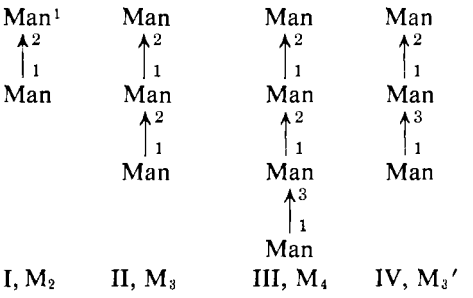
# A Comparison of Yeast Mannans and Phosphomannans by Acetolysis*

T. S. Stewart and Clinton E. Ballou

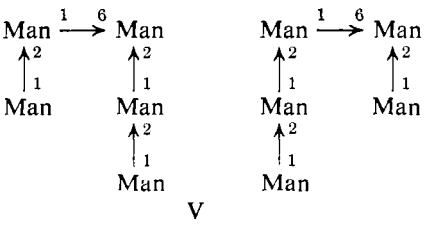
ABSTRACT: Controlled acetolysis of yeast mannan yields a mixture of relatively stable oligosaccharides containing 1→2 and 1→3 linkages, which result from the cleavage of the 1→6 linkages of the backbone. The composition of the mixture of oligosaccharides is characteristic of the strain of yeast from which the mannan was obtained. Acetolysis thus provides a useful tool for comparing the structures of the different mannans. The phosphorus content of different yeast mannan preparations varies widely. If the isolation is carried out under neutral conditions, the phosphate is diesterified. If the mannan is exposed to alkali or acid during isolation, the phosphate is converted into the monoester form. Acetolysis of phosphomannan yields mannobiose phosphate and mannotriose phosphate, both of which have the phosphate on the reducing unit of the oligosaccharide. Thus, the phosphate must be on position 3 or 4 of the mannose units, since all other positions are involved in glycosidic linkages in the original polysaccharide. A bacterial $\alpha$-mannosidase has been shown to remove most of the 1→2- and 1→3-linked side chains from the phosphomannan of *Kloeckera brevis*, leaving the backbone structure of 1→6-linked mannose units intact and greatly enriched in phosphate.

An important component of the yeast cell wall is the polysaccharide mannan. Numerous investigations on the mannans from a variety of yeast strains have been published and the proposed structures have differed considerably. However, all seem to share $\alpha$-1→2, $\alpha$-1→3, and $\alpha$-1→6 linkages as common structural features. A structure for the mannan from *Saccharomyces cerevisiae* was first published by Haworth *et al.* (1941), and modified later by Peat *et al.* (1961a,b). From methylation studies on the intact polymer, coupled with the isolation of a series of 1→6-linked oligosaccharides following acid hydrolysis, the latter proposed that the mannan had a "backbone" of 1→6-linked mannose units with 1→2- and 1→3-linked side chains that averaged about two sugar units in length.

In studies on *S. cerevisiae* mannan (commercial bakers' yeast), we have employed acetolysis (Gorin and Perlin, 1956) as a procedure for the selective cleavage of the 1→6 linkages and have obtained excellent yields of the relatively stable 1→2- and 1→3-linked oligosaccharides (Lee and Ballou, 1965). The main products are the di-, tri-, and tetrasaccharides I, II, and III, the first two having been described previously by Gorin and Perlin (1956). Recently, we have described the isolation of another trisaccharide, IV, which was shown to be an integral part of the mannan molecule (Stewart *et al.*, 1968). We have refined the conditions of acetolysis to minimize the further degradation of these products, and we believe that the ratio of small oligosaccharides obtained in this manner represents that in the intact mannan.




As stated above, these units are thought to be assembled *via* 1→6 linkages involving the mannose at the reducing end. As direct evidence for this we have shown that controlled acetolysis of the mannan produces oligosaccharides which contain intact 1→6 linkages. Penta- to heptasaccharides were obtained (Stewart *et al.*, 1968), and the structure of the pentasaccharide fraction is shown in V as an illustration of the nature of these products. Although we do not imply any particular ratio or order of the units in the chain, the mannan from *S.*

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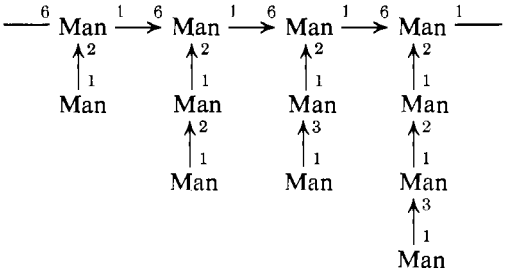
[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: Man, mannose.

*cerevisiae* probably has a structure like that depicted in VI.



VI

Peat *et al.* (1961a) reported that bakers' yeast mannan contained equal amounts of 1→2- and 1→3-linked mannose units. From our own methylation studies, we calculated a ratio of 2.5:1. Therefore, we came to wonder whether the mannans might not vary considerably between different strains of *S. cerevisiae*. Such variation is also suggested by the known specificity of antibodies that are formed to mannans from different strains of yeast (Summers *et al.*, 1964). It became clear, after the examination of several *S. cerevisiae* mannans, that acetolysis is an excellent tool whereby one can compare in a unique way the structures of these polysaccharides. With the aid of gel permeation chromatography, separation of the small oligosaccharides is achieved, enabling quantitation of the degradation products. In addition to the mannans of *S. cerevisiae*, we have compared the mannans from *Saccharomyces carlsbergensis*, *Kloeckera brevis*, *Candida albicans* (792), and *Candida stellatoidea*. For several of the mannans, methylation studies were performed on the intact polysaccharide in conjunction with degradation by acetolysis. All results suggest that the mannan structure is a highly characteristic feature of each organism.

