Preparation of a Mannopentaose, Mannohexaose, and Mannoheptaose from *Saccharomyces cerevisiae* Mannan*

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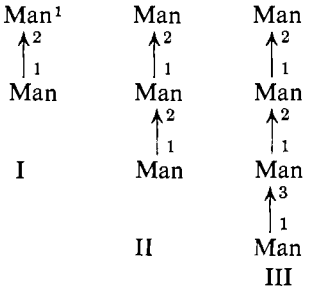
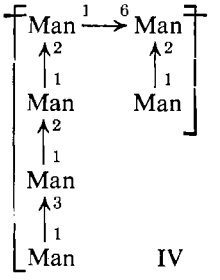
ABSTRACT: By a controlled acetolysis of yeast mannan (*Saccharomyces cerevisiae*) penta-, hexa-, and heptasaccharides have been obtained. Further acetolysis of these oligosaccharides yields di-, tri-, and tetrasaccharides with 1→2 and 1→3 linkages, suggesting that these units are connected by 1→6 linkages to give the larger fragments. This suggestion has been confirmed by standard methylation studies. It is concluded that the pentasaccharide is composed of two isomers with structures Va and b of the text, the relative amounts of which have been determined by studies on the reduced oligosaccharide. Similar structures have been assigned to the hexa- and heptasaccharides. The isolation of these oligosaccharides provides the first direct proof that the small 1→2-linked oligosaccharides are connected together by 1→6 linkages, and the significance of these acetolysis products with respect to the over-all structure of the mannan is discussed.

A polysaccharide, composed entirely of D-mannose, can be extracted from the cell wall of bakers' yeast, *Saccharomyces cerevisiae*. Kessler and Nickerson (1959) found that this mannan is closely associated with cell wall protein, and a recent report by Neumann and Lampen (1967) has indicated that a fraction of yeast invertase is covalently linked to the polysaccharide. It appears that this polysaccharide could be a structural unit which anchors proteins in the cell wall.

Several investigations on the structure of yeast mannan have been reported. Methylation studies show that the molecule is highly branched, with one end group and one branch point for every three mannose units; and that the polysaccharide contains 1→2, 1→3, and 1→6 linkages. From such studies, Haworth *et al.* (1941) proposed several structures for the mannan, each having a backbone of 1→2-linked D-mannose units with side chains linked through the 6 position of residues in the backbone.

In studies on the structure of *Saccharomyces rouxii* mannan, Gorin and Perlin (1956) showed that acetolysis gave good yields of the 1→2-linked di- and trisaccharides, from which they concluded that the mannan could have a backbone structure either of 1→6 linkages or of alternating 1→2 and 1→6 linkages. Later, Peat *et al.* (1961a,b) isolated in minute yields a series of 1→6-linked oligosaccharides that were produced by acid hydrolysis of baker's yeast mannan, and they suggested a structure for this polysaccharide of 1→6-linked D-mannose units, with 1→2- and 1→3-linked oligosaccharides attached to the 2 positions of the backbone. The acetolysis of *S. cerevisiae* mannan has also been investigated by Lee and Ballou (1965), using the general techniques of Gorin and Perlin (1956) but improving the ease of isolation of the products by the use of gel filtration. In addition to the di- and trisaccharides with 1→2 linkages (I and II, henceforth called M_2 and M_3), Lee and Ballou isolated a tetrasaccharide (M_4) which contained two 1→2 linkages and a 1→3 linkage at the non-





reducing end of the molecule (III). Structure IV was proposed to represent the repeating unit of mannan, on the assumption that II was an artifact produced by the degradation of III.

We have continued the study of the structure of yeast mannan by isolating acetolysis fragments which contain intact 1→6 linkages. It was hoped that these oligosac-

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: man, mannose.

charides could provide evidence as to how the relatively stable 1→2- and 1→3-linked units are connected in the mannan molecule. Penta, hexa-, and heptasaccharides were obtained and we report here their characterization. These fragments enable a more detailed structure to be proposed for yeast mannan, but they also suggest that it is more complex than had been proposed previously. A preliminary account of this work has appeared (Stewart *et al.*, 1966). A brief survey of mannans from several sources by the acetolysis technique has shown that the structure of this polysaccharide varies considerably from one strain of yeast to another, a fact which may help to explain the immunochemical specificity of different mannan preparations (Summers *et al.*, 1964).

Experimental Procedure

Carbohydrate was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956). This assay was streamlined by using Repipettes (Labindustries, Berkeley, Calif.) to deliver both the phenol solution and concentrated sulfuric acid. For monitoring carbohydrate from Sephadex columns, aliquots of fractions were taken using a Dilumat (Van Waters and Rogers, San Francisco, Calif.), the sample simultaneously being washed from the instrument and diluted with the phenol solution. Phosphorus was determined by the method of Bartlett (1959) and total nitrogen according to Long and Staples (1961).

Descending paper chromatography was carried out on Whatman No. 1 filter paper for analytical work and No. 3MM paper for preparative chromatography. The No. 3MM paper was prepared by eluting the sheet with water for 1 day, drying, then eluting another day with the solvent system to be used. The following solvent systems were used (in volume ratios): (A) ethyl acetate–pyridine–water (5:3:2), (B) ethyl acetate–pyridine–water (10:4:3), (C) 1-butanol–ethanol–water (3:1:1), and (D) ethyl acetate–acetic acid–formic acid–water (18:3:1:4).

Thin-layer chromatography was carried out using silica gel G or H (Merck, Darmstadt, Germany). For analytical work, the thickness of the layer was 0.25 mm, and 0.5 mm thickness was used for preparative thin layers. Silica gel G was preferred because it contained fewer contaminants than silica gel H. For chromatography of free oligosaccharides, 1-butanol–ethanol–water (2:1:1) was used (solvent E). The following solvent systems were used for chromatography of acetylated oligosaccharides and partially methylated mannose derivatives on thin-layer plates; (F) benzene containing 5–7% methanol, and (G) chloroform containing 5–7% methanol.

Sugars were detected on paper chromatograms either with the silver nitrate–sodium hydroxide or the periodate–benzidine reagents (Gordon *et al.*, 1956), and on thin-layer chromatograms by charring with 50% sulfuric acid or by the hydroxylamine–ferric chloride spray described by Tate and Bishop (1962) for acetylated derivatives.

Formaldehyde, from the periodate oxidation of glycols, was determined with chromotropic acid (Hanahan and Olley, 1958). However, oxidation of cellibiitol under strongly acidic conditions did not release the expected 2 moles of formaldehyde/mole of sugar. When the periodate oxidation reaction mixture was buffered with 0.2 M sodium acetate, the calculated yield of formaldehyde was obtained in 10 min.

Reductions were carried out in 0.2% sodium borohydride at room temperature for 16 hr. The reduced product was isolated by acetylation and extraction into chloroform. Deacetylation was followed by gel filtration on Sephadex G-25 for final purification.

Methylation of oligosaccharides was carried out as follows. The sample (5–10 mg) was dissolved in 0.5 ml of anhydrous dimethylformamide, and 0.2 ml of methyl iodide was added. If the solution became turbid, dimethylformamide was added dropwise until the sample had redissolved. Powdered silver oxide (0.2 g) was added and the mixture was shaken vigorously for 4 days. Further additions of 0.1 g of silver oxide and 0.1 ml of methyl iodide were made each day. Benzene (10 ml) was added and the precipitated salt was removed by centrifugation. The benzene extract was washed several times with water, dried over sodium sulfate, and evaporated. The residue was taken up in 1.0 ml of 2 N methanolic hydrogen chloride. Methanolysis was done in a sealed ampule for 8 hr at 100°. The methanolic hydrogen chloride was removed by placing the sample in a desiccator which contained potassium hydroxide pellets and anhydrous calcium chloride. Although this technique required 2 days for removal of the methanol, there was no loss of methyl tetra-*O*-methylmannoside, which did occur if the desiccator was evacuated. The methanolysis products were stored at 5° to avoid loss by evaporation.

Trimethylsilyl derivatives of partially methylated methylmannosides were prepared by a modification of the method of Sweeley *et al.* (1963). For 10–100 μg of sample, 4 drops of pyridine, 8 drops of hexamethyldisilazane, and 3 drops of trimethylchlorosilane were used, and the mixture was left at room temperature for 60 min with periodic shaking.

