PARTIAL CHARACTERIZATION OF MUCORAN: THE GLUCURONOMANNAN COMPONENT

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ABSTRACT

Mucoran, an acidic heteropolysaccharide isolated from the cell walls of the fungus *Mucor rouxii*, was purified by DEAE-Sephadex chromatography. It consists mainly of D-glucuronic acid, D-mannose, and L-fucose in a 5:3:2 ratio plus small proportions of galactose and glucose. Mucoran was subjected to methylation by the Hakomori procedure. Only about 60–70% of the polysaccharide was recovered as fully methylated material. A large proportion of this methylated material was dialyzable, indicating extensive depolymerization, probably via β-elimination, during exposure to dimethylsulfinyl carbanion. The fully methylated fraction of mucoran (both dialyzable and nondialyzable portions) consists of unbranched glucuronomannan chains, with equal proportions of 4-linked D-glucuronic acid residues and 3-linked D-mannose residues. The aldobiouronic acid, α-D-glucopyranosyluronic acid-(1→3)-D-mannose, was a major product of partial acid hydrolysis of mucoran. The principal structural feature of mucoran is the following alternating sequence of D-glucuronic acid (GlcA) and D-mannose (Man) residues: D-Man-(1→[4)-α-D-GlcA-(1→3)-D-Man-(1]$_n$-.

INTRODUCTION

Polysaccharides rich in D-glucuronic acid residues comprise a significant portion of the cell walls of the fungus *Mucor rouxii*[1]. One such polymer, termed mucoran, was isolated from the yeast cells of *Mucor rouxii* by extraction with M potassium hydroxide, and purified by several precipitations as a copper complex[1]. Initial studies indicated that mucoran contained D-glucuronic acid, D-mannose, L-fucose, and small proportions of D-galactose. There were roughly equal proportions of uronic acid and neutral sugars.

In the present communication we describe the characterization of the glucuronomannan chains that were found to be the main structural component of mucoran, and also report on the extensive depolymerization encountered in the methylation of mucoran by the Hakomori procedure.

EXPERIMENTAL

Preparation of mucoran. — Mucoran was isolated as described earlier[1] from

yeast cells of *M. rouxii* by extraction with M KOH and precipitation as a copper complex. Radioactive ^{14}C-mucoran was prepared in a similar manner except that the yeast cells were grown in 2 liters of yeast extract–peptone–D-glucose medium containing uniformly labeled ^{14}C-D-glucose (specific activity: 2.25 mCi/mole). An additional purification step was introduced prior to precipitation of the copper complex: the dialyzed KOH extract was passed through a 25 × 160-mm column of *O*-(carboxymethyl)cellulose (pH 7.0) to remove contaminating protein. A higher recovery of mucoran–copper complex was obtained by using a modified copper tartrate reagent containing 4 g of $CuSO_4 \cdot 5H_2O$, 4 g of potassium sodium tartrate·$4H_2O$, and 40 g of NaOH in 1 liter of water.

Final purification of both radioactive and non-radioactive mucoran was achieved by chromatography on DEAE-Sephadex.

DEAE-Sephadex fractionation of mucoran. — A 15 × 300-mm column of DEAE-Sephadex A-25 was equilibrated with 0.05M potassium acetate. The mucoran sample (238 mg) was introduced and the column was eluted with a linear gradient (1500 ml) of 0.05M to 2.0M potassium acetate. Fractions (10 ml) were collected and the elution of the polymer monitored by polarimetry.

