Characterization of a Yeast Mannan Containing N-Acetyl-D-glucosamine as an Immunochemical Determinant†

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ABSTRACT: The cell wall mannan of the yeast *Kluyveromyces lactis* is characterized by a high content of *N*-acetylglucosamine, most of which is located in pentasaccharide side chains containing four mannose units and one *N*-acetylglucosamine [$(Man)_4GlcNAc$]. This side chain is released from the mannan by controlled acetolysis along with mannose, mannobiose, mannotriose, mannotetraose, and a fragment containing 1 mole of *N*-acetylglucosamine and 3 of mannose. The latter was shown to be an acetolysis degradation product of $(Man)_4GlcNAc$. The presence of the *N*-acetylglucosamine protected the latter side chain from digestion with α-mannanase, while all others in the mannan were removed by the action of the enzyme. The structure of $(Man)_4GlcNAc$ was shown by methylation analysis to consist of a mannotetraose unit, αMan-(1→3)αMan(1→2)αMan(1→2)Man, with an α-linked *N*-acetyl-D-glucosamine residue attached to position 2 of the penultimate mannose in the chain. Rabbit antiserum formed against intact *K. lactis* cells had two specificities, one for the $(Man)_4GlcNAc$ structure and the other for the mannotetraose side chain. A mixture of these two mannan fragments was necessary to give complete inhibition of the precipitin reaction with purified mannan. A weaker precipitin reaction was observed with mannan after enzymatic removal of the mannotetraose side chain, and $(Man)_4GlcNAc$ alone gave complete inhibition of this reaction.

The serological relationships between pathogenic and nonpathogenic yeasts have long been of interest in medicine and yeast taxonomy. For example, studies on the pathogenic genus *Candida* have revealed two antigenically distinct groups, with *C. albicans* group A yeast containing all the determinants found on group B cells, plus one or more in addition (Hasenclever and Mitchell, 1961a,b, 1963). Such differences have been reviewed as the basis for establishing new criteria for classification of yeasts in general (Tsuchiya *et al.*, 1965).

The principal antigen of the yeast cell surface has been identified as the cell wall mannan, since this material completely inhibited the agglutination of whole yeast cells by the homologous antiserum (Hasenclever and Mitchell, 1964). The serological relationships established between *Candida* species using agglutination techniques were consistent with the observed cross-reactions of the isolated mannans (Summers *et al.*, 1964), although the heterologous precipitation reactions between *Candida* antisera and mannans from other yeasts did not yield to a straightforward interpretation.

The differences in the precipitin reactions of mannans, and ultimately the serological characteristics of the yeasts, reflect structural differences between the mannans. Structural analysis of the cell wall mannan of *Saccharomyces cerevisiae* baker's yeast has established that the polysaccharide has an α-1→6-linked D-mannose backbone with side chains containing 1→2- and 1→3-linked D-mannose units (Lee and Ballou, 1965; Stewart *et al.*, 1968; Jones and Ballou, 1968). This general structure holds for a majority of the yeast mannans studied to date (see reviews by Phaff, 1971, and Gorin and Spencer, 1970), with variability arising most commonly from differences in the lengths of the side chains and in the anomeric configurations of the mannose units.

Several studies have demonstrated that the side chains are the main antigenic determinants in the mannans. The isolated fragments are excellent inhibitors of the mannan–antibody precipitin reaction, while 1→6-linked oligosaccharides from the backbone inhibit poorly (Suzuki *et al.*, 1968; Suzuki and Sunayama, 1968, 1969). The antibodies apparently recognize single side chains since fragments containing two adjacent chains joined by a backbone 1→6 linkage were no better as inhibitors (Ballou, 1970).

The structural features of the mannan side chains which are responsible for the differences in serological cross-reactions have only recently been investigated. In mannan from *S. cerevisiae* baker's yeast, as well as strains S288C and X2180, the tetrasaccharide side chain[1] was the strongest inhibitor of the homologous precipitin reaction (Suzuki *et al.*, 1968; Ballou, 1970), and the terminal 1→3 linkage in this side chain was identified as the immunodominant structure (Suzuki *et al.*, 1968). The general chemotype of this mannan is illustrated in Figure 1A. A second mannan chemotype (Figure 1B) was first recognized from studies on the cell wall phosphomannan of *Kloeckera brevis*. The phosphate was shown to be present as α-D-mannosyl 1-phosphate units attached in diester linkage to position 6 of the middle mannose residue of a trisaccharide side chain (Thieme and Ballou, 1971). We were able to demonstrate that the α-D-mannosyl 1-phosphate group was immunodominant in this mannan (Raschke and Ballou, 1971), and the same mannan chemotype has been found in certain *S. cerevisiae* brewer's yeast species (N. Cawley and C. E. Ballou, unpublished results).

In this paper we describe a third mannan chemotype which appears to be characteristic of the genus *Kluyveromyces*. A previous study had suggested the presence of two distinct immunological determinants in *Kluyveromyces lactis* (Ballou, 1970), one of them being identical with the tetrasaccharide side chain of *S. cerevisisiae* and the other having the

† From the Department of Biochemistry, University of California Berkeley, California 94720. Supported by grants from the National Science Foundation (GB-19199) and the U. S. Public Health Service (AM-884 and TI-GM31).

‡ Holder of a National Science Foundation predoctoral fellowship.

[1] All "side chains" we describe contain one D-mannose residue from the backbone.

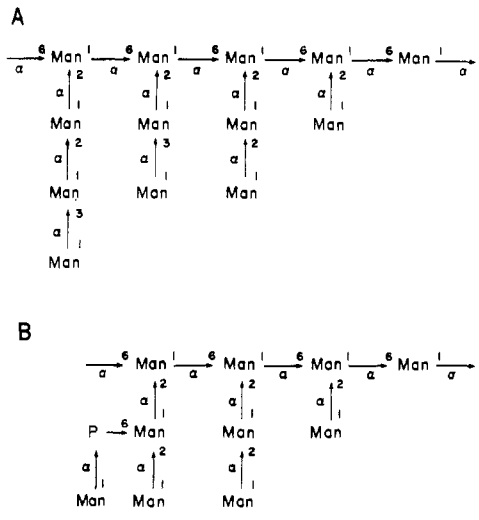

FIGURE 1: Mannan chemotypes of (A) *Saccharomyces cerevisiae* X2180 and (B) *Kloeckera brevis* 55-45.

size of a pentasaccharide. The complete characterization of the second immunogenic structure in *K. lactis* mannan is reported here, and it is shown to consist of a mannotetraose unit substituted by *N*-acetyl-D-glucosamine. A preliminary report of this finding has appeared (Raschke and Ballou, 1972).

Experimental Section

Materials. Bio-Gel, P-2 (−400 mesh), P-4 (−400 mesh), and A-5m (200–400 mesh) were obtained from Bio-Rad Corp. Phenyl 2-acetamido-2-deoxy-α-D-glucopyranoside and phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside were purchased from Sigma, and *p*-nitrophenyl α-D-mannopyranoside from Calbiochem. Sodium borotritide (specific activity 200 Ci/mole) was obtained from New England Nuclear. Dr. D. E. Koshland kindly provided chitobiose and chitotetraose.

General Procedures. Total carbohydrate was measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956), and hexosamine by the Elson–Morgan method (as described by Kabat and Mayer, 1964). Hexosamine was also determined by amino acid analysis using a Beckman Model 120C analyzer. Glucosamine, mannosamine, and galactosamine were resolved on the 50-cm cation-exchange column of the analyzer giving retention times of 193, 211, and 217 min, respectively.