Gas chromatography was carried out on an Aerograph Hi-Fi Model 600-B (Wilken Instrument Co.,) fitted with a flame ionization detector. Peak area was determined with a digital integrator Model CRS11 HSB (Infotronics) connected directly to the electrometer of the gas chromatograph. The 5 ft. × 1/8 in. stainless-steel column was packed with 10% Carbowax 20 M on Aeropak 30 (Wilken Instrument Co.), unless otherwise stated, and flow rates of 20–30 cc/min of N_2 were used. The identity of a peak on the gas chromatogram was confirmed by injecting internal standards.

Acetylation of oligosaccharides in acetic anhydride–pyridine (1:1) was carried out on a steam bath for 1–3 hr. The reagents were then evaporated and chloroform was added to the residue. The chloroform solution, after centrifugation to remove solids, was washed several times with water and the dried extract was evaporated on a rotary evaporator. Acetylation of mannan (1.0 g) was performed in 25 ml of a mixture of acetic anhydride–pyridine–dimethylformamide (1:1:1). The finely powdered suspension was mixed with a magnetic

stirrer and heated to 75° in an oil bath. After 2 days, the solution was poured into ice water and the precipitated acetate was removed by filtration.

The acetolysis medium was a mixture of acetic anhydride, acetic acid, and sulfuric acid (10:10:1, v/v). For acetolysis of free sugars, the finely powdered suspension (0.1 g) was stirred in 10 ml of the acetolysis medium at 40° (unless otherwise stated). The reaction was stopped by addition of pyridine to the cooled solution, and the reagents were evaporated. The products were extracted with chloroform and the precipitated salts were removed by centrifugation. The chloroform solution was washed with water and evaporated to dryness.

Acetylated sugars (5–100 mg), dissolved in 1 ml of the acetolysis medium, were heated at 40°. The reaction was followed by thin-layer chromatography by removing a sample, neutralizing with pyridine, and spotting a chloroform solution onto the plate. Quick assay could be made using thin layers prepared by dipping two microscope cover slides, back to back, into a well-stirred slurry of silica gel G, 30 g in 100 ml of chloroform.

Deacylation was carried out by dissolving the sample in dry methanol and adding a catalytic amount of barium methoxide in methanol. The reaction was continued at room temperature for 15 min, when solid carbon dioxide was added to neutralize the base. After evaporation of methanol, the products were dissolved in water and insoluble barium carbonate was removed by centrifugation.

Sephadex G-25 (Pharmacia) in the nonbead (irregular) form was used in all gel filtrations. Its properties seemed comparable with the bead form of G-15. A 2 × 150 cm column was used for less than 100 mg of material, and 3 × 150 cm for quantities up to 1.0 g. A jacketed, 2 × 150 cm column was tried at temperatures between 30 and 60°. No improvement in resolution was achieved, although greatly increased flow rates resulted.

Results and Discussion

Isolation of Mannan. Mannan was isolated from bakers' yeast (Red Star Co., Oakland, Calif.) by two procedures; one similar to that outlined by Peat *et al.* (1961a), and the other as described by Cifonelli and Smith (1955). The crumbled yeast cake (4.54 kg) was suspended in citrate buffer (0.19 mM, pH 7.0) and autoclaved at 120° for 2 hr. After centrifugation the supernatant was poured into absolute ethanol and the cell residue was further extracted with water using the same procedure. Mannan was isolated from the combined ethanol precipitate by utilizing three precipitations with Benedict's solution. The yield of mannan ($[\alpha]_D^{23°}$ +72.7°) was 35 g, which contained 0.16% organic phosphorus and 0.13% total nitrogen. The cell residue from the above extractions was autoclaved at 120° for 30 min in 6% sodium hydroxide. After centrifugation, the supernatant was poured into ethanol and the mannan in the precipitate was purified by formation of the copper complex (Cifonelli and Smith, 1955). This second procedure yielded 15 g of mannan, $[\alpha]_D^{23°}$ +79.5°, containing 0.13% organic phosphorus and 0.17% total nitrogen.

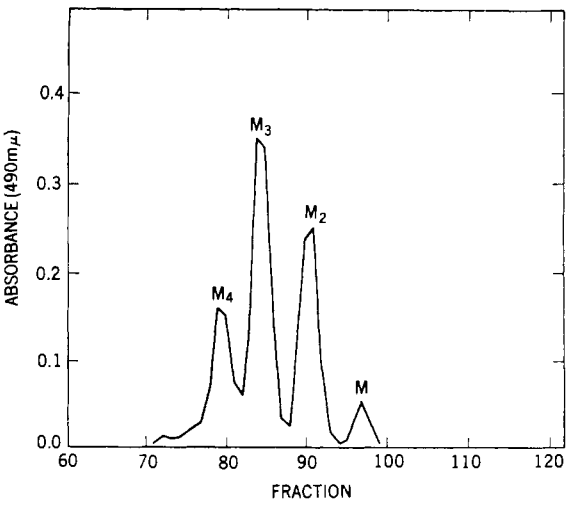


FIGURE 1: Oligosaccharides from the acetolysis of mannan for 13 hr at 40°. Separation on Sephadex G-25. Molar ratios: M = 0.47, M_2 = 1.05, M_3 = 1.00, and M_4 = 0.43.

Mannose was the only monosaccharide detected on paper chromatography (solvent D) of an acid hydrolysate. The acetolysis products from these two mannan preparations were identical, indicating that there was no difference in structure between them.

Isolation of Oligosaccharides.

Prolonged Acetolysis of Mannan. Acetolysis of powdered mannan, for 13 hr at 40°, yielded a mixture of the same small oligosaccharides previously reported (Lee and Ballou, 1965); no 1→6 linkage remained intact. The deacetylated products were separated by gel filtration on Sephadex G-25 (Figure 1). By comparison with acetolysis for 5 days at room temperature, conditions previously used (Lee and Ballou, 1965), the amount of mannose was reduced significantly to less than 1 mole/mole of M_3. Moreover, in short-term acetolysis of mannan acetate, M_3 was produced at a rate that equaled or exceeded the rate of formation of M_4 (Figure 2). These results indicate that M_3 is an integral unit of the mannan rather than a degradation product of M_4.

Further evidence for this view was obtained by studying the acetolysis of M_4. Acetolysis for 70 hr at 48–49°, conditions far more severe than those required for release of M_3 from mannan, degraded less than one-half of the M_4. The ratio of mannose to M_2 to M_3, produced by acetolysis of M_4, suggests that there is no significant difference in the rate of cleavage of 1→2- and 1→3-linked mannosides (see Figure 3). The ratio of M_2, M_3, and M_4 (5:5:2), obtained after acetolysis of mannan for 13 hr at 40°, must be quite close to that present in the polysaccharide itself.

The origin of the free mannose formed during acetolysis is uncertain. It could come from cleavage of 1→6 linkages or from a small amount of random degradation of 1→2 and 1→3 linkages. In the experiment shown in Figure 1, the ratio of free mannose to the total is 1:15; while in other acetolyses, the ratio was as low as 1:45. The yield of 2,3,4-trimethylmannose from methylated mannan (see later results) approximates 1 in 45 of the mannose units. No more than this number of

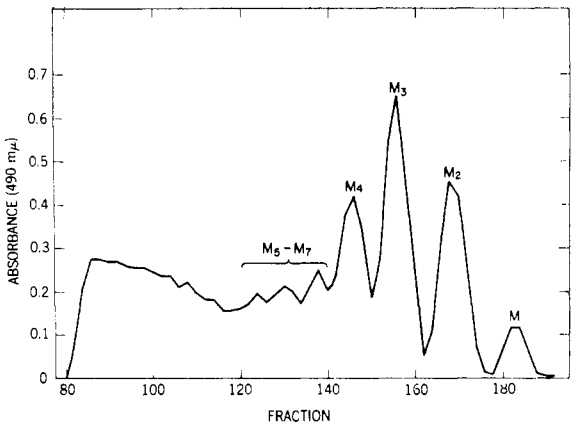


FIGURE 2: Oligosaccharides from the acetolysis of yeast mannan acetate for 1.25 hr at 40°. Separation on Sephadex G-25.

