. tuberculosis	M. leprae
1	Ara	5	2,3	1,4	5-Ara*f*	1	8	8
2	Ara	3	2	1,4,5	3,5-Ara*f*[d]	1 & 5	3	ND[e]
3	Ara	1,4	2,3,5	—	t-Ara*f*	—	ND	10
4	Ara	5	2	1,3,4	3,5-Ara*f*	1 & 3	1	ND
5	Ara	—	2,3	1,4,5	5-Ara*f*[f]	1 & 5	15	5
6	Ara	1,4	2,3	5	5-Ara*f*	5	10	10
7	Ara	1,4	2	3,5	3,5-Ara*f*	3 & 5	ND	1
8	Ara	3,5	2	1,4	3,5-Ara*f*	1	ND	1
9	Ara	1,2,4	3,5	—	2-Ara*f*	—	ND	6
10	Gal	—	2,3,6	1,4,5	5-Gal*f*[g]	1 & 5	11	—
11	Gal	5	2,3,6	1,4	5-Gal*f*	1	3	0.3
12	Ara	1,4,5	2,3	—	5-Ara*f*[f]	—	10	34
13	Gal	6	2,3,5	1,4	6-Gal*f*	1	10	8
14	Man	1,5	2,3,4,6	—	t-Man*p*	—	0.4	ND
15	Gal	1,4	2,3,5,6	—	t-Gal*f*	—	2	ND
16	Gal	6	2,3	1,4,5	5,6-Gal*f*[h]	1 & 5	ND	2
17	Gal	1,4	2,3,6	5	5-Gal*f*	5	17	8
18	Gal	1,4	2,3,5	6	6-Gal*f*	6	2	ND
19	Gal	1,4,5	2,3,6	—	5-Gal*f*[g]	—	6	4
20	Gal	1,4	2,3	5,6	5,6-Gal*f*	5 & 6	2	2
21	Gal	1,4,6	2,3	5	5,6-Gal*f*	5	0.4	0.5

[a]Refer to Fig. 2. [b]Defines the linkage of the residues in the native intact arabinogalactan. [c]Defines the position cleaved by the partial acid hydrolysis step; the symbol — indicates that no cleavage took place. [d]The linkage and ring form of the arabinosyl residue that yielded this derivative cannot be unequivocally identified. However, because of the presence of compounds **4, 7**, and **8**, it apparently should be 3,5-Ara*f*. [e]ND = not detected. [f]The linkage and ring form of the arabinosyl residue that yielded these derivatives cannot be unequivocally identified. However, because of the presence of compounds **1** and **6**, it apparently should be 5-Ara*f*. [g]The linkage and ring form of the galactosyl residue that yielded these derivatives cannot be unequivocally identified. However, because of the presence of compounds **11** and **17**, it apparently should be 5-Gal*f*. [h]The linkage and ring form of the galactosyl residue that yielded this derivative cannot be unequivocally identified. However, because of the presence of compounds **20** and **21**, it apparently should be 5,6-Gal*f*.

