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THE CHEMICAL STRUCTURE OF A FRAGMENT OF *Micrococcus lysodeikticus* CELL-WALL*

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ABSTRACT

A fragment of *Micrococcus lysodeikticus* cell-wall obtained by cetylpyridinium precipitation from the nondialyzable portion of the degradation products of egg-white lysozyme was studied by the periodate oxidation and methylation procedures. The fragment consists of a polysaccharide chain composed of about 40 repeating (1→4)-*O*-(2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid)-(1→6)-*O*-(α-D-glucopyranosyl) residues with D-glucopyranosyl residues at both ends. The α-D-glucopyranose residue at the reducing end is linked to a phosphate group that is also linked to C-6 of a 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residue of a peptidoglycan chain composed of four repeating (1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl] residues. The peptidoglycan chain has, as nonreducing group, a 2-acetamido-2-deoxy-β-D-glucopyranosyl group, and, as reducing residue, a 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucose residue.

INTRODUCTION

Perkins[6] has described the isolation in low yield of the external polysaccharide chains of *Micrococcus lysodeikticus* cell-wall by trichloroacetic acid treatment, and the chemical structure of these chains has been partially elucidated by Hase *et al.*[7]. A fragment representing 26% of the total cell-wall, 10% of the peptidoglycan, and 50% of the external polysaccharide chains was isolated from *Micrococcus lysodeikticus* cell-wall[8]. This fragment, obtained by degradation with egg-white lysozyme, precipitation with cetylpyridinium chloride, and Bio-gel chromatography, was shown[8] to

*Amino Sugars CII and *Micrococcus lysodeikticus* cell-wall V (for preceding papers in this series, see Refs. 1–4). This is publication No. 694 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This investigation was supported by a research grant from the National Institute of Allergy and Infectious Diseases (Grant AI-06692). A preliminary communication has been presented[5].

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consist of a peptidoglycan moiety composed of four alternating, (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl (*N*-acetylmuramic acid) residues to which is attached an external, polysaccharide chain composed of ~40 *O*-(β-D-mannopyranosyluronic acid)-*O*-(α-D-glucopyranosyl) repeating units linked through a phosphate group to C-6 of one of the muramic acid residues of the peptidoglycan chain; in addition, all the carboxyl groups of the muramic acid residues are substituted by peptide chains. The present paper describes the elucidation of the chemical structure of the external polysaccharide chain and of the glycan part of the peptidoglycan moiety by the periodate and methylation procedures.

RESULTS AND DISCUSSION

Extensive, periodate oxidation at 4° in the dark, which has been shown to cause no overoxidation of 2-acetamido-2-deoxy sugar residues[9], gave an amount of formic acid (0.9 mol/D-glucose residue) corresponding to the oxidation of all of the D-glucose residues, suggesting that these residues are linked at C-6 (see Table I). The

TABLE I

SEQUENTIAL PERIODATE OXIDATION, SODIUM BOROHYDRIDE REDUCTION, AND HYDROLYSIS OF FRACTION CPC_{A-2} [a]

Components[b], *reagent, and compounds formed*	*Fraction* CPC_{A-2}	*Sequential degradation*			
		First		*Second*	
		Dl[c]	*Ndl*[c]	*Dl*[c]	*Ndl*[c]
D-Glucose[d]	28		1		0
2-Amino-2-deoxy-D-mannuronic acid	37[e]		12[f]		0
2-Amino-2-deoxy-D-glucose[g]	7		9		13
Muramic acid[g]	9		14		20
Muramic acid 6-phosphate[g]	1.4		2.2		
Glycerol		3.3[h]	7	0	0
Alanine[i]	4		10		14
Glutamic acid[i]	3		8		6
Glycine[i]	2		4		6
Lysine[i]	3		5		8
Periodate consumed[j] (μmol/mg)		3.65			
Formic acid released[k] (μmol/mg)		1.3			
Formaldehyde released[l] (nmol/mg)		65			

[a]For conditions, see Experimental section. [b]Component contents expressed in %. [c]Abbreviations: Dl, dialyzable fraction; Ndl, nondialyzable fraction. [d]Determined by the anthrone colorimetric method[20]. [e]Determined by g.l.c. as 2-amino-2-deoxy-D-mannose, relative to the D-glucose content. [f]Determined by g.l.c. as 2-amino-2-deoxy-D-mannose. [g]Determined by a modified Elson–Morgan, colorimetric method[21]. [h]Expressed in %, relative to fraction CPC_{A-2}. [i]Determined with an amino acid analyzer (see Experimental section). [j]Determined by u.v. absorption at[26] 223 nm. [k]Determined by titration with sodium hydroxide. [l]Determined by the chromotropic acid, colorimetric method[27].