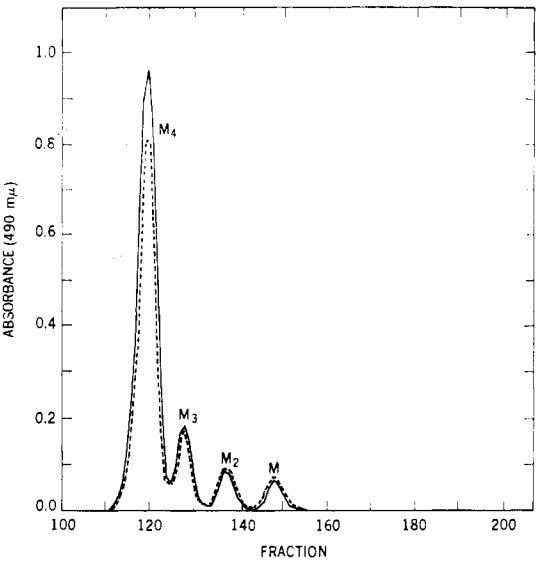


FIGURE 3: Products from the acetolysis of mannotetraoses obtained by a 2-hr (solid line) and a 13-hr (dashed line) acetolysis of mannan. For M_4 from 13-hr acetolysis, the molar ratios were: M = 1.04, M_2 = 0.96, M_3 = 1.0, and M_4 = 4.3. For M_4 from 2-hr acetolysis, the molar ratios were: M = 0.92, M_2 = 0.72, M_3 = 1.0, and M_4 = 5.7.

sugar units of the mannan molecule could yield mannose directly by acetolysis of only 1→6 linkages. Therefore, we assume that much of the mannose found in the acetolysis products of mannan, or in the products from the larger acetolysis fragments (penta- to heptasaccharide), originated from random degradation of 1→2 and 1→3 linkages.

Isomeric M_3 from Prolonged Acetolysis of Mannan. The major trisaccharide from mannan acetolysis contained two 1→2 linkages. However, gas chromatography of the methylation products obtained from the trisaccharide revealed approximately 10% methyl 2,4,6-tri-*O*-methylmannoside, indicating the presence of some 1→3-linked oligosaccharide. Thin-layer chromatography of an acetylated trisaccharide fraction revealed a spot with R_F slightly greater than normal M_3, which was isolated by preparative thin-layer chromatography (solvent F). The deacetylated product was purified by gel filtration. Methylation analysis of this isomeric M_3 (referred to as M_3') gave methyl 2,3,4,6-tetra-*O*-methylmannoside, methyl 2,4,6-, and methyl 3,4,6-tri-*O*-methylmannosides in the ratio 1.0:0.84:1.34. (The slightly high proportion of methyl 3,4,6-tri-*O*-methylmannoside reflects contamination by normal M_3.) Reduction of M_3', followed by methylation, greatly diminished the peak corresponding to methyl 3,4,6-tri-*O*-methylmannoside (a small peak remained owing to contamination by normal M_3), and a new peak appeared corresponding to the partially methylated mannitol derivative. Thus, M_3' was shown to be $M \xrightarrow{1} \xrightarrow{3} M \xrightarrow{1} \xrightarrow{2} M$. It constitutes 20% of the trisaccharide fraction. Since we have shown that the acetolysis of M_4 proceeds at a very slow rate, only a small part of the M_3' could come from this source. Consistent with this conclusion, the acetolysis of mannan acetate yields M_3' at the same rate as M_4.

The trimethylsilyl derivative of M_3' could be separated from that of M_3 by gas chromatography (2% QF-1 on Aeropak 30, column temperature 250°), and its appearance in the products of acetolysis of reduced oligosaccharides was followed by this technique. Confirmation of the structure of M_3' was obtained by studying the acetolysis of M_4. Both M_3 and M_3' are possible products of M_4 acetolysis. Gas chromatography of the trimethylsilyl derivative of the trisaccharide fraction from M_4 acetolysis showed two peaks, one with the retention time of trimethylsilyl-M_3 and the other with the retention time of trimethylsilyl-M_3'.

Short-Term Acetolysis of Mannan. These experiments were aimed at isolation of oligosaccharides with intact 1→6 linkages. Initial studies were performed with powdered mannan. After a 2-hr acetolysis at 40°, the unreacted material was removed by centrifugation and the supernatant was neutralized by addition of pyridine. This procedure was repeated with the undissolved residue until a major portion of the mannan was solubilized. Later studies were done on acetylated mannan, since this was soluble in the acetolysis medium and the acetylated products could be examined immediately by thin-layer chromatography. The reaction was usually terminated after 1–1.5 hr at 40° by neutralization with pyridine. The mixture was poured onto ice–water and the products were recovered by chloroform extraction. The deacetylated products were passed through a Sephadex G-25 column (3 × 150 cm) in 1.0-g batches; and fractions 120–140 (Figure 2), corresponding to the higher oligosaccharides, were combined.

Preparative chromatography on prewashed Whatman No. 3MM paper was then carried out. Approximately 150 mg of material was applied onto a 46 × 57 cm paper sheet which was eluted for 5–7 days with solvent A. The bands recovered from the paper chromatograms were rerun on Sephadex G-25 (2 × 150 cm, 40 mg each filtration) for further purification. These steps were sufficient to purify the penta- and hexasaccharides, but further preparative paper chromatography followed by gel filtration was necessary to obtain a homogeneous heptasaccharide. From 8.0 g of mannan, 6.6 g of deacetylated acetolysis products was obtained which yielded

TABLE I: Properties of Oligosaccharides and Reduced Oligosaccharides.

Oligosaccharides	M_2	RM_2	M_3	RM_3	M_4	RM_4	M_5	RM_5	M_6	RM_6	M_7
R_{Man} (solvent A)	0.64	0.48	0.38	0.27	0.22	0.185	0.11	0.088	0.066	0.060	0.040
R_{Man} (solvent F[a])	0.68	0.77	0.45	0.55[b]	0.36	0.45	0.20	0.24	0.165	0.22	0.145
$[\alpha]_D^{22}$ (water)°			+60.9[c]		+74.2[c]		+73.5		+76.3		+81.3
Elution volume[d] (ml)	350		325		305		290		275		262

[a] Thin-layer chromatography of peracetylated samples. [b] M_3' acetate gives the same value. [c] Lee and Ballou (1965) report M_3, $[\alpha]_D$ +59.3°; M_4, $[\alpha]_D$ +76.2°. [d] Elution volume from 2 × 150 cm column of Sephadex G-25 (irregular).

0.94 g of the penta- to heptasaccharide mixture. After th several purification steps, 112 mg of pentasaccharide (henceforth referred to as M_5), 78 mg of hexasaccharide (M_6), and 20 mg of heptasaccharide (M_7) were isolated. Paper chromatography (solvent D) of the acid hydrolysates of these oligosaccharides showed mannose to be the only monosaccharide constituent. Paper chromatography of the free sugars (solvent A) and thin-layer chromatography of the acetates (solvent F) indicated that the isolated fragments were homogeneous (Table I). The gel filtration elution pattern of a synthetic mixture of oligosaccharides (Figure 4) demonstrates the relationship between these fragments.

High molecular weight material from a short-term mannan acetolysis (fractions 80–119, Figure 2) was reisolated and used for the preparation of M_5, M_6, and M_7 by further acetolysis. The products were identical with those isolated from mannan, and the yields were the same.

Molecular Weights of Oligosaccharides. Molecular weights were determined by sedimentation equilibrium. The partial specific volume of 0.618 for sucrose was used, and gave correct molecular weights for the well-characterized M_2, M_3, and M_4. The results are given in Table II and they confirm that the products were penta-, hexa-, and heptasaccharides. In plots of log C against X^2 for each measurement, only M_7 deviated slightly from a straight line.

TABLE II: Molecular Weights of Oligosaccharides.[a]

Oligosaccharide	Calcd (g)	Measured (g)	Deviation (%)
M_2	342	345	+0.9
M_3	504	503	−0.2
M_4	666	682	+2.4
M_5	828	844	+1.9
M_6	990	964	−3.6
M_7	1142	1100	−3.8

[a] By sedimentation equilibrium, assuming a $\bar{V}$ of 0.618.

