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STRUCTURAL AND IMMUNOCHEMICAL CHARACTERIZATION OF THE ACIDIC ARABINOMANNAN OF *Mycobacterium smegmatis**

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ABSTRACT

A serologically active, acidic arabinomannan has been isolated from *Mycobacterium smegmatis*. The polysaccharide contains approximately 56 arabinosyl and 11 mannosyl residues, and 2 phosphate, 6 monoesterified succinate, and 4 ether-linked lactate groups. After saponification to remove succinyl groups, the polysaccharide can be separated into phosphorylated (55%) and nonphosphorylated (45%) forms, the former containing a little more arabinose and a little less mannose than the latter. The structures of these polysaccharides were investigated by ^{1}H- and ^{13}C-n.m.r. spectroscopy and methylation analysis, before and after selective cleavage of furanosyl linkages. The phosphorylated and nonphosphorylated forms of the polysaccharide were found to have similar, if not identical, structures. The main structural feature of the polysaccharides is the presence of chains of contiguous arabinofuranosyl residues linked α-(1→5). These chains are attached at O-4 of arabinopyranosyl residues that are present in a core region of the polysaccharide that also contains mannopyranosyl residues. Immunochemical studies demonstrated that the polysaccharide is an effective, precipitating antigen with antisera from rabbits immunized with cell walls or heat-killed cells of *M. smegmatis*. The polysaccharide is, however, more effective as a precipitating antigen after removal of the succinate groups, and completely ineffective after removal of arabinofuranosyl residues. The polysaccharide therefore contains an important antigen in common with the arabinogalactan lipopolysaccharide of the cell wall of the bacterium, *i.e.*, chains of contiguous α-(1→5)-linked arabinofuranosyl residues.

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INTRODUCTION

The presence of serologically active polysaccharides in the cell walls, cytoplasm, and culture filtrates of *Mycobacteria* has long been recognized[1-8]. Four different types of polysaccharide are typically found in all *Mycobacteria*: a mannan, a glucan, an arabinomannan, and an arabinogalactan; however, only the arabinomannans and arabinogalactans have been found to possess significant antigenic activity in precipitation, complement-fixation, and passive hemagglutination tests with antisera to mycobacterial cells[8].

The extraction of these polysaccharides from the mycobacterial cell has usually been accomplished under alkaline conditions at elevated temperatures[8], conditions that would, however, cause saponification of any ester groups present in these polysaccharides and, perhaps, also cause structural alteration of the polysaccharides themselves. Under these circumstances, the serological activity observed for a polysaccharide would probably not reflect its true activity as an immunizing antigen.

During the isolation of the *O*-methylmannose-containing polysaccharide and *O*-methylglucose-containing lipopolysaccharides of *Mycobacterium smegmatis*, an acidic (a) arabinomannan (AM) was frequently observed as a contaminant[9]. A closer examination of the arabinomannan revealed the presence of esterified succinic acid, but further chemical and structural characterization of this polysaccharide was not undertaken. As the presence of ester groups in this polysaccharide could drastically alter its antigenic properties, we have re-examined its structure and serological activity. Reported herein are the isolation, composition, structure, and serological activity of this acidic arabinomannan (aAM).

EXPERIMENTAL

Materials. — Sephadexes G-50 (fine), G-150 (fine), and DEAE A-25 were obtained from Pharmacia. Bio-Gel P-2 was purchased from Bio-Rad, and Freund's incomplete adjuvant was a product of Difco. Column packings for gas–liquid chromatography were obtained from Supelco.

Analytical procedures. — Total carbohydrate was determined by the phenol–sulfuric acid procedure[10], and reducing sugar was measured by the procedure of Park and Johnson[11]. Organic phosphate was determined by the method of Bartlett[12], protein by the method of Bradford[13], and ester by the hydroxamic assay as modified by Lee[14].

Chromatography. — The following stainless-steel columns were used for gas–liquid chromatography: Column 1, 3% of SP-2340 on 100/120 Supelcoport, 183 cm × 3.2 mm; Column 2, same as Column 1, 366 cm × 3.2 mm; Column 3, 10% of SP-2401 on 100/120 Supelcoport, 366 cm × 3.2 mm; Column 4, 10% of SE-30 on 80/100 Supelcoport, 183 cm × 3.2 mm; and Column 5, 3% of OV-225 on 100/120 Supelcoport, 304.8 cm × 3.2 mm. Descending paper-chromatography was conducted

on Whatman No. 1 paper in 8:2:1 (v/v/v) ethyl acetate–pyridine–water and, after drying, carbohydrate components were detected with alkaline silver nitrate[15].

Immunochemical methods. — *M. smegmatis* cells were heat-killed by autoclaving for 0.5 h at 130°, and cell walls were prepared as described under "Isolation of aAM". The cell walls were washed several times by centrifugation with water to remove soluble components. Rabbit antisera to whole cells were prepared by giving two animals an initial injection of 1.3–1.6 mg of heat-killed cells in 0.7–0.8 mL of Freund's incomplete adjuvant and 0.9% NaCl solution (1:1, v/v, emulsified), followed by two additional injections at intervals of one week. Rabbit antisera to cell walls were prepared by giving a single animal an initial injection of 1.1–1.9 mg of lyophilized cell-walls in 0.5–1.0 mL of Freund's incomplete adjuvant and 0.9% NaCl solution (1:1, v/v, emulsified), followed by additional injections after 1, 1.5, 2, and 3 weeks. All injections were administered intracutaneously near the inguinal and axillary lymph-nodes. Blood (~2 mL) was collected from the ear vein of each animal at 2 and 3 weeks after the initial injection, and ~80 mL by cardiac puncture at 4 weeks after the initial injection.

Sera were titered as follows: using titer plates having hemispherical wells (Scientific Products), 50 μL of 0.9% NaCl solution was placed in each of ten wells, and then 50 μL of the serum to be titered was added to the first well and serially diluted through the remaining nine wells. After the addition, with stirring, of 50 μL of a suspension (2 mg/mL) of heat-killed cells to each well, the titer plate was covered and incubated for 1 h at 37°, and then overnight at 4°. Cell agglutination was indicated by the appearance of a disc of precipitate having feathered edges, visible under a microscope.

Quantitative precipitin-reactions were performed in the following way. After placing 250 μL of 0.9% NaCl solution in each of ten test tubes (12 × 74 mm), 250 μL of a solution containing 0.7–1.0 mg of polysaccharide per mL was added to the first tube, and the remaining tubes were serially diluted from the first. Serum (250 μL) was added to each tube, and the tubes were mixed, covered, and incubated for 48 h at 4°. At the end of this period, the tubes were centrifuged for 20–30 min in a clinical centrifuge, the supernatant liquors were carefully removed, and the precipitates were washed twice by centrifugation with 500-μL aliquots of 0.9% NaCl solution. The amount of protein in the precipitates was determined by the method of Bradford[13], using a reagent blank that contained serum but not polysaccharide.

Instrumentation. — ^{1}H-Nuclear magnetic resonance (n.m.r.) spectra were recorded with Varian XL-100-15 and Bruker 270-MHz, n.m.r. spectrometers. The ^{13}C-n.m.r. spectrum was recorded with a Varian XL-100-15 spectrometer, and referenced to sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propanoate, sodium salt (TSP; 10% solution in D_2O) contained in a coaxial, capillary tube. Under these conditions, the methyl carbon signal of internal acetone was δ 32.66; this value differs by +1.46 p.p.m. from that obtained with tetramethylsilane as the external reference standard[16], and literature values cited herein that had been referenced to tetramethylsilane are corrected by that amount.