Phosphomannans have been isolated from several strains of yeast (Jeanes and Watson, 1962; Slodki, 1962). These have usually been exocellular, although Mill (1966) has reported a cell wall mannan from *S. cerevisiae* to have a mannose to phosphorus ratio of 15. We have reported a much lower phosphorus content for bakers' yeast mannan (Stewart *et al.*, 1968), as have several other investigators (Northcote and Horne, 1952; Falcone and Nickerson, 1956). In the present study, we have observed a great variation in the phosphorus content of different mannans, the highest being in *K. brevis* mannan which has one phosphate for every nine mannose units. We have investigated the structure of this phosphomannan and report here our observations.

## Experimental Procedure

*Materials. S. cerevisiae* (238C), *S. carlsbergensis*, and *K. brevis* were grown at 30°, by aeration or with shaking, on the following medium: glucose (50 g), Difco Casamino acids (3 g), yeast extract (5 g), citric acid (1 g), sodium citrate (5 g), $KH_2PO_4$ (0.55 g), KCl (0.425 g), $MgSO_4 \cdot 7H_2O$ (0.125 g), $CaCl_2 \cdot 2H_2O$ (0.125 g), $MnSO_4$ (2.5 mg), $FeCl_3 \cdot 6H_2O$ (2.5 mg), $(NH_4)_2SO_4$ (3.75 g), myoinositol (10 mg), and water to 1 l. The harvested cells were stored at −10°. The two bakers' yeasts, strains 1 and 2, were supplied by the Red Star Co., Oakland, Calif.

Mannans were isolated, either by extraction with pH 7 citrate buffer (Peat *et al.*, 1961a) or by alkali extraction followed by copper complex formation (Cifonelli and Smith, 1955). Isolation of *K. brevis* (H. Phaff 5545) mannan with intact diesterified phosphate groups is described later in the text. We are indebted to Dr. H. F. Hasenclever for gifts of *C. albicans* (792) and *C. stellatoidea* mannans.

Alkaline phosphatases (from chicken intestine and *Escherichia coli*) were from the Worthington Biochemical Corp., and were used without further purification. Bacterial α-mannosidase, the description of which will appear elsewhere (G. H. Jones and C. E. Ballou, unpublished data), was prepared in this laboratory.

Sephadex G-25 (fine bead and irregular), G-50 (fine bead), and DEAE Sephadex A-25 (medium) were obtained from Pharmacia. DEAE Sephadex was converted into its bicarbonate form. Sephadex G-25 gel permeation chromatography was done on a 2 × 150 cm column.

*General Procedures.* Analytical procedures for total carbohydrate (Dubois *et al.*, 1956) and phosphorus (Bartlett, 1959) have been described (Stewart *et al.*, 1968). Reducing sugar was determined as described by Palog (1959) and protein by the Lowry method (Lowry *et al.*, 1951).

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) 1-butanol–ethanol–water (3:1:1), (C) 1-butanol–pyridine–water (3:1:1), (D) 2-propanol–ammonia–water (7:1:2), and (E) isobutyric acid–ammonia–water (66:1:33). Thin-layer chromatography was carried out on silica gel G (Merck, Damstadt, Germany) using solvent, F, benzene containing 5–7% methanol.

Sugars were detected on paper chromatograms with the silver nitrate–sodium hydroxide or periodate–benzidine reagents (Gordon *et al.*, 1956). On thin-layer chromatograms, acetylated sugars were detected by the hydroxylamine–ferric chloride spray described by Tate and Bishop (1962). Phosphorus was detected on paper chromatograms with the Hanes and Isherwood (1949) spray or with the dip reagent described by Harrap (1960).

Methylation and trimethylsilylation procedures, together with gas chromatographic analysis of the methanolyzed products, have been described (Stewart *et al.*, 1968). Mannan samples were methylated by the technique of Hakomori (1965), and then were further methylated, either by refluxing in benzene with sodium wire and then with methyl iodide, or by using silver oxide and methyl iodide in dimethylformamide.

Acetylation, reduction, acetolysis, both of the free oligomer or peracylated samples and deacetylation procedures have been described (Stewart *et al.*, 1968). In the presence of phosphorylated oligosaccharides, deacetylation with sodium methoxide, rather than with barium methoxide, was preferred to eliminate the possibility of forming insoluble barium salts.