Structure of M_4 from Short-Term Acetolysis of Mannan. If present in mannan, an isomeric M_4 composed of two M_2 units connected by a 1→6 linkage should appear in the M_4 fraction obtained after short-term acetolysis. Further acetolysis of this M_4 should cleave the 1→6 linkage and produce characteristic degradation products. Figure 3 shows the gel filtration elution pattern of the products of acetolysis (5 hr at 40°) of M_4 isolated after long- and short-term acetolysis of mannan. Since M_4, from short-term acetolysis of mannan, does not yield the degradation products to be expected of an isomer containing a 1→6 linkage, we conclude that there are no adjacent M_2 units connected by 1→6 linkages in the mannan.

A comparison of Figure 3 with Figure 5 or 6 shows the dramatic difference between rates of acetolysis of 1→2 or 1→3 linkages and that of 1→6 linkages. There

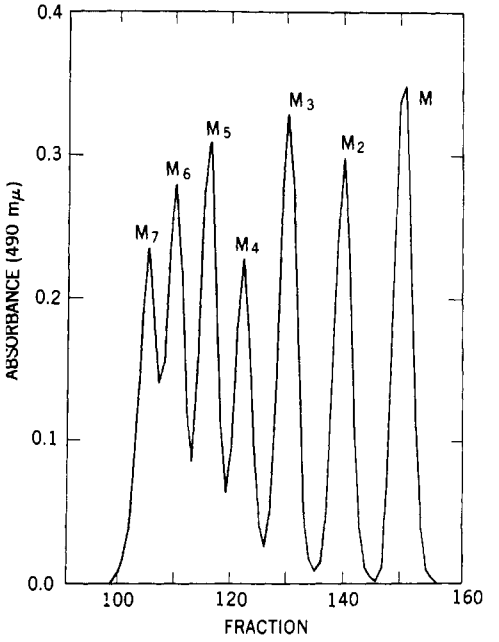


FIGURE 4: Gel filtration pattern of a synthetic mixture of mannooligosaccharides isolated by acetolysis of mannan.

TABLE III: Methylation Studies.

Mannose Derivatives				Ratio of Products after Methylation and Methanolysis[d]				
				M_5		M_6		Mannan
CH_3O-	$(CH_3)_3$-SiO-	Rel Retention Time[a]	Thin-Layer Chromatography[b]	Calcd	Obsd	Calcd	Obsd	Obsd
1,2,3,4,6-		1.00 (1.29)	1.00	2.00	2.00	1.00	1.00	1.00
1,3,4,6-		2.03	0.34 (0.77)[c]	2.00	1.77[e]	1.15	1.17[e]	0.66[e]
1,3,4,6-	2-	0.48 (0.54)			2.08		1.20	0.60
1,2,3,4-		2.03	0.25		*d*		*d*	*d*
1,2,3,4-	6-	0.66			0.04		0.045	0.06
1,2,4,6-		2.35	0.46		0.20	0.35	0.43	0.24
1,2,4,6-	3-	0.73			0.21		0.44	0.23
1,3,4-		4.85	0.068 (0.64)[c]	1.00		0.50		
1,3,4-	2,6-	0.35 (0.43)			1.06		0.54	0.89

[a] Relative to methyl tetramethyl-α-D-mannoside from 10% Carbowax 20 M on Aeropak 30 (Wilkins Instrument Co.) at a column temperature of 195° for methylated mannoses, and 175° for trimethylsilyl derivatives of methylated mannoses. Figures in brackets refer to β anomers, other values refer to α anomers. [b] R_F values relative to methyl tetramethyl-α-D-mannoside using solvent F. [c] R_F values with solvent G. [d] Detector response relative to methyl tetramethyl-α-D-mannoside was determined with standard methylated samples and used in the quantitations quoted here. [e] Methyl 3,4,6-tri-*O*-methylmannoside and methyl 2,3,4-tri-*O*-methylmannoside have identical retention times. Hence the values quoted here are the combined totals of these two isomers. Trimethylsilylation enables separation of these compounds.

is little difference between the rates of cleavage of 1→2 and 1→3 linkages.

Characterization of Oligosaccharides M_5, M_6, and M_7

Acetolysis. Since these large fragments are only present after short-term acetolysis of mannan, it can be assumed that they contain one or more 1→6 linkages. This was confirmed by controlled acetolysis of the oligosaccharides.

A. M_5. The gel filtration elution pattern of the deacet-

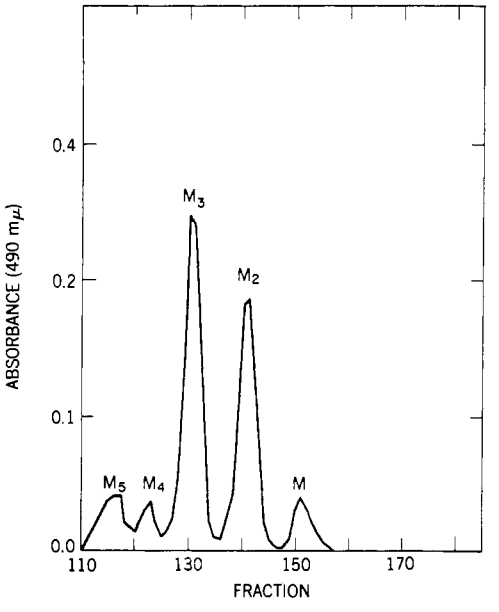


FIGURE 5: Oligosaccharides from the acetolysis of mannopentaose. Molar ratios: M = 0.45, M_2 = 1.12, M_3 = 1.00, and M_4 + M_5 = 0.25.

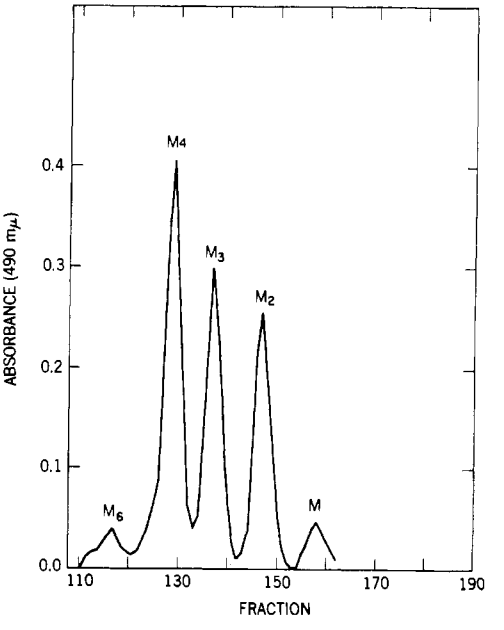


FIGURE 6: Oligosaccharides from the acetolysis of mannohexaose. Separation on Sephadex G-25 for 5 hr at 40°. Molar ratios: M = 0.44, M_2 = 1.17, M_3 = 0.875, M_4 = 1.00, and M_6 = 0.09.

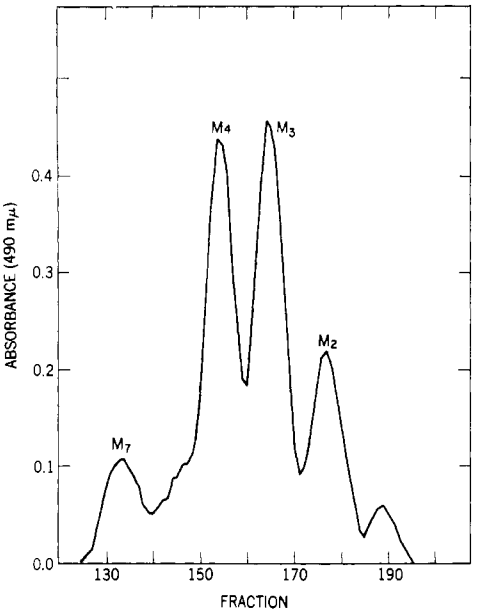


FIGURE 7: Oligosaccharides from the acetolysis of manno-heptaose. Molar ratios: mannose = 0.48, M_2 = 1.05, M_3 = 1.48, M_4 = 1.00, and M_7 = 0.17.