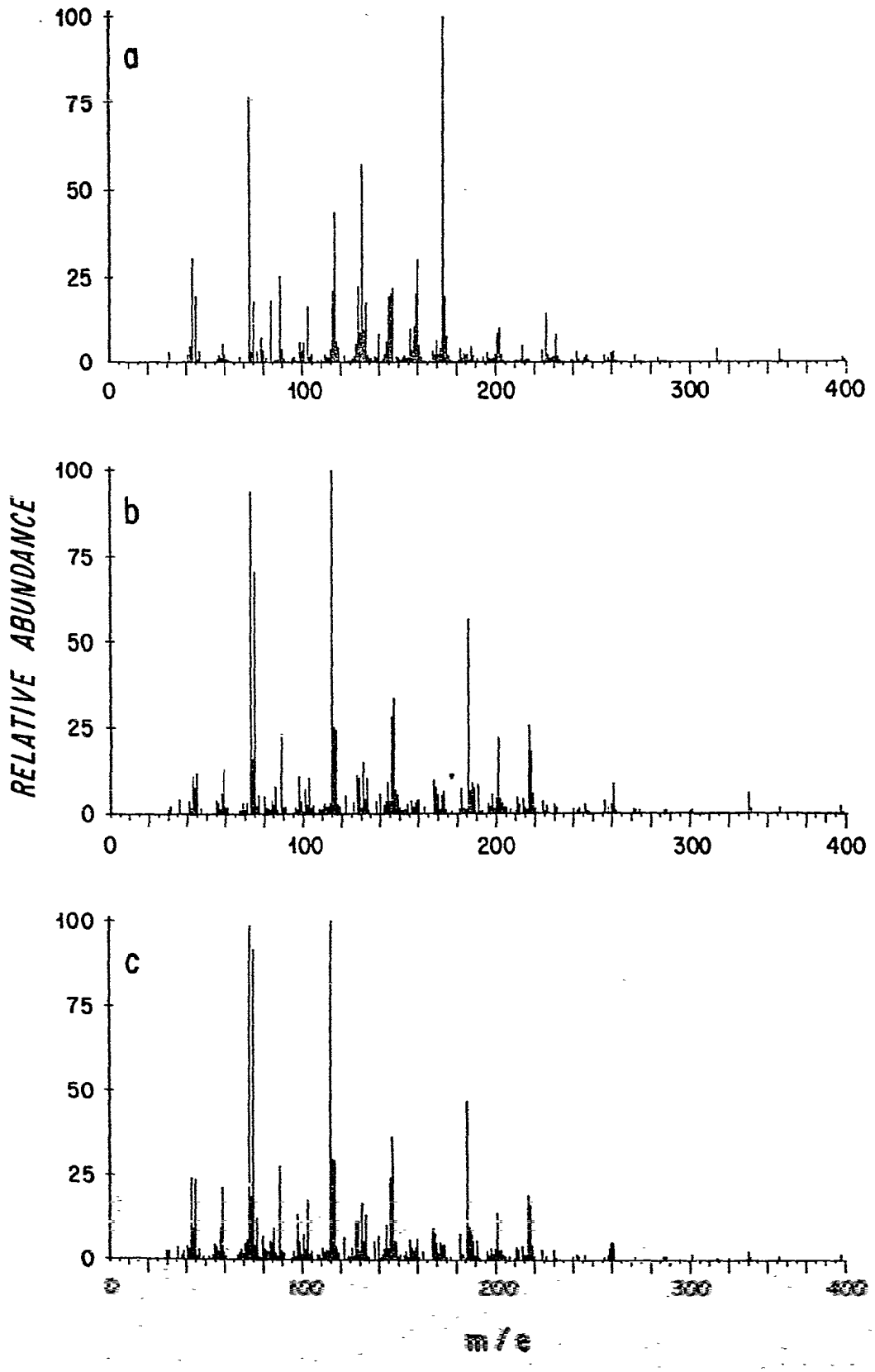
release of formaldehyde (65 nmol/mg) corresponds to the oxidation of the reducing, terminal, muramic acid residue of the peptidoglycan fragment. The oxidized fragment was reduced with sodium borohydride, the resulting polyalcohol hydrolyzed by mild hydrolysis with acid, and the hydrolyzate dialyzed. The dialyzable fraction contained only one quarter of the expected amount of glycerol, and the remainder consisted of 2-amino-2-deoxy-D-mannuronic acid. This compound, after treatment by methanolic hydrogen chloride and reduction, was identified by g.l.c. as methyl 2-acetamido-2-deoxy-α,β-D-mannopyranoside, and, after hydrolysis and *N*-acetylation, as crystalline 2-acetamido-2-deoxy-D-mannose.

The presence of about 1% of unoxidized D-glucose and 7% of glycerol in the oxidized, hydrolyzed, and nondialyzable material indicates clearly a limitation of the periodate–sodium borohydride–mild acid hydrolysis method for structure determination of complex carbohydrate molecules. The resistance of the D-glucose residues to periodate oxidation may suggest a (1→3)-linkage, but this observation was not confirmed by the methylation procedure, and it is more probable that steric effects were responsible for this inhibition of the periodate oxidation. The presence of glycerol shows that the conditions of hydrolysis were not sufficient to hydrolyze the oxidized, reduced, external chains completely, but stronger conditions would have hydrolyzed the phosphate bonds and, possibly, the 2-acetamido-2-deoxy-D-glycopyranosyl linkages. The total amount of glycerol found in the dialyzable and nondialyzable fractions corresponds to about half of the value expected, the loss probably being due to hydrolysis during purification of the reduced fractions. No erythrose was observed, thus confirming that all of the D-glucose residues are linked at C-6. A second periodate oxidation of the oxidized, reduced, nondialyzable fraction degraded the remaining D-glucose residues.

Methylation was performed by a modified Haworth procedure[10], in order to avoid degradation of alkali-sensitive bonds. Methanolysis, followed by acetylation and hydrolysis, gave a mixture that was fractionated on a column of Dowex 50 ion-exchange resin. The total recovery from the column was ~70%, from which 86% was further fractionated by paper chromatography.

The D-glucose component was recovered in 52% yield as 2,3-di-, 2,3,4-tri-, and 2,3,4,6-tetra-methyl ethers. In addition to identification by g.l.c. and paper chromatography, the first two compounds were identified, after reduction, as the crystalline *p*-phenylazobenzoyl derivatives, and the last-mentioned compound was obtained in crystalline form. Direct methanolysis and g.l.c. indicated that the 2,3-dimethyl ether was the product of hydrolysis of the 2,3,4-trimethyl ether. The ratio of 2,3,4-tri- to 2,3,4,6-tetra-methyl ether suggested a chain-length of ~50 repeating units, in good agreement with the value of ~40 found by determination of the reducing end linked to the phosphate group[8].