TABLE I: Neutral Stable Acetolysis Products of Various Mannans.

| Mannan | Oligosaccharides[a] | | | | | |
|---|---|---|---|---|---|---|
| | M | $M_2$ | $M_3$ | $M_4$ | $M_5$ | $M_6$ |
| Bakers' yeast (Red Star, strain 1) | 0.44 | 1.05 | 1.00 | 0.43 | | |
| Bakers' yeast (Red Star, strain 2) | 0.75 | 1.15 | 1.00 | 0.50 | | |
| *S. cerevisiae* (238C), 7-hr culture | 1.06 | 1.60 | 1.00 | 0.65 | | |
| *S. cerevisiae* (238C), 48-hr culture | 0.81 | 1.50 | 1.00 | 0.66 | | |
| *S. carlsbergensis* (ATCC 9080) | 1.25 | 1.75 | 1.00 | 0.28 | | |
| *K. brevis* (H. Phaff 5545) | 0.87 | 2.16 | 1.00 | | | |
| *C. albicans* (792) | 12.0 | 2.16 | 1.00 | 1.20 | 0.65 | 0.36 |
| *C. stellatoidea* | 3.9 | 2.92 | 1.00 | 1.00 | 0.73 | 0.79 |

[a] Molar ratios relative to $M_3$.

The separation of acidic and neutral products of acetolysis was achieved by batchwise elution from DEAE Sephadex A-25 ($HCO_3$ form). A sample (10–200 mg) was applied to a small DEAE Sephadex column (50-ml bed volume), and the neutral components were eluted with 0.01 M $NH_4HCO_3$. The $NH_4HCO_3$ was removed from the fractions by repeated evaporations at 40° on a rotatory evaporator. Sephadex G-25 gel permeation chromatography of phosphorylated sugars was carried out in 0.2 M $NH_4HCO_3$ to lessen the "ion effect" which caused the components to spread when water was the eluent.

α-Mannosidase incubation was performed at 37° in the following mixture: 0.01 M phosphate buffer (pH 6.7), 50 ml; mannan, 20 mg; bovine serum albumin, 5 mg; $Ca^{2+}$, to $10^{-4}$ M; and mannosidase, 2 mg of total protein. The digestion was followed by assaying for the reducing sugar that was released. On completion, the mixture was concentrated, boiled, and then centrifuged. The supernatant was applied to a Sephadex G-25 column (bead form) and eluted with 0.2 M $NH_4HCO_3$.

## Results

*Prolonged Acetolysis of Mannans.* Acetolysis of each mannan was carried out for 13 hr at 40°. The products were isolated, separated into neutral and acidic (phosphorylated) components where necessary, then the neutral oligosaccharides were separated on Sephadex G-25 (irregular). The elution patterns of the neutral products of acetolysis of the several mannans are shown in Figure 1. All components of the mixtures are 1→2- and 1→3-linked mannooligosaccharides, except peak VII of Figure 1E, which appears to be unreacted *C. albicans* mannan.

A comparison of the mannan structures was made by examining the ratios of oligomers present after acetolysis. For example, the several mannans show a large variation in the amount of tetrasaccharide (peak IV). *K. brevis* mannan (Figure 1C) contains no tetrasaccharide, but the amount increases progressively with the mannans of *S. carlsbergensis* (Figure 1D), *S. cerevisiae* (bakers' yeast) (Figure 1A), and *S. cerevisiae* (238C) (Figure 1B). The *Candida* mannans show patterns that are quite distinct from the other four mannans, both yielding 1→2- and 1→3-linked penta- and hexasaccharides as acetolysis products (peaks V and VI, Figure 1E,F). The areas under each of the peaks in Figure 1 were measured and the molar ratio of products was calculated. The results are given in Table I. The detailed structures of the acetolysis products of the mannans are given in a later section.

*Methylation of Mannans.* The gas chromatographic tracings of the methanolyzed products from four of the methylated mannans are shown in Figure 2. A comparison of these tracings, together with those shown in the previous report for products of methylated mannan from commercial bakers' yeast (Stewart *et al.*, 1968, Figure 8C), confirms the conclusions based on the acetolysis products (Figure 1, Table I).

Methylated mannan from *S. carlsbergensis* gave a pattern (Figure 2A) very similar to that for mannan

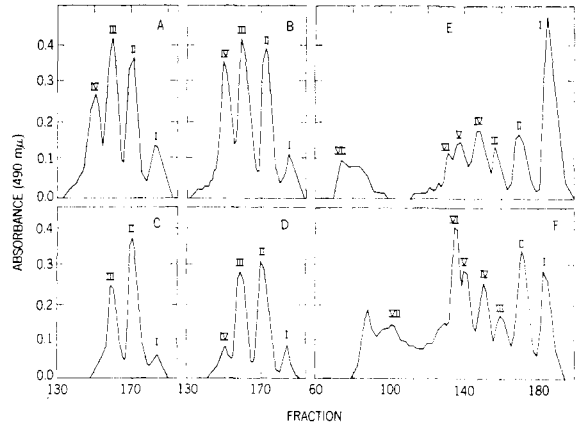



FIGURE 1: Elution patterns from Sephadex G-25 of neutral acetolysis products from several different mannans: (A) commercial bakers' yeast, Red Star strain 2; (B) *S. cerevisiae* (238C), (C) *K. brevis*, (D) *S. carlsbergensis*, (E) *C. albicans* (792), and (F) *C. stellatoidea*. The peaks were identified as follows: I, mannose; II, $M_2$; III, $M_3(+M_3')$; IV, $M_4$ for A to D and isomeric tetrasaccharide with only 1→2-linkages for E and F; V, pentasaccharide; VI, hexasaccharide; VII, high molecular weight products.