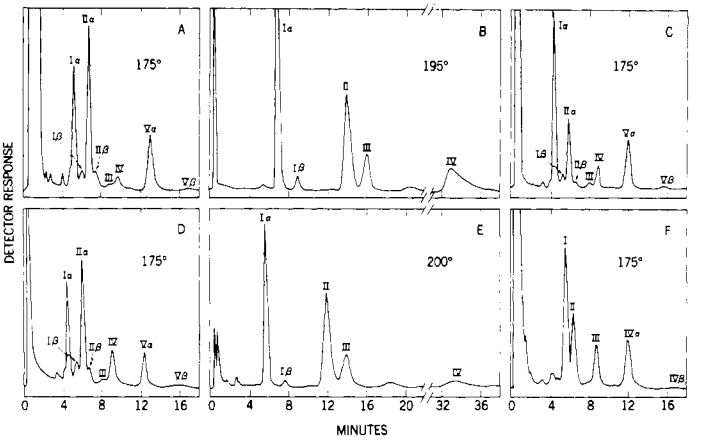


FIGURE 8: Gas chromatographic tracing of the separation of partially methylated methylmannosides. (A) Trimethylsilyl derivatives of the methanolysate of methylated M_5: I, methyl 3,4-di-*O*-methyl-2,6-di-*O*-trimethylsilylmannoside; II, methyl 3,4,6-tri-*O*-methyl-2-*O*-trimethylsilylmannoside: III, methyl 2,3,4-tri-*O*-methyl-6-*O*-trimethylsilylmannoside; IV, methyl 2,4,6-tri-*O*-methyl-3-*O*-trimethylsilylmannoside; and V, methyl 2,3,4,6-tetra-*O*-methylmannoside. (B) Methanolysate of methylated mannan: I, methyl 2,3,4,6-tetra-*O*-methylmannoside; II, methyl 3,4,6-tri-*O*-methylmannoside; III, methyl 2,4,6-tri-*O*-methylmannoside; and IV, methyl 3,4-di-*O*-methylmannoside. (C) Trimethylsilyl derivatives of (B): I, methyl 3,4-di-*O*-methyl-2,6-di-*O*-trimethylsilylmannoside; II, methyl 3,4,6-tri-*O*-methyl-2-*O*-trimethylsilylmannoside; III, methyl 2,3,4-tri-*O*-methyl-6-*O*-trimethylsilylmannoside; IV, methyl 2,4,6-tri-*O*-methyl-3-*O*-trimethylmannoside; and V, methyl 2,3,4,6-tetra-*O*-methylmannoside. (D) Trimethylsilyl derivatives of methylated M_6: I, methyl 3,4-di-*O*-methyl 2,6-di-*O*-trimethylsilylmannoside; II, methyl 3,4,6-tri-*O*-methyl-2-*O*-trimethylsilylmannoside; III, methyl 2,3,4-tri-*O*-methyl-6-*O*-trimethylsilylmannoside; IV, methyl 2,4,6-tri-*O*-methyl-3-*O*-trimethylsilylmannoside; and V, methyl 2,3,4,6-tetra-*O*-methylmannoside. (E) Methanolysate of methylated RM_6: I, methyl 2,3,4,6-tetra-*O*-methylmannoside; II, methyl 3,4,6-tri-*O*-methylmannoside; III, methyl 2,4,6-tri-*O*-methylmannoside; and IV, 1,3,4,6-tetra-*O*-methylmannitol. (F) Trimethylsilyl derivatives of (E): I, methyl 3,4,6-tri-*O*-methyl-2-*O*-trimethylsilylmannoside; II, 1,3,4,6-tetra-*O*-methyl-2,5-di-*O*-trimethylsilylmannitol; III, methyl 2,4,6-tri-*O*-methyl-3-*O*-trimethylsilylmannoside; and IV, methyl 2,3,4,6-tetra-*O*-methylmannoside.

ylated products of acetolysis of M_5 is shown in Figure 5. Acetolysis for 5 hr at 40° cleaved M_5 almost quantitatively into equimolar amounts of M_2 and M_3, indicating that this pentaose is composed of an M_2 and M_3 unit connected by a 1→6 linkage. Methylation analysis of the products of acetolysis of M_5 confirmed that they were M_2 and M_3 containing a small amount of M_3'.

B. M_6. The gel filtration pattern of the products from the acetolysis of M_6 is shown in Figure 6. Equimolar amounts of M_2 and M_4 plus some M_3 (0.75 mole/mole of M_2) were obtained. The structures of these products were identical with those isolated from prolonged acetolysis of mannan. There was also some M_3' in the products of M_6 acetolysis. We conclude that M_6 is a mixture of isomers, one composed of M_2 and M_4 units and the other of two M_3 units, each with a 1→6 linkage connecting the two smaller oligosaccharides. From Figure 6, it is calculated that there are 2.3 moles of the former type to 1.0 mole of the latter M_6 isomer in the mixture.

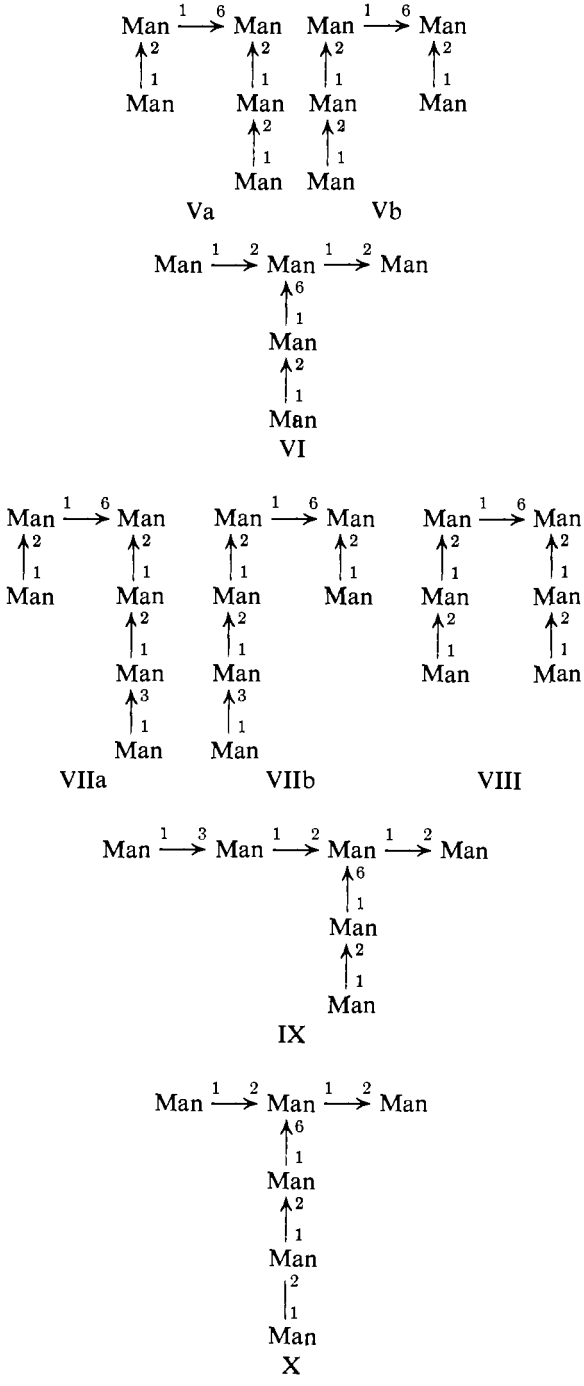
C. M_7. Acetolysis of this oligosaccharide for 5 hr at 40° gave a mixture of M_2–M_4 (Figure 7). Therefore, M_7 is a mixture of isomers, one composed of M_3 and M_4 units connected by a 1→6 linkage, and the other of two M_2 units and an M_3 unit connected by two 1→6 linkages. From the molar ratio of products, we calculate that M_7 contains 2 moles of the former isomer to 1 mole of the latter. The products of M_7 acetolysis were identical with the oligosaccharides from mannan acetolysis.