Complete hydrolysis of mucoran. — This was attained by a modification of the procedure used by Aspinall *et al.*[2] Freeze-dried mucoran (5 mg) was soaked for 1 h in 0.1 ml of 13.5M H_2SO_4. Methanol (30 ml) was added and the samples heated at 70° in a sealed tube for 24–48 h until the polysaccharide had dissolved. The acid was carefully neutralized with aqueous NaOH. D-Arabinose (1.0 mg) was added as internal standard and the methanolyzate evaporated to dryness over P_2O_5 *in vacuo* at 60°. The product was dissolved in pyridine (3 ml) and Tri-Sil (1 ml of a ready-mixed formulation of hexamethyldisilazane and chlorotrimethylsilane; Pierce Chemical Co., Rockford, Ill. U. S. A.). After 2 h at room temperature, the mixture was evaporated to dryness and redissolved in 10 ml of tetrahydrofuran. $LiAlH_4$ (200 mg) was added and the mixture was refluxed for 4 h. After addition of several ml of dilute acetic acid, the tetrahydrofuran was evaporated off, and the aqueous suspension was adjusted to pH 1.0 with H_2SO_4. The molarity of the acid was adjusted to 0.25 with concentrated acid, and the sample heated for 8 h at 97°. The sample was then neutralized with $Ba(OH)_2$, deionized [Dowex-50 (H^+) and Dowex-1 (AcO^-)], evaporated, and the sugars converted into alditol acetates as described later.

Partial acid hydrolysis of mucoran. — Mucoran (121 mg) was heated with 0.01M H_2SO_4 (10 ml) for 1 h at 100° and then dialyzed against water. The non-dialyzable material was further hydrolyzed with 0.5M H_2SO_4 for 1 h at 100° and separated into dialyzable and non-dialyzable fractions. The former was neutralized, concentrated to low volume, and fractionated on a Sephadex G-15 column (2.5 × 180 cm). The fractions were analyzed for uronic acid by the carbazole procedure[3] and neutral sugar by Dreywood's anthrone method[4].

Characterization of aldobiouronic acids. — The aldobiouronic acid (5 mg) was transformed into its corresponding methyl glycoside methyl ester[5] and subsequently reduced with borohydride[6]. The methyl glycoside of the reduced aldobiouronic acid

was hydrolyzed (0.25M H_2SO_4, 12 h, 100°) and the resulting neutral monosaccharides converted into alditol acetates as described later.

Hakomori[7] *methylation of mucoran.* — Freeze-dried mucoran (10–40 mg) was placed in a serum vial (rubber stopper + aluminum cap) and dissolved in 0.1 or 0.2 ml of water. Two ml of Me_2SO were added and the vial was swept out with N_2 and sealed. About 10–15 ml of 4M dimethylsulfinyl sodium in Me_2SO was introduced[11]. This amount was sufficient to decompose all water present and leave a large excess of reagent. The vial was partially immersed in a sonic oscillator bath (MEL type L-368-1 40 MHz), treated for 1 h, and then kept for 4 h, or longer if so indicated. An excess of methyl iodide was injected dropwise while the bottle was cooled in cold water. The mixture was sonicated for 1 h, evaporated to near dryness, and the methylated mixture resuspended in water and dialyzed three times against 10–20 volumes of water. The dialyzable and non-dialyzable fractions were separately concentrated to low volume, acidified with H_2SO_4, and extracted three times with $CHCl_3$. (The chloroform phase of the dialyzable fraction was washed three times with water to remove as much Me_2SO as possible). The methylated carbohydrates in these two chloroform extracts were reduced and hydrolyzed as follows.

Reduction and hydrolysis of methylated products of mucoran. — The methylated material, dissolved in $CHCl_3$, was evaporated to dryness, desiccated over P_2O_5 and then redissolved in a few ml of tetrahydrofuran. The solution was treated with 50–100 mg of $LiAlH_4$ (or $LiAlD_4$) and refluxed for 4 h. Excess hydride was decomposed by dropwise addition of ethanol, and the mixture was evaporated to dryness. The reduced, methylated carbohydrate was extracted with hot $CHCl_3$ and the extract was evaporated to dryness. The residue was treated with 0.2 ml of 13.5M H_2SO_4 for 1 h. The acid was next diluted to 0.5M and the hydrolysis continued for 4 h in a boiling water-bath. The solution was neutralized ($BaCO_3$) and concentrated to low volume.