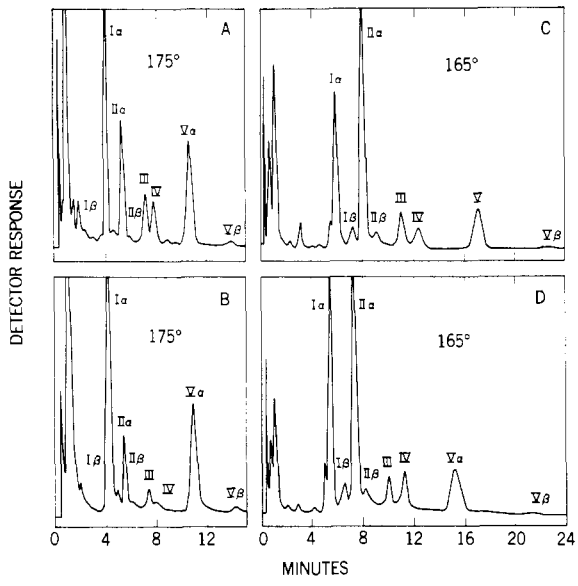



FIGURE 2: Gas chromatographic tracings of the trimethylsilyl derivatives of the methanolysis products from methylated mannans. The sources were: (A) *S. carlsbergensis*, (B) *K. brevis*, (C) *C. albicans*, and (D) *C. stellatoidea*. The peaks were identified as the $\alpha$ and $\beta$ anomers of: I, 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*-trimethylsilylmannoside; and V, methyl 2,3,4,6-tetra-*O*-methylmannoside.

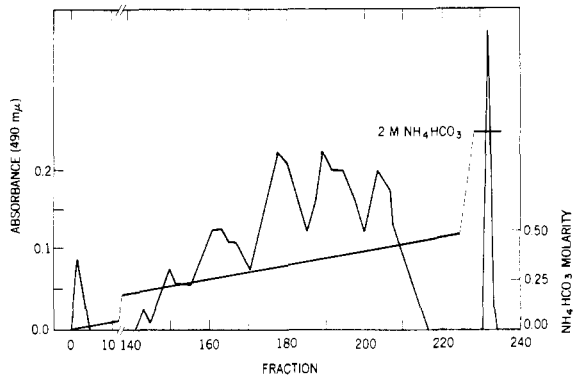



FIGURE 3: Elution pattern of *K. brevis* mannan from a column of DEAE Sephadex A-25 ($HCO^-_3$ form) with a linear gradient of 0–0.5 M $NH_4HCO_3$.

from commercial bakers' yeast. There is a high degree of branching as indicated by the amount of methyl 3,4-di-*O*-methylmannoside (peak I, Figure 2A). The most noticeable difference is in the amount of methyl 2,3,4-tri-*O*-methylmannoside (peak III, Figure 2A), which is obtained in considerable quantity from the mannan of *S. carlsbergensis*, but which was found only in trace amounts with commercial bakers' yeast mannan. This trimethylmannose comes from the 1→6-linked mannose units that are otherwise unsubstituted. The data in Table I confirm the presence of such units, since the proportion of mannose formed as an acetolysis product of *S. carlsbergensis* mannan is higher than that from commercial bakers' yeast. Thus, unsubstituted 1→6-linked mannose units appear to make up a significant part of the mannan structure of *S. carlsbergensis*.

Mannan from *K. brevis* is also highly branched as indicated by the large amount of methyl 3,4-di-*O*-methylmannoside (peak I, Figure 2B). However, there are very few 1→3 linkages in this mannan, as shown by the presence of only a trace of methyl 2,4,6-tri-*O*-methylmannoside (peak IV, Figure 2B). The small proportion of methyl 3,4,6-tri-*O*-methylmannoside (peak II, Figure 2B) is a consequence of the small amount of $M_3$ in this mannan. The presence of methyl 2,3,4-tri-*O*-methylmannoside (peak III, Figure 2B) indicates that some of the 1→6-linked mannose is not associated with branch points. As with mannan from *S. carlsbergensis*, the high proportion of mannose as an acetolysis product (Table I) must mean that there are single 1→6-linked mannose units in the mannan from *K. brevis*.

The tracings for the methylated mannans from *C. albicans* (Figure 2C) and *C. stellatoidea* (Figure 2D) are very different from those for mannans from *Saccharomyces* and *K. brevis*. There is a very high proportion of 1→2 linkages in these mannans, as indicated by the peak corresponding to methyl 3,4,6-tri-*O*-methylmannoside (peak II). This is correlated with the acetolysis-stable penta- and hexasaccharides (peak V and VI, Figure 1E, F) obtained from these mannans. For mannan from *C. stellatoidea*, the area of peak II of Figure 2D, is even greater than for the mannan from *C. albicans* (Figure 2C). Again, this is consistent with the large amount of high molecular weight acetolysis-stable products (peak VII, Figure 1F) which must contain predominantly 1→2 linkages. There are only a few 1→3 linkages in these mannans, as indicated by the amount of methyl 2,4,6-tri-*O*-methylmannoside (peak IV), *C. stellatoidea* containing somewhat more than *C. albicans*. The presence of methyl 2,3,4-tri-*O*-methylmannoside (peak III) shows that each mannan has 1→6 linkages not associated with branch points. Mannose is a major product of acetolysis of mannan from *C. albicans* (Table I), while *C. stellatoidea* yields considerably less.

