Carbohydrate Research, 48 (1976) 225–237

METHYLATION AND ACETOLYSIS OF EXTRACELLULAR D-MANNANS FROM YEAST*

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(Received February 14th, 1976, accepted for publication, March 19th, 1976)

ABSTRACT

Methylation–fragmentation analyses were conducted on a series of extracellular, yeast α-D-linked mannans representing six different structural types D-Mannans of low degree of branching were produced by *Hansenula capsulata* strains and by species related to *H holstii* The former consisted primarily of (1→2)- and (1→6)-linked D-mannosyl residues; the latter, of (1→2)- and (1→3)-linked D-mannosyl residues Although the remaining structural types were highly branched, each gave distinct methylation-patterns indicative of (1→6)-linked backbones to which are appended non-(1→6)-linked side-chains Acetolysis studies were correlated with the methylation analyses, and the correlation demonstrated that each branched polymer possesses side chains of heterogeneous length

INTRODUCTION

Neutral, extracellular D-mannans and glucomannans are formed when orthophosphate is omitted from the culture medium of yeasts that synthesize *O*-phosphono-D-mannans Alternative synthesis of D-mannans was first observed[1] for *Hansenula capsulata* NRRL Y-1842 and *H. holstii* Y-2448 This observation was extended to other strains of these species and to related genera that also produce extracellular *O*-phosphono-D-mannans[2] All yeasts capable of excreting *O*-phosphono-D-mannan can alternatively make either neutral D-mannans or, in certain low-yielding strains, lightly phosphorylated D-mannans Similar yields result when a given strain produces either D-mannan or *O*-phosphono-D-mannan Earlier studies[3,4] had indicated that various strains belonging to a given species of yeast produce characteristic *O*-phosphono-D-mannans We now find that characteristic D-mannans are also formed. The various species make α-D-linked D-mannans that differ in apparent degree of branching and in proportion and type of sugar–sugar linkage

*Presented before the Division of Carbohydrate Chemistry, 169th American Chemical Society Meeting, Philadelphia, Pennsylvania, April 6–11, 1975

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We have developed a procedure[5] for g l c. separation, as peracetylated aldononitriles, of all possible methyl ethers of D-mannose that could arise from methylation–fragmentation analysis of D-mannans In confirmation and extension of the work of Dmitriev *et al* [6], distinctive mass spectra were observed for the various peracetylated aldononitriles of tetra-, tri-, and di-*O*-methyl-D-mannose We used combined g l c.–m s of peracetylated aldononitriles for the analysis of hydrolyzates of methylated, extracellular D-mannans from yeast In addition, acetolysis patterns, obtained by high-pressure, liquid chromatography (h p l c), have been correlated with the results of methylation analysis to provide additional information about various structures

Some yeast strains previously studied make *O*-phosphonoglucomannans and, alternatively, produce neutral glucomannans on media from which orthophosphate is omitted[3] Only D-mannans will, however, be discussed here, the glucomannans being the subject of a later report

RESULTS

Methylation analyses — Methylation–fragmentation analyses were made of extracellular α-D-linked D-mannans produced by various genera and species of yeast (see Table I) With the exception of *Pachysolen tannophilus* D-mannan, organic phosphate (P) could not be detected in any of the polymers examined. Strain NRRL Y-2461 D-mannan has a molar ratio of D-mannose P of 94 1, the corresponding *O*-phosphono-D-mannan has a molar ratio of D-mannose P of 4 2 1

A single Hakomori methylation was sufficient to methylate the D-mannans completely Completeness of methylation was judged by the criteria of (*1*) close correspondence between the amounts of tetra- and di-methyl ethers, representing nonreducing end-groups and points of branching, respectively, and (*2*) the presence of only a few types of methyl ether in each permethylated polymer No 2,3,6-tri-*O*-methyl derivative, representing (1→4)-linked D-mannosyl residues, was detected in any of the polymers. Furthermore, only the 2,4- and 3,4-di-*O*-methyl derivatives were found, representing 1,3,6- and 1,2,6-tri-*O*-substituted D-mannosyl residues at branch points The absence of (1→4)-linked D-mannosyl residues and the involvement of (1→6)-linkages at all points of branching suggest that the extracellular mannans resemble yeast cell-wall D-mannans examined by others[7,8]. The latter D-mannans are believed to consist of α-D-(1→6)-linked, backbone chains to which side chains of non-(1→6)-linked units are attached through either (1→2)- or (1→3)-linkages[9 10] For purposes of comparison, the results described here are set forth in terms of the cell-wall D-mannan model