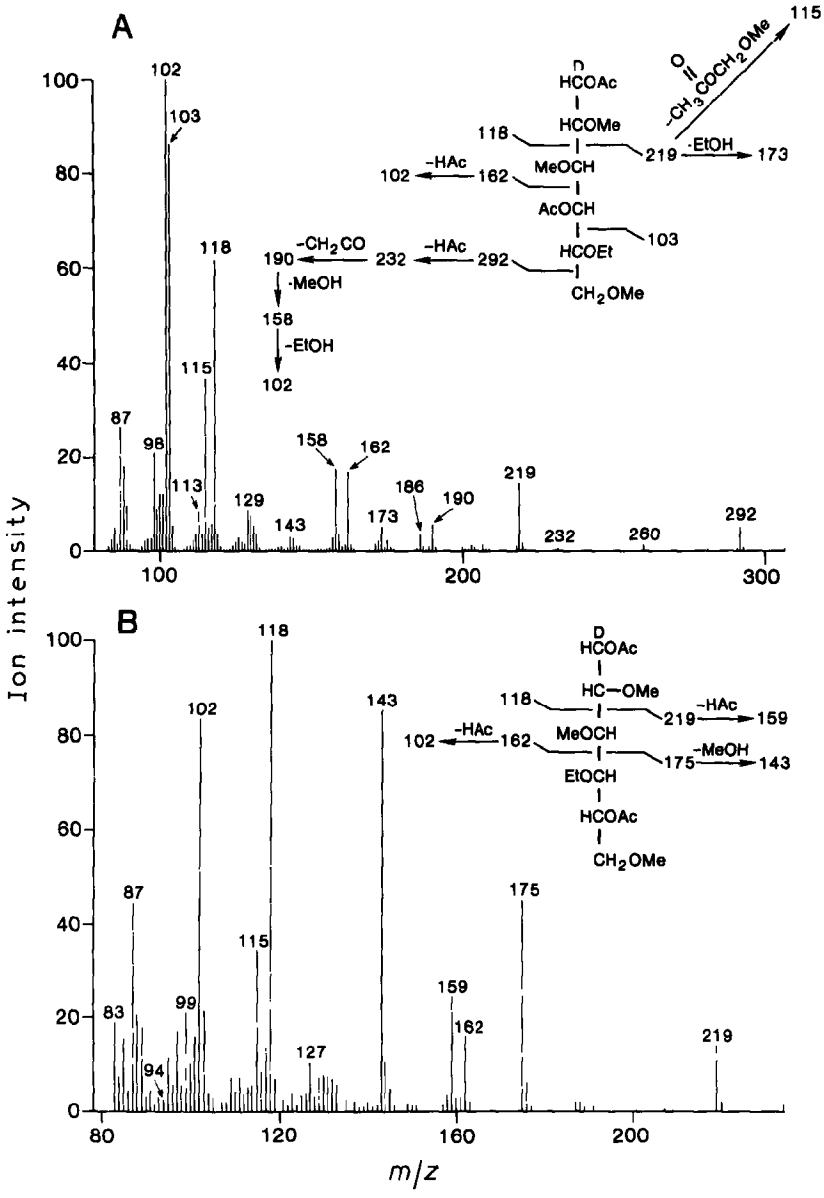

Fig. 3. The mass spectrum of compound **17** (Fig. 2 and Table III), 1,4-di-*O*-acetyl-1-deuterio-5-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol, is presented in (A). The mass spectrum of the corresponding product which would have resulted had the arabinogalactan contained a 4-linked galactopyranosyl unit, namely, 1,5-di-*O*-acetyl-1-deuterio-4-*O*-ethyl-2,3,6-tri-*O*-methylgalactitol, is presented in (B); it was obtained from citrus-pectin galactan [HAc refers to CH_3CO_2H, not CH_3CHO].

In the light of the knowledge gained from this comparison, further examination of the data in Table III revealed that all of the glycosyl residues of arabinogalactan, from both *M. tuberculosis* and *M. leprae*, whose ring form could not be determined by direct methylation analysis (see Table II) are in fact in the furanose form. Thus, compounds **1** and **6** show the existence of 5-Ara*f* (rather than 4-Ara*p*); compounds **4**, **7**, and **8** show the presence of 3,5-Ara*f* (rather than 3,4-Ara*p*); and compounds **20** and **21** show the existence of 5,6-Gal*f* (rather than 4,6-Gal*p*). These results, in combination with the ring-form data that can be deduced from direct methylation analysis, demonstrated that all of the residues in the cell-wall arabinogalactan from both *M. leprae* and *M. tuberculosis* are in the furanose form. It should be pointed out that if small proportions of residues are present in the pyranose form they might be missed by the type of analyses described.

From the evidence presented here, and by others[8-11,16], it may be concluded, without much equivocation, that both the arabinosyl and galactosyl residues of the mycobacterial arabinogalactan are in the furanosyl form, and it is probable, based on general chemotaxonomic principles[1,2], that the same consideration applies to all actinomycetes and related bacteria. Heteropolysaccharides in which all of the glycosyl residues are furanosidic are highly unusual[17]. This feature, and the resulting acid-lability, should explain a well known and useful characteristic of cell-wall arabinogalactan, namely, the felicity with which it can be cleaved by mild acid[3]. An added peculiarity of mycobacterial arabinogalactan may also be explained in such structural terms. Although arabinanases capable of cleaving the arabinosyl residues are known[8], no appropriate galactanases have been so described, and this now seems not unreasonable considering that about one-third of the galactosyl residues are 6-linked galactofuranosides, and the rest are 5-linked galactofuranosides, a unique combination.