Hakomori methylation and hydrolysis of aldobiouronic acid derivatives. — The methyl glycoside of the reduced aldobiouronic acid was evaporated to dryness in a serum vial and permethylated by the Hakomori[7] procedure as already described, except that the sample was dissolved directly in 1 ml of Me_2SO, treated with only 2 ml of dimethylsulfinyl carbanion reagent[11] and sonicated for 2 h. After addition of methyl iodide and sonication, the mixture was poured into 20 ml of water and the solution acidified with a few drops of M H_2SO_4. The water phase was extracted three times with chloroform. The chloroform layer was washed twice with water and flash-evaporated as thoroughly as possible (traces of Me_2SO remained). The methylated disaccharide was hydrolyzed with 2 ml of 0.5M H_2SO_4 overnight. After neutralization ($BaCO_3$), alditol acetates of the resulting methylated sugars were prepared.

Preparation of alditol acetates. — Sugars (free or methylated), dissolved in a few ml of water, were treated with 100 mg of $NaBH_4$ for at least 2 h. Dowex-50 (H^+) was added to decompose excess $NaBH_4$ and the resin was filtered off. The filtrate was evaporated and the residue was redissolved in methanol and evaporated again. The procedure was repeated 2–3 more times to eliminate all boric acid. The residue was thoroughly dried and treated with dry pyridine (0.5 ml) and acetic anhydride (0.5 ml)

for 20 min in a boiling water bath. These solvents were evaporated off by evaporation with toluene. The final residue was dissolved in a few drops of $CHCl_3$, any insoluble material discarded, and the liquid injected into a gas chromatograph.

Gas–liquid chromatography and mass spectrometry (g.l.c.–m.s.). — Alditol acetates of sugars, or their methylated derivatives[8], were separated on a glass column (200 × 0.3 cm) packed with 3% (w/w) ECNSS-M on Gas Chrom Q (100–120 mesh). A Perkin–Elmer gas chromatograph, model 881, fitted with a flame-ionization detector was employed routinely. Retention times (T values) were calculated by interpolation with the alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose ($T = 1.00$) and 2,3,6-tri-*O*-methyl-D-glucose ($T = 2.50$) as references. The identity of methylated sugar derivatives was confirmed by mass spectrometry[9] with an interfaced g.l.c.–m.s. instrument (Perkin–Elmer, model 270).

Miscellaneous. — Zone electrophoresis was conducted on glass-fiber sheets with 0.1M acetate buffer (pH 4.0 or 7.2) at 1500 V for 2 h. Carbohydrates were visualized by spraying with 8% H_2SO_4 and heating in an oven at 100°. Paper chromatography of aldobiouronic acids was performed in descending fashion on Whatman 1 paper. Optical rotation was measured in a 10-cm cell at 589 nm with a Perkin–Elmer polarimeter Model 141. Radioactivity was counted in a Packard liquid-scintillation spectrometer. Absolute counting rates were determined with an internal standard of ^{14}C-toluene.

RESULTS AND DISCUSSION

Purification and properties. — Mucoran $[\alpha]_D^{23}$ +48° (*c* 1.0, water) was isolated as a copper complex as earlier described[1] and further purified by ion-exchange chromatography on a column of DEAE-Sephadex. Most (81%) of the applied material was eluted as a single sharp peak; zone electrophoresis of this purified material on glass-fiber sheets showed a single, moving component. The homogeneity

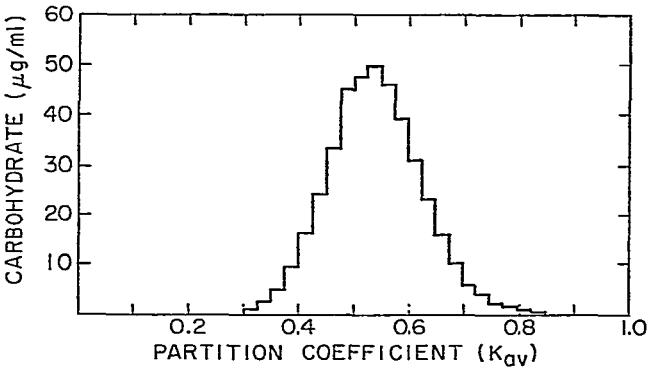

Fig. 1. Gel chromatography of mucoran on sepharose 4B; carbohydrate concentration was determined with anthrone.