Two major categories of D-mannan are evident In accord with the small number of branch points and nonreducing end-groups, D-mannans belonging to the first category apparently have low degrees of branching These D-mannans are produced by strains of *H capsulata*, and by *H holstii* strains and species related to it The corresponding *O*-phosphono-D-mannans are largely poly(phosphoric diesters) of

TABLE I

MOLE PERCENTAGE OF METHYLATED D-MANNOSE COMPONENTS IN HYDROLYZATES OF PERMETHYLATED D-MANNANS

Mannan	*NRRL No*	*Methyl ethers of* D-*mannose*					
		2,3,4,6-	*3,4,6-*	*2,4,6-*	*2,3,4-*	*3,4-*	*2,4-*
Hansenula capsulata	Y-1842	2 5	51 1		44 3	2 0	
	Y-1889	1 2	53 8		42 3	2 4	
	YB-4661	2 6	58 3		38 6	1 2	
Hansenula sp n	YB-3070	2 3	59 1	33 4		1 9[a]	
	YB-1443	1 3	59 4	32 7		3 7	
Selenotila peltata	Y-6888	3 0	50 3	42 7		2 4	
Hansenula holstii	YB-347	3 4	57 9	34 0		4 0	
	Y-2154	2 2	60 8	35 4		1 7	
	Y-2448	3 9	48 3	44 0		3 9	
	Y-7178	2 3	57.7	32 5		1 7	
Hansenula holstii[b]	Y-7415	1 9	51 4	41 3	1 7	2 3	
Pachysolen tannophilus	Y-2460	26 4	42 9	Trace	2 1		26 1
	Y-2461	23 5	37 8		2 5		36 2
Torulopsis pinus	Y-2023	19 2	54 1		9 7	16 9	
Pichia sp	YB-2097	27 7	20 4	25 2			23 8
Pichia mucosa	YB-1344	22 8	4 2	48 0	2 6	2 3	20 1

[a]Trace of 3,6-dimethyl ether also present [b]Near *Torulopsis wickerhamii* (Dr C. P Kurtzman, personal communication)

D-mannose oligosaccharides, and have low molar ratios of D-mannose P ranging[11,12] from 2 5 1 to 6 1 More-highly branched D-mannans are found in the second category, which includes *Pachysolen tannophilus*, *Torulopsis pinus*, *Pichia* sp , and *Pichia mucosa* D-mannans The corresponding *O*-phosphono-D-mannans are polysaccharides having hexosyl phosphoric diester end-groups[3,13] and, except for *P tannophilus*, have higher molar ratios of D-mannose P *P tannophilus* *O*-phosphono-D-mannan has a molar ratio of D-mannose P of 4 2 1, but nothing is known about its molecular structure

The *H capsulata* polymers consist mainly of (1→2)- and (1→6)-linked D-mannosyl residues with (1→3)-linked D-mannosyl residues absent, even at points of branching Gorin and Spencer[14] obtained similar results for methylation analysis of a D-mannan extracted from the cell wall of *H capsulata* The presence of a large proportion of (1→6)-linked D-mannosyl residues accounts for the significant amounts of formic acid released during periodate oxidation Erroneously, this formic acid had been ascribed to (nonreducing) D-mannosyl end-groups in a more highly branched polymer[2]

Similar D-mannans consisting mainly of (1→2)- and (1→3)-linked D-mannosyl residues, but devoid of (1→6)-linked residues except at points of branching, are produced by *H holstii* and related species *Hansenula* sp n *O*-phosphono-D-mannan has a molecular architecture resembling that of *H holstii* strains[3] Strain NRRL