No methyl ethers of the 2-acetamido-2-deoxy-D-mannuronic acid residues were recovered as such, probably because of extensive decarboxylation during the hydrolysis. On reduction of the product of methanolysis, the 3-methyl ether of the corresponding 2-amino-2-deoxy-D-mannose residue was obtained in good yield by g.l.c.,


a
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25
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RELATIVE ABUNDANCE
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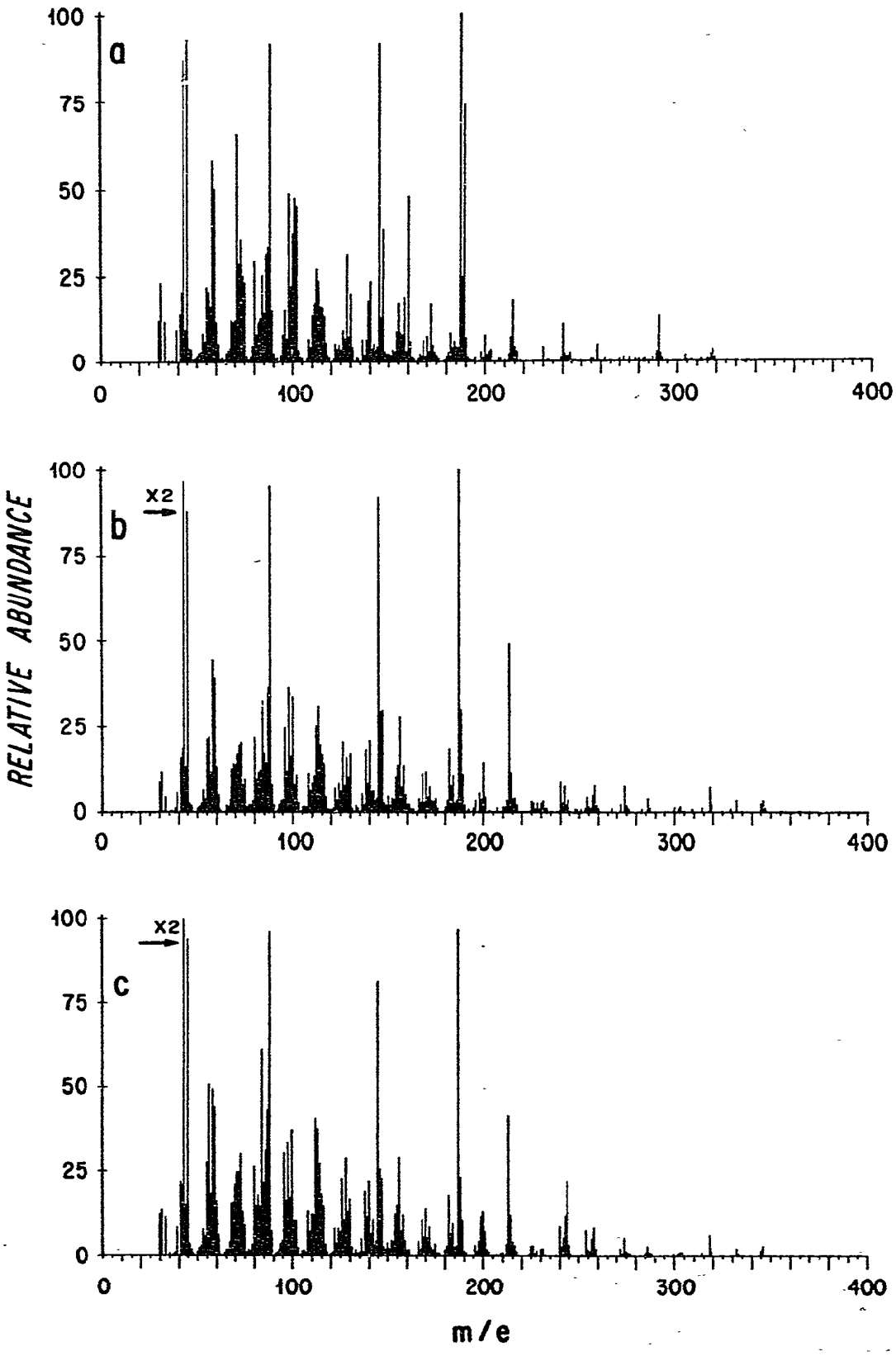
a
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x2
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75
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25
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200
300
400
RELATIVE ABUNDANCE
m / e

Fig. 1. Mass spectra of: (a) methyl 2-acetamido-2-deoxy-4-*O*-methyl-3,6-di-*O*-(trimethylsilyl)-α-D-mannopyranoside; (b) methyl 2-acetamido-2-deoxy-3-*O*-methyl-4,6-di-*O*-(trimethylsilyl)-α-D-mannopyranoside; and (c) fraction from methylated, carboxyl-reduced fraction CPC_{A-2}. (See fig. page 248.)

Fig. 2. Mass spectra of: (a) methyl 2-acetamido-6-*O*-acetyl-2-deoxy-4-*O*-methyl-3-*O*-[D-1-(methyl ethylcarboxylate)]-α-D-glucopyranoside; (b) methyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-methyl-α-D-glucopyranoside; and (c) fraction d(*i*) obtained from methylated fraction CPC_{A-2}. (See fig. page 249.)

and it was identified by g.l.c.–m.s. and comparison with an authentic sample[11] (see Fig. 1).

The 6-methyl ether of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-6-*O*-methyl-D-glucose] was obtained in ~50% yield, and characterized by comparison of the g.l.c.–m.s. data with those for an authentic sample[12] (see Fig. 2), and muramic acid 6-phosphate was recovered in a similar yield.

The 2-amino-2-deoxy-D-glucose component was recovered in excellent yield as the 3-mono-, 3,6-di-, and 3,4,6-tri-*O*-methyl derivatives. In addition to characterization by paper chromatography and by g.l.c. of the methyl 2-acetamido-2-deoxy-α,β-D-glucopyranosides, the compounds were characterized by degradation with ninhydrin[13] to the corresponding D-arabinose derivatives, and by crystallization of 2-acetamido-2-deoxy derivatives. Examination by g.l.c. of the product of methanolysis indicated that the 3-methyl ether obtained resulted from the hydrolysis of the 3,6-dimethyl ether. The proportion of 3,6-di- to 3,4,6-tri-methyl ethers indicated a chain-length, for the glycan portion of the peptidoglycan moiety, of four disaccharide units, in good agreement with the value obtained by determination of the reducing, muramic acid end-group residue[8].