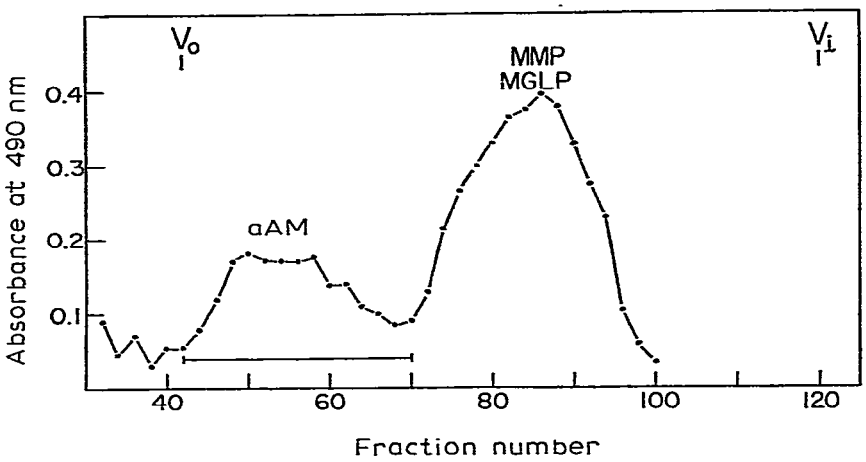

Fig. 1. Fractionation of the water-soluble carbohydrates from *M. smegmatis* on a column (4 × 100 cm) of Sephadex G-50. [Fractions (10 mL) were collected at a flow rate of 48 mL/h. The void volume (V_0) and inclusion volume (V_i) are the positions of elution of Blue Dextran 2000 and glucose, respectively. MGLP and MMP are the *O*-methylglucose-containing lipopolysaccharides and *O*-methylmannose-containing polysaccharide first isolated by Lee[14] and Gray and Ballou[28], respectively. Fractions labeled aAM were combined for further purification.]

Gas–liquid chromatography (g.l.c.) was performed with Varian Aerograph model 1400 and F and M model 810 chromatographs, both equipped with flame-ionization detection. Combined g.l.c.–mass spectrometry (m.s.) was conducted with a Hitachi RMU-6D mass spectrometer, renovated and converted to g.l.c.–m.s. operation. Mass chromatography[17] was performed by generating spectra at 8-sec intervals throughout the gas–liquid chromatography experiment. These data were processed with an AEI DS-30 data system, and the relative abundances of characteristic *m/e* values were plotted to give mass chromatograms. Each component emerging from the gas–liquid chromatograph thus gave rise to a peak in the mass chromatogram at each *m/e* value in its mass spectrum.

Isolation of aAM. — Moist, *M. smegmatis* cell-paste (50 g) was suspended in water (4 vol.) in a Sorvall Omni-mixer that was set at low speed, and the suspension was then passed through a Sorvall RM1 Cell Fractionator at 40,000 lb.in.$^{-2}$ at 0–15°. The resultant, viscous solution was centrifuged for 30 min at 27,000*g*, and the supernatant liquor was separated from the pellet, which consisted primarily of cell walls. The supernatant liquor was then lyophilized to a powder which was subsequently stirred vigorously with 50% ethanol (250 mL). The mixture was centrifuged for 20 min at 10,000*g* to remove insoluble protein and nucleic acid, the supernatant liquor was evaporated under vacuum to a syrup, and this was partitioned, with vigorous agitation, between the two layers of 8:4:3 (v/v/v) chloroform–methanol–water (300 mL). To break the emulsion, the mixture was centrifuged for 20 min at 10,000*g*, and then the upper, aqueous layer was removed, evaporated under vacuum (to remove methanol and traces of chloroform), and dialyzed against distilled water overnight at 4°.

The nondialyzable solution was concentrated by evaporation under vacuum,

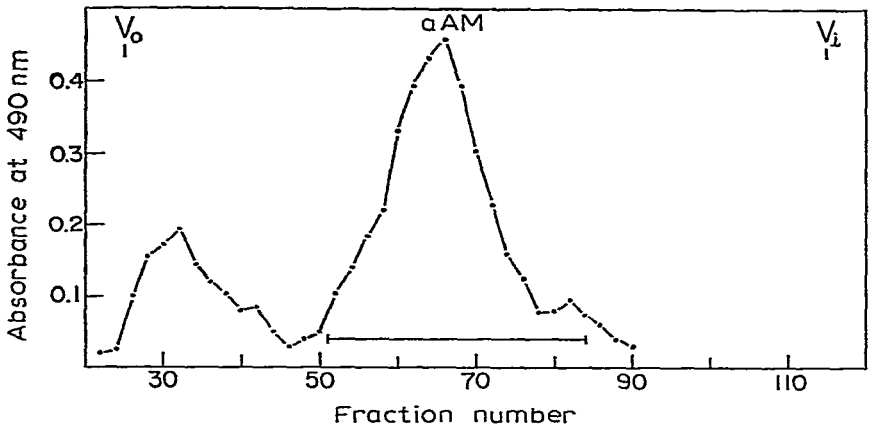

Fig. 2. Fractionation of aAM from Fig. 1 on a column (2.5 × 98 cm) of Sephadex G-150. [Fractions (5 mL) were collected at a flow rate of 18 mL/h. The void volume and inclusion volume were determined as in Fig. 1. The indicated fractions were combined for further purification.]

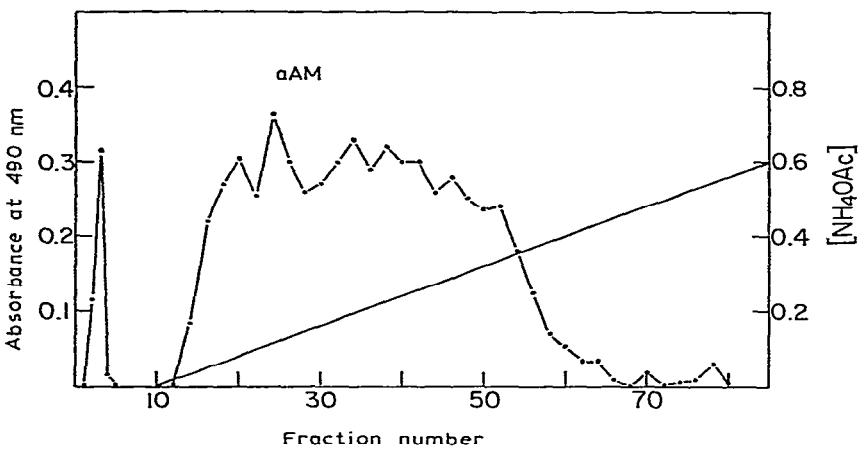

Fig. 3. Fractionation of the aAM fractions from Fig. 2 on a column (2 × 10 cm) of DEAE-Sephadex A-25 (acetate). [aAM was applied to the column in water, and the column was eluted, first with water (10 fractions), and then with a linear gradient of 0.0–1.0M NH_4OAc (1 L). Fractions were 8 mL each. Fractions labeled aAM were used for further studies.]

and fractionated on a column (4 × 100 cm) of Sephadex G-50 in 0.1M ammonium acetate (containing 0.02% of sodium azide as a preservative). Assay for total carbohydrate gave the pattern shown in Fig. 1, and the fraction labeled aAM was collected. After dialysis against water at 4° to remove column buffer, the nondialyzable portion was concentrated, and applied to a column (2.5 × 98 cm) of Sephadex G-150. Analysis for total carbohydrate (see Fig. 2) revealed the presence of two polysaccharides, aAM and a larger polysaccharide composed mostly of glucosyl residues.