Y-6888, unlike members of the genus *Hansenula*, cannot assimilate NO_3^- Designated *Selenotila peltata* by Yarrow[15], the species produces *O*-phosphono-D-mannans resembling those of *H.* sp n Strain NRRL Y-7178 was classified as *H holstii* primarily on the basis its *O*-phosphono-D-mannan structure[16]; this conclusion is reinforced by the results of methylation analysis of its D-mannan Strain NRRL Y-7415*, also of doubtful classification on morphologic and nutritional grounds, forms *H holstii*-type *O*-phosphono-D-mannan and D-mannan

Turning to the category of highly branched D-mannans, the results of methylation analysis suggest that *T pinus* Y-2023 mannan has side chains composed of (1→2)-linked D-mannosyl residues, which are attached to a (1→6)-linked backbone through (1→2)-linkages The presence of 2,3,4-tri-*O*-methyl-D-mannose in the hydrolyzate of the permethylated D-mannan suggests that almost one-third of the (1→6)-linked D-mannosyl residues are not involved in branching *P tannophilus* D-mannans are also highly branched, with (1→2)-linked side-chains attached primarily through (1→3)-linkages to (1→6)-linked, backbone D-mannosyl residues Some 2,3,4-tri-*O*-methyl-D-mannose is also present, in addition to 3,4-di-*O*-methyl-D-mannose

The D-mannan of *Pichia* sp YB-2097 is the most highly branched Side chains that contain (1→2)- and (1→3)-linked D-mannosyl residues are apparently attached to (1→6)-linked D-mannosyl residues of a backbone chain through (1→3)-linkages Evidently, all of the main-chain residues are involved in branching

Because *P mucosa* produces only neutral D-mannan, even in the presence of orthophosphate[2], it does not truly belong to the family of yeasts that make extracellular *O*-phosphono-D-mannans Its extracellular D-mannan obviously differs from the other, highly branched D-mannans Here, (1→3)-linked side-chains are attached mainly through (1→3)-linkages to the (1→6)-linked, main chain Also present are small proportions of 1,2- and 1,6-di-, as well as 1,2,6-tri-, *O*-substituted D-mannosyl residues

Acetolysis — Acetolysis of yeast cell-wall D-mannans has been employed by Ballou and co-workers[8] to prepare oligosaccharides composed of non-(1→6)-linked side-chains attached to (reducing) D-mannose residues that were formerly part of the (1→6)-linked D-mannosyl backbone Rosenfeld and Ballou[17] found the relative rate of acetolysis of the α-D-(1→6)-mannobiose to be far greater than that of either the α-D-(1→2)- or α-D-(1→3)-mannose disaccharides The data in Table I suggest that the extracellular D-mannans structurally resemble cell-wall D-mannans As the latter possess side chains varying in length and in linkage composition, acetolysis was performed on extracellular D-mannans, representing the general polymer types listed in Table I, to determine whether they, likewise, possess these structural features

H p l c of the acetolysis products (see Fig 1) yielded a series of symmetrical peaks whose retention times are listed in Table II The retention times of a mixture

*This strain was provided by Dr J P Van der Walt, South African Council for Scientific and Industrial Research, Pretoria

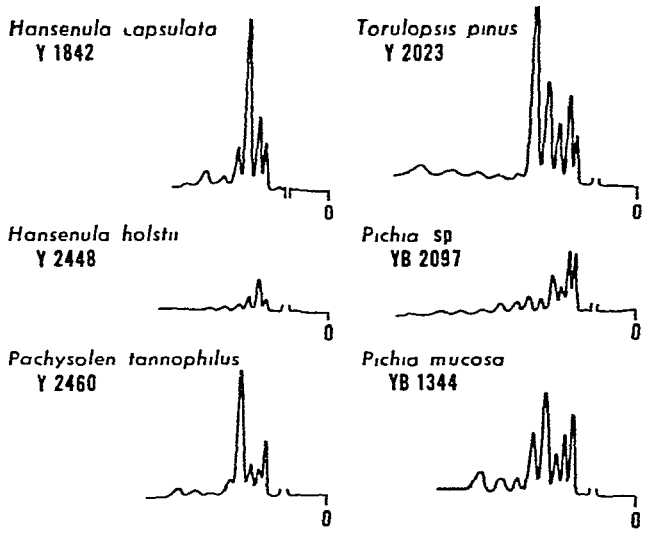


Fig 1 High-pressure, liquid chromatograms of acetolysis products of D-mannans [*O* designates the point of sample injection The broken, base-line peak represents the solvent front Column (4 mm i d × 30 cm) μBondapak-NH_2 (particle size 10 μm) Solvent 7 13 water–acetonitrile pumped at 1 ml min^{-1}]