Descending paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems (in volume ratios): (A) ethyl acetate–pyridine–water (5:3:2), (B) ethyl acetate–pyridine–water (2:1:2, upper phase), and (C) 1-butanol–ethanol–pyridine–water (40:11:2:19). For preparative chromatograms, Whatman No. 3MM paper was used. Paper electrophoresis was performed at 100 V for 90 min on Whatman No. 1 paper in 0.86 M acetic acid adjusted to pH 3.5 with pyridine.

Reducing sugars were detected on paper chromatograms with the aniline hydrogen phthalate reagent (Partridge, 1949), and amino sugars and their methylated derivatives with 1% ninhydrin in acetone. Paper chromatograms were scanned for radioactivity by cutting a 2-cm wide strip into 1-cm horizontal bands which were counted in 10 ml of Bray's solution (Bray, 1960).

Gas chromatography of partially methylated alditol acetates at 220° on a 2.5 ft Carbowax 20M column was carried out on a Varian Aerograph 1400 instrument equipped with a DuPont 21-491 mass spectrometer, operating at an ionizing voltage of 70 eV.

Proton magnetic resonance spectra were measured at room temperature with a Varian A-60 spectrometer using tetramethylsilane as an external standard. Samples were lyophilized twice from 99.5% D_2O and dissolved in the same solvent for the spectral measurements.

Optical rotation was measured with a Bendix 1100 polarimeter, while optical rotatory dispersion spectra were obtained with a Cary 60 spectropolarimeter. Absorbances were read on a Zeiss PMQ spectrophotometer. Sedimentation equilibrium measurements on mannan in 0.20 M KCl were made with a Spinco Model E analytical ultracentrifuge using interference optics. The partial specific volume of mannose in a polysaccharide (0.625) was used in calculations (Gray and Ballou, 1971).

Preparation of Mannan. K. lactis (NRRL 1140) was grown with aeration at 30° in the medium previously described (Stewart and Ballou, 1968). Growth was followed by measuring turbidity at 660 nm. Mannan was extracted from the harvested cells by autoclaving them in 0.02 M citrate buffer (pH 7.0) for 90 min. The solids were removed by centrifugation, resuspended in the citrate buffer, and the suspension autoclaved again for 90 min. Carbohydrate was precipitated from the combined supernatants by the addition of two volumes of 100% ethanol. The gummy precipitate was dissolved in water and an equal volume of 2 N acetic acid was added to precipitate protein, which was removed by centrifugation and discarded. Dialysis of the supernatant against water removed the acetic acid, and the mannan was precipitated by Fehling's solution as described by Kocourek and Ballou (1969).

Acetolysis. Acetylation and acetolysis of the mannan followed the procedure of Kocourek and Ballou (1969). The addition of pyridine terminated the reaction, and the solvents were removed by rotary evaporation at 50°. The residue was dissolved in benzene and the solution was extracted successively with water, 1 N HCl, 1 M NH_4HCO_3, and water. The benzene layer was dried with anhydrous sodium sulfate followed by azeotropic distillation of benzene on a rotary evaporator.

The acetylated acetolysis fragments were dissolved in dry methanol and deacetylated with a few drops of 1 N sodium methoxide. After 20 min at room temperature, the reaction was stopped by the addition of solid carbon dioxide or Dowex 50 (H^+).

Acid Hydrolysis. Acid hydrolysis was carried out with 1 N HCl for 4 hr at 100°. For estimation of glucosamine, hydrolysis was done on 2–5 mg of mannan in 5.0 ml of 4 N HCl *in vacuo* at 110° for 6 hr. Complete release of glucosamine resulted with very little destruction.

Methylation Procedure. Methylation of oligosaccharides was performed by the Hakomori (1964) procedure. The methylsulfinyl anion was prepared according to Sandford and Conrad (1966), while the methylation conditions followed those of Hellerqvist *et al.* (1968). Methylated oligosaccharides were extracted into chloroform from the reaction mixture and the solution was washed twice with water. Hydrolysis of methylated samples was accomplished with 90% formic acid at 100° for 2 hr. After rotary evaporation at 30–35° to remove the formic acid, the residue was dissolved in 0.27 N HCl and kept at 100° for 12 hr. For analysis by gas chromatography–mass spectrometry, the products of hydrolysis were converted to alditol acetates by reduction with sodium borohydride followed by acetylation with acetic anhydride and pyridine.

TABLE I: Molar Ratios of Mannose to *N*-Acetylglucosamine.

Material	Analytical Procedure			
	Pmr[a]	Amino Acid Analyzer (Ninhydrin)	Elson–Morgan	From Acetolysis Pattern
$(Man)_3GlcNAc$	2.96	2.87	3.20	
$(Man)_4GlcNAc$	4.04	4.09	4.02	
Log-phase mannan	17.3	16.9		19.8
Early stationary phase mannan	13.4	13.2		13.9
Late stationary phase mannan	12.1	11.9		12.4

Enzymes. α-Mannanase, purified from *Arthrobacter* GJM-1 through the 60% ammonium sulfate precipitation step, was obtained from Dr. T. R. Thieme. The α-mannosidase and β-*N*-acetylglucosaminidase from jack bean meal were isolated by the method of Li (1967). No α-*N*-acetylglucosaminidase activity was observed at any stage of preparation. This enzyme activity was obtained from rabbit testes by the procedure of Roseman and Dorfman (1951). Testes from six New Zealand white rabbits (143 g) were homogenized with a Waring blender in 100 ml of 0.075 M citrate phosphate buffer (pH 4.6). The crude extract contained α-mannosidase and β-*N*-acetylglucosaminidase activities as well as α-*N*-acetylglucosaminidase.

α-Mannanase activity was assayed by the procedure of Jones and Ballou (1968). α-Mannosidase and α- and β-*N*-acetylglucosaminidase activities were assayed using *p*-nitrophenyl α-D-mannopyranoside (Li, 1967) and the phenyl glycosides of both α- and β-*N*-acetylglucosamine (Kerr *et al.*, 1948) as enzyme substrates. The liberated *p*-nitrophenol was estimated from the absorbance at 420 nm (ϵ 1.5×10^4 M^{-1} cm^{-1}). For the assay using the phenyl glycosides, the enzyme reaction was stopped by immersing the tubes in a boiling-water bath for 3 min. After removing precipitated protein by centrifugation, 2.0 ml of a 1:5 dilution of the Folin phenol reagent was added, followed by 4.0 ml of 0.05 M sodium carbonate. The absorbance was read after 20 min at 680 nm (ϵ 8.6×10^3 M^{-1} cm^{-1}). All enzyme assays were performed at 37°, with enzyme and substrate blanks being run concurrently. One unit of activity is defined as the release of 1 mole of the free sugar/hr.

The digestion of mannan with α-mannanase followed the conditions of Jones and Ballou (1968). Mannan (100 mg) was incubated at 37° with 500 units of the enzyme in 100 ml of 0.05 M potassium phosphate buffer (pH 6.8), containing 10 μmoles of $CaCl_2$. The release of reducing sugar had ceased after 10 hr and did not resume with further additions of enzyme.

Immunological Methods. Anti-*K. lactis* and anti-*S. cerevisiae* sera were prepared as reported (Ballou, 1970). The procedures for precipitin and inhibition reactions are described in detail elsewhere (Raschke and Ballou, 1971).