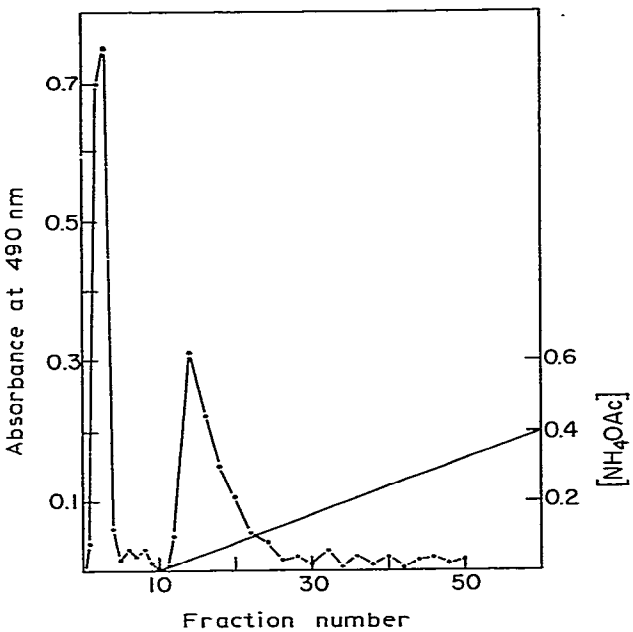

Fig. 4. Fractionation of saponified aAM on a column (2 × 10 cm) of DEAE-Sephadex A-25 (acetate). [The elution conditions were the same as in Fig. 3. The materials eluted with water (nonphosphorylated AM) and the salt gradient (phosphorylated AM) were used for further studies.]

Finally, the aAM fraction (Fig. 2) was dialyzed against water at 4°, and then applied to a column (2 × 10 cm) of DEAE-Sephadex A-25 (acetate) at 4°. Elution with water, followed by a linear gradient of 0.0–1.0M ammonium acetate, and assay for total carbohydrate (see Fig. 3) gave pure aAM. The aAM fractions were dialyzed three times against water at 4°, and then lyophilized, to yield 48 mg of aAM; $[\alpha]_D^{22}+77.4°$.

O-Deacylated aAM (phosphorylated AM) and nonphosphorylated AM. — Purified aAM (65 mg) was dissolved in 0.1M sodium hydroxide (8 mL), and, after 4 h at room temperature, the solution was dialyzed against water (1 L) at 4°. The dialyzate was lyophilized, and saved for further analysis, and the nondialyzable material was concentrated, and applied to a column (2 × 10 cm) of DEAE-Sephadex A-25 (acetate). Elution with water, followed by a linear gradient of 0.0–1.0M ammonium acetate (see Fig. 4) gave two components. The material eluted with water, referred to as nonphosphorylated arabinomannan (nonphosphorylated AM), constituted 45% of the total carbohydrate applied to the column, and the material eluted with ammonium acetate, referred to as phosphorylated AM, constituted 55% of the total carbohydrate.

Composition and general properties of aAM, phosphorylated AM, and nonphosphorylated AM. — The chemical compositions of these polysaccharides are summarized in Table I. Arabinose (Ara) and mannose (Man) were identified as constituents of the polysaccharides by hydrolysis and paper chromatography of the free monosaccharides, and by g.l.c. of their alditol acetate derivatives on Column 1

TABLE I

THE CHEMICAL COMPOSITIONS OF aAM, PHOSPHORYLATED AM, AND NONPHOSPHORYLATED AM

Residues or groups per molecule	*aAM*	*Phosphorylated AM*[a]	*Nonphosphorylated AM*[b]
Arabinose	56	59	53
Mannose	11	8	14
Phosphate	1.6	2.3	0.4
Lactate[c]	4	4	4
Succinate	6	0	0

[a]Constitutes 55% of aAM. [b]Constitutes 45% of aAM. [c]Variable in different preparations; values obtained by integration of the n.m.r. spectrum.

(40 mL of N_2/min, 190–240° at 2°/min). The ratio of arabinose to mannose (Ara/Man), was determined by dividing the area of the arabinitol pentaacetate peak at 14.5 min by the area of the mannitol hexaacetate peak at 22.0 min, and multiplying by the molar response factor (1.157). The Ara/Man ratio was found to be 5.3:1 for aAM, 7.2:1 for phosphorylated AM, and 3.7:1 for nonphosphorylated AM. The Ara/Man ratio was related to the phosphate content by analyzing the polysaccharides for total carbohydrate (using solutions of arabinose and mannose combined in the foregoing ratios as standards) and for organic phosphate. The Ara/Man/P ratios, normalized to phosphate, were 34.8:6.6:1 for aAM, 26.1:3.5:1 for phosphorylated AM, and 132.5:35.0:1 for nonphosphorylated AM.

The approximate numbers of arabinosyl and mannosyl residues and phosphate groups per mol of polysaccharide were determined from the foregoing ratios and the results of reducing-end analysis, assuming one mol of reducing end per mol of polysaccharide (identified as mannose by reduction of the polysaccharide with sodium borotritide, followed by hydrolysis, and chromatographic identification of radioactive mannitol). Analysis of phosphorylated AM for reducing end gave a ratio of 2.27:1.0 for phosphate relative to reducing sugar (see later), which corresponds to 59 arabinosyl and 8 mannosyl residues, and 2.3 phosphate groups per molecule of polysaccharide. Nonphosphorylated AM contains a little less arabinose, and more mannose, than phosphorylated AM, and, as expected, the contents of arabinose and mannose in aAM are intermediate between those of phosphorylated AM and nonphosphorylated AM. Unexpectedly, a small proportion of organic phosphate was found in nonphosphorylated AM by analysis, possibly the result of experimental error due to the much smaller amount of phosphate that was analyzed.

Analysis of aAM for ester content (with ethyl acetate as the standard) relative to organic phosphate revealed the presence of 3.92 ester groups per phosphate group, which corresponds to ~6 ester groups per molecule of aAM. The identity of the ester substituents was established by the hydroxamic acid procedure[18]. aAM containing ~22 μmol of ester was added to 0.5 mL of alkaline hydroxylamine (prepared by

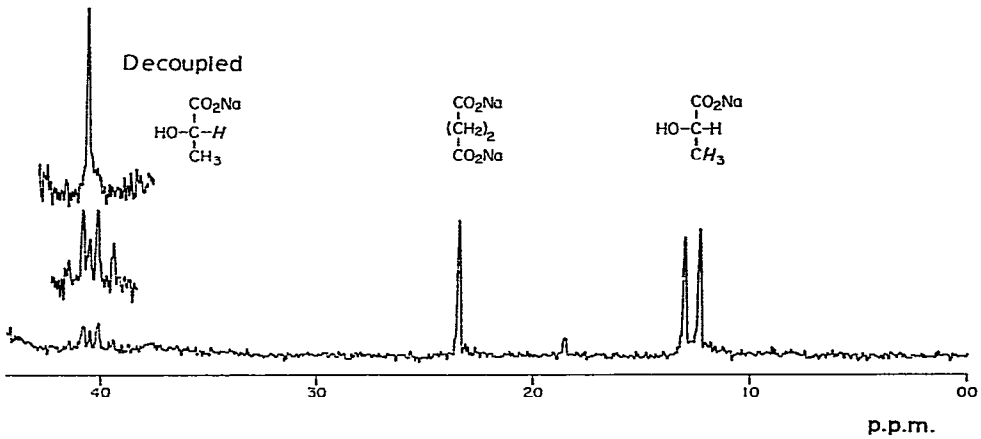

Fig. 5. The 100-MHz, ^{1}H-n.m.r. spectrum of dialyzable components released by treatment of aAM with 0.1M NaOH. [The lower, inset spectrum was obtained at higher amplification, and the upper, inset spectrum, by decoupling the upfield doublet. The spectrum was obtained in D_2O at 27°, with tetramethylsilane as the external standard (in a coaxial, capillary tube).]

mixing equal volumes of 2M hydroxylamine hydrochloride and 3.5M sodium hydroxide). After 40 min at room temperature, the mixture was made neutral by passage through Dowex 50 (H^+) ion-exchange resin in 50% methanol (3 mL), concentrated, and applied to a column (2.5 × 100 cm) of Bio-Gel P-2. Elution with water yielded the deacylated polysaccharide at the void volume, and the hydroxyamic acid derivative at the inclusion volume, as determined by treating aliquots of each fraction with 0.5 mL of 0.37M ferric chloride–0.1M hydrochloric acid, and measuring the absorbance at 520 nm. The 60-MHz, ^{1}H-n.m.r. spectrum (not shown) of a solution of the inclusion-volume material in D_2O contained a single resonance, at δ 2.80, consistent with the presence of only the succinyl derivative (see later).