TABLE II

RELATIVE MOLE AMOUNTS OF OLIGOSACCHARIDES PRESENT IN ACETOLYZATES

Mannan	*NRRL No*	*Degree of polymerization*										
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Torulopsis pinus	Y-2023	5 0	5 4	3 2	4 8	7 2	0 4	0 4	0 5	0 5	0 6	
Pachysolen tannophilus	Y-2460	10 0	3 7	3 6	11 9	1 3	0 7	1 0	0 9			
Pichia mucosa	YB-1344	13 7	4 9	3 0	7 4	4 2	0 6	0 7	0 1			
Pichia sp	YB-2097	15 0	10 7	3 2	4 0	1 2	1 4	0 9	0 9	0 4	0 4	0 3
Hansenula capsulata	Y-1842	8 1	9 3	14 3	4 1	1 0	1 3	0 2				
Hansenula holstii	Y-2448	12 0	22 8	6 7	3 6	1 6						
Average retention-time (min)		4 4	4 9	5 7	6 5	7 4	8 7	10 1	11 8	13 3	15 7	

of D-glucose, maltose, maltotriose, and maltopentaose were, respectively, equal to those of compounds 1, 2, 3, and 5 in the mixtures of acetolysis products Therefore, the first five acetolysis peaks were considered to represent, respectively, D-manno-oligosaccharides of degree of polymerization (d p) of 1–5 In addition, the various acetolysis mixtures yielded regularly spaced, succeeding peaks, and these were,

tentatively, assigned d p values of 6–11 The refractive indices per unit weight of D-glucose oligomers of high d p are approximately equal[18] For the standard series of D-gluco-oligosaccharides, we found that D-glucose and maltose give greater response than the higher oligomers as regards refractive index Assuming that the same relationship holds for the D-mannose oligomers, we multiplied the refractive index responses of D-mannose and apparent D-mannose disaccharide by 0 86 and 0 90, respectively

For each chromatogram, the area under each peak was measured and, after the correction factors had been applied to D-mannose and the disaccharide, was expressed as a percentage of the total area This percentage area represented the percentage of D-mannose units in the chromatogram The percentage area in each peak was divided by its assigned d p value, to give the relative mole amounts of the oligosaccharides present (see Table II)

Because acetolysis breaks down the α-D-(1→6)-linked, backbone chain, the oligomers resulting represent the non-(1→6)-linked side-chains of the original polymers plus the single backbone units to which they are attached This hypothesis may be tested by determining, from acetolysis data, the degree of branching of a polymer and comparing it to the degree of branching determined from methylation data

In the generalized D-mannan structures, each (1→6)-linked, nonbranching, backbone unit was acetolyzed to a monomer Therefore, the proportion of sugar (d p 1) should correspond to that of 2,3,4-tri-*O*-methyl-D-mannose given in Table I. Each oligomer of d p $\geqslant 2$ represents a branching D-mannosyl residue and a (non-reducing) D-mannosyl group The sum of the relative, mole quantities of all oligomers of d p $\geqslant 2$, therefore, represents both the number of branch points and nonreducing end-groups Oligomers of d p $\geqslant 3$ contain, in addition, non-(1→6)-linked, internal, D-mannosyl residues Therefore, the sum of the quantity of all oligomers multiplied by their corresponding value of d p minus 2 represents the total amount of non-(1→6)-linked D-mannosyl residues These calculations were made for each acetolysis mixture, and are expressed in Table III as the 2,3,4,6-tetra-, 2,3,4-tri-, non-2,3,4-tri-, and di-*O*-methyl derivatives expected from methylation of the assumed structures that would lead to the acetolysis products Appreciable cleavage of any (1→2)- or (1→3)-linkages would lead to elevated values for the amounts of free D-mannose (d p 1), which is a measure of the 2,3,4-tri-*O*-methyl component