The low yields of methylated derivatives obtained are explained by the complexity of the mixture composed of seven different *O*-methyl derivatives and four amino acids, and by the instability of some of the components. The results of the sequential, periodate degradation and of the methylation procedure, although not quantitative, are both in agreement with the structure shown in Fig. 3. This structure agrees with the "external" polysaccharide chain having the partial structure proposed by Hase *et al.*[7], based on the periodate oxidation of the polysaccharide and the isolation of disaccharides in low yield, and also with that proposed for the glycan part of the peptidoglycan moiety based on the isolation of di- and tetra-saccharides[1]. No difference in structure between the part susceptible to lysozyme degradation and that resistant to this enzyme is observed, suggesting that the linkage at C-6 of one of the muramic acid residues is responsible for this resistance. Removal of the carbohydrate chain by periodate oxidation did not increase the degradation by lysozyme, which suggests that the phosphate group is mainly responsible for this lack of effect; this observation is in agreement with the specificity of egg-white lysozyme[14]. The exact location of the phosphate group could not be determined, except that it is not linked to the reducing muramic acid residue[8]. The proportion of α-D-glucopyranosyl (nonreducing) end-groups is also in good agreement with the mol. wt. ($22–27 \times 10^3$) observed by physical methods[8]. As the fragment CPC_{A-2} represents a large propor-

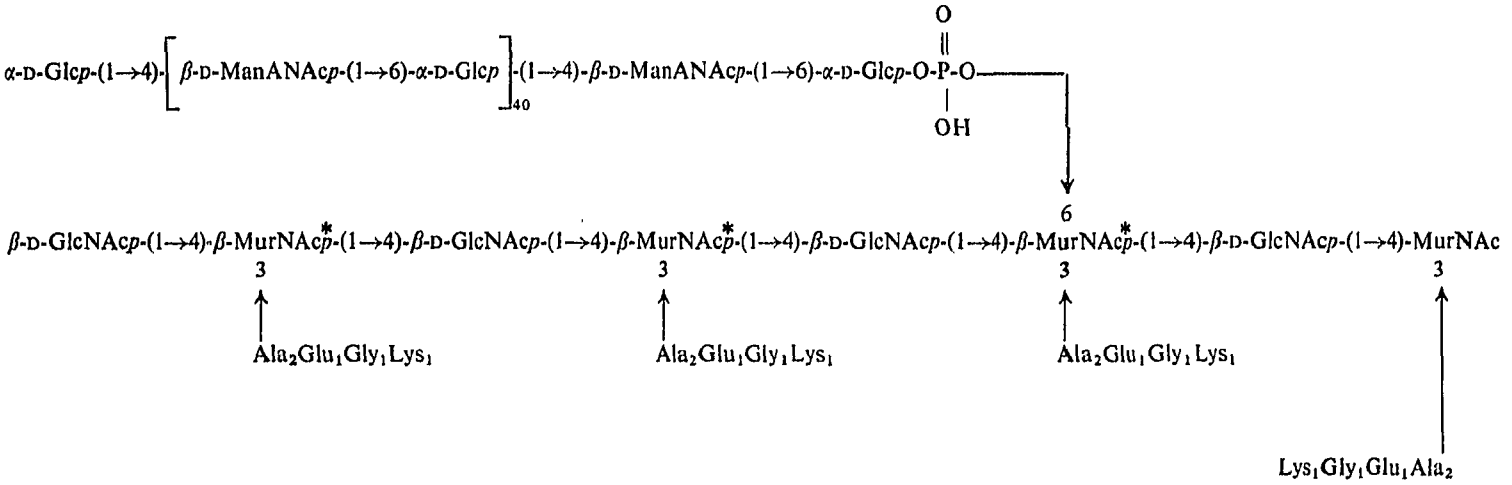

Fig. 3. Tentative structure of fraction CPC_{A-2} of the *Micrococcus lysodeikticus* cell-wall. [The chain composed of ManANAc*p* and Glc*p* residues can be linked to any of the three MurNAc*p* residues marked by an asterisk. Abbreviations: Glc*p*, glucopyranosyl; ManANAc*p*, 2-acetamido-2-deoxy-D-manno-pyranosyluronic acid; GlcNAc*p*, 2-acetamido-2-deoxy-D-glycopyranosyl; MurNAc*p*, 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucopyranosyl.]

tion of the cell wall, it may be assumed that most of the lysozyme-resistant part of the cell wall of *M. lysodeikticus* has the chemical structure depicted in Fig. 3.

EXPERIMENTAL

General methods. — Evaporations were performed under diminished pressure, and the temperature of the bath was maintained at or below 40°. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter. Melting points were recorded with a Mettler FP-2 hot-stage equipped with a microscope, and correspond to corrected melting points. The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

General analytical methods. — Paper chromatography on Whatman Nos. 1 and 3MM papers, and thin-layer chromatography on plates of cellulose F (E. Merck A. G., Darmstadt) were performed with the following solvent-systems (v/v): (*A*) 10:4:3 ethyl acetate–pyridine–water; (*B*) 7:7:6 pentanol–pyridine–water; (*C*) 4:1:5 (upper layer) butanol–ethanol–water; (*D*) 4:1:5 (upper layer) butanol–acetic acid–water; and (*E*) 6:4:3 butanol–pyridine–water. R_{Me_4Glc}, R_{Me_3GlcN}, and R_{GlcN} refer to the mobilities relative to 2,3,4,6-tetra-*O*-methyl-D-glucose, 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose, and 2-amino-2-deoxy-D-glucose, respectively. Amino sugars and amino acids were detected with ninhydrin[15], reducing sugars with the aniline phthalate reagent[16], reducing and nonreducing sugars with the alkaline silver nitrate[17] and periodate–benzidine reagents[18], lactones and esters with the hydroxylamine–ferric chloride reagent[19], and uronic acids with Bromothymol Blue indicator. Hexoses were quantitatively determined by the anthrone colorimetric method[20], and phosphate groups by the procedure of Chen *et al.*[21]. Mixtures of muramic acid 6-phosphate, muramic acid, and 2-amino-2-deoxy-D-glucose were separated on Dowex 50 (H^+) ion-exchange resin, and quantitatively estimated by use of a modified Elson–Morgan reaction[22]. Amino acids in acid hydrolyzates were quantitatively determined with a Beckman Model 116 amino acid analyzer.