The identities of base-labile substituents were established from the ^{1}H-n.m.r. spectrum of the dialyzable residues released by saponification (see Fig. 5). This spectrum contains a singlet at δ 2.33, assigned to the methylene groups of succinate, and a doublet at δ 1.25 and a quartet at δ 4.05, assigned to the methyl group and H-2 of lactate, respectively. The quartet at δ 4.05 collapsed to a singlet upon irradiation of the δ-1.25 doublet, confirming these assignments. The presence of lactate and succinate in this mixture was confirmed by combined g.l.c.–m.s. of the butyl ester derivatives formed by refluxing the sodium salts for 0.5 h in 1-butanol saturated with HCl gas. After neutralization of the acid with barium carbonate, the resulting butyl esters were analyzed by g.l.c.–m.s. in Column 2 (30 mL of N_2/min, 100° for 5 min, and then 100–180° at 2°/min). Components having retention times of 12.8 and 35.0 min were observed, and these components gave mass spectra corresponding to authentic butyl lactate and dibutyl succinate, respectively.

Analysis of the polysaccharides for amino acids, 2-amino-2-deoxyglucose, and muramic acid was accomplished by adding a known amount of norleucine to solutions containing a known amount of the polysaccharide. The solutions were diluted with

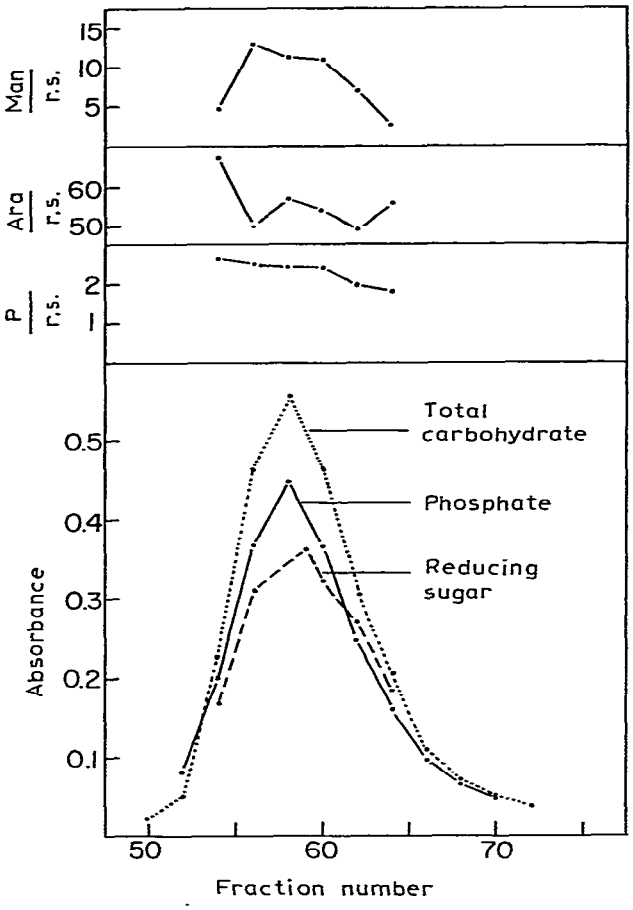

Fig. 6. Chromatography of phosphorylated AM on a column (2.5 × 98 cm) of Sephadex G-150. [The column was eluted with 0.1M ammonium acetate containing 0.02% of NaN_3, and 5-mL fractions were collected at a flow rate of 18 mL/h. Alternate fractions were assayed for total carbohydrate, phosphate, and reducing sugar, and, from the ratios of Ara:Man as determined by gas–liquid chromatography, the Ara:reducing sugar, Man:reducing sugar, and phosphate:reducing sugar ratios were calculated.]

an equal volume of 12M HCl, degassed under vacuum, and hydrolyzed for 21 h at 110°. After removal of HCl by evaporation, and four evaporations with water, the solutions were subjected to the standard conditions of amino acid analysis. The three polysaccharides were found to contain no muramic acid, and <0.1% of 2-amino-2-deoxyglucose (by weight). A broad spectrum of amino acids was observed in each of the polysaccharides, with the acidic amino acids, aspartic and glutamic acid, preponderating. The total amino acid content (by weight) was 11% in aAM, 7% in phosphorylated AM, and 1% in nonphosphorylated AM.

Phosphorylated AM was examined for heterogeneity of size and composition by chromatography on a column (2.5 × 98 cm) of Sephadex G-150, as shown in Fig. 6. Given in Fig. 6 are the results of assays for total carbohydrate, phosphate, and

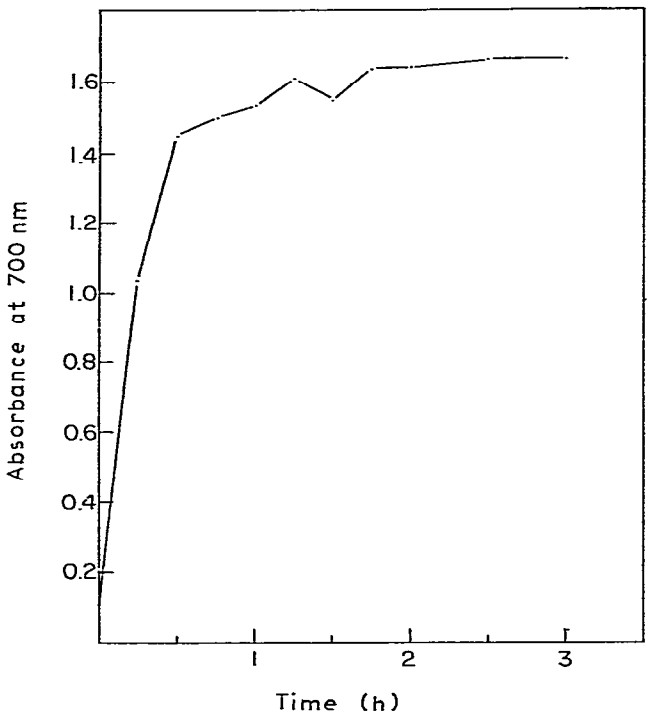

Fig. 7. The appearance of reducing sugar (absorbance at 700 nm) as a function of time of hydrolysis of aAM in 0.1M hydrochloric acid at 100°.

reducing sugar performed quantitatively on every other tube. In addition, aliquots of the same fractions were subjected to acid hydrolysis, and the resulting monosaccharides were converted into their alditol acetate derivatives, and these analyzed by g.l.c. as before, to give the Ara/Man ratios. From these data, the absolute amounts of arabinose, mannose, phosphate, and reducing sugar in each fraction were determined, and these were used to calculate the phosphate:reducing sugar (P/r.s.), arabinose: reducing sugar (Ara/r.s.), and mannose:reducing sugar (Man/r.s.) ratios. In general, it may be seen that the ratios of phosphate and arabinose to reducing sugar are fairly constant across the peak, averaging ~2.4:1 and ~53:1, respectively. The ratio of mannose to reducing sugar decreases with decreasing molecular size of the polysaccharide, however. Fraction 54 gave an unusually large arabinose:mannose ratio; it is not known whether this is a true representation of larger molecular forms of the polysaccharide, or the error introduced into the assays because of the smaller amounts of samples available. However, the major forms of the polysaccharide contain approximately 2 phosphate groups, and 55 arabinosyl and 11 mannosyl residues per molecule.