In general, if acetolysis indicates a degree of branching greater than that shown by methylation, the extra side-chains could arise from original ones containing internal (1→6)-linkages that have been cleaved However, should methylation show branching greater than that indicated by acetolysis, it reveals clearly that the side chains are internally branched

For the highly branched D-mannans produced by strains Y-2023, Y-2460, and YB-1344, close correspondence between acetolysis and methylation data indicates that the assumptions of (*a*) linear, (1→6)-linked backbone, and (*b*) linear, non-(1→6)-linked side-chains are probably correct Methylation and acetolysis data on D-

TABLE III

CORRELATION OF METHYLATION AND ACETOLYSIS DATA

Mannan	*NRRL No*	*Data source*[a]	*Calculated percentages of methyl ethers*			
			Tetra-	*2,3,4-Tri-*	*Non-2,3,4-tri-*	*Di-*
Torulopsis pinus	Y-2023	Me	19 2	9 7	54 1	16 9
		Ac	22 8	5 0	49 3	22 8
Pachysolen tannophilus	Y-2460	Me	26 4	2 1	42 9	26 1
		Ac	23 0	10 1	44 5	23 0
Pichia mucosa	YB-1344	Me	22 8	2 6	52 2	22 4
		Ac	21 7	13 8	42 9	21 7
Pichia sp	YB-2097	Me	27 7		45 6	23 8
		Ac	23 2	15 1	38 6	23 2
Hansenula capsulata	Y-1842	Me	2 5	44 3	51 1	2 0
		Ac	30 0	8 1	31 8	30 0
Hansenula holstii	Y-2448	Me	3 9		92 3	3 9
		Ac	34 5	12 2	19 0	34 5

[a]Me = methylation analysis, Ac = acetolysis

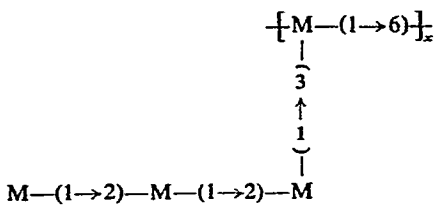
mannan from strain YB-2097 are also in general agreement, except for the large proportion of (1→6)-linked units indicated by acetolysis

Divergent data are given by acetolysis and methylation of the more-linear D-mannans produced by strains Y-1842 and Y-2448 The chromatogram of the acetolysis products of Y-2448 D-mannan gave only about 10% of the peak area given by the other D-mannans, this result accords with the paucity of (1→6)-linked units in the D-mannan and with the concept that acetolysis affords a large number of oligomers of d p >11, which are retained on the column

DISCUSSION

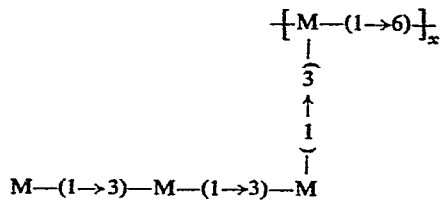
Characteristics of individual classes of D-*mannans.*

Pachysolen tannophilus D-*mannans* — Methylation analyses of *P tannophilus* D-mannans give component ratios indicating a terminal D-mannosyl group, two (1→2)-linked D-mannosyl residues, and branching through either a 1,3,6- or a 1,2,6-tri-*O*-substituted sugar residue Assuming the backbone chain to be exclusively (1→6)-linked, the structure can be drawn as shown (where M is a D-mannopyranosyl



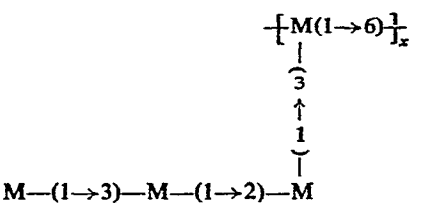
group or residue, or a D-mannopyranose residue), with a three-unit side-chain linked to all backbone sugars Although this structure represents the average chain-length, it does not imply that all side-chains are exactly three units long On the basis of acetolysis data, the d p of the side chain ranges from 1 to 7 As each acetolysis oligomer is assumed to contain one sugar molecule from the backbone, the length of each side chain resulting from an oligomer is d p minus 1 When the products of the relative mole amounts of each oligomer multiplied by the respective value of (d p minus 1) are summed, and when this value is divided by the sum of the relative mole amounts, the average chain-length, as given by acetolysis, is obtained For Y-2460 mannan, this value is 2 9, in excellent agreement with the average chain-length of 3 determined by methylation analysis The least-accurate acetolysis value, d p 1, which could partially arise from side-chain cleavage, does not directly enter into this calculation