*Phosphorus Content of Mannans.* Molar ratios of mannose to phosphorus are given in Table II, which shows the wide variation in phosphorus content of the mannans investigated. *K. brevis* mannan had the greatest

TABLE II: Phosphorus Content of Mannans.

| Mannan | Moles of Mannose/Mole of Phosphorus |
|---|---|
| Baker's yeast (strain 1) | 120 |
| Bakers' yeast (strain 2) | 78 |
| *S. cerevisiae* (238C), 7-hr culture | 120 |
| *S. cerevisiae* (238C), 48-hr culture | 144 |
| *S. carlsbergensis* (ATCC 9080) | 21 |
| *K. brevis* (H. Phaff 5545) | 9 |
| *C. albicans* (792) | 18 |
| *C. stellatoidea* | 46 |

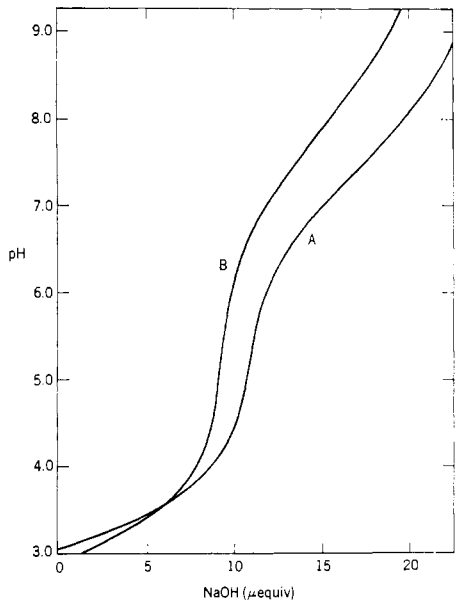



FIGURE 4: Titration of phosphomannans with 0.0025 N NaOH. Each sample was prepared by passage through a column of Dowex 50 ($H^+$ form). Mannan from *S. carlsbergensis* (A) contains 7.8 μequiv of phosphorus. Mannan from *K. brevis* (B) contains 6.7 μequiv of phosphorus.

amount of phosphorus, whereas the *S. cerevisiae* mannans had very little.

The DEAE Sephadex elution pattern for mannan from *K. brevis* is shown in Figure 3. This demonstrates that, although there was only a trace of neutral carbohydrate, there was great heterogeneity in the phosphorus content of the mannan molecules. The spread of carbohydrate on the DEAE Sephadex elution pattern is due to charge differences, not differences in molecular size, since sedimentation studies indicate that the mannan is homogeneous with respect to size.

Titration curves for mannans from *K. brevis* and *S. carlsbergensis* are shown in Figure 4. As expected, these indicate that the phosphate groups are monoesterified, since any labile phosphate groups should have been degraded by the procedure used to isolate the mannans. Even when extraction with citrate buffer (pH 7.0) was used, strong base is employed for copper complex formation and strong acid to decompose this complex. As shown in later studies on the mannan from *K. brevis*, a milder isolation procedure yields diesterified phosphomannan.

*Structural Studies on Mannan from S. cerevisiae* (238C). The gel permeation chromatography elution patterns of the acetolysis products are shown in Figure 1B. These were shown by chromatography and methylation analysis to be identical with mannose, $M_2$, $M_3$, and $M_4$. The trisaccharide fraction contained some $M_3'$. Table I gives the ratio of these small oligosaccharides.

Short-term acetolyses (30 and 75 min at 40°) were run concurrently both of peracetylated mannan from strain 238C and commercial bakers' yeast. The deacylated products were examined by Sephadex G-25. Each was degraded at the same rate which is further confirmation that the mannan structures are very similar.

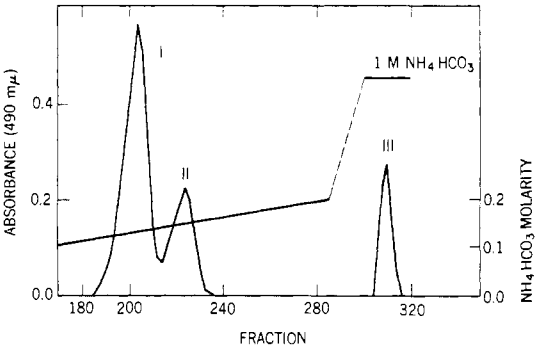



FIGURE 5: Separation on DEAE Sephadex of the acidic components from the acetolysis of *K. brevis* mannan. A linear gradient from 0 to 0.2 M $NH_4HCO_3$ (1 l. each chamber) was employed on a 2 × 70 cm column of DEAE Sephadex A-25 ($HCO_3^-$ form). Peak I is $M_3$ phosphate and peak II is $M_2$ phosphate. Peak III was not characterized.

To determine whether the ratio of oligosaccharides varied with growth conditions or the period of the growth cycle, two experiments were performed. *S. cerevisiae* was grown both with efficient aeration and in erlenmeyer flasks with shaking. The mannan samples were extracted from each lot of cells and the products of acetolysis were compared. There was no difference in the ratio of products. In a second experiment, cells nearing the end of log phase (7 hr) were harvested, and the acetolysis products of the mannan were compared with those from mannan derived from cells harvested after 48 hr. The results in Table I again reveal no significant difference in the mannans.