Methylation of Oligosaccharides. Figure 8A,D shows the gas chromatographic patterns of the trimethylsilyl derivatives of the methanolyzed products from methylated M_5 and M_6, respectively. The presence of methyl 3,4-di-*O*-methylmannoside confirms that these oligosaccharides are branched. Quantitative results are given in Table III. Consistent data for the amount of methyl 3,4-di-*O*-methylmannoside in the mixtures were obtained only after trimethylsilylation. Only traces of methyl 2,3,4-tri-*O*-methylmannoside (Peak III, Figure 8A,D) were observed in M_5 and M_6, which is consistent with the results of mannan methylation (see later). The ratio of methyl tetra-*O*-methylmannoside to methyl 2,4,6-tri-*O*-methylmannoside in M_5 is at least 10:1, indicating that about 20% of the triose units is M_3'. For M_6, the excess of 2,4,6-*O*-methylmannose, over that expected from the M_4 units present in this oligosaccharide, suggests a similar ratio of M_3 to M_3'.

The calculated ratio of methylated products for an M_5 of structures V or VI agrees very well with the observed figures (Table III). However, from these methylation studies alone, it is not possible to distinguish between an M_5 with the reducing group associated with the branch point (V), or a structure in which the reduc-

ing group is at the end of an M_3 unit not associated with the branch point (VI).

A similar problem is faced in distinguishing between the various models (VII and VIII) for M_6. For a mixture of 2.3 parts M_2–M_4 (VII or IX) and 1 part M_3–M_3 (VIII or X), the calculated ratios of methylated products agrees with the observed results (Table III). The following studies on reduced oligosaccharides were designed to distinguish between these possibilities.



Reduced Oligosaccharides

Oligosaccharide samples were reduced with sodium borohydride for 16 hr at room temperature and the reduced products were purified through acetate formation. Reduced M_2 (henceforth referred to as RM_2), RM_3, and RM_4 were prepared as standards. On thin-layer chromatograms (solvent F), the acetylated samples all had R_F values slightly faster than the parent oligosaccharides. On paper chromatograms (solvent A), the reduced oligosaccharides had R_F values slower than the corresponding parent compound (see Table I). All gave single spots except RM_7 acetate, which appeared as two spots on thin-layer chromatography.

Periodate Oxidation. The reduced product of structures V, VII, and VIII should yield no formaldehyde on periodate oxidation; whereas the reduced product of structure VI, IX, and X should yield 1 mole of formaldehyde/mole of reduced oligosaccharide. Figure 9 shows the plot of formaldehyde released and reduced oligosaccharide oxidized. Very little formaldehyde was obtained from RM_5 and RM_6, a comparable amount being produced by M_5 and M_6 themselves.

Methylation. Methylation of reduced M_5 from V and reduced M_6 from VII and VIII would yield a tetra-*O*-methylmannitol together with methyl tetra- and methyl tri-*O*-methylmannosides. Reduced VI, IX, and X oligosaccharides would yield a penta-*O*-methylmannitol together with methyl tetra-, methyl tri-, and methyl di-*O*-methylmannosides.

The reference 1,2,3,4,6-penta-*O*-methylmannitol was prepared by methylation and methanolysis of RM_2. Gas chromatography of the products showed a peak retention time 1.79[2] which corresponded to the penta-*O*-methylmannitol. It had a retention time identical with the penta-*O*-methylmannitol derivative obtained on methylation of reduced M_3'. The trimethylsilyl derivative of 1,2,3,4,6-penta-*O*-methylmannitol had a retention time value of 0.53; 1,3,4,5- (or 2,3,4,6-) tetra-*O*-methylmannitol was prepared by sodium borohydride reduction of 2,3,4,6-tetra-*O*-methylmannose. Gas chromatography showed a peak (retention time 6.0). On trimethylsilylation, a peak appeared with a retention time of 0.52. Therefore, although the tetra- and penta-*O*-methylmannitols are easily separated by gas chromatography, their trimethylsilyl ethers have the same retention times.

Methylation and methanolysis of RM_6 gave the results in Figure 8E,F. There is no peak (retention time 1.79) corresponding to penta-*O*-methylmannitol. There is a peak at retention time 6.0 (peak IV, Figure 8E) corresponding to 2,3,4,6-tetra-*O*-methylmannitol, which indicates that RM_6 was derived from an M_6 having structures VII and VIII.

As confirmation, the peak corresponding to methyl 3,4-di-*O*-methylmannoside (peak Iα, Figure 8D) is eliminated by reduction of the oligosaccharide and replaced by a peak (retention time 0.52) corresponding to 2,3,4,6-tetra-*O*-methylmannoside (trimethylsilyl derivative) (peak II, Figure 8F).

Similar results were obtained for the analysis of products from RM_5 methylation. No peak was observed at

[2] Retention time relative to methyl tetra-*O*-methyl-α-D-mannopyranoside.

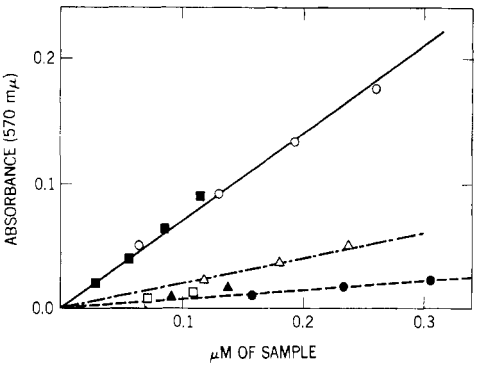
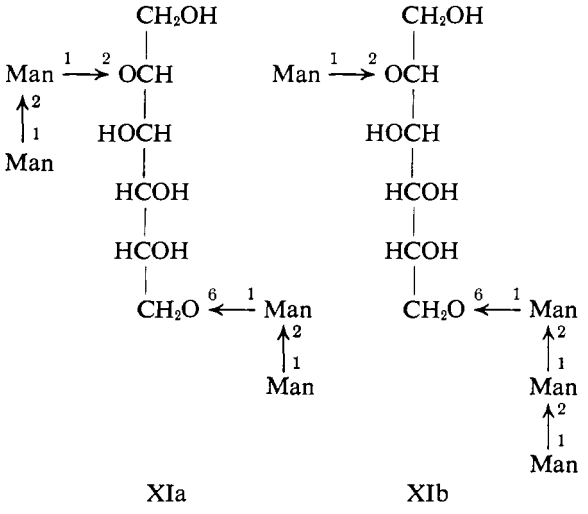


FIGURE 9: Periodate oxidation of oligosaccharides to yield formaldehyde as determined by the chromatropic acid assay. Mellibiitol, open circles; RM_3, closed squares; M_5, open triangles; M_6, open squares; RM_5, closed triangles; RM_6, closed circles.

retention time 1.79. On trimethylsilylation, a peak appeared at retention time 0.52, with no evidence of any peak (retention time 0.35) corresponding to the trimethylsilyl derivative of methyl 3,4-di-*O*-methylmannoside.

Acetolysis. From the products of acetolysis of reduced M_5, the ratio of the amounts of structures Va and b present in the mixture may be estimated. Assuming selective cleavage of the 1→6 linkage (the linkage to the



primary hydroxyl of mannitol), acetolysis of RM_5 with structure XIa would yield M_2 and RM_3, while RM_5 with structure XIb would yield RM_2 and M_3. The assumption that there is still selective cleavage of the 1→6 linkage, when the ring to which it is attached is opened to form a mannitol derivative, was confirmed. The acetolysis of RM_3, which has M_2 attached to a secondary hydroxyl of mannitol, proceeded at a rate comparable with that of M_4, which is much slower than cleavage of 1→6 linkages in mannan or the larger oligosaccharides.