Pichia mucosa D-*mannan* — D-Mannan YB-1344 is indicated by methylation analysis to have one D-mannosyl group, two (1→3)-linked D-mannosyl residues, and a 1,3,6-tri-*O*-substituted branching sugar residue A small proportion of 1,2,6-tri-*O*-substituted D-mannose residue is also present Using the (1→6)-linked backbone assumption, the structure is as follows Again, acetolysis shows that a range of side-



chain lengths exists and that an average length of side chain of 2 8 can be calculated, a value still in good agreement with methylation data

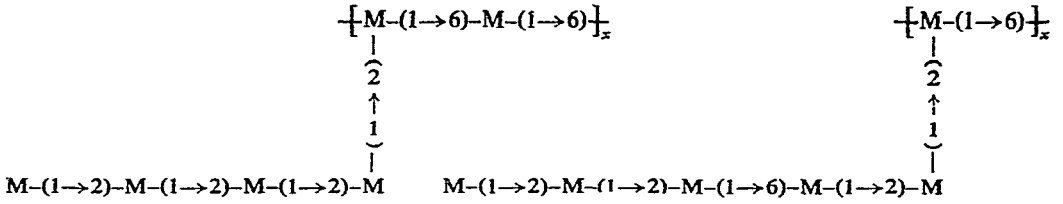
Pichia *sp* D-*mannan* — According to the results of methylation analysis, YB-2097 D-mannan has a D-mannosyl group, a (1→2)-linked D-mannosyl residue, a (1→3)-linked D-mannosyl residue, and a branching 1,3,6-tri-*O*-substituted residue This analysis agrees with the structure shown, in which the order of the (1→2)-



and (1→3)-linkages in the side chain are not known In addition, a small degree of (1→2)-branching occurs from the backbone chain Acetolysis data again indicate a size distribution for the chain length, with an average length of 2 7

Torulopsis pinus D-*mannan* — D-Mannan Y-2023 apparently differs from

the aforementioned polymers, because it contains some (1→6)-linked D-mannosyl residues not involved in branching The ratio of sugars present may be approximately expressed as one D-mannosyl group, three (1→2)-linked D-mannosyl residues, one (1→6)-linked D-mannosyl residue, and a 1,2,6-tri-*O*-substituted, branching sugar residue Two general structures (A and B) may be drawn The position of the (1→6)-linkage in structure B may be at any nonbranching position in the side chain, with the exception of the nonreducing, terminal position



Acetolysis data indicate a range of d p of the side chain of 1 to 9 The acetolysis data also suggest that structure B is the more likely, because an average side-chain length of 3 1 units is calculated This calculated value accords with a single, (1→6)-linked D-mannosyl residue in the side chain, which would be broken on acetolysis, together with a smaller amount of monomer, which would arise from nonbranched, (1→6)-linked units in the backbone chain

Hansenula capsulata D-*mannans* — For the class of polymers represented by Y-1842 D-mannan, methylation analysis indicates little branching, with a ratio of one D-mannosyl group, 22 (1→6)-linked D-mannosyl residues, 25 (1→2)-linked D-mannosyl residues, and one 1,2,6-tri-*O*-substituted, branching sugar residue A structure resembling that of the Y-1842 D-mannan, yet accommodating the (1→6)-linked backbone concept, would have long sequences of unbranched, (1→6)-linked, D-mannosyl residues in the backbone, and side chains consisting of extended sequences of (1→2)-linked D-mannosyl residues Acetolysis analysis shows that such a structure cannot be correct A large number of oligomers of low d p are observed that cannot be reconciled with this structure In fact, according to acetolysis, the average chain length is only 2 1 This value suggests that a large number of (1→6)-linkages must occur in the side chains, and renders unlikely an exclusively (1→6)-linked, backbone chain