Gas–liquid chromatography and mass spectrometry. — G.l.c. of reducing and nonreducing sugars was performed according to the procedure of Reinhold[23]. The fragments resulting from sequential periodate oxidation–sodium borohydride reduction–mild acid hydrolysis (Smith degradation)[24] were separated on columns of OV-1 and OV-11, and methyl glycosides of methyl ethers of hexoses and hexosamines on columns of OV-1, OV-11, OV-17, OV-25, and H1-EFF-8BP, with a Perkin–Elmer gas chromatograph equipped with dual ionization detector and integrator. G.l.c.–m.s. was performed with an analytical system consisting of an IBM 1800 computer fed raw data generated by a single-focusing, Hitachi–Perkin–Elmer RMU-6 mass spectrometer interfaced with a gas chromatograph (Perkin–Elmer Model 990). In all cases except those involving fully methylated sugars and muramic acid derivatives, analyses were performed on the trimethylsilyl derivatives of the sugars.

N-*Acetylation.* — *N*-Acetylation of small samples of amino sugars was performed by dissolution in methanol and addition of a 3-molar excess of acetic anhydride.

After 4 h at room temperature, the mixture was evaporated under a stream of nitrogen.

Hydrolysis. — Small-scale hydrolyses were performed on 1–2 mg of material with 4M hydrochloric acid for 16 h at 100°. The acid was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of ethanol and toluene.

Methanolysis. — Small-scale methanolysis was performed by treating the dried material (1–5 mg) with 0.5–1M methanolic hydrogen chloride (0.5–1.5 ml) for 8–12 h at 100°. The methanolic hydrogen chloride was removed by evaporation under a stream of nitrogen, followed by repeated addition and evaporation of methanol and toluene.

Reduction with sodium borohydride. — Ester and aldehyde groups were reduced by treating the cooled, aqueous solution of the substance (2–4 mg in 100–200 µl of water) with a 3–4M sodium borohydride solution (100–200 µl). The excess of borohydride was decomposed by addition of acetic acid or of Dowex 50 (H^+) ion-exchange resin. The solution was de-ionized with Dowex 50 ion-exchange resin (when necessary), and the borate ions were removed as methyl borate by repeated addition and evaporation of methanol.

Demethylation. — Methylated sugars were demethylated with boron tribromide or boron trichloride according to the procedure of Bourne and assoc.[25].

Ninhydrin degradation of hexosamines. — This degradation was performed according to Stoffyn and Jeanloz[13].

Materials. — The CPC_{A-2} fraction was obtained from *Micrococcus lysodeikticus* cell-walls, prepared according to Sharon and Jeanloz[2], by lysozyme degradation and cetylpyridinium fractionation, as described by Nasir-ud-Din *et al.*[8].

Sequential degradation by periodate oxidation–sodium borohydride reduction of fraction CPC_{A-2}. — *First treatment.* Fraction CPC_{A-2} (0.69 g in 100 ml of water, pH 3.8) was treated with sodium metaperiodate (1.925 g) for 24 h at 4° in the dark. The consumption of periodate, measured spectrophotometrically[26] at 223 nm, was constant after 8 h (2.3 mol/D-glucose residue). The formic acid released (0.9 mol/D-glucose residue) was estimated by titration with 20µM sodium hydroxide, and the formaldehyde released (1.35 mg), with the chromotropic acid reagent[27] (2-acetamido-2-deoxy-D-glucitol as the control). The excess of periodate was decomposed with 1,2-ethanediol, and the solution was dialyzed for three days at 4° against distilled water, to give a nondialyzable fraction (0.546 g after lyophilization). To a portion of this material (0.525 g) in water (25 ml) at 4° was added sodium borohydride (0.2 g) in four portions. An additional quantity of sodium borohydride (0.03 g) was added, and the solution was kept for 24 h in the cold. The excess of borohydride was decomposed with M acetic acid, and the solution was dialyzed for two days against distilled water. The nondialyzable portion was concentrated, and borate ions were removed as methyl borate by repeated addition and evaporation of methanol (6 × 50 ml). The residue was dissolved in water, the solution lyophilized (0.52 g), and the product treated with 0.5M sulfuric acid (15 ml) for 2 h at room temperature; the solution was then dialyzed against distilled water for three days. The nondialyzable fraction

was lyophilized, to give a periodate-resistant fraction (0.256 g), and kept for further treatment with periodate. The composition of this fraction is reported in Table I.

The dialyzable fraction was concentrated, and the solution passed through a column (1.8 × 16 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin (200–400 mesh), which was eluted with water (80 ml) and 10mM acetic acid (80 ml). The eluate was concentrated to 40 ml, and lyophilized (0.23 g). Thin-layer chromatography on cellulose, and paper chromatography in Solvents *B*, *C*, and *D*, with detection with alkaline silver nitrate, Bromothymol Blue, and the hydroxylamine–ferric chloride reagent, indicated the presence of glycerol, 2-acetamido-2-deoxy-D-mannuronic acid, and 2-acetamido-2-deoxy-D-mannuronolactone. G.l.c. on columns of OV-1 and OV-17 of the derived methyl glycosides showed the presence of glycerol (3.3%), in addition to unidentified compounds.

Hydrolysis of a portion of the dialyzable fraction with M hydrochloric acid for 16 h at 100°, and examination by paper chromatography in Solvent *D*, indicated the presence of glycerol and 2-amino-2-deoxy sugars. Another portion of the dialyzable fraction was treated with 0.5M methanolic hydrogen chloride for 6 h at 100°. The acid-free residue was *N*-acetylated, and the product treated in aqueous methanol (1:1) with sodium borohydride in the usual way. The reduced material was hydrolyzed with M hydrochloric acid for 4 h at 100°, and the product *N*-acetylated. Examination by paper chromatography in Solvent *D* (detection with alkaline silver nitrate), and in Solvent *E* on borate-treated paper[28] (Whatman No. 3MM) with detection with 0.5M sodium hydroxide in ethanol (u.v. light)[28], showed the presence of 2-acetamido-2-deoxy-D-mannose and glycerol. This result was confirmed by g.l.c. of the methyl glycosides.