Inhibitors. Acetolysis of the mannan yielded six fragments which were separated by gel filtration on a 2 × 200 cm Bio-Gel P-4 (−400 mesh) column. The products were mannose, mannobiose, mannotriose, mannotetraose, and two oligosaccharides with a smaller elution volume than mannotetraose. These two were not resolved by gel filtration, but were easily separated by descending paper chromatography in solvent A. The chromatographic properties of these larger fragments are discussed in the Results section.

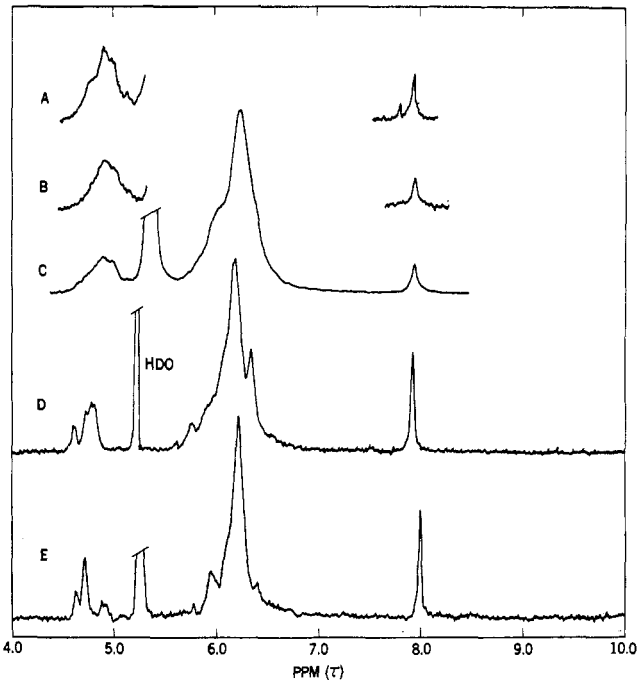

FIGURE 2: The 60-MH_z proton magnetic resonance spectra of (A log-phase mannan, (B) early stationary phase mannan, (C) late stationary phase mannan, (D) $(Man)_4GlcNAc$, and (E) $(Man)_3GlcNAc$. Ratios were estimated from the integration of the *N*-acetyl protons at 8 ppm and the α-anomeric protons at 4.5–5 ppm.

A mixture of methyl 2-acetamido-2-deoxy-α,β-D-glucosides was synthesized by the procedure of Zillikan *et al.* (1955), and was resolved by descending paper chromatography in solvent B as described by Dahlquist *et al.* (1969). Methyl 2-acetamido-2-deoxy-α-D-glucoside showed an R_{GlcNAc} of 1.38, while the β anomer had an R_{GlcNAc} of 1.19. Gel filtration on a 2 × 200 cm column of Bio-Gel P-2 (−400 mesh) in water also resolved the products, with the β-glycoside being eluted 13 ml before that of the α anomer and 42 ml ahead of *N*-acetylglucosamine. The products, after elution from paper, were passed over the gel column to remove contaminants from the paper chromatography.

Results

Mannan was isolated from cells harvested at log phase, early stationary phase, and late stationary phase (suspension turbidities of 8.7, 15.6, and 18.8, respectively). Paper chromatography of acid hydrolysates of the preparations revealed, in addition to mannose, significant amounts of an amino sugar which was identified as glucosamine by its retention time on the amino acid analyzer.

The proton magnetic resonance spectra of the mannan preparations (Figure 2) had a signal at 2.0 ppm (τ) which is characteristic of the *N*-acetyl group. The *N*-acetylglucosamine content, estimated from integration of the *N*-acetyl signal relative to the anomeric proton signals, increased as the cells went from log phase to late stationary phase (Table I).

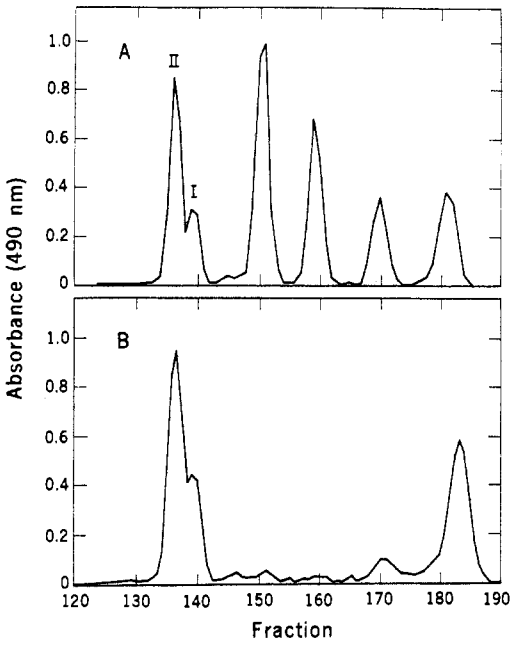

FIGURE 3: Acetolysis pattern of (A) mannan from late stationary phase *K. lactis* cells, and (B) of the same mannan after digestion with α-mannanase. Peaks I and II were shown to be $(Man)_3GlcNAc$ and $(Man)_4GlcNAc$, respectively. The other peaks, from right to left, correspond to Man, $(Man)_2$, $(Man)_3$, and $(Man)_4$. A 2 × 200 cm column of Bio-Gel P-4 (−400 mesh), eluted with water, was used.

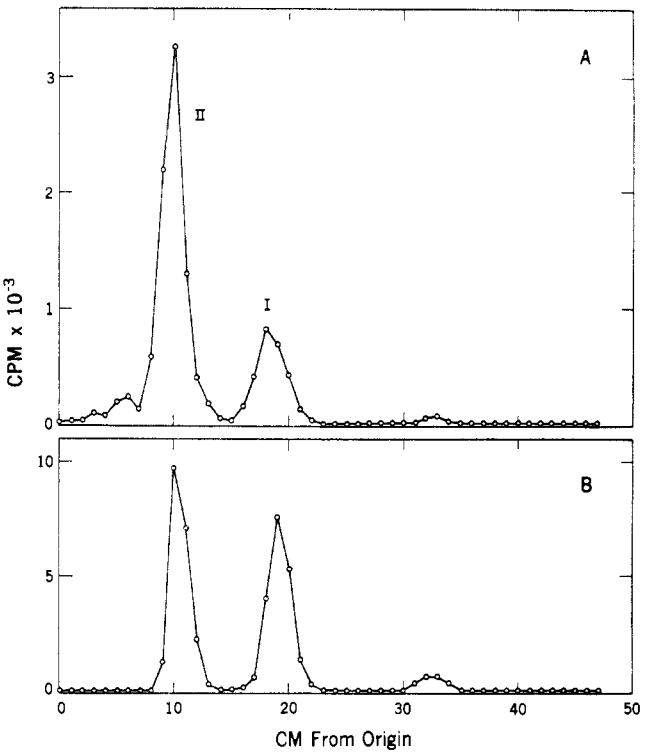

FIGURE 4: The conversion of $(Man)_4GlcNAc$ to $(Man)_3GlcNAc$ during acetolysis. Aliquots from the acetolysis reaction of *K. lactis* mannan taken at various times were deacetylated and separated on a Bio-Gel P-4 (−400 mesh) column. The fractions containing $(Man)_4GlcNAc$ and $(Man)_3GlcNAc$ were pooled, reduced with $NaBT_4$, and chromatographed in solvent A. Data for the (A) 3-hr and (B) 13-hr reactions are shown. Peaks I and II are $(Man)_3$-GlcNAc and $(Man)_4GlcNAc$, respectively.

TABLE II: Molar Ratios of Acetolysis Products.