*Structural Studies on Mannan from S. carlsbergensis.* The separation of neutral acetolysis products by gel permeation chromatography is shown in Figure 1D. The products are identical with the $M_2$, $M_3(M_3')$, and $M_4$. This was established by methylation studies on the oligosaccharides and from their chromatographic properties. Table II shows that the phosphorus content of this mannan is relatively high, and titration (Figure 4) indicates that the phosphate is monoesterified. However, no conclusion can be drawn as to the state of the phosphate groups in the intact cell wall because the mannan was purified by copper complex formations.

The acidic acetolysis products had a mannose to phosphorus ratio of 3.3:1. Digestion with chicken intestinal phosphatase for 90 hr released 65% of the phosphorus. The neutral sugars were separated by passing the incubation mixture through a mixed-bed resin (Dowex 501, carbonate form). Gel permeation chromatography showed that the major product was $M_3$, with only a small amount of $M_2$ and a trace amount of $M_4$. This result agrees with the mannose to phosphorus ratio.

*Phosphomannan from K. brevis Isolated by Copper Complex Formation.* Methylation studies on mannan from *K. brevis* (Figure 2B) indicate that this mannan lacks 1→3 linkages. Methylation of $M_2$ and $M_3$, the neutral products of acetolysis (Figure 1D), confirmed the above result. This is consistent with the absence of $M_4$, the tetrasaccharide containing a 1→3 linkage at the nonreducing terminus.

The acidic components of the acetolysis products

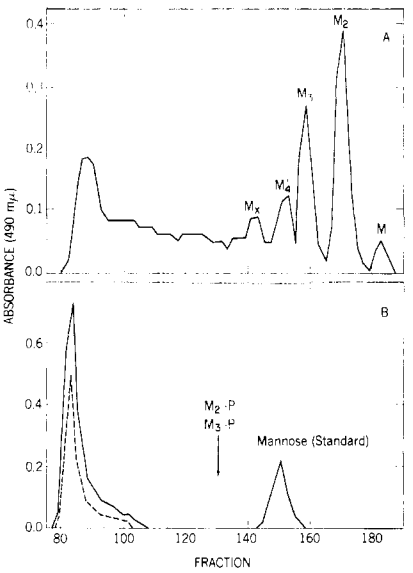



FIGURE 6: Elution patterns from Sephadex G-25 of products from a short-term acetolysis of *K. brevis* mannan acetate. Acidic and neutral fractions were separated on DEAE Sephadex A-25. (A) Elution pattern of neutral products from Sephadex G-25 (irregular form). (B) Elution pattern of acidic products from Sephadex G-25 (bead from) with 0.2 M $NH_4HCO_3$. A mannose standard was added to the acidic fraction to determine the expected position of $M_2$-P and $M_3$-P. The solid line is carbohydrate and the dashed line is phosphorus.

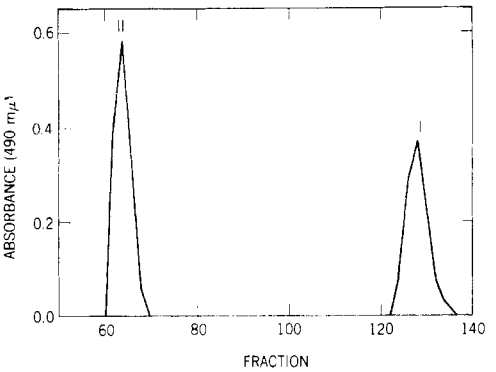



FIGURE 7: Gel permeation chromatography of products obtained by digestion of *K. brevis* mannan with a bacterial α-mannosidase. A Sephadex G-25 column was used with 0.2 M $NH_4HCO_3$ as eluent. I is mannose, II is excluded polysaccharide.

were separated from neutral products by ion exchange on DEAE Sephadex A-25 ($HCO_3^-$). The ratio of acidic to neutral carbohydrate was 1:2.3. The acidic components were separated on DEAE Sephadex A-25. The elution pattern (Figure 5) shows three components in the mixture. The mannose to phosphorus ratios were 2:7 in peak I, 2:1 in peak II, and 4:2 in peak III. Alkaline phosphatase (*E. coli*) digestion released all of the phosphorus of each peak. The neutral carbohydrate produced was desalted and examined by paper chromatography and gel filtration on Sephadex G-25. Peak I (Figure 5) gave only $M_3$ while peak II gave $M_2$. Peak III gave no substance which reacted on the paper chromatogram with the silver nitrate–sodium hydroxide reagent. No further study was made on this component. Methylation of the other two products of alkaline phosphatase digestion confirmed that they were normal $M_2$ and $M_3$. Thus, the acidic products of acetolysis of mannan from *K. brevis* were $M_2$ and $M_3$ monophosphates, and these products are obtained in a ratio of 1:1.6. This ratio contrasts with that obtained for the neutral components in mannan from *K. brevis* ($M_2$ to $M_3$ is 2:1; Figure 1 and Table I). Therefore, the over-all ratio of $M_2$ to $M_3$ in this mannan is calculated to be 1:0.7.