H holstii D-*mannans* — The class of polymers represented by Y-2448 D-mannan also points up differences between methylation and acetolysis data Methylation data indicate a ratio of one D-mannosyl group, 12 (1→2)-linked D-mannosyl residues, 11 (1→3)-linked D-mannosyl residues, and one branching, 1,2,6-tri-*O*-substituted sugar residue The presence in *H holstii* D-mannans of (1→6)-linkages solely in the low proportion of branch points suggests deviation from the (1→6)-linked backbone model Other possible structures might involve repeating disaccharide sequences of (1→2)- and (1→3)-linked D-mannosyl residues, or else extended sequences of (1→2)- or (1→3)-linked D-mannosyl residues Occasional side-branching

would occur. Acetolysis gave only a weak chromatogram (~10% of the total intensity of the other acetolysis chromatograms) of oligomers of small, average d p (1 5) A probable explanation is that the column retained the bulk of the long side-chains, and consequently, we detected only the internally cleaved products of non-(1→6) bonds in the side chains Therefore, with no information available on the distribution of side-chain lengths, except that most must be quite long, the structure proposed represents the likeliest possibility

All discussion of acetolysis is predicated on the complete and exclusive hydrolysis of (1→6)-bonds If some hydrolysis of non-(1→6) bonds occurs, the percentage of fractions of low d p will increase Indeed, in some of the h p l chromatograms, the proportion of sugar of d p 1 is larger than can be accounted for by the (1→6)-linked D-mannosyl residues as determined by methylation analysis Conversely, if some (1→6)-linkages are not ruptured, larger oligomers in the chromatograms could result from two adjacent side-chains remaining linked by a backbone (1→6)-bond The patterns of certain of the h p l chromatograms suggest this possibility, *e g*, Y-2460 D-mannan gives strong peaks for oligomers of d p 1, 2, 3, and 4, followed by much weaker peaks for those of d p 5, 6, 7, and 8 The actual determination of whether either under- or over-acetolysis occurs awaits a careful examination of the structures represented in the oligomer fractions

Although there are distinctly different classes of extracellular, α-D-linked D-mannans, we have confirmed that, in general, the (1→6)-linked backbone structure, previously demonstrated by others[8–10] to occur in yeast cell-wall D-mannans, also occurs in extracellular D-mannans having a high degree of branching Two classes of extracellular D-mannan, from *Torulopsis pinus* Y-2023 and from *H capsulata*, apparently also contain (1→6)-linkages in the side chains Acetolysis further demonstrates that, even though each species of yeast produces a distinctive type of polysaccharide, great heterogeneity apparently exists within the side chains of any given D-mannan A similar situation exists in cell-wall D-mannans and *O*-phosphono-D-mannans[8] It is, therefore, remarkable (considering, also, the yeasts which elaborate extracellular glucomannans) that such diverse polysaccharide structures are specified by organisms belonging to a narrow phylogenetic area[19].

Is there a relationship between the extracellular D-mannans and cell-wall D-mannan? Only one example is available that bears on this question Gorin and Spencer[14] extracted with hot aqueous alkali an α-D-linked D-mannan from *H capsulata* Y-1842 cells grown in a medium low in phosphate This polysaccharide resembles the extracellular D-mannan structurally, except for certain differences in the ratios of components. The cell-wall D-mannan contained one nonreducing end-unit in 23 (excluding the branch point), and (1→2)- and (1→6)-linked D-mannosyl residues in the ratio of 2 1·1. We find that the extracellular D-mannan contains one nonreducing end-unit in 47, and (1→2)- and (1→6)-linked D-mannosyl residues in an almost 1 1 ratio Is the phosphoric diester form of the polysaccharide conserved in the walls of yeast grown on a medium low in phosphate? This question cannot be answered by study of material prepared by hot alkaline extraction, because this

treatment removes esterified phosphate[8], but a solution may be given by studies that employ ^{31}P nuclear magnetic resonance spectroscopy[20] on intact cells

Our experience, as well as that reported by Jeanes *et al* [21], has been that the extracellular D-mannans and *O*-phosphono-D-mannans, when purified by mild procedures[22], contain only traces (less than 1%) of protein In contrast, D-mannans extracted from cell walls by mild procedures contain significant proportions of covalently bound protein[23]. This difference may be related to the mechanism whereby D-mannans and *O*-phosphono-D-mannans are liberated into a medium