Mannan	Oligosaccharide				
	Man[a]	$(Man)_2$	$(Man)_3$	$(Man)_4$	$(Man)_4$-GlcNAc[a]
Log phase	1.50	1.46	1.00	1.29	0.80
Early stationary phase	1.77	0.99	1.00	1.29	1.21
Late stationary phase	1.92	0.93	1.00	1.15	1.35
α-Mannanase-treated late stationary phase	4.42	0.04	0.04	0.31	1.35

[a] The values for mannose and $(Man)_4GlcNAc$ are corrected for the partial breakdown of the latter to $(Man)_3GlcNAc$ and mannose.

Isolation of Acetolysis Fragments. The acetolysis of *K. lactis* mannan has been reported (Kocourek and Ballou, 1969; Ballou, 1970) to yield mannose, mannobiose, mannotriose, mannotetraose, and what appeared to be a mannopentaose. Improved resolution of the acetolysis products (Figure 3) by using a column of Bio-Gel P-4 (−400 mesh), revealed two components with a larger effective size than $(Man)_5$,[2] both of which gave mannose and glucosamine upon hydrolysis. The increase in *N*-acetylglucosamine content with the age of the culture was correlated with an increase in these two acetolysis fragments (Table II).

The mannose to *N*-acetylglucosamine ratio in the two peaks was determined from the proton magnetic resonance (pmr) spectra (Figure 2), and by a combination of the phenol–H_2SO_4 assay for mannose (*N*-acetylglucosamine did not give a color with this reagent) and various colorimetric assays for hexosamine. The results in Table I showed that peak II was a pentasaccharide composed of four mannose residues and one *N*-acetylglucosamine, while peak I was a tetrasaccharide containing one less mannose.

A combination of gel filtration and paper chromatography provided an excellent means for purifying the two fragments. The chromatographic properties of the two fragments were affected by the presence of the *N*-acetylglucosamine, both being eluted ahead of $(Man)_5$ on gel filtration, and each moving faster than $(Man)_5$ when chromatographed on paper in solvent A. The $(Man)_3GlcNAc$, with an R_{Man} of 0.278, appeared ahead of $(Man)_4$, while $(Man)_4GlcNAc$ had an R_{Man} of 0.155 in this solvent.

The sugar at the reducing end of each oligosaccharide was determined by reduction with sodium borotritide followed by hydrolysis and paper electrophoresis. In both cases, the radioactivity remained near the origin with the mannitol standard, while glucosaminitol moved toward the cathode in the pH 3.5 buffer.

A time study of the acetolysis reaction suggested that $(Man)_3GlcNAc$ was a chemical degradation product of $(Man)_4$-

[2] Abbreviations used are: Man, D-mannose; $(Man)_2$, mannobiose; $(Man)_3$, mannotriose; $(Man)_4$, mannotetraose; $(Man)_5$, mannopentaose; $(Man)_3GlcNAc$, $(Man)_3$ substituted by *N*-acetyl-D-glucosamine; $(Man)_4GlcNAc$, $(Man)_4$ substituted by *N*-acetyl-D-glucosamine.

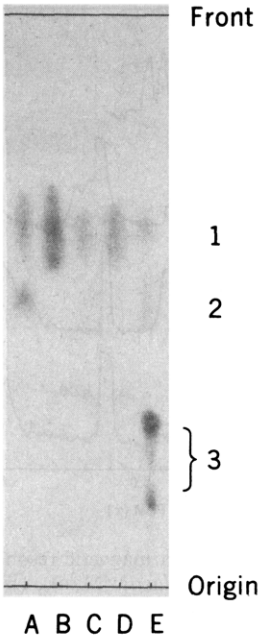

FIGURE 5: Paper chromatogram of methylated glucosamine derivatives. The $NaBT_4$-reduced substance was methylated, hydrolyzed, and chromatographed in solvent C, and the color was developed with ninhydrin. The products are from (A) chitotetraose, (B) chitobiose, (C) $(Man)_4GlcNAc$, (D) $(Man)_3GlcNAc$, and (E) *K. lactis* mannan. The products are (1) a mixture of pentamethylglucosaminitol-T and tetramethylglucosamine, (2) trimethylglucosamine, and (3) amino acids resulting from the mannan–protein.

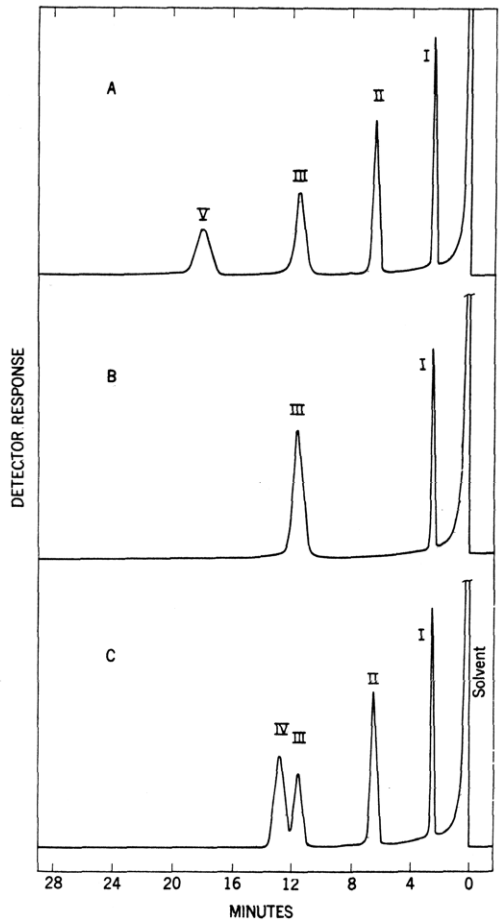

FIGURE 6: Gas chromatography of partially methylated alditol acetates from (A) $(Man)_4GlcNAc$, (B) $(Man)_3GlcNAc$, and (C) $(Man)_4$. The peaks identified by mass spectrometry are (I) 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylmannitol, (II) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, (III) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol, (IV) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, and (V) 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylmannitol.

GlcNAc. The results in Figure 4 show that during acetolysis $(Man)_4GlcNAc$ was converted to $(Man)_3GlcNAc$. The reaction had a half-time of about 13 hr. The kinetics of the reaction with isolated fragments was followed using sodium borotritide reduced $(Man)_4GlcNAc$, and the radioactivity was found to shift from reduced $(Man)_4GlcNAc$ to reduced $(Man)_3GlcNAc$ at the same rate observed with intact mannan. Ninety per cent remained in these two fragments after 13-hr acetolysis, indicating that the mannose removed by acetolysis must have come from a nonreducing terminus.

Methylation Analysis of Acetolysis Fragments. The linkages in the two oligosaccharides were investigated by methylation using the Hakomori procedure which is reported to give N-methylation (Kärkkäinen, 1970). Following reduction with $NaBT_4$, methylation, and acid hydrolysis, the products were chromatographed in solvent C, which separated tetramethylglucosamine from trimethylglucosamine (Gorin *et al.*, 1971). Chitobiose and chitotetraose were treated similarly as standards. The chromatogram, developed with ninhydrin (Figure 5), shows that chitotetraose gave two ninhydrin-positive spots. The slower component was identified as trimethylglucosamine since it was absent from the products of reduced chitobiose. The faster spot contained both radioactivity and a reducing compound (aniline phthalate positive), and must have been a mixture of pentamethylglucosaminitol-T and tetramethylglucosamine. The products from both $(Man)_4GlcNAc$ and $(Man)_3GlcNac$ contained tetramethylglucosamine, thereby proving the *N*-acetylglucosamine in these two components was at a nonreducing terminus. The same result was obtained with intact mannan (Figure 5), in agreement with the results of Gorin *et al.* (1971) for other mannans. The small amount of trimethylglucosamine from the mannan probably came from the linkage point between mannan and the protein which is attached to the polysaccharide (Sentandreu and Northcote, 1968).