Reduction of the phosphorylated oligosaccharides (henceforth referred to as $M_2$-P and $M_3$-P) was carried out. The products were hydrolyzed in 1 N HCl at 100° for 2 hr. The hydrolysis products were separated into neutral and acidic components by batchwise elution from DEAE Sephadex A-25. Paper chromatography (solvent system C) showed that the neutral component was mannose, not mannitol. ($RM_3$-P gave a trace of mannitol as a hydrolysis product.) The sugars were detected with the periodate–benzidine dip which is more sensitive to mannitol than mannose. The isolation of mannose as the sole neutral product of $RM_2$-P hydrolysis and the major product of $RM_3$-P hydrolysis is strong evidence that the phosphate groups in $M_2$-P and $M_3$-P are attached to the terminal reducing mannose residue. Confirmation of this result was obtained by the following experiments on the short-term acetolysis, total acid hydrolysis, and α-mannosidase digestion of the mannan.

*Short-Term Acetolysis of K. brevis Mannan.* Acetolysis of the acetylated mannan was carried out for 2 hr at 40°. After deacylation, the neutral and acidic components were separated on DEAE Sephadex by batchwise elution. While short-term acetolysis yields small neutral oligosaccharides (Figure 6A), $M_2$-P and $M_3$-P are not released (Figure 6B). (The expected position of $M_2$-P on the elution pattern is shown in Figure 6B.) This result indicates that the phosphate groups stabilize the 1→6 linkages, an effect which is understandable if the phosphate groups are located close to the 1→6-linked "backbone" in the mannan.

The Sephadex G-25 elution pattern of neutral acetolysis products (Figure 6A) shows a tetrasaccharide component $M_4'$. Since the normal $M_4$ is not obtained as a product of prolonged acetolysis of *K. brevis* mannan (Figure 1C), we suspected that the tetrasaccharide isolated here had an intact 1→6 linkage. This was confirmed by further acetolysis of this product (as the acetate) for 5 hr at 40°, which resulted in its conversion into $M_2$. Thus, the tetrasaccharide $M_4'$ is composed of two $M_2$ units connected by a 1→6 linkage. This $M_4$ isomer has never been detected as a product of short-term acetolysis of bakers' yeast mannan (Stewart *et al.*, 1968).

The other major product of short-term acetolysis (designated $M_x$, Figure 6A) is not a pentasaccharide, but probably somewhat larger. Acetolysis of this oligosaccharide gave $M_2$ and $M_3$ in the ratio of 2:1. Thus, $M_x$ appears to be a heptasaccharide containing one $M_3$ and two $M_2$ units connected by two 1→6 linkages.

*Total Acid Hydrolysis of K. brevis Mannan.* Acid hydrolysis of mannan was carried out with 1 N HCl for 4 hr at 100°. The products were separated into neutral

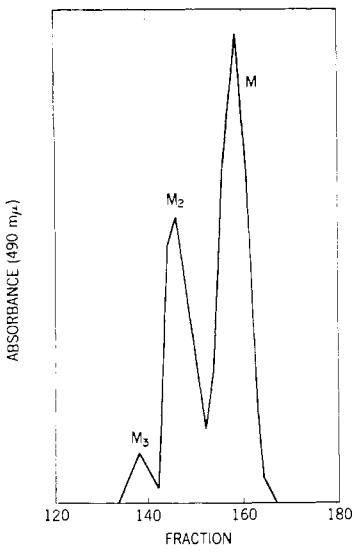



FIGURE 8: Sephadex G-25 elution pattern of neutral products of acetolysis of high molecular weight residue from α-mannosidase digestion of *K. brevis* mannan.

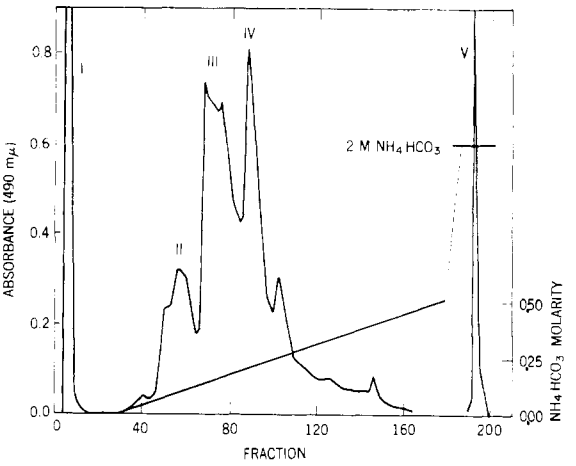



FIGURE 9: Elution pattern from DEAE Sephadex A-25 of carbohydrate material isolated from *K. brevis* by citrate extraction. Fraction I is glucan. Fractions II–III–IV are phosphomannans. Fraction V contains mannose, glucose, and an unknown sugar. The column dimensions were 1.5 × 40 cm and a linear gradient with 0–0.5 M $NH_4HCO_3$ was used (1 l. in each chamber).

and acidic fractions on DEAE Sephadex and examined by paper chromatography. The only neutral monosaccharide was mannose. Paper chromatograms (solvent systems D and E) of the acidic fraction showed many sugar phosphate-containing spots, none with the $R_F$ of mannose-6-P. Mill (1966) has reported the isolation of mannose-6-P from a phosphomannan from *S. cerevisiae* cell wall. Our inability to detect mannose-6-P is consistent with the evidence which indicates that the phosphate groups are esterified to hydroxyl groups of the mannose units in the 1→6-linked backbone of the polysaccharide.