of mucoran was also analyzed by gel chromatography as described by Granath and Kvist[10] on a column of Sepharose 4B. The elution curve (Fig. 1) was unusually symmetric for a polysaccharide and the molecular-weight distribution was correspondingly narrow (Fig. 2). By using a dextran calibration curve, the following values were computed:, weight-average molecular weight ($\overline{M}_w$) = 119,000; number-average molecular weight ($\overline{M}_n$) =91,800; homogeneity index ($\overline{M}_w/\overline{M}_n$) = 1.3.

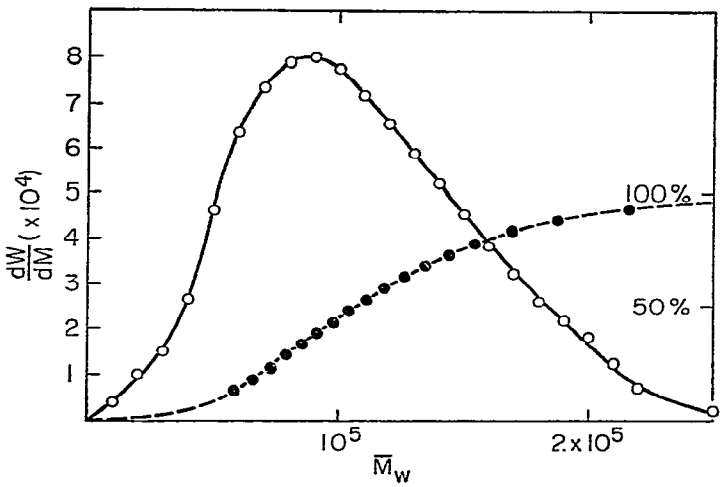

Fig. 2. Molecular-weight distribution of mucoran computed from gel chromatographic values (Fig. 1). Data analyzed as described by Granath and Kvist[10]. ○———○, Differential distribution; ●———●, integral distribution.

These findings show a high degree of molecular homogeneity in the sample and minimize, but do not rule out, the possibility that mucoran may consist of two different polysaccharide species of similar molecular-weight distribution.

Monomer composition. — A near quantitative recovery of monosaccharides was attained by an elaborate procedure that included the following consecutive treatments: hydrolysis in concentrated sulfuric acid, methanolysis, silylation, reduction of uronic acid derivatives with lithium aluminum hydride, and hydrolysis with dilute acid. The g.l.c. determination of alditol acetates in the final hydrolyzate showed that mucoran consists mainly of D-glucuronic acid, D-mannose, and L-fucose in an approximate molar ratio 5:3:2. There was also a small proportion of galactose (<5%) that could not be quantitated because of the large glucose peak. Almost all of the glucose detected was derived from reduced D-glucuronic acid residues; however, as indicated later, a minute proportion of glucose residues is present in the mucoran sample. The chromatogram showed other minor peaks, but these were not characterized.

Hakomori methylation of mucoran. — The Hakomori[7] procedure is the simplest and most effective method presently available for methylating polysaccharides[16]. It has been successfully employed for the methylation of various polysaccharides, seemingly without any significant decomposition of the polysaccharide (e.g. refs.

11–15). However, Anderson *et al.*[17] found that acidic polysaccharides, if subjected to repeated methylation undergo severe β-elimination.

We found that mucoran was extensively depolymerized during a single Hakomori methylation. Furthermore, only part of the carbohydrate was amenable to full methylation. An additional difficulty was the insolubility of the polysaccharide in methyl sulfoxide. This, however, was circumvented by first dissolving the sample in a minute amount of water and then adding it to the methyl sulfoxide. Visual inspection indicated that the polysaccharide was still in solution when the dimethylsulfinyl carbanion was added. Because of the turbidity of the latter, it was not usually possible to assess whether any subsequent precipitation occurred.