The hydrolysis products of the methylated oligosaccharides were converted to alditol acetates for analysis by combined gas chromatography (Figure 6) and mass spectrometry. In addition to pentamethylmannitol from the reducing end mannose unit, the $(Man)_4GlcNAc$ products contained a tetramethylmannitol, a trimethylmannitol, and a dimethylmannitol. The dimethylmannitol must have come from a branch point in the oligosaccharide, presumably a mannose substituted by the *N*-acetylglucosamine and the acetolysis-labile mannose. This conclusion was confirmed by analysis of $(Man)_3GlcNAc$. The tetramethylmannitol, which resulted from a nonreducing end in $(Man)_4GlcNAc$, and the dimethylmannitol from the branch point were both absent. The size of the trimethylmannitol peak from $(Man)_3GlcNAc$ was doubled, as expected if the previously disubstituted mannose had lost one substituent. The methylated glucosamine was not observed in these patterns, having been removed by the Dowex 50 (H^+) treatment after the reduction step.

The $(Man)_4$ side chain obtained by acetolysis of *K. lactis* mannan was treated in the same manner (Figure 6). In addition to a pentamethylmannitol and a tetramethylmannitol, two trimethylmannitols were observed (peaks III and IV).

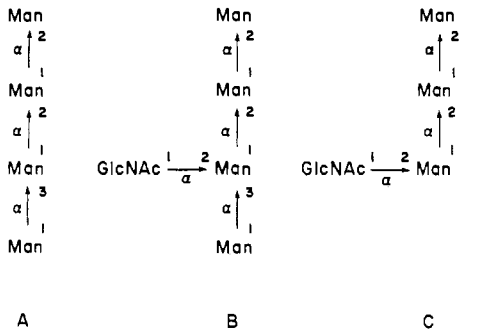

FIGURE 7: Structure of the three largest fragments from acetolysis of *K. lactis* mannan.

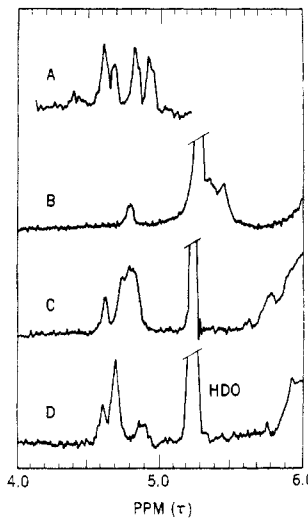

FIGURE 8: The 60-MH_z proton magnetic resonance spectra of the anomeric protons of (A) *K. lactis* $(Man)_4$, (B) chitotetraose, (C) $(Man)_4GlcNAc$, and (D) $(Man)_3GlcNAc$. Tetramethylsilane was used as an external standard. The α-anomeric signals are at 4.5–5 ppm and the β-anomeric signals at 5.2–5.5 ppm.

These products were identical with those from the mannotetraose of *S. cerevisiae* S288C mannan, which is known to give equal amounts of 2,4,6- and 3,4,6-trimethylmannitol upon methylation (Lee and Ballou, 1965). The $(Man)_2$ and $(Man)_3$ gave expected ratios of 2,3,4,6-tetramethyl- and 3,4,6-trimethylmannitol confirming that all linkages are 1→2 in these side chains.

The peaks from gas chromatography were identified by mass spectrometry. In all cases, peak I gave the characteristic fragments of 1,3,4,5,6,-pentamethylmannitol (*m/e* 205, 161, 133, and 89; with secondary fragments at *m/e* 145, 129, and 101). Peak II was derived from 2,3,4,6-tetramethylmannitol, and gave the characteristic fragments at *m/e* 205, 161, 145, 129, 117, and 101.

The absence of a fragment at *m/e* 117 characterized peak III as 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol. This fragment is obtained when C1 is acetylated and C2 is methylated, and it is observed in all other possible trimethyl derivatives (Björndal *et al.*, 1967). The spectrum for peak III from $(Man)_3GlcNAc$ lacked the *m/e* 117 fragment, eliminating the possibility that a second trimethylmannitol cochromatographed with the 3,4,6 derivative. Fragments from this peak had *m/e* 189, 161, 129, and 101.

Peak IV was derived only from $(Man)_4$. Since this side chain has the same structure as the mannotetraose from *S. cerevisiae* (Ballou, 1970), it was expected that this peak was 2,4,6-trimethylmannitol resulting from the 1→3 linkage. Fragments at *m/e* 233, 189, 161, 129, and 117 confirmed this assignment. The dimethylmannitol (peak V) from $(Man)_4GlcNAc$ was characterized by fragments at *m/e* 261, 161, 129, and 101, and a 4,6-dimethylglucitol standard gave an identical spectrum.

From these data, the linkages in $(Man)_4GlcNAc$ and $(Man)_3GlcNAc$ can be assigned (Figure 7B, C). The structure of the tetrasaccharide was not unequivocally established by this study, since the mannose substituted at position 3 could be in either internal position. However, previous immunochemical data (Ballou, 1970) confirm the structure presented in Figure 7A.

Determination of Anomeric Configurations. The pmr spectra of the anomeric protons of $(Man)_4$, $(Man)_4GlcNAc$, $(Man)_3GlcNAc$, and chitotetraose are shown in Figure 8. The $(Man)_4$ had a spectrum identical with that reported previously for the α-linked mannotetraose of *S. cerevisiae* (Lee and Ballou, 1965). Gorin and Spencer (1970) demonstrated that the signal at τ 4.61 came from the reducing end mannose, the τ 4.69 signal from the second mannose, the τ 4.95 signal from the third mannose, and the τ 4.83 signal from the nonreducing end mannose.

The chemical shifts of the anomeric protons of *N*-acetylglucosamine were determined from the spectrum of chitotetraose. The β-anomeric signals appeared beneath the HDO line and slightly upfield from it, while the α signal from the unit at the reducing end had a chemical shift of τ 4.80. Neither $(Man)_4GlcNAc$ nor $(Man)_3GlcNAc$ had an anomeric proton signal near the HDO line. The proton at τ 4.89 in $(Man)_3GlcNAc$ was clearly from an α-linked *N*-acetylglucosamine. In both oligosaccharides, the reducing end anomeric proton was characteristically observed at τ 4.61, since free mannose occurs mainly in the α configuration. The peak at τ 4.70 in D with the area of two protons was assigned to the two internal mannose units of $(Man)_3GlcNAc$. The additional mannose in $(Man)_4GlcNAc$ apparently deshielded the *N*-acetylglucosamine anomeric proton, while the anomeric signal for the mannose at the nonreducing end was also deshielded by the close proximity of the *N*-acetylglucosamine. These interactions led to the unresolved lines in C between τ 4.72 and 4.83, which included the anomeric proton signals from 3 mannose units and 1 *N*-acetylglucosamine.

The $(Man)_4$ and $(Man)_3GlcNAc$ had almost identical specific rotations, $[\alpha]_{546}$ +111 and +110°, respectively, values which are consistent for oligosaccharides with α linkages. $(Man)_4GlcNAc$ had a specific rotation of $[\alpha]_{546}$ +132°. Since the addition of the *N*-acetylglucosamine to the $(Man)_4$ structure gave a larger positive rotation, the *N*-acetylglucosamine must also have had an α linkage.