A. RM_5 ACETOLYSIS. An acetolysis of RM_5 acetate for 5 hr at 40° gave the deacetylated products in Figure 10. Acetolysis was slower than that of M_5 (0.47 mole of RM_6 remained after 5-hr acetolysis), which supports

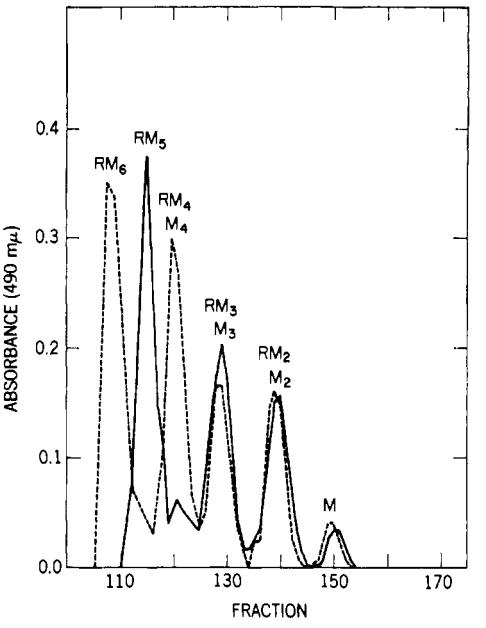


FIGURE 10: Products of acetolysis of reduced oligosaccharides separated on Sephadex G-25. RM_6 acetolysis, dashed line; RM_5 acetolysis, solid line.

the previous evidence that M_5 is of type V structure. In a reduced type VI structure, the 1→6 linkage would still be attached to a mannose unit and would be very labile. The disaccharide fraction was predominantly M_2 and the trisaccharide fraction mainly RM_3. Therefore, the predominant isomer of RM_5 must be XIa, and M_5 must be mainly Va with the reducing group associated with the M_3 unit.

B. RM_6 ACETOLYSIS. Similar results were obtained for RM_6 acetolysis (Figure 10). The reaction proceeded at a slower rate than for M_6 (0.44 mole of RM_6 remained after 5-hr acetolysis) supporting type VII and VIII structures. The products of acetolysis were mainly M_2 and RM_4. Therefore, the predominant isomer in M_6 is VIIa with the reducing group associated with the M_4 unit.

RM_6 from M_6 having two M_3 units (VIII) connected by a 1→6 linkage should yield an equal mixture of M_3 and RM_3. Chromatographic evidence did not support this but showed a more intense spot for RM_3 than M_3.

Quantitation of Reduced and Nonreduced Products of Acetolysis of Reduced Oligosaccharides. Three methods were tried. First, the ratio of mannose to mannitol was determined on acid hydrolysates, using the phenol–sulfuric acid assay for mannose and formaldehyde released on periodate oxidation for mannitol. This technique proved unsatisfactory because the mannose in each hydrolysate gave a high blank reading for formaldehyde. As a second method, formaldehyde released on periodate oxidation of the intact reduced oligosaccharides was related to the phenol–sulfuric acid color. It was assumed that 1 mole of reduced oligosaccharide released 1 mole of formaldehyde, that is, that all the reduced oligosaccharides were 1→2 linked. Once again the nonreduced oligosaccharides

interfered by their high blanks. Finally, the ratios were determined successfully by gas chromatography of the trimethylsilyl derivatives (2% QF-1 on Aeropak 30, column temperature 210° for disaccharides and 250° for trisaccharides), although the tetrasaccharide fraction could not be examined in this way. Results for the di- and trisaccharide fractions are given in Table IV.

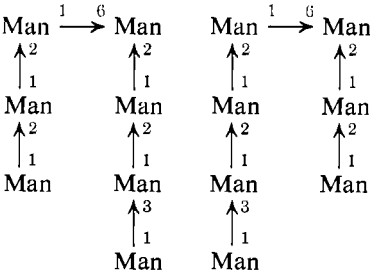
TABLE IV: Ratios of Acetolysis Products of Reduced Oligosaccharides.

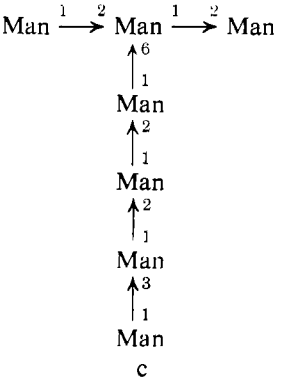
Products	Oligosaccharides		
	RM_5	RM_6	RM_7
RM_2	1.0	1.0	1.0
M_2	5.6	3.1	4.5
RM_3	5.0	1.0	1.4
M_3	1.0	1.3	0.3
M_3'	Trace	0.5	0.7

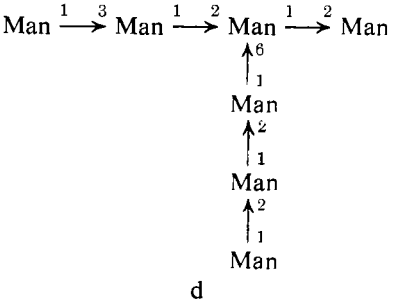
The results show that the major component of M_5 is Va. From RM_6 acetolysis, we conclude that the major component of M_6 is the isomer with the reducing group associated with M_4 (VIIa). For some reason, the ratio of M_3 to RM_3 from RM_6 acetolysis was almost 2:1. This did not result from differences in the rates of acetolysis of RM_3 and M_3, since they were degraded at equal rates in a control experiment.

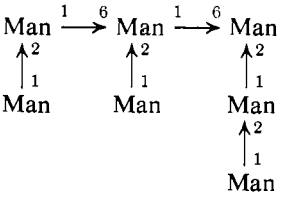
Very little M_3' appeared as a product of RM_5 acetolysis, an appreciable amount was found in RM_6 acetolysis products (approximately 30%); and, in RM_7 acetolysis, the major nonreduced trisaccharide was M_3' (approximately 70%). Thus, there is a tendency for this isomeric trisaccharide to be concentrated at the nonreducing part of the molecule, and to increase going from M_5 to M_7.

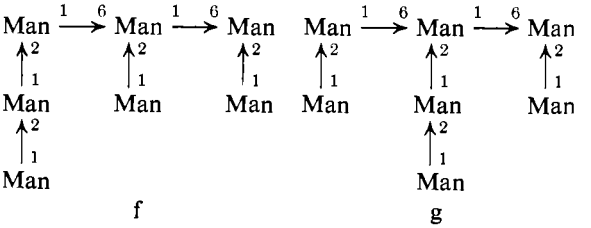
Structure of M_7. M_7 is a complex mixture of isomers, two-thirds composed of one M_3 and one M_4 unit connected by a 1→6 linkage, and one-third consisting of two M_2 units and one M_3 unit connected by two 1→6 linkages. There are several possible arrangements of this heptasaccharide, even excluding any isomer containing M_3'. Because of the limited amount available, it was not possible to carry out a complete structural determination of the isomers present. However, several possibilities were eliminated. The methanolysate of methylated RM_7 gave no peak on gas chromatography corresponding to penta-*O*-methylmannitol (retention time 1.79) thus eliminating XIIc and d as possibilities. As confirmation, gas chromatograms after trimethylsilylation of the methylated products showed the peak (retention time 0.35) corresponding to methyl 3,4-di-*O*-methylmannoside to be less than half the area of the peak (retention time 0.53) corresponding to the methylated mannitol derivative. If

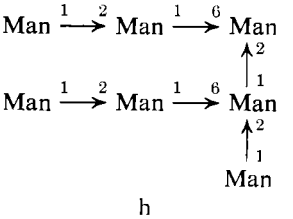












XII

XIIc or d was a major M_7 isomer, the ratio of these products would be 1:1.

The products of acetolysis of RM_7 were mainly M_2, RM_4, and approximately equal amounts of M_3-($+M_3'$) and RM_3. These ratios are given in Table IV. The high percentage of M_2 suggested that isomers e and/or h were present. A ratio of 1.75 e and/or h to 1.0 f and/or g would give the amount of M_2 formed by acetolysis of RM_7. Previous studies had shown that M_4, consisting of two M_2 units linked by a 1→6 linkage, was not detectable in short-term acetolysis of mannan. This suggests that h, rather than e, was the M_7 isomer that gave rise to the large amount of M_2.

The acetolysis of RM_5 and RM_6 is slower than the acetolysis of M_5 and M_6 (compare Figures 5 and 6 with Figure 10). Thus, a mannose residue attached to the 6 position of mannitol was more resistant to acetolysis than was a mannose attached to the 6 position of mannose. Therefore, in RM_7 obtained from an M_7 with two 1→6 linkages, the rates of cleavage of the two acetal linkages should be different. Of interest would be the RM_5 isomer that should be formed initially. We have examined the products of RM_7 (acetate) acetolysis after 1- and 3-hr reaction at 40° and have found no significant differences in the rate of release of the small units; all appeared after 1 hr. We conclude that the major isomer of M_7 is XIIa which contains a high proportion of M_3' at the nonreducing end; and for isomers with three subunits, the ratio of e and/or h to f and/or g is 1.75 to 1.0.