Characterization of furanosyl linkages. — In order to determine whether furanosyl linkages were present in the aAM, the polysaccharide was subjected to hydrolysis in 0.1M HCl at 100°, and aliquots were withdrawn at various times and

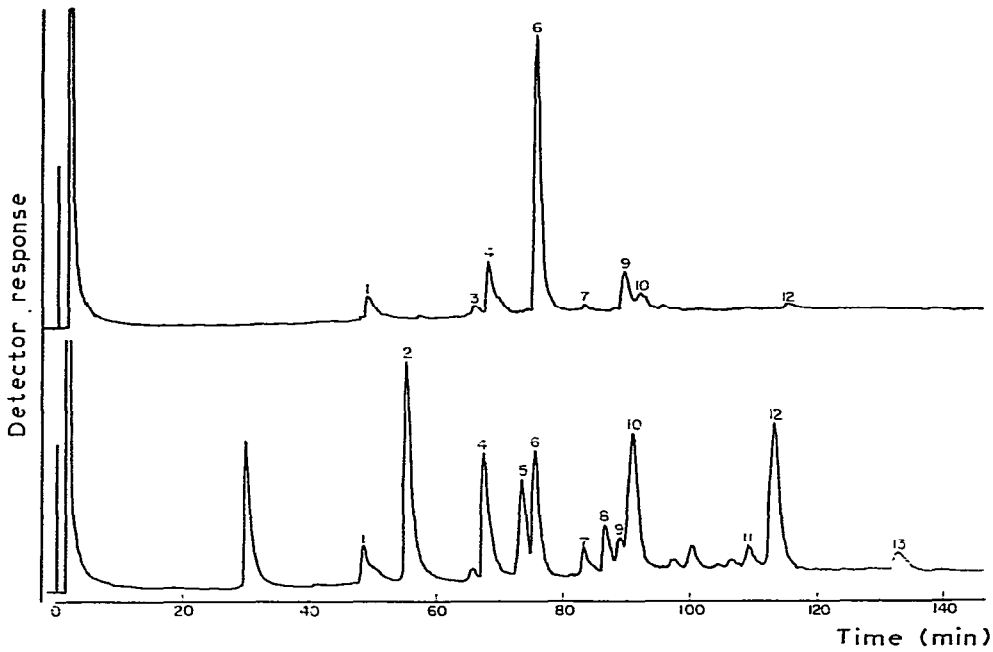

Fig. 8. Gas–liquid chromatograms (Column 3, 160° for 20 min, and then programmed to 230° at 1°/min, 30 mL of N_2/min) of the partially methylated alditol acetates derived after complete methylation of phosphorylated AM (upper) and the core resistant to mild hydrolysis with acid (lower). [The numbered peaks represent the derivatives of 2,3,5-tri-*O*-methylarabinose (1), 2,3,4-tri-*O*-methylarabinose (2), 3,5-di-*O*-methylarabinose (3), 2,3,4,6-tetra-*O*-methylmannose (4), 2,4-di-*O*-methylarabinose (5), 2,3-di-*O*-methylarabinose (6), 2,4,6-tri-*O*-methylmannose (7), a mixture of 2,3,6- and 3,4,6-tri-*O*-methylmannose (8), 2-*O*-methylarabinose (9), 2,3,6-tri-*O*-methylglucose (leading edge of peak 10), 2,3,4-tri-*O*-methylmannose (tailing edge of peak 10), 2,4-di-*O*-methylmannose (11), 3,4-di-*O*-methylmannose (12), and 3-*O*-methylmannose (13).]

assayed for the presence of reducing sugar. The appearance of reducing sugar as a function of time of hydrolysis (see Fig. 7) is characteristic of the presence of furanosyl linkages[19], *i.e.*, a rapid release of reducing sugar occurs within the first 30 min, due to cleavage of furanosyl linkages, and a much slower release of reducing sugar after that time, due to cleavage of the (more stable) pyranosyl linkages. Phosphorylated AM and nonphosphorylated AM were subjected to selective cleavage of furanosyl residues (30-min hydrolysis), and the products were examined by gel-filtration chromatography on Bio-Gel P-2 (data not shown). Both polysaccharides were found to give "cores" resistant to mild acid (which were eluted at the void volume), small proportions of oligosaccharides that consisted primarily of arabinosyl residues, and larger amounts of free arabinose. The Ara/Man ratios of these polysaccharides, as determined by g.l.c. of the alditol acetate derivatives as already described, were 3.8 : 1 for the phosphorylated AM core and 3 : 2 for the nonphosphorylated AM core. Examined by gel-filtration chromatography on Sephadex G-50, the cores were found to be much smaller in size, chromatographing as only slightly larger than the *O*-methylglucose lipopolysaccharides (see Fig. 1).

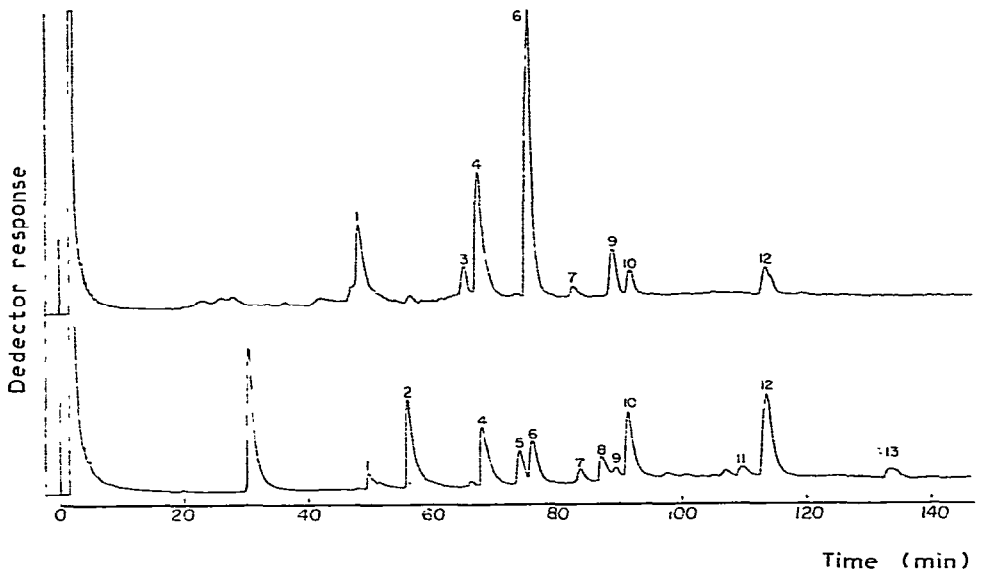

Fig. 9. Gas–liquid chromatograms (Column 3, 160° for 20 min, and then programmed to 230° at 1°/min, 30 mL of N_2/min) of the partially methylated alditol acetates derived after complete methylation of nonphosphorylated AM (upper) and the core resistant to mild hydrolysis with acid (lower). [The numbered peaks represent the derivatives of 2,3,5-tri-*O*-methylarabinose (1), 2,3,4-tri-*O*-methylarabinose (2), 3,5-di-*O*-methylarabinose (3), 2,3,4,6-tetra-*O*-methylmannose (4), 2,4-di-*O*-methylarabinose (5), 2,3-di-*O*-methylarabinose (6), 2,4,6-tri-*O*-methylmannose (7), a mixture of 2,3,6- and 3,4,6-tri-*O*-methylmannose (8), 2-*O*-methylarabinose (9), 2,3,6-tri-*O*-methylglucose (leading edge of peak 10), 2,3,4-tri-*O*-methylmannose (tailing edge of peak 10), 2,4-di-*O*-methylmannose (11), 3,4-di-*O*-methylmannose (12), and 3-*O*-methylmannose (13).]