Optical rotatory dispersion measurements confirmed this assignment (Beychok and Kabat, 1965; Kabat *et al.*, 1969). The 2-acetamido group gives a negative Cotton effect with a trough at 220–230 nm. *N*-Acetylglucosamine in β linkage exhibits a deep trough, while the α anomer shows only a slight depression (Figure 9). The spectra of $(Man)_4$ and $(Man)_4GlcNAc$ are also shown in Figure 9, with $(Man)_4$ giving a simple dispersion curve with no Cotton effect. The difference spectrum gave a curve that was almost identical with that of methyl 2-acetamido-2-deoxy-α-D-glucoside.

Enzymatic Digestion of Mannan and Its Acetolysis Fragments. The α-mannanase excreted by *Arthrobacter* GJM-1 (Jones and Ballou, 1968, 1969a,b) released 50% of the mannose from *K. lactis* mannan, leaving a polysaccharide

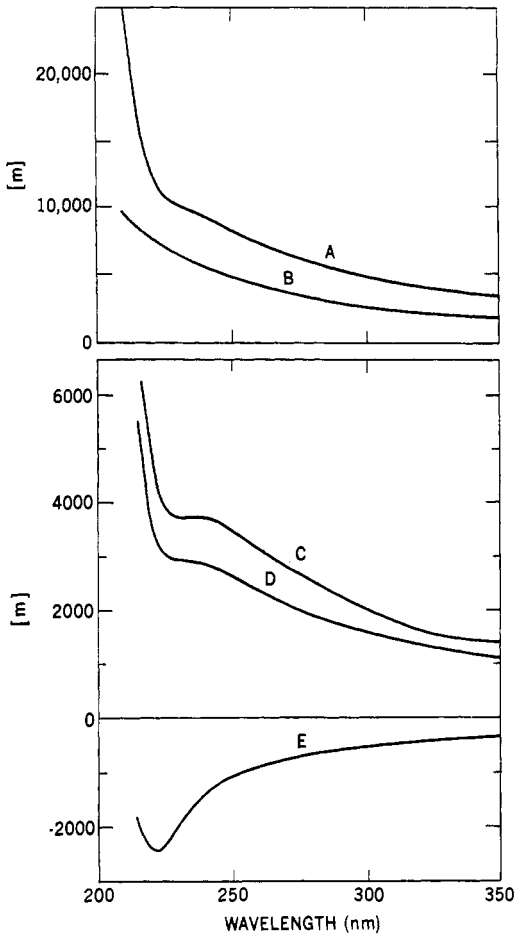

FIGURE 9: Optical rotatory dispersion spectra of (A) $(Man)_4GlcNAc$ and (B) $(Man)_4$; the methyl glycosides of (D) α- and (E) β-*N*-acetylglucosamine; and (C) the difference spectrum obtained by subtracting the curve of $(Man)_4$ from that of $(Man)_4GlcNAc$.

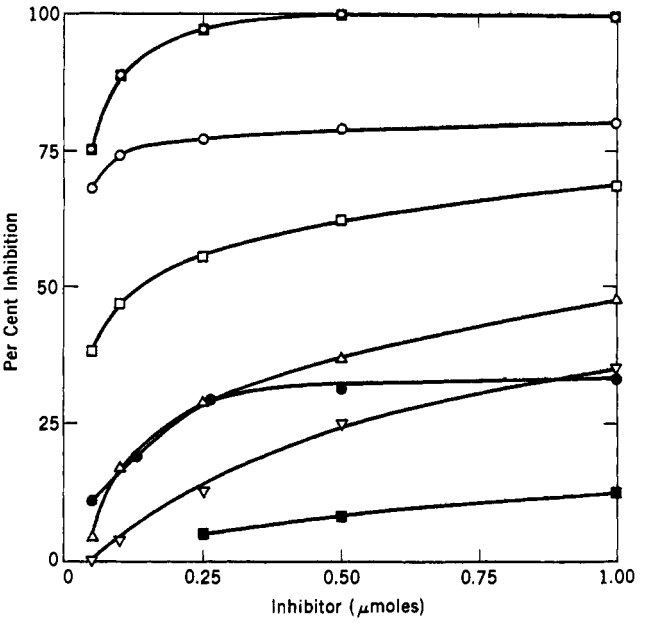

FIGURE 11: Inhibition of the precipitin reaction between anti-*K. lactis* serum and *K. lactis* mannan from late stationary phase cells. Inhibitors are $(Man)_4GlcNAc$ + $(Man)_3$ (⊙), $(Man)_4GlcNAc$ (○), $(Man)_3GlcNAc$ (●), $(Man)_4$ (□), $(Man)_3$ (△), $(Man)_2$ (▽), and Man (■). For each assay, 50 μl of antiserum and 10 μg of mannan were used.

residue that gave the acetolysis pattern shown in Figure 3B. The $(Man)_4GlcNAc$ side chain had remained intact, while the $(Man)_4$, $(Man)_3$, and most of $(Man)_2$ were eliminated (see also Table II). Apparently the *N*-acetylglucosamine hindered the action of the enzyme on the terminal mannose unit of $(Man)_4GlcNAc$. The alternative possibility that this oligosaccharide formed a part of the backbone can be discounted by the immunochemical data presented below as well as the observation that free $(Man)_4GlcNAc$ was not hydrolyzed by the α-mannanase.

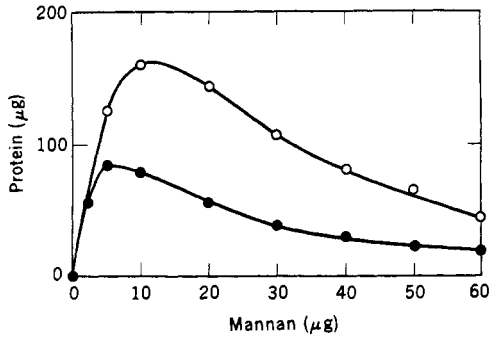

FIGURE 10: Anti-*K. lactis* precipitin curves with late stationary phase *K. lactis* mannan before (○) and after (●) digestion with α-mannanase. Each assay contained 50 μl of anti-*K. lactis* serum.

Purified $(Man)_4GlcNAc$ was resistant to all enzyme preparations tested. These included the bacterial α-mannanase, α-mannosidase, and β-*N*-acetylglucosaminidase from jack bean meal, and extracts of rat epididymis and rabbit testes. The latter two sources are reported to contain an α-*N*-acetylglucosaminidase in addition to α-mannosidase and β-*N*-acetylglucosaminidase (Findlay *et al.*, 1958; Roseman and

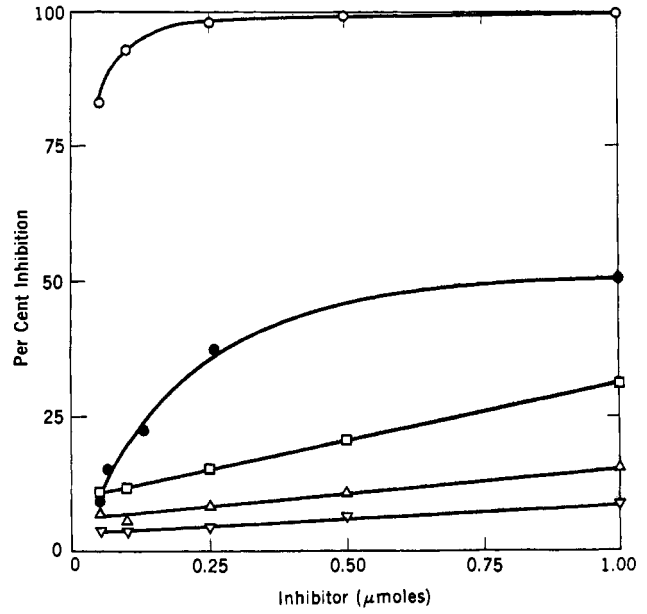

FIGURE 12: Inhibition of the precipitin reaction between anti-*K. lactis* serum and *K. lactis* mannan digested by α-mannanase. Inhibitors are $(Man)_4GlcNAc$ (○), $(Man)_3GlcNAc$ (●), $(Man)_4$ (□), $(Man)_3$ (△), and $(Man)_2$ (▽). For each assay, 100 μl of antiserum and 15 μg of digested mannan were used.