Methylation of Mannan

Mannan was methylated twice by the technique described by Hakomori (1965). The product was further methylated three times by refluxing in benzene with sodium wire followed by removal of sodium, addition of methyl iodide, and further refluxing. A 3% solution of the methylated mannan in carbon tetrachloride showed no hydroxyl absorption in the infrared spectrum. About 0.8 g of methylated product was obtained from 1.0 g of mannan.

The methylated mannan was methanolyzed in 2 N hydrogen chloride in anhydrous methanol. Thin-layer chromatography of the products (solvents F and G) showed methyl 2,3,4,6-tetra-*O*-methylmannoside, methyl 2,4,6-tri-*O*-methylmannoside, methyl 3,4,6-tri-*O*-methylmannoside, and methyl 3,4-di-*O*-methylmannoside. Gas chromatographic analysis (column temperature 195°) gave the pattern shown in Figure 8B, and confirmed the results from thin-layer chromatography. Since methyl 2,4,6-tri-*O*-methylmannoside has the same retention time as methyl 3,4,6-tri-*O*-methylmannoside, and since methyl 3,4,di-*O*-methylmannoside tails badly, good quantitative results were obtained only with the trimethylsilyl derivatives of the methylated sugars. As a precaution, loss of the highly volatile methyl 2,3,4,6-tetra-*O*-methylmannoside was avoided by careful evaporation of the methanol after methanolysis.

Because the trimethylsilyl derivative of methyl 3,4-di-*O*-methylmannoside has a short retention time, it was necessary to eliminate the large reagent peak. Excess hexamethyldisilazane and trimethylchlorosilane were removed by careful evaporation on a rotary evaporator. However, removal of pyridine by this technique could not be achieved without loss of methyl 2,3,4,6-tetra-*O*-methylmannoside. Pyridine was removed by treating an ethereal solution of the reaction with anhydrous Chelex 100 (H^+ form) or Bio-Rex 70 (H^+ form) (Bio-Rad) ion-exchange resins (dried by washing with anhydrous methanol and anhydrous diethyl ether). After removal of the ether, the mixture of products was dissolved in carbon disulfide and examined by gas chromatography. Figure 8C shows the separation of the methylated products as their trimethylsilyl derivatives and Table III gives the ratios. Figure 8B,C shows that there was a much higher proportion of methyl 3,4,6-tri-*O*-methylmannoside than of methyl 2,4,6-tri-*O*-methylmannoside (2.5:1). Previous investigators have all reported a 1:1 ratio for these derivatives.

Assuming that M_2, M_3, M_3', and M_4 are the building units of the mannan and they are 1→6 linked to each other, that the ratio of M_3 to M_3' is 4:1, that the ratio of M_2:M_3($+M_3'$):M_4 is 5:5:2, the expected ratio of 2,3,4,6-tetra- to 3,4,6-tri- to 2,4,6-tri- to 3,4-di-*O*-methylmannose would be 1.0:0.50:0.25:1.0. As seen from Table III, there is good agreement with the observed results.

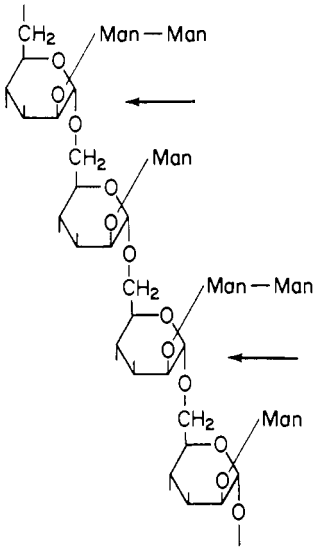
Structures of Mannan

The following facts have to be accommodated in any proposed structure of bakers' yeast mannan. (a) M_3 and M_3' are integral units of the polymer and are not derived from degradation of M_4. (b) There is little difference in the rates of acetolysis of 1→2 and 1→3 linkages, and the rates are very much slower than that for cleavage of a 1→6 linkage. (c) The ratio of oligosaccharides M_2, M_3($+M_3'$), and M_4, obtained from long-term acetolysis of mannan, is 5:5:2, and this probably represents the ratio that exists in the parent mannan. (d) No isomeric M_4 composed of two M_2 units connected by a 1→6 linkage was detected, and it is assumed that this arrangement of M_2 units does not exist in the *S. cerevisiae* mannan molecule. (e) Penta-, hexa-, and heptasaccharides were isolated containing one or more 1→6 linkages. The structure of M_5 was shown to be as depicted in Va and b, with a ratio of 5:1 for these two isomers. The structure of M_6 is more complex. There are two isomers, VIIa and b, containing an M_4 and M_2 unit connected by a 1→6 linkage; and one isomer, VIII, composed of two M_3 units. The ratio of $M_{6(4+2)}$ to $M_{6(3+3)}$ is approximately 2.3:1. The ratio of the isomer VIIa to VIIb is 3:1. M_7 contained isomers composed of M_4 and M_3 units, as well as isomers of two M_2 and one M_3 units (see XII for the several possibilities). The ratio of the two types was 2:1. The major isomer with three subunits appears to be that in which the reducing function is associated with the M_3 unit. (f) M_3' was concentrated at the nonreducing portion of the larger oligosaccharides M_6 and M_7. (g) Methylation studies of the intact mannan are consistent with the oligosaccharides derived from the parent molecule by acetolysis. The molecule is highly branched, with one branch point per three

mannose residues. The proportion of 1→3-linked mannose units is much lower than earlier investigators had reported, but the results we have obtained are consistent with the amounts of M_4 and M_3' obtained after acetolysis.

It is apparent that simple repeating units do not satisfy the structural requirements outlined above. Assuming that there is a "backbone" of 1→6-linked mannose units and that the structure is similar to that shown in IV, it is not obvious how to arrange the M_2, M_3, and M_4 units (in 5:5:2 ratio) and obtain a linear structure that would yield the observed ratios of isomers of the higher oligosaccharides (M_5, M_6, and M_7) by a random acetolysis process. That is, there must be a 5:1 ratio for the two M_5 isomers and a 3:1 ratio for the M_6 isomers containing M_4 and M_2 units.

Suprisingly, we find that, in all the larger oligosaccharides which contain a 1→6 linkage, the reducing function in the major isomer is associated with the larger of the two subunits. It may be that stereochemical factors lead to preferential cleavage of certain 1→6 linkages during acetolysis. That is, a disaccharide attached to the 2 position of a mannose unit could inhibit by steric interaction the glycosidic cleavage of a residue attached to the 6 position of that unit much more than would a single mannose attached to the 2 position. Thus, in XIII, the major M_5 isomer, Va, would



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be the preferred product as a result of cleavage at the linkages indicated by arrows. Such a preferential cleavage by acetolysis could also explain why the isomeric M_4 composed of two M_2 units with a 1→6 linkage was not found. However, in studies on another mannan (T. S. Stewart and C. E. Ballou, unpublished) we have detected such an M_4, so if such units are present in a mannan they can be obtained by the partial acetolysis technique.

Conclusions

The acetolysis of yeast mannans can yield an accurate estimate of the proportions of 1→2- and 1→3-linked oligosaccharides (M_2, M_3, and M_4) that form the building units of the polymer structure. Comparative studies on several different mannans (T. S. Stewart and C. E. Ballou, unpublished) show that the proportion of these oligosaccharides is characteristic of a particular strain of yeast.

Partial acetolysis of yeast mannan yields higher oligosaccharides in which the smaller units (M_2, M_3, and M_4) are connected by 1→6 linkages. The actual amounts of these higher oligosaccharides that are obtained in this manner may not have structural significance, since the acetolysis process may be subject to steric control and thus selective. However, the higher oligosaccharides that are obtained do provide a direct clue to the relationships of the smaller units that must exist in the polysaccharide. The partial acetolysis technique also provides a valuable tool for the preparation of oligosaccharides for use in the study of enzyme specificity and the identification of the immunochemical determinants of mannan antigens.

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