The dialyzable fraction remaining was separated into two fractions on Whatman paper No. 3MM in Solvent *C*. The first fraction (5 mg) (R_{GlcN} 0.26) of the chromatogram contained glycerol, as indicated by t.l.c. The second fraction (38 mg) was composed of two compounds, respectively having $R_{\text{D-Glucuronolactone}}$ 1.0 and 1.21, positive to Bromothymol Blue and to the hydroxylamine–ferric chloride reagent. It was eluted from the paper, glycosidated and esterified with 0.5M methanolic hydrogen chloride (5 ml) for 6 h at 100°, and the product acetylated with pyridine and acetic anhydride. The product was reduced with sodium borohydride (25 mg) in the usual way, and the product hydrolyzed with M hydrochloric acid (1.5 ml) for 6 h at 100°. The hydrolyzate was adsorbed on a column (1 × 8 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin, and the column was eluted with aqueous methanol. The eluate was evaporated, the residue *N*-acetylated, and the product crystallized from aqueous ethanol (1:1, v/v) and acetone. Recrystallization from a mixture of aqueous ethanol and acetone gave platelets having m.p. 102–103°, and mixed m.p. with authentic 2-acetamido-2-deoxy-D-mannose, 102–105°; $[\alpha]_D^{20}$ −32° [after 5–10 min; *c* 0.42, 1:1 (v/v) methanol–water]; lit.[29] m.p. 105–108°, $[\alpha]_D$ −21 → +10° (*c* 1.0, water).

Second treatment. A small portion of the nondialyzable material resulting from the first treatment (12.0 mg) was dissolved in 0.1M sodium metaperiodate (5 ml), and the solution kept for 24 h at 4°. The consumption of periodate was constant

after 4 h, and then corresponded to 26 mmol/D-glucose residue. The solution was processed as described for the first treatment, except that the duration of the acid hydrolysis with 0.5M sulfuric acid was extended to 4 h. The nondialyzable material was lyophilized (11.0 mg). Examination of the dialyzate by paper chromatography showed the absence of reducing and nonreducing sugars.

Lysozyme degradation of periodate-degraded CPC_{A-2} fraction. — Twice periodate-treated fraction CPC_{A-2} (10 mg) in 10mM ammonium acetate (4 ml) was treated with lysozyme (50 mg; General Biochemicals, Division of the Mogul Corporation, Laboratory Park, Chagrin Falls, Ohio 44022) for 24 h at 37° in the presence of a drop of toluene. The resulting solution was dialyzed to give a nondialyzable fraction (9 mg). Examination of the dialyzable fraction by paper chromatography in Solvent *D* showed the absence of reducing and nonreducing sugars.

Application of the methylation procedure to fraction CPC_{A-2}. — Methylation. A cooled solution of fraction CPC_{A-2} (0.45 g) in water (8 ml) was treated at 4° with M sodium hydroxide (2 ml) and dimethyl sulfate (2 ml), added in small portions, while the mixture was vigorously stirred under an atmosphere of nitrogen. Sodium hydroxide (6 ml, 30%) and dimethyl sulfate (6 ml) were further added dropwise at 4°, and the mixture was stirred for 16 h. After seven additions of 6 ml of 30% sodium hydroxide and 6 ml of dimethyl sulfate, the mixture was dialyzed against distilled water, and the nondialyzable material was further methylated by three repetitions of the whole procedure just described, to give 0.4 g, $[\alpha]_D^{20}$ +9° [*c* 0.32, 1:1 (v/v) methanol–water].

Anal. Calc. for fully methylated polymer: OCH_3, 20.0. Found: OCH_3, 20.7.

Attempts to raise the methoxyl content further by methylation with silver oxide and methyl iodide[30], or with barium oxide–barium hydroxide–methyl iodide in *N*,*N*-dimethylformamide[31], showed no increase in methoxyl content, but excessive degradation took place. The i.r. spectrum indicated complete methylation.

Hydrolysis. A portion (0.325 g) of the methylated material just described was dissolved in M methanolic hydrogen chloride (15 ml), and the mixture was boiled under reflux for 16 h, and evaporated. The residue was freed of hydrochloric acid by repeated addition and evaporation of methanol, and treated with anhydrous pyridine (4 ml) and acetic anhydride (3 ml) at 4° and then for 2 h at room temperature. The solution was evaporated, the residue was hydrolyzed with M hydrochloric acid (6 ml) for 6 h at 100°, and the solution was diluted with water (to 10 ml) and applied to a column (0.9 × 20 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin. Elution with water (25 ml) and then with 0.1M acetic acid (45 ml) gave, after evaporation, a residue (0.30 g) that was dried by several additions and evaporations of water, methanol, and toluene.

Identification of methyl ethers of monosaccharides by isolation. A solution of the residue in water was applied to a column (1.8 × 38 cm) of Dowex 50 X-8 (H^+) ion-exchange resin. The carbohydrate components were eluted with water (500 ml) and then with a gradient of 0.05–2.5M hydrochloric acid (1 liter); 8-ml fractions were

collected, examined by t.l.c. and paper chromatography, and combined into seven fractions (*a–g*).

(*a*). This fraction (11 mg) was separated into two compounds (2.0 and 5.0 mg, respectively) on Whatman 3MM paper in Solvent *D*.

(i) The first compound had R_{Me_4Glc} 0.11, identical with that of muramic acid 6-phosphate. Treatment of 0.5 mg with acid phosphatase (wheat-germ, Sigma Chemical Co., St. Louis, Mo. 63178) in 50mM sodium acetate–acetic acid buffer (400 μg/ml; pH 5.0) for 4 h at 37°, followed by heating for 1 min, treatment with Dowex 50 X-8 (H^+) ion-exchange resin, and elution of the resin with 10mM hydrochloric acid, gave a residue showing in t.l.c. (Solvent *D*) the same rate of migration as that of muramic acid (R_{GlcN} 2.02).