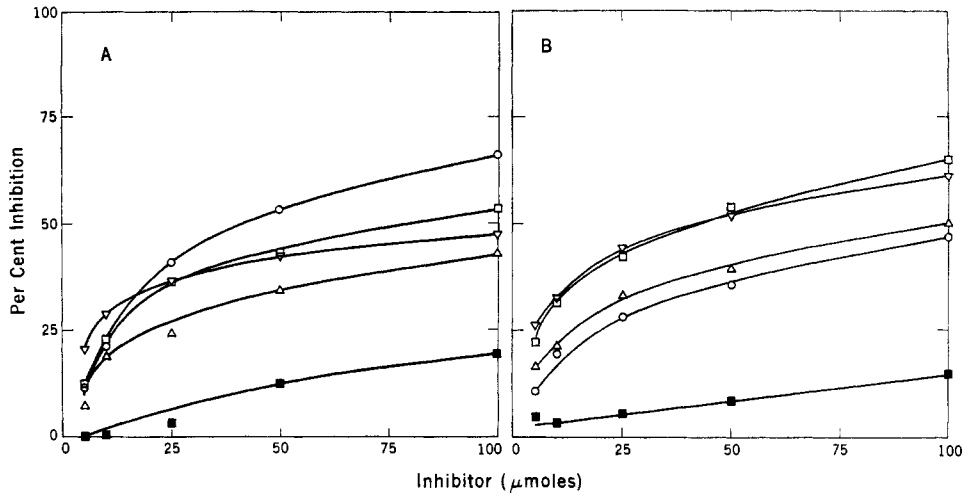

FIGURE 13: Inhibition of the precipitin reaction between anti-*K. lactis* serum and (A) intact *K. lactis* mannan, or (B) α-mannanase-digested *K. lactis* mannan. Inhibitors are Man (○), GlcNAc (□), Me-α-GlcNAc (▽), and Me-β-GlcNAc (△). The degree of nonspecific inhibition is illustrated with glucose (■). Each assay in part A contained 50 μl of antiserum and 10 μg of mannan, and in part B 100 μl of antiserum and 15 μg of digested mannan.

Dorfman, 1951). While significant α-mannosidase and β-*N*-acetylglucosaminidase activities were found, the α-*N*-acetylglucosaminidase activity was low. A 25-hr incubation of the rabbit testes extract, containing 0.512 unit of α-*N*-acetylglucosaminidase activity, failed to cleave any linkages in 0.073 μmole of reduced $(Man)_4GlcNAc$. A similar digestion using 0.112 μmole of $(Man)_3GlcNAc$ also failed to remove any residues.

Immunochemical Studies. Some of the immunochemical properties of *K. lactis* mannan were reported earlier (Ballou, 1970). The precipitin reaction of anti-*K. lactis* serum with mannan from cells in late stationary phase is illustrated in Figure 10. The mannans from log phase and early stationary phase cells, while having higher *N*-acetylglucosamine content, gave the same precipitin curve. All immunochemical data are for mannan from late stationary phase cells.

Of the purified products of acetolysis, $(Man)_4GlcNAc$ was the best inhibitor of the precipitin reaction (Figure 11), but complete inhibition was obtained only with a mixture of $(Man)_4GlcNAc$ and $(Man)_4$. Thus, the antiserum contained antibodies directed against both of these structures in the mannan. The relatively low inhibition exhibited by $(Man)_3$-GlcNAc demonstrates the importance of the terminal mannose unit in $(Man)_4GlcNAc$ for antibody binding.

Treatment of the mannan with α-mannanase led to a weaker precipitin reaction (Figure 10), reflecting the removal of the $(Man)_4$ side chain. Precipitation now became dependent solely on the antibody population which recognized the $(Man)_4$-GlcNAc determinant, since this fragment gave 100% inhibition while $(Man)_4$ inhibition was greatly diminished (Figure 12).

The complete loss of the tetrasaccharide determinant was demonstrated with antiserum against *S. cerevisiae* S288C cells. This antiserum reacted strongly with intact *K. lactis* mannan, reflecting the similar structures of the $(Man)_4$ side chains in the two yeast mannans. On the other hand, enzymatic removal of the mannose side chains from the *K. lactis* mannan resulted in complete loss of reaction with *S. cerevisiae* antiserum. Clearly, antibodies against $(Man)_4$ did not recognize the similar structural features of $(Man)_4GlcNAc$, so that the addition of the *N*-acetylglucosamine created a completely distinct antigenic determinant.

Any inhibition by $(Man)_4$ of the anti-*K. lactis* precipitin reaction with mannanase-digested mannan must have been due to cross-reaction with antibodies against the $(Man)_4$-GlcNAc determinant. This must also be the case for the inhibition by $(Man)_3GlcNAc$, since this structure probably does not exist in the mannan. In fact, this study provides an assessment of the immunological importance of the various features of the $(Man)_4GlcNAc$ structure. Although both of the nonreducing end units of the oligosaccharide must be important for antibody recognition, the greater inhibition by $(Man)_3$-GlcNAc than by $(Man)_4$ indicates that the α-linked *N*-acetylglucosamine was more critical to the binding of antibody than was the terminal mannose residue. This conclusion was substantiated by the greater inhibition in this system by *N*-acetylglucosamine (and its methyl glycoside) than by mannose (Figure 13B). The immunological activity of both of these units confirms that $(Man)_4GlcNAc$ was a side chain in the polysaccharide and that its resistance to α-mannanse degradation was due to the presence of the *N*-acetylglucosamine.

Since mannose contributes to the antigenic structure of both $(Man)_4$ and $(Man)_4GlcNAc$, it was expectedly a better inhibitor than *N*-acetylglucosamine in the precipitin reaction with intact mannan (Figure 13A). However, when enzyme-digested mannan was used (Figure 13B), specificity for the *N*-acetylglucosamine residue predominated. The relative inhibitions by the methyl glycosides of α- and β-*N*-acetylglucosamine provided additional support for the α-anomeric linkage of *N*-acetylglucosamine in the mannan.