Methylation analysis. — Phosphorylated AM, nonphosphorylated AM, and their cores resistant to mild acid were exhaustively methylated[20,21], and the products hydrolyzed[22]; the resultant, partially methylated monosaccharides were separated by g.l.c. of their alditol acetate derivatives on Column 3 (see Figs. 8 and 9). By use of g.l.c.–m.s., all components were identified by a comparison of the primary fragments observed in their mass spectra with those observed by Björndal *et al.*[23,24] for authentic, partially methylated alditol acetates. With the exception of peak 8 in Figs. 8 and 9, all peaks were found, by mass chromatography[17] of characteristic fragments, to comprise a single, partially methylated, alditol acetate derivative. The mass spectrum of peak 8 was that expected for a mixture of 2,3,6- and 3,4,6-tri-*O*-methylmannose derivatives. Chromatography was also conducted in three other columns commonly used for the separation of partially methylated alditol acetates [Column 2 (30 mL of N_2/min, 165–230°, 1°/min), Column 4 (30 mL of N_2/min, 140–230°, 1°/min), and Column 5 (30 mL of N_2/min, 130–210°, 1°/min)], in order to separate the poorly resolved components in the 80–95-min range of Figs. 8 and 9. Using molar-response factors calculated by the effective-carbon-response method[25], the molar percentage of each component was determined (see Table II). These values are less useful than they should be, because of the high volatility (and loss prior to g.l.c.) of tri-*O*-

TABLE II

THE MOLAR PERCENTAGE OF *O*-METHYLATED SUGARS DERIVED FROM PERMETHYLATED PHOSPHORYLATED AM, NONPHOSPHORYLATED AM, AND THEIR (MILD ACID-RESISTANT) CORES[a]

	Phosphorylated AM		*Nonphosphorylated AM*	
	Intact	*Core*	*Intact*	*Core*
O-methylarabinose				
2,3,5-tri-	9.5	4.2	17.1	3.4
2,3,4-tri	—	23.2	—	23.3
3,5-di-	2.3	—	4.0	—
2,4-di-	—	9.0	—	6.4
2,3-di-	58.1	10.9	42.5	9.5
2-	9.2	2.8	6.6	1.5
O-methylmannose				
2,3,4,6-tetra-	13.6	12.5	20.0	13.8
2,4,6-tri-	1.1	2.4	1.8	2.7
2,3,6- and 3,4,6-tri-	—	4.0	—	4.6
2,3,4-tri-[b]	4.9	9.9	3.6	11.9
2,4-di-	—	1.5	—	2.0
3,4-di-	1.5	13.7	4.4	18.4
3-	—	2.0	—	2.0
O-methylglucose				
2,3,6-tri-[b]	—	3.9	—	0.6

[a]All percentages determined from peak areas in Figs. 8 and 9, except those indicated by footnote *b*.
[b]These two components were resolved on Column 2, and the percentages given were determined from these peak areas.

methylarabinose and tetra-*O*-methylmannose derivatives. From the data in Table II, however, the major structural features of these polysaccharides are apparent. The arabinosyl residues occur in both the furanose and the pyranose form, but the mannosyl residues are present exclusively in the pyranose form. The major methylated sugar derived from both phosphorylated and nonphosphorylated AM is 2,3-di-*O*-methylarabinose (Peak 6, Figs. 8 and 9); this could have arisen from either (1→5)-linked arabinofuranosyl or (1→4)-linked arabinopyranosyl residues. It is apparent, however, that 2,3-di-*O*-methylarabinose arises mainly from (1→5)-linked arabinofuranosyl residues, as it is derived in much lower proportion from the cores of the polysaccharides that are resistant to mild acid. Significant amounts of 2,3,5-tri-*O*-methylarabinose (Peak 1, Figs. 8 and 9) were derived from both phosphorylated and nonphosphorylated AM, indicative of the presence of terminal (nonreducing) arabinofuranosyl groups in these polysaccharides. 2,3,4-Tri-*O*-methylarabinose (Peak 2, Figs. 8 and 9) and 2,4-di-*O*-methylarabinose (Peak 5, Figs. 8 and 9) were derived in large proportions from the cores (resistant to mild acid) of both phosphorylated and nonphosphorylated AM, but these methylated sugars were not derived from the intact polysaccharides. Conversely, 2-*O*-methylarabinose (Peak 9, Figs. 8

and 9) was derived in significant proportions from both of the intact polysaccharides, but not from their cores (resistant to mild acid).

Mannose is mainly located at (nonreducing) termini as mannopyranosyl groups, as evidenced by the large proportion of 2,3,4,6-tetra-*O*-methylmannose derived by methylation, but a substantial proportion of (1→6)-linked mannopyranosyl residues is also present. In addition, smaller but significant proportions of (1→3)-linked and 1,2,6-linked mannopyranosyl units are present, as evidenced by the presence of 2,4,6-tri-*O*-methylmannose and 3,4-di-*O*-methylmannose, respectively. Greater proportions of *O*-methylmannoses are present in the cores (resistant to mild acid), as expected, due to the selective removal of arabinofuranosyl residues from the intact polysaccharides; however, the increased proportion of 3,4-di-*O*-methylmannose was greater than expected. This result cannot be explained by selective removal of arabinofuranosyl residues from O-3 or O-4 of (2→6)-linked mannopyranosyl residues, as 3- or 4-*O*-methylmannose was not derived by methylation of the intact polysaccharides. It is possible, however, that arabinofuranosyl residues, or other acid-labile residues, were present at both O-3 and O-4 of the (2→6)-linked mannopyranosyl residues, although the presence of a tetrasubstituted mannopyranosyl residue is considered unlikely.

Nuclear magnetic resonance studies. — Given in Fig. 10 are the 270-MHz, ^{1}H-n.m.r. spectra of aAM, phosphorylated AM, and the phosphorylated AM core, all in D_2O at 27°. The spectra of nonphosphorylated AM and its (mild acid-resistant) core were virtually identical to those of phosphorylated AM and its (mild acid-resistant) core, and are not shown. Although all three spectra contain weak, unidentified resonances, most of the resonances can be readily assigned. Exhibited in all three spectra is a doublet (J 8 Hz) at δ 1.34, assigned to the methyl group of lactate. The presence of this doublet in the spectrum of phosphorylated AM, which was derived by alkaline hydrolysis of aAM, demonstrates that lactate is linked to the polysaccharide *via* its hydroxyl group, not its carboxyl group. Treatment of phosphorylated AM under more vigorous conditions of alkaline hydrolysis (0.5M NaOH, 16 h at room temperature) resulted in a further decrease in the intensity of the δ-1.34 doublet; thus, although not esterified to aAM, lactate is slowly removed by alkaline hydrolysis. Two resonances of equal intensity, at δ 2.54 and 2.67, are present in the spectrum of aAM (see Fig. 10A), but are absent from the spectra of phosphorylated AM and its (mild acid-resistant) core. These resonances can be assigned to the methylene protons of monoesterified succinic acid. The absence of these resonances from the spectrum of phosphorylated AM demonstrates that the succinate groups had been completely removed by alkaline hydrolysis. Integration of the spectrum of aAM gave ratios of 1.0:1.83:29 for the peak areas due to the methyl protons of lactate (δ 1.34), the methylene protons of succinate (δ 2.54 and 2.67), and the ring protons (excluding the anomeric) of monosaccharide residues (δ 3.6–4.5), respectively; these correspond to molar ratios of 1.0:1.37:16.8. Given an average composition of 67 monosaccharide residues in aAM (see Table I), these ratios corresponds to 4 lactate and 5.5 succinate groups per molecule of polysaccharide. The succinate

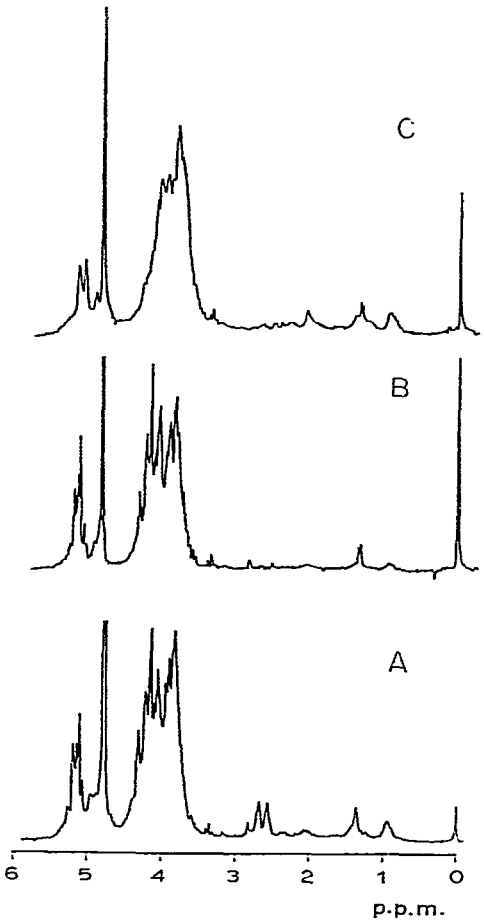

Fig. 10. The 270-MHz, ^{1}H-n.m.r. spectra of aAM (Frame A), phosphorylated AM (Frame B), and the phosphorylated AM core (Frame C), in D_2O at 27°, with TSP as the internal standard.

composition determined in this way is in close agreement with the value (~6) determined by direct analysis for ester.