(ii) A portion of the second compound (R_{Me_4Glc} 0.73) was demethylated to give a compound that showed in t.l.c. (Solvent *A*) R_{Glc} 1.0, identical with that of D-glucose. A second portion was methanolyzed; g.l.c. of the methanolyzate on a column of OV-11 showed R_T 8.7 and 10.9, identical with the R_T values of methyl 2,3-di-*O*-methyl-α,β-D-glucopyranoside. Reduction of a third portion with sodium borohydride, and treatment of the product with *p*-phenylazobenzoyl chloride in pyridine[32] gave 2,3-di-*O*-methyl-1,4,5,6-tetra-*O*-(*p*-phenylazobenzoyl)-D-glucitol, m.p. 181–184° and mixed m.p. 183–185°; lit.[32] m.p. 181°.

Anal. Calc. for $C_{60}H_{50}N_8O_{10}$: C, 69.06; H, 4.83; N, 10.77. Found: C, 69.06; H, 4.91; N, 10.80.

(*b*). The fraction (34 mg) was purified on Whatman 3MM paper (Solvent *C*) to give a compound (26 mg) showing $[\alpha]_D^{20}$ +68° (*c* 0.41, water) and R_{Me_4Glc} 0.92. A portion of this compound was demethylated to give a product showing in t.l.c. (Solvent *C*) the same migration as that of D-glucose. The compound was identified by g.l.c. on a column of OV-11 (R_T 9.4 and 10.7) as methyl 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside; this was confirmed by reduction with sodium borohydride, and treatment of the product with *p*-phenylazobenzoyl chloride in pyridine[32] to give 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-(*p*-phenylazobenzoyl)-D-glucitol[32], m.p. 88–90° and mixed m.p. 88–91°; lit.[32] m.p. 85°.

Anal. Calc. for $C_{48}H_{44}N_6O_4$: C, 67.91; H, 5.22; N, 9.90. Found: C, 67.88; H, 5.26; N, 9.88.

(*c*). The fraction (32 mg) was separated on Whatman 3MM paper (Solvent *C*) into two compounds (10 mg and 4 mg, respectively).

(i) The first compound (R_{Me_4Glc} 0.92) was methanolyzed, and the product identified by g.l.c. on an OV-11 column (R_T 9.4, 10.7) as methyl 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside.

(ii) A portion of the second compound (R_{Me_4Glc} 1.0) was demethylated to give a compound showing in t.l.c. (Solvent *A*) R_{Glc} 1.0, identical with that of D-glucose. Methanolysis of a second portion, and g.l.c. of the product on an OV-11 column showed R_T values of 8.9 and 9.2, identical with those of authentic methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-glucopyranoside. The compound crystallized, and was recrys-

tallized from warm hexane to give 2,3,4,6-tetra-*O*-methyl-D-glucose, m.p. 86–88°; lit.[33] m.p. 96°.

(*d*). This fraction (40 mg) was separated on Whatman 3MM paper (Solvent *D*) into two components (R_{Me_3GlcN} 0.95 and 0.14).

(i) The first compound (14 mg) showed, in t.l.c. (Solvent *D*), R_{Glc} 2.5 identical with that of 2-amino-3-*O*-(D-1-carboxyethyl)-6-*O*-methyl-D-glucose (6-*O*-methylmuramic acid)[12]. Demethylation of this compound gave a compound showing, in t.l.c. (Solvent *D*), R_{GlcN} 1.0, identical with that of 2-amino-2-deoxy-D-glucose. A portion of the compound was *N*-acetylated, the product methanolyzed, and the product acetylated with acetic anhydride–pyridine. G.l.c.–m.s. showed an R_T 19.0 and mass spectrum corresponding to those of methyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-methyl-α,β-D-glucopyranoside, but different from those of the 4-*O*-methyl analogue (see Fig. 2). On periodate oxidation of the *N*-acetylated, sodium borohydride-reduced compound, 1.0 mol of oxidant/mol of 2-acetamido-3-*O*-(D-1-carboxyethyl)-6-*O*-methyl-D-glucitol was used up, and no formaldehyde was detected by the chromotropic acid method[27].

(ii) The second compound (8 mg) showed, in t.l.c. (Solvent *D*), R_{Me_3GlcN} 0.14. It was reducing, and showed the presence of an amino group and the absence of a carboxyl group. A portion of this compound was demethylated, and paper chromatography (Solvent *D*) of the product showed the presence of 3 compounds, having R_{GlcN} 0.8, 1.15, and 2.0, respectively. This compound was not further investigated.

(*e*). This fraction (48 mg) was separated on Whatman 3MM paper (Solvent *D*) into 3 compounds.

(i) The first compound (4 mg) showed an R_{GlcN} value of 1.7 (Solvent *D*), identical with that of 2-amino-2-deoxy-3-*O*-methyl-D-glucose. A portion of this compound was demethylated to give a compound showing, in t.l.c. (Solvent *D*), an R_{GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-D-glucose. Another portion was degraded with ninhydrin to give a compound showing, in t.l.c. (Solvent *D*), an R_F value identical with that of authentic 2-*O*-methyl-D-arabinose. Another fraction was *N*-acetylated; the crystalline compound resulting was recrystallized from methanol–acetone, m.p. 190–192°, m.p. on admixture with 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose[34], m.p. 191–193°; lit.[34] m.p. 195–198°.

(ii) The second compound (8 mg; R_{GlcN} 2.9, Solvent *D*) showed an R_{GlcN} value identical with that of 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose[35]. A portion of the compound was demethylated to give a compound showing, in t.l.c. (Solvent *D*), an R_{GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-D-glucose. G.l.c. of the product obtained after treatment with methanolic hydrogen chloride showed an R_T value of 9.1, identical with that of methyl 2-acetamido-2-deoxy-3,6-di-*O*-methyl-α,β-D-glucopyranoside[35,36].