The introduction of water probably did not interfere with methylation, since the large proportion of dimethylsulfinyl carbanion added was more than sufficient to decompose any water present, leaving at least a 5-fold molar excess over the quantity of carbanion needed to ionize all hydroxyl groups in the polysaccharide. On occasion, triphenylmethane was added to the methylation mixture; the red color of its carbanion[18] verified the presence of excess dimethylsulfinyl carbanion.

The products of methylation were not homogeneous, and could be fractionated by dialysis and solubility in chloroform into three separate categories: (*1*) non-dialyzable, chloroform-soluble fraction, (*2*) a non-dialyzable, chloroform-insoluble but water-soluble fraction, and (*3*) a dialyzable fraction soluble in chloroform. Only fractions *1* and *3* were fully methylated.

The relative amounts of the methylated fractions were readily determined by using ^{14}C-mucoran (Table I).

TABLE I

FRACTIONATION OF METHYLATION PRODUCTS OF ^{14}C-MUCORAN[a]

Fraction	*Incubation time with dimethylsulfinyl carbanion*		
	4 h	*4 h*[b]	*12 h*
	(% of radioactivity)		
1, Non-dialyzable, chloroform-soluble	30.2	33.5	11.3
2, Non-dialyzable, chloroform-insoluble	40.0	39.1	31.7
3, Dialyzable (chloroform soluble)	29.8	27.4	57.0

[a]Methylation as described in experimental section except that only about 5 mg (11,000 d.p.m.) of ^{14}C-mucoran were utilized and the polysaccharide was treated with dimethylsulfinyl carbanion for 4 h or 12 h as shown. [b]Sonication omitted.

Nearly 30% (initial dry wt) of mucoran was degraded to dialyzable products by a routine 4-h exposure to dimethylsulfinyl carbanion; twice as much was degraded after 12 h (Table I). The u.v. spectrum of the non-dialyzable, chloroform-insoluble fraction showed a marked peak at 226 nm. This peak, which was present neither in the chloroform-soluble methylated fractions nor in the original polysaccharide is

TABLE II

ANALYSIS OF METHYLATED MUCORAN (CHLOROFORM-SOLUBLE FRACTIONS) BY G.L.C.–M.S.

Retention time (T)	*Alditol acetate of*	*Fraction (%)*		*Mass-spectral lines*[a]
		non-dialyzable	*dialyzable*	
1.00	2,3,4,6-tetra-*O*-methyl-D-mannose[b]	4.6	9.1	43, 101, 117, 129, 45, 161, 145, 87, 71, 57, 205
1.16	2,3-di-*O*-methyl-L-fucose	0.5	[c]	43, 117, 101, 143, 57, 55, 58, 45, 203
1.24	2,3,4,6-tetra-*O*-methyl-D-galactose	0.6	[c]	43, 101, 117, 145, 129, 161, 45, 71, 57, 55, 205
2.08	2,4,6-tri-*O*-methyl-D-mannose	47.3	40.2	43, 117, 129, 45, 101, 161, 87, 99, 71, 233, 58,., 201
2.42	2,3,4-tri-*O*-methyl-D-mannose[d]	1.7	5.8	43, 129, 101, 117, 161, 45, 87, 99, 71, 145,., 189, 233
5.46	2,3-di-*O*-methyl-D-glucose[e]	45.2	44.8	43, 117, 101, 87, 129, 45, 161, 261, 44

[a]Major lines in decreasing order of intensity. Determined only for the non dialyzable sample. [b]Presence of small proportion of the glucose isomer cannot be ruled out. [c]More than 2 unresolved components appeared in this region; the total proportion was less than 3%. [d]Tentative characterization. [e]Derived from 2,3-di-*O*-methyl-D-glucuronic acid.

probably due to unsaturated uronic acid residues produced by β-elimination[19–21]. β-Elimination would explain the disintegration of the polysaccharide into short chains, some of them of dialyzable size.