The chemical basis of the antigenicity of many mycobacterial species, notably carbohydrate-containing antigens[18-20] and particularly those in the leprosy bacillus[21], is nearing complete definition. A major exception is the mycolyl-arabinogalactan–peptidoglycan complex. A lesson to be learned from a re-examination of the structure of the "arabinomannan" of mycobacteria, also a highly immunogenic cross-reactive antigen, is that many supposed structures derived in the past should be re-examined in the light of recent technological developments; arabinomannan contains a considerable proportion of *myo*-inositol 1-phosphate, some apparently in the form of a phosphatidylinositol[22] "anchor". Likewise, the arabinogalactan complex may yield further surprises. The arrangements of the 2-Ara*f*, the 5-Ara*f*, and the 3,5-Ara*f* are unknown at this time. Also, nothing is known about the arrangement of the 6-Gal*f*, the 5-Gal*f*, and the 5,6-Gal*f* residues, or the manner in which the arabinose- and galactose-containing regions are attached to each other. Neither has the issue of the mode of attachment of arabinogalactan to peptidoglycan been resolved. Again, the secondary literature suggests a phosphoric diester format[23], although the original evidence, based on the isolation of muramic acid 6-phosphate[24,25] and a presumed arabinosyl phosphate[26] from cell walls, is tentative, and, in view of the evidence for a glycosidic link in a *Nocardia*[27], the question

should be reopened. Yet, to the mycolyl-arabinogalactan–peptidoglycan complex is attributed much of the immunogenicity, including delayed-type hypersensitivity and protective immunity, pathogenicity, including granuloma formation, peculiar persistence, and staining properties of mycobacteria[28]. It may be the last of the major antigenic, carbohydrate-containing complexes of *Mycobacterium* spp. awaiting molecular definition.

EXPERIMENTAL

Cell walls. — Lyophilized *M. leprae* (1.42 g) prepared from infected armadillo livers[22] was exhaustively extracted with 2:1 (v/v) $CHCl_3$–CH_3OH to remove all lipids, disrupted in a French Press followed by sonication, and further extracted with refluxing 70% ethanol to remove lipoarabinomannan, lipomannan, other soluble carbohydrates, and some soluble proteins[22]. The residue was extensively washed with water to yield 671 mg of a cell-wall-containing insoluble fraction.

M. tuberculosis $H_{37}R_a$ was obtained from Dr. Kuni Takayama[29] (William S. Middleton Memorial Veterans Hospital, Madison, WI), and cultured on a glycerol–L-alanine–salts medium[29]. The cells (30 g, wet weight) were extensively washed with water, and suspended in 50 mL of phosphate-buffered saline containing 0.1% of Tween 80, mM $MgCl_2$, and mM benzamidine (Sigma Chemical Co., St. Louis, MO). The cells were sonicated by using a Heat Systems-Ultrasonic, Inc. (Farmingdale, NY) sonicator at maximum power with a 1.9-cm (0.75-in.) tip at a 10% duty cycle for ~5 min of sonicator "on" time. The disrupted cells were treated overnight at 4° with 10 mg each of ribonuclease (Catalog No. R4875; Sigma) and deoxyribonuclease (Catalog No. D5025, Sigma). Upon centrifugation, cell walls were found within the 27,000-*g* pellet. These were washed three times with the buffer already described and three times with water. Cell walls (5 g, wet weight) were recovered.

Analytical procedures. — The glycosyl residues were analyzed as described[18]. Methylation was performed as described by Stellner *et al.*[30], with modifications[13]. For glycosyl-linkage analysis, the methylated polysaccharide was hydrolyzed, the sugars reduced, the alditols acetylated, and the acetates analyzed by g.l.c. and g.l.c.–m.s. as described[18]. Partial hydrolysis, and reduction with sodium borodeuteride, followed by complete hydrolysis, further sodium borodeuteride reduction, and acetylation were accomplished as previously described[13,14]. The resulting, partially *O*-acetylated, partially *O*-ethylated, partially *O*-methylated alditols were analyzed by g.l.c. and g.l.c.–m.s. described previously[18].

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