(iii) A mixture of compounds (14 mg; R_{GlcN} <1.68) was purified by chromatography on Whatman 3MM paper, first in Solvent *B* and then in Solvent *C*, to give a homogeneous product (5 mg) showing R_{GlcN} 1.7 (Solvent *C*), the presence of reducing and amino groups, and the absence of carboxyl groups. A portion of

the compound was *N*-acetylated, and the product treated with 0.1M sodium metaperiodate for 4 h at 4°; t.l.c. (Solvent *C*) of the resulting compound showed unchanged starting-material. A second portion of the compound (1.5 mg) was *N*-acetylated, the product reduced with sodium borohydride, and the product treated with 10mM sodium metaperiodate (3 ml) for 4 h at 4°; ~1 mol of periodate (relative to 1 mol of hexose) was consumed, and t.l.c. (Solvent *C*) showed a reducing spot having R_{Me_3GlcN} 0.62, and a trace of a nonreducing compound having R_{Me_3GlcN} 0.4. A third portion of the compound was treated with ninhydrin[13]; t.l.c. (Solvent *C*) showed an R_{Glc} value of 3.1, different from that of 3-*O*-methyl-D-arabinose (obtained by ninhydrin degradation of 2-amino-2-deoxy-4-*O*-methyl-D-glucose). The compound showed properties different from those of known methyl ethers of 2-amino-2-deoxy-D-glucose, suggesting that it was a 2-amino-2-deoxy-3-*O*-methylpentose, possibly arising from the decarboxylation of 2-amino-2-deoxy-D-mannuronic acid.

(*f*). This compound (24 mg) was purified further by chromatography on Whatman 3MM paper (Solvent *D*). It showed an R_{GlcN} value of 2.9, identical with that of 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose[35]. A portion of the compound was treated with ninhydrin, and t.l.c. (Solvent *D*) showed an $R_{2,5\text{-}Di\text{-}O\text{-}methylarabinose}$ value of 1.0, identical with that of 2,5-di-*O*-methylarabinose and different from the values for 2,3- (0.85), 3,4- (0.70), and 3,5-di-*O*-methylarabinose (1.10). The compound was *N*-acetylated, and the product was crystallized from ethanol–ether–pentane to give 2-acetamido-2-deoxy-3,6-di-*O*-methyl-D-glucose, m.p. 214–216°, mixed m.p. 212–214°; lit.[35] m.p. 232–233° (α anomer).

(*g*). This compound (18 mg) was purified by chromatography on Whatman 3MM paper (Solvent *D*), to give a compound (8 mg) that showed an R_{Me_3GlcN} value of 1.0, identical with that of 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose. A portion of the compound was demethylated, and t.l.c. (Solvent *D*) of the resulting compound (R_{GlcN} 1.0) indicated that it was identical with 2-amino-2-deoxy-D-glucose. G.l.c. of the *N*-acetylated methyl glycosides on an OV-25 column showed R_T 6.4, identical with that of methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α,β-D-glucopyranoside[36]. The compound was *N*-acetylated, and the product crystallized from ethanol, to give 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose as needles, m.p. 231–233°, mixed m.p. 230–233°; lit.[37] m.p. 234°.

*Identification of methyl ethers of monosaccharides by gas–liquid chromatography. — Methyl ethers of 2-amino-2-deoxy-*D*-mannuronic acid.* These methyl ethers were identified as methyl ethers of 2-amino-2-deoxy-D-mannose after reduction as follows. A solution of fraction CPC_{A-2} (55 mg) in 10mM methanolic hydrogen chloride (5 ml) was kept for 14 h at 22°, and then evaporated. The residue was treated by several additions and evaporations of toluene, and then dissolved in 3:2 (v/v)water–ethanol (5 ml). Potassium borohydride (10 mg) was added, and the solution was kept for 4 h at 4°, and then for 2 h at 22°; it was processed in the usual way, to give carboxyl-reduced fraction CPC_{A-2} (42 mg).

A portion of this fraction (20 mg) was methanolyzed with M methanolic hydrogen chloride (4 ml) for 16 h at 100°, and the solution evaporated. A solution

of the residue in water (0.5 ml) was applied to a column (0.8 × 12 cm) of Dowex 50 X-8 (H^+) ion-exchange resin, which was eluted with water (50 ml) and then with M hydrochloric acid (40 ml). The hydrochloric acid eluate was concentrated to 15 ml, and the solution applied to a column (0.8 × 7 cm) of Dowex 1 X-8 (AcO^-) ion-exchange resin. The column was successively eluted with water and 0.1M acetic acid (15 ml), the eluates were combined and lyophilized. and the residue was *N*-acetylated. G.l.c. of the trimethylsilyl ethers of the *N*-acetylated methyl glycosides on a column of OV-17 showed a component having R_T 10, identical with that of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[11], and different from that of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α,β-D-glucopyranoside. The mass spectrum of the component having R_T 10 was identical with that of authentic methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside[11], and different from that of the 4-*O*-methyl analogue[11].

A second portion (3 mg) of the methylated, reduced, and methanolyzed fraction $CPC_{A-2}$was freed of hydrogen chloride by repeated addition and evaporation of toluene (1 ml) and methanol (1 ml), and then *N*-acetylated. Quantitative g.l.c. of the trimethylsilyl ether on an OV-1 column showed the presence of 32% (w/w of methylated, carboxyl-reduced fraction CPC_{A-2}) of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-mannopyranoside.

Methyl ethers of D-*glucose and 2-acetamido-2-deoxy*-D-*glucose.* A sample of methylated fraction CPC_{A-2} (8 mg) was methanolyzed. A solution of the acid-free methanolyzate in the minimal volume of water was adsorbed on a column (0.8 × 8 cm) of Dowex 50 X-8 (H^+) ion-exchange resin. Elution was successively conducted with water (35 ml), 50mM acetic acid, and 2.5M hydrochloric acid. The water and acetic acid eluates were combined and evaporated. G.l.c. of the residue on a column of OV-11 showed the presence of methyl 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-α,β-D-glucopyranoside in the ratio of 1:49, and the absence of methyl 2,3-di-*O*-methyl-α,β-D-glucopyranoside. The hydrochloric acid eluate was evaporated to dryness, and the acid removed as previously described. The residue was *N*-acetylated, and g.l.c. on a column of OV-25 showed the presence of methyl 2-acetamido-2-deoxy-3,4,6-tri- and 3,6-di-*O*-methyl-α,β-D-glucopyranoside in the ratio of 1:3, and the absence of methyl 2-acetamido-2-deoxy-3-*O*-methyl-α,β-D-glucopyranoside.

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