Additional Properties of K. lactis Mannan. The possibility was considered that *K. lactis* cells contained two types of mannan, one with $(Man)_4GlcNAc$ side chains and one similar to *S. cerevisiae* mannan with side chains lacking *N*-acetylglucosamine. Therefore, the mannan was fractionated by gel filtration on Bio-Gel A-5m (Thieme and Ballou, 1972) and assayed for mannose and *N*-acetylglucosamine. The results (Figure 14) revealed the presence of three components, one appearing at the void volume of the column and two included. *N*-Acetylglucosamine was found in all three, although the ratio of mannose to aminohexose varied slightly from peak to peak. The various components of the log-phase mannan

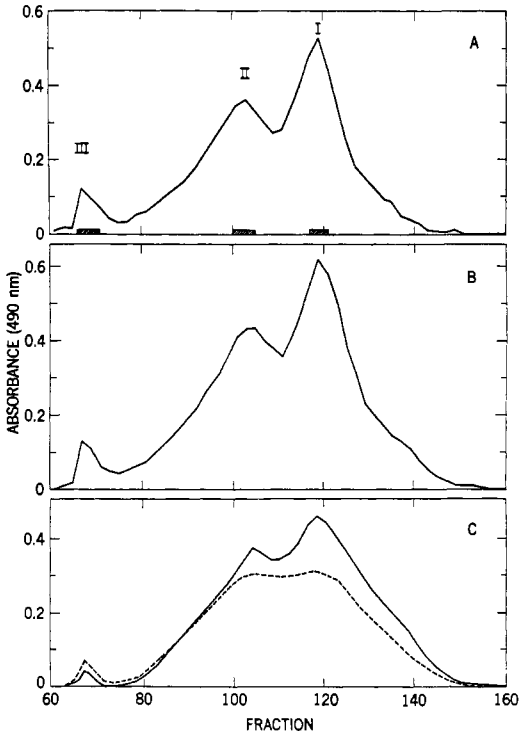

FIGURE 14: Gel filtration on Bio-Gel A-5m of *K. lactis* mannans. The patterns represent (A) log-phase mannan, (B) early stationary phase mannan, and (C) late stationary phase mannan. The solid lines represent carbohydrate, and the dashed line in part C represents glucosamine. The fractions indicated in part A were pooled for sedimentation analysis. A 2 × 200 cm column eluted with 0.2 M KCl was used.

separated in this way were found by sedimentation velocity and equilibrium to range in molecular size from 60,700 to over five million. This observation is similar to that reported for the phosphomannan from *Kloeckera brevis* (Thieme and Ballou, 1972).

Discussion

The unique serological properties of the yeast *K. lactis* are related to the *N*-acetylglucosamine residues which occur in high amounts in the cell wall mannan, most of which is attached to mannose side chains in the polysaccharide. Rabbit antisera against intact *K. lactis* cells had two major specificities, one for the mannan side chain containing *N*-acetylglucosamine and the other for a mannotetraose side chain which is also found in *S. cerevisiae* baker's yeast. While neither hapten alone gave 100% inhibition of the precipitation reaction, together complete inhibition was obtained. Further evidence for the distinct antigenic nature of $(Man)_4GlcNAc$ was that *S. cerevisiae* antiserum, which contained antibodies specific for the $(Man)_4$ side chain, cross-reacted negligibly with the $(Man)_4GlcNAc$ structure. The *K. lactis* chemotype is illustrated in Figure 15.

The acetolysis of *K. lactis* mannan yielded two fragments containing *N*-acetylglucosamine, $(Man)_3GlcNAc$ and $(Man)_4$-GlcNAc, the former being a degradation product of the latter. The structures of the fragments were determined by methylation analysis. Mannose and *N*-acetylglucosamine were shown to be at nonreducing terminals of $(Man)_4GlcNAc$, while $(Man)_3GlcNAc$ had only *N*-acetylglucosamine at this position. All linkages in $(Man)_3GlcNAc$ were identified as 1→2,

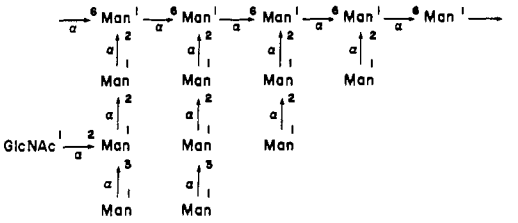

FIGURE 15: Representative structure of *K. lactis* mannan.

while the additional mannose in $(Man)_3GlcNAc$ was attached in 1→3 linkage to the third mannose residue of the chain. All linkages in these fragments were determined to have the α configuration by proton magnetic resonance, optical rotation, optical rotatory dispersion, and antiserum inhibition analyses. Thus, $(Man)_4GlcNAc$ has the structure of the mannotetraose side chain of the mannan with an *N*-acetylglucosamine attached to the third mannose residue by an α-1→2 linkage.

The lability to acetolysis of the 1→3 linkage of $(Man)_4$-GlcNAc was unexpected since this same linkage in the $(Man)_4$ side chain was comparatively stable to this treatment (Stewart *et al.*, 1968). Clearly, the difference resulted from the presence of the *N*-acetylglucosamine attached to position 2 of the mannose residue and could be due to steric factors or to participation in the reaction by the acetamido group.

The structural identity of $(Man)_4$ and the mannotetraose part of $(Man)_4GlcNAc$ suggests that the latter may be formed by a transfer of *N*-acetylglucosamine to a preexisting mannotetraose unit. Excluding the *N*-acetylglucosamine-containing side chain, *K. lactis* mannan is very similar to the mannan of *S. cerevisiae* S288C, and it is possible that the pathways for biosynthesis of mannan in the two strains differ only in the presence or absence of an enzyme capable of transferring *N*-acetylglucosamine to the mannotetraose side chain. The change in *N*-acetylglucosamine content of the mannan with the age of the culture is an interesting but unexplained phenomenon.

Other yeasts in the *Kluyveromyces* genera, *Kluyveromyces marxianis* and *Kluyveromyces dobzhanskii*, also possess the $(Man)_4GlcNAc$ side chain (P. Hsiao and C. E. Ballou, unpublished results). If this structure is present in all *Kluyveromyces* strains, it may provide a useful taxonomic test for this group. The report of unusually high amounts of *N*-acetylglucosamine in the mannans of a variety of yeasts (Gorin *et al.*, 1971) suggests that this structure may be widespread.

Several serologically active groups have now been defined in yeast mannans (see Figure 16). The terminal 1→3-mannosyl unit has been shown to be an important determinant in *S.*

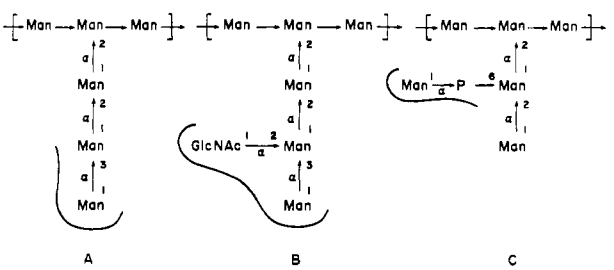

FIGURE 16: Immunodominant structures in yeast mannans. The structures presented are the representative side chains for (A) *Saccharomyces cerevisiae* S288C, (B) *Kluyveromyces lactis* NRRL 1140, and (C) *Kloeckera brevis* 55-45.

cerevisiae baker's yeast (Suzuki *et al.*, 1968) and in *K. lactis* (Ballou, 1970). Both α-1→2- and α-1→3-linked D-mannose units appear to be important determinants in *Candida* species (Suzuki and Sunayama, 1968; Sunayama, 1970; Sunayama and Suzuki, 1970). The α-D-mannosyl 1-phosphate group is immunodominant in *Kloeckera brevis* (Raschke and Ballou, 1972) and in *S. cerevisiae* brewer's strains obtained from Guinness Laboratories (N. Cawley and C. E. Ballou, unpublished data). Finally, the immunological importance of the α-1→2-linked *N*-acetylglucosamine units in the side chains of *K. lactis* mannan has been demonstrated in this study. From this and other work, there is being revealed for yeasts a whole new family of cell surface determinants which parallel in many ways the bacterial O-antigen and the blood group substances (Nikaido and Hassid, 1971). What role they play in yeast physiology is still undetermined.

Acknowledgments

We thank Dr. Gary Gray for assistance in obtaining and interpreting the proton magnetic resonance spectra and Michael Stallcup for his help with the optical rotatory dispersion measurements.

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