The i.r. spectra of the two chloroform-soluble fractions (non-dialyzable and dialyzable) showed no indication of the OH band. By contrast, the chloroform-insoluble fraction exhibited a pronounced, broad band at 3500 cm^{-1} (KBr pellet), indicating it was highly undermethylated. This undermethylated fraction amounted to 30–40% of the initial polysaccharide. A second methylation (no water added) failed to yield a fully methylated product, and this fraction was not further studied.

The two chloroform-soluble fractions, representing 60–70% of the initial polysaccharide yielded the same kinds of methylated sugars (Table II), indicating that they have similar structures. In both the non-dialyzable and dialyzable fractions, D-glucuronic acid residues substituted at C-4 and D-mannose residues substituted at C-3 occur in an approximately 1:1 molar ratio and together comprise 85–95% of the total carbohydrate in these fractions. That these two fractions were fully methylated was shown by the absence of monomethyl ethers or unmethylated sugars according to g.l.c. of the hydrolyzates (Table II). After reduction with lithium aluminum deuteride, the non-dialyzable, chloroform-soluble fraction was remethylated, and as a result the entire peak of 2,3-di-*O*-methyl-D-glucose was converted into 2,3,6-tri-*O*-methyl-D-glucose ($T = 2.50$). Mass spectroscopy of the latter indicated two deuterium atoms at C-6, thus confirming that the di-*O*-methyl sugar arose from D-glucuronic acid residues substituted at C-4. The high proportion of terminal mannose residues and the absence of branching residues indicates that both fractions consist of rather short, linear chains of glucuronomannan having an average $\overline{d.p.}$ of 20 for the non-dialyzable fraction and an understandably lower $\overline{d.p.}$ of10 for the dialyzable fraction.

Partial acid hydrolysis. — Hydrolysis with 0.01 M acid had little effect. Hydrolysis with 0.5M H_2SO_4 for 1 h at 100° degraded most of the mucoran into dialyzable

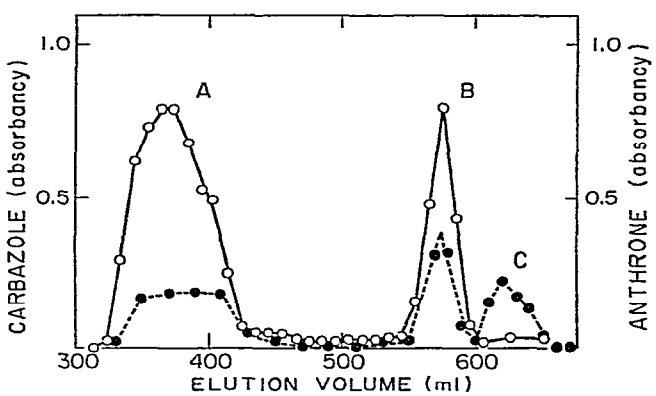

Fig. 3. Separation of products of 0.5M acid hydrolysis of mucoran by gel chromatography on Sephadex G-15. ○———○, uronic acid content by carbazole assay; ●———●, neutral sugar by anthrone assay. Absorbancy readings are for 0.1-ml samples.

fragments. These fragments, which amounted to 88% (carbazole assay) or 95% (anthrone assay) of the initial polysaccharide, were separated by gel filtration into three distinct non-overlapping peaks: (*A*) oligosaccharides, (*B*) disaccharides and (*C*) monosaccharides (Fig. 3 and Table III). Trisaccharides were conspicuously absent.

The oligosaccharide peak (*A*) was broad and contained several components, as revealed by paper electrophoresis on fiberglass sheets. These were not characterized further.

TABLE III

SEPARATION OF PRODUCTS OF 0.5M ACID HYDROLYSIS OF MUCORAN BY GEL FILTRATION (SEE FIG. 3)

Peak	*Percent initial dry wt.*	
	Uronic acid[b]	*Neutral sugar*[c]
A Oligosaccharides	28.7	8.2
B Disaccharides	8.9	5.0
C Monosaccharides	[a]	4.5

[a]Negligible. [b]Carbazole determination with glucuronolactone as standard; uncorrected values. [c]Anthrone determination with glucose as standard; uncorrected values. The high color coefficient of D-glucose makes the values underestimations of the actual content of the main neutral sugars (mannose and fucose).