Several resonances are present in the anomeric region of the spectra of aAM (see Fig. 10A) and phosphorylated AM (see Fig. 10B), including intense resonances at δ 5.10 and 5.18, three less-intense resonances at δ 4.91 (poorly resolved), 5.05, and 5.13, and very weak resonances at δ 5.15 and 5.25. Resonances at δ 5.10 and 5.18 are absent from the spectrum of the (mild acid-resistant) core of phosphorylated AM (see Fig. 10C), indicative of their derivation from arabinofuranosyl residues in the former polysaccharides. Further assignments of the anomeric-hydrogen resonances cannot be made with certainty, however, due to the present lack of suitable standards.

The ^{13}C-n.m.r. spectrum of aAM (see Fig. 11) is, however, more informative with regard to the configuration of anomeric linkages. The anomeric region of this spectrum contains six resonances: a very intense resonance at δ 110.2, less-intense

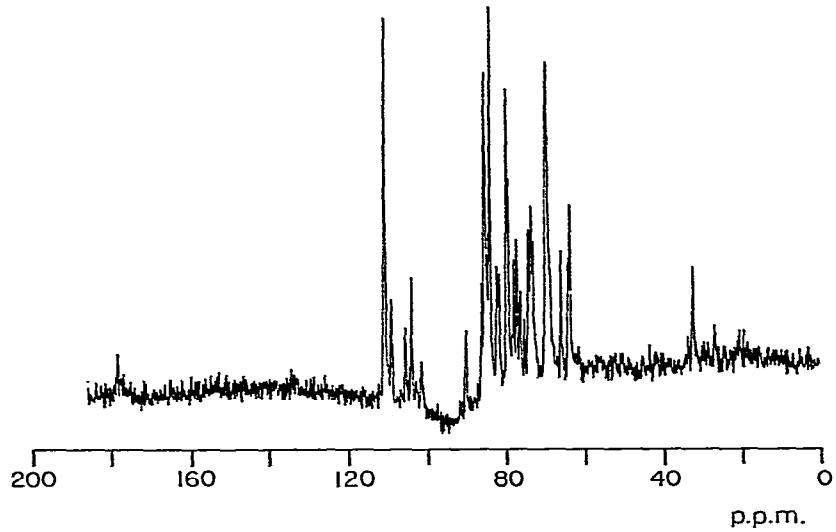

Fig. 11. The 25.2-MHz, ^{13}C-n.m.r. spectrum of aAM in D_2O at 34°, with TSP as the external standard (in a coaxial, capillary tube).

resonances at δ 101.1, 103.3, 104.8, and 108.4, and a very weak resonance at δ 102.2. The intense resonance at δ 110.2 can be unequivocally assigned to C-1 of terminal (nonreducing) and internal, (1→5)-linked, α-arabinofuranosyl residues. The chemical shift of the C-1 resonance of the L-arabinofuranosyl group of 6-*O*-α-L-arabinofuranosyl-α,β-D-glucose has been reported[26] to be δ 108.9 (relative to tetramethylsilane), which corresponds to a chemical shift of δ 110.4 relative to TSP (see the Experimental section), and the C-1 chemical shift of (1→5)-linked α-arabinofuranosyl residues has been reported[27] to be δ 110.35 (relative to TSP). A further indication of the presence of (1→5)-linked arabinofuranosyl residues in aAM is the presence of an intense resonance at δ 69.3 (most-intense, high-field resonance). The C-5 resonance of 5-linked arabinofuranosyl residues had been found to occur at δ 69.8, whereas the C-5 resonance of arabinofuranosyl residues not linked at C-5 has been shown[27] to lie at δ 64.1. Anomeric-carbon resonances having lower intensity cannot be assigned with certainty, but it is probable that the resonance at δ 108.4 is also due to C-1 of arabinofuranosyl residues. Resonances in the region δ 100–105 can be due to C-1 of arabinopyranosyl[27] or mannopyranosyl[16] residues, or (terminal) β-arabinofuranosyl[26] groups.

Immunochemical studies. — Sera from two rabbits immunized with heat-killed, *M. smegmatis* cells, and from a rabbit immunized with *M. smegmatis* cell-walls, were collected at two, three, and four weeks after the initial injection, and were titered against heat-killed cells. All sera showed an increase in titer with time, and, as the sera from the fourth week were the most reactive with antigens, they were used for subsequent studies. Quantitative precipitin-reactions were performed with all three sera and aAM (see Fig. 12), phosphorylated AM (see Fig. 13), and the (mild acid-resistant) phosphorylated AM core (not shown). In these studies, the amount of

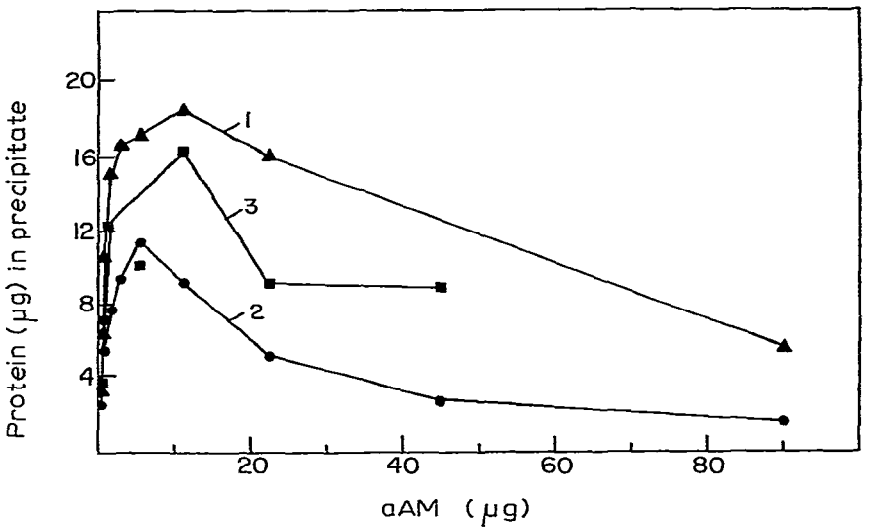

Fig. 12. Quantitative precipitin-reactions of aAM with sera from rabbits immunized with heat-killed *M. smegmatis* cells (curves 1 and 2) and cell walls (curve 3). (Each reaction-mixture contained 250 μL of antiserum and the indicated amount of aAM in a total volume of 500 μL.)