EXPERIMENTAL

General — Growth of yeasts, as well as isolation and purification of D-mannans, has been described earlier[2,3] Lyophilized D-mannan preparations were used exclusively

Methylation and hydrolysis — Permethylation of D-mannans was performed by the procedure of Hakomori[24] The dimethylsulfinylsodium reagent was prepared either as described by Sandford and Conrad[25] or by Sjoberg[26] D-Mannan (5–20 mg) was dried *in vacuo* over phosphorus pentaoxide for 2 h at 60° and then dissolved in dimethyl sulfoxide (4 ml, dried over 4A molecular sieve) with the aid of magnetic stirring for 1 h under nitrogen Hakomori's reagent (0 4 ml) was added, the mixture was stirred for 30 min, and methyl iodide (0 4 ml) was added The mixture was stirred for 30 min, and then dialyzed for 12 h against running tap-water

The dialyzed, aqueous suspension of permethylated D-mannan was evaporated to dryness in a rotary, vacuum evaporator at 50°, and the product dried by adding and evaporating absolute ethanol (2 × 5 ml) Alternatively, the permethylated D-mannans were dried by lyophilization To the dried residue were added 16 drops of 0 5M sulfuric acid in 95% acetic acid [solution made by adding 5M sulfuric acid (5 ml) to glacial acetic acid (95 ml)], this mixture was stirred and heated[27] for 12 h at 80° Water (16 drops) was added to the solution, and heating and stirring were continued for 5 h Sulfate ion was removed from the hydrolyzate by passing it through a column (0 5 × 5 cm) of Dowex AG 2-X8 (AcO^-) anion-exchange resin, and the column was eluted and washed with successive portions (4 ml) of water and methanol The eluate was evaporated to dryness at 40° in a rotary, vacuum evaporator, and the product dried by adding and evaporating absolute ethanol (2 × 5 ml)

Derivatization of the methylated sugars to form peracetylated aldononitriles, and the analysis of the latter by g l c–m s has been described[5]. The identity of each methyl ether was established by comparison of its relative retention-time in g l c with that of a known, methylated-peracetylated aldononitrile As the gas chromatograph was coupled to a mass spectrometer, the identities of the emerging peaks were confirmed by their characteristic mass spectra, these were identical to those given by known reference-compounds Unidentified, extraneous peaks amounted to less than a few percent of each g l chromatogram The area under each g l c peak was measured, and expressed as a percentage of the total area of the peaks in the chromatogram The detector response was considered to be equal for each compound, and the

relative proportions of peracetylated aldononitrile were taken to reflect the ratios of corresponding, underivatized methyl ethers of D-mannose in the hydrolyzate Equivalent results were obtained by measuring the total, fragment-ion output for each g l c peak as measured in the computer-assisted, mass spectrometer Response of the mass spectrometer to the various methylated, peracetylated aldononitriles is similar to that of a g l c , hydrogen-flame ionization-detector[5].

Acetolysis — Acetolysis was conducted on D-mannan (100 mg), essentially as described by Kocurek and Ballou[28]. The products of acetolysis were deacetylated according to the procedure of Torii *et al* [29] The resulting solution of oligosaccharides was evaporated to dryness, so that the residue could be dissolved in water (1 ml) before filtration through a 0 45-μm Millipore filter (type HA) in a Swinny hypodermic-syringe adaptor.

Analysis of the acetolysis products was performed with a Waters Associates μBondapak-NH_2 (particle size, 10 μm) "carbohydrate" column (4 mm i d $\times$ 30 cm) in a Waters Associates ALC-100 liquid chromatograph equipped with a differential refractometer. The solvent system was 7 13 water–acetonitrile[30] pumped at 1 0 ml. min^{-1} Results were excellent with (*a*) 7-μl samples that contained 0 1 to 0 3 mg of carbohydrate, (*b*) detector attenuation settings of 8X, and (*c*) resolution observed for all components, base-line separation was complete with the solvent system of 3 7 water–acetonitrile

ACKNOWLEDGMENTS

We thank Robert D Stubblefield for assistance in recording the high-pressure, liquid chromatograms, and Paul A Sandford for aid in preparing the Hakomori methylation reagent

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