The monosaccharides of peak *C* (Fig. 3) were converted into alditol acetates and separated by g.l.c. Xylose was added to the hydrolyzate as an internal standard. Fucose, 3.0% (initial dry wt), mannose, 2.5%, galactose, 0.95% and glucose, 0.35% were found. This minute proportion of glucose was overlooked in an earlier report[1].

Paper chromatography revealed one major component in the disaccharide peak having an $R_{glucuronic\ acid}$ value of 0.93 in 5:1:5:3 ethyl acetate–acetic acid–pyridine–water and 0.82 in 3:1:1 ethyl acetate–acetic acid–water; $[\alpha]_D^{20}$ +70° (*c* 1.0, water). On the basis of carbazole and anthrone assays (Table III, Fig. 3), the disaccharide peak appeared to be an aldobiouronic acid(s). This was confirmed by reduction to a neutral disaccharide, via the methyl glycoside methyl ester. After hydrolysis, g.l.c. determination yielded glucose, mannose, and fucose in the relative proportions 1.0:0.93:0.07. Accordingly, 93% of the aldobiouronic acid material was disaccharide of D-glucuronic acid and D-mannose, the rest being a disaccharide of D-glucuronic acid and L-fucose. Methylation analysis of the methyl ester methyl glycosides from the aldobiouronic acid peak gave a major component corresponding to the 2,3,4,6-tetramethyl ethers of D-glucose and D-mannose, and a smaller proportion of 2,4,6-tri-*O*-methyl-D-mannose. Theoretically, equimolar amounts of tetra- and tri-*O*-methyl hexoses should have formed from the presumed aldobiouronic acid. The unduly large proportion of tetra-*O*-methyl hexose probably reflects premature hydrolysis of the aldobiouronic acid during preparation of the methyl glycoside methyl ester. The foregoing data indicate that the major aldobiouronic acid in the acid hydrolyzate of

mucoran is α-D-glucopyranosyluronic acid-(1→3)-D-mannose. The same disaccharide was earlier isolated from a bacterial polysaccharide[22].

The high recovery of aldobiouronic acid (nearly 20% of the initial dry wt.) proves that a major feature of mucoran is an alternating sequence of D-glucuronic and D-mannose residues.

CONCLUSION

Assuming that the sample of mucoran studied was a single polysaccharide species, we propose the following interpretation for our findings. On exposure to the strong base used in the Hakomori methylation, mucoran precipitates and undergoes extensive β-elimination, with the release of unbranched glucuronomannan chains of different lengths. These chains are soluble and become fully methylated. Upon dialysis, they separate into two fractions having average $\overline{\text{d.p.}}$ values of ~10 and 20. Since mucoran has $\overline{\text{d.p.}}$ ~600, both glucuronomannan fractions are clearly the products of extensive depolymerization. Their structure **1** is an alternating sequence of D-mannose and D-glucuronic acid residues terminated by a D-mannose residue.

D-Man-(1→[4)-α-D-GlcA-(1→3)-D-Man-(1-]$_n$→ ? **1**

These chains represent 60–70% of the dry weight of mucoran.

The rest of the mucoran sample probably remained undissolved and undermethylated. This fraction should contain mostly L-fucose and D-glucuronic acid residues.

The structure and significance of minor sugars found in mucoran (galactose, glucose) is uncertain.

Our experience with mucoran demonstrates that polysaccharides containing uronic acid residues substituted at C-4 may be extensively degraded during methylation by the Hakomori procedure. In spite of this, considerable information can be acquired on the structure of the polysaccharide, but one must bear in mind that the methylated material recovered after routine dialysis and chloroform extraction may not be a representative part of the initial polysaccharide.

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