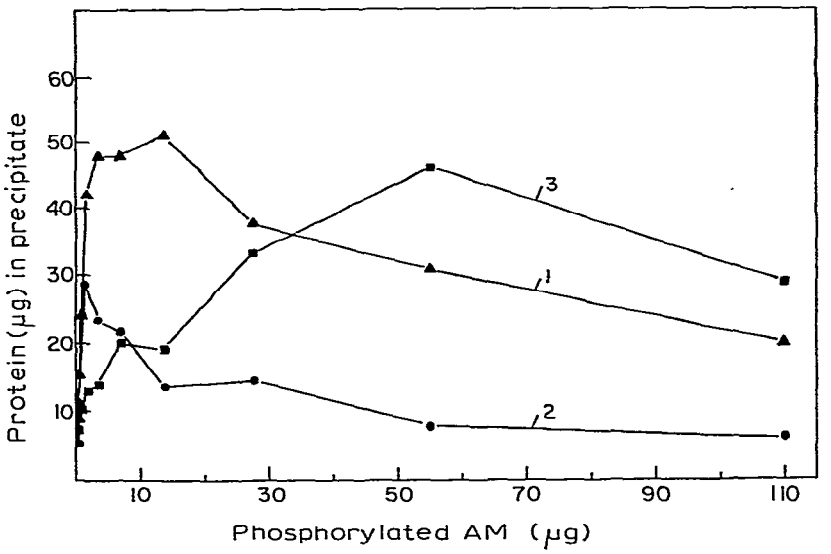

Fig. 13. Quantitative precipitin reactions of phosphorylated AM with sera from rabbits immunized with heat-killed *M. smegmatis* cells (curves 1 and 2) and cell walls (curve 3). (Each reaction-mixture contained 250 μL of antiserum and the indicated amount of phosphorylated AM in a total volume of 500 μL.)

protein in the precipitate was determined, using bovine serum albumin as the standard, and the amount of polysaccharide added was determined by the phenol–sulfuric procedure[10] using a standard solution containing arabinose and mannose in the appropriate ratios. Both aAM (see Fig. 12) and phosphorylated AM (see Fig. 13) formed precipitates with all three antisera over a broad range of concentration, but,

interestingly, phosphorylated AM was approximately three times as effective in precipitating antigen as aAM, from which it was derived. The (mild acid-resistant) core of phosphorylated AM failed to form a precipitate with any of the antisera, however (data not shown).

DISCUSSION

The *M. smegmatis* arabinomannan isolated as described herein is similar, both in its size and relative composition of arabinose and mannose, to an arabinomannan previously isolated from this organism[8]. It is different, however, in its immunochemical properties and ester content, a reflection of the fact that the polysaccharide had previously been isolated by alkaline extraction. When isolated under chemically nondegrading conditions, the polysaccharide is very acidic, containing approximately 2 phosphate, 6 monoesterified succinate, and 4 ether-linked lactate groups per molecule. The polysaccharide is, however, very heterogeneous with respect to charge, as judged by its elution profile on DEAE-Sephadex A-25, and somewhat heterogeneous with respect to size, as judged by its elution profile in gel-permeation chromatography. The average composition of aAM corresponds to a molecular weight of 10,000.

After saponification to remove succinate groups, the polysaccharide can be separated into phosphorylated and nonphosphorylated forms, with average molar compositions of Ara:Man of 59:8 and 53:14, respectively. Mild hydrolysis of these polysaccharides with acid selectively removes arabinofuranosyl residues, giving (mild acid-resistant) cores comprised of arabinopyranosyl and mannopyranosyl residues. The Ara:Man ratios of these cores were 3.8:1 for the phosphorylated AM core, and 3:2 for the nonphosphorylated AM core. Assuming that these cores contain all of the mannosyl residues of the original polysaccharides (no release of mannose during mild hydrolysis with acid was observed), these ratios correspond to molar compositions of Ara:Man of 30:8 for the phosphorylated AM core, and 21:14 for the nonphosphorylated AM core. Therefore, ~30 arabinofuranosyl residues were removed from each of the polysaccharides by mild hydrolysis with acid. This high content of arabinofuranosyl residues (45%) is in agreement with the results of methylation analysis.

The types of linkages present in phosphorylated AM and nonphosphorylated AM were determined by methylation analysis, through a comparison of the partially methylated aldoses derived from the intact polysaccharides and their (mild acid-resistant) cores. These results are consistent with the presence of the following structural unit in both polysaccharides.

$$\text{Ara}f\text{-}(1\rightarrow5)\text{-}[\text{Ara}f\text{-}(1\rightarrow5)]_n\text{-}\begin{cases}\text{Ara}f\text{-}(1\rightarrow4)\text{-Ara}p\text{-}(1\rightarrow4)\text{-Ara}p\text{-}\\ \qquad\qquad\qquad\quad 3\\ \qquad\qquad\qquad\quad \uparrow\\ \qquad\qquad\qquad\quad 1\\ \text{Ara}f\text{-}(1\rightarrow4)\text{-Ara}p\end{cases}$$

The major feature of this representation is the presence of chains of contiguous,

(1→5)-linked arabinofuranosyl residues that are attached to O-4 of arabinopyranosyl residues. The Ara*p* residue that is substituted at O-4 by Ara*f* and at O-3 by Ara*p* gives rise to 2-*O*-methylarabinose from the intact polysaccharide, but to 2,4-di-*O*-methylarabinose after selective cleavage of the 4-linked Ara*f* group. The presence of this structural unit would explain the formation of 2-*O*-methylarabinose from the intact polysaccharide but not from the core resistant to mild hydrolysis with acid, and conversely, the formation of 2,4-di-*O*-methylarabinose from the core, but not from the intact polysaccharide. The Ara*p* residue that is linked to O-3 of the aforementioned Ara*p* residue gives rise to 2,3-di-*O*-methylarabinose from the intact polysaccharide, but to 2,3,4-tri-*O*-methylarabinose after selective cleavage of the 4-linked Ara*f* group. The fact that no 2,3,4-tri-*O*-methylarabinose is derived from the intact polysaccharide would indicate that Ara*f* groups are always linked at O-4 of these Ara*p* residues. It should be pointed out, however, that this proposed structure is only an average structure, and that it may not be representative of the sequence or molar ratios of the monosaccharide units actually present.

The sequence of mannosyl residues in the polysaccharides is less well-established, although most, if not all, of these residues are located within the cores resistant to hydrolysis by dilute acid. The mannosyl units are located primarily at nonreducing termini as mannopyranosyl groups, but substantial proportions of (1→6)-linked mannopyranosyl residues are also present.

The anomeric configurations of all of the residues in aAM could not be determined, due to the present lack of suitable standards for ^{13}C chemical-shift correlations; however, the (1→5)-linked Ara*f* residues were shown to have the α configuration (through a comparison of their C-1 and C-5 chemical-shifts with literature values for similar polysaccharides).

Immunochemical studies demonstrated that aAM is serologically active, *i.e.*, rabbits immunized with either heat-killed, whole cells, or cell walls, of *M. smegmatis* elicit antibodies that cross-react with the polysaccharide. Interestingly, aAM is a precipitating antigen against these sera, and the precipitin reaction can, therefore, be used to explore the structural features of the polysaccharide that are recognized by the antibodies. For example, although the intact polysaccharide is a precipitating antigen, the core of the polysaccharide that is resistant to mild hydrolysis with acid is not, demonstrating that the arabinofuranosyl side-chains of the polysaccharide are important in antibody recognition. Surprisingly, however, after saponification of the polysaccharide to remove succinate groups, the effectiveness of the polysaccharide in precipitating antibody is increased! It is possible that, to some as-yet-unknown degree, precipitates formed with aAM and phosphorylated AM are in response to contaminating protein antigens (11 and 7%, respectively), but the difference in the precipitating effectiveness of these two polysaccharides cannot be due to contaminating protein, as phosphorylated AM, which is considerably more effective as a precipitating antigen, actually contains less protein. This result, combined with the failure of the (mild acid-resistant) core of phosphorylated AM to form a precipitate at all, suggests that *exposed* arabinofuranosyl residues are important in antibody recognition. This

possibility is particularly attractive in view of the results obtained by Misaki *et al.*[29] from an immunochemical study of the mycobacterial cell-wall arabinogalactan. These workers concluded[29] that six or more consecutive D-arabinofuranosyl residues present in the side chains of the arabinogalactan are responsible for its immunological activity. Thus, it is probable that antibodies which recognize the arabinofuranosyl side-chains of phosphorylated AM were actually "raised" against the same antigen of the cell wall. Consequently, although aAM is "serologically active", it appears to be so mainly by virtue of the fact that it shares an important antigen in common with the cell-wall, arabinogalactan lipopolysaccharide.

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