*α-Mannosidase Digestion of K. brevis Mannan.* Digestion of *K. brevis* mannan was carried out at 37° for 24 hr, after which time 52% of the total carbohydrate was reducing sugar. The concentrated incubation mixture was boiled and centrifuged, and the supernatant was passed through a Sephadex G-25 column (eluting with 0.2 M $NH_4HCO_3$). The elution pattern of products is shown in Figure 7, which indicates that mannose and high molecular weight material, excluded from Sephadex G-25, are the only products. This enzyme has been shown to be an exoglycosidase, particularly active on 1→2-linked mannose units. This preliminary study indicates that the enzyme degrades the side chains in the mannan molecule, yielding mannose and a high molecular weight residue which contains the 1→6-linked mannose units. Acetolysis of the high molecular weight residue yields the neutral products shown in Figure 8, which are mannose and $M_2$ with only a trace of $M_3$. In comparison with the products from the parent mannan (Figure 1C) it is evident that the high molecular weight residue from mannosidase digestion contains a high proportion of unsubstituted 1→6-linked mannose residues. This would result from removal of the 1→2-linked side chains. No mannose phosphate was detected as a product of mannosidase digestion.

Table III gives the mannose to phosphorus ratios of mannan before digestion and of the high molecular weight residue after mannosidase digestion. There is a concentration of phosphate in the latter, indicating that these phosphate groups are not in the side chains but are situated on the backbone of the polymer.

*Isolation of K. brevis Mannan Containing Diesterified Phosphate Groups. K. brevis* cells were extracted with pH 7 citrate buffer at 120° in the autoclave for 2 hr. The supernatant, after centrifugation, was poured into ethanol and the precipitate was redissolved in water. A further ethanol precipitation of the mannan was followed by precipitation of the contaminating protein at 5° by careful addition of acetic acid. The supernatant from centrifugation was concentrated, dialyzed against water, then put onto a DEAE Sephadex A-25 column ($HCO_3^-$ form) and the neutral material was removed by a water wash. The elution pattern, using a linear gradient of $NH_4HCO_3$, is shown in Figure 9. A portion of each peak was hydrolyzed with 1 N HCl and the prod-

TABLE III: Action of Bacterial α-Mannosidase on *K. brevis* Mannan.

| Fraction of *K. brevis* Mannan | Reducing Sugar Released (% of total carbohydrate) | Moles of Mannose/Mole of Phosphorus in Polysaccharide Fraction | |
|---|---|---|---|
| | | Before Digestion | After Digestion |
| Peak II (Figure 9) | 64 | 23 | 7.5 |
| Peak III (Figure 9) | 64 | 12 | 6.1 |
| Peak IV (Figure 9) | 49 | 7 | 4.6 |
| Mannan purified *via* copper complex | 52 | 9 | 5.5 |

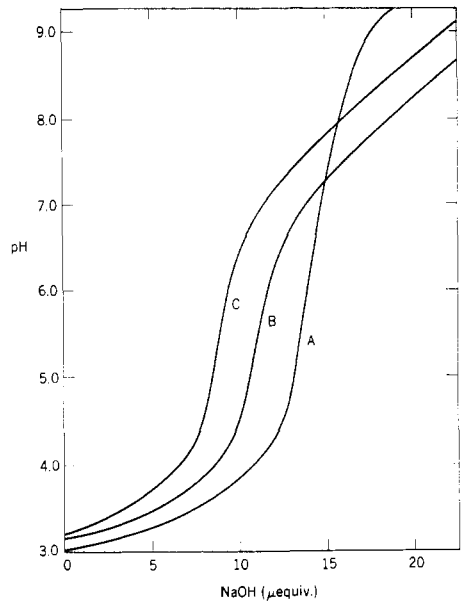



FIGURE 10: Titration curves of *K. brevis* mannan fraction IV with 0.0025 N NaOH. Samples were desalted on Sephadex G-25, then passed through a Dowex 50 ($H^+$) column. The titration of intact fraction IV (A) containing 12.8 μequiv of phosphorus. The titration curve of fraction IV, which had been treated with 1 N HCl for 15 min at 100°, contained 10.25 μequiv of phosphorus (B). The titration curve of fraction IV, treated with 1 N NaOH for 1 hr at 100°, contained 9.6 μequiv of phosphorus (C).

ucts were examined by paper chromatography (solvent system C). Glucose was the major product of fraction I, which contained only a trace of mannose. Mannose was the major product from fractions II to IV, with only trace amounts of glucose being present. Fraction V contained mannose, glucose, and another component with a high $R_F$ value (solvent system C). Thus, this column procedure yielded several fractions of mannan which were relatively free from glucose.

Phosphorus content of the mannan fractions (Table III) shows a steady increase in going from fractions II to IV, as expected from the ion-exchange behavior. Titration of each of the phosphomannan fractions with 0.0025 N NaOH indicated that the phosphorus is diesterified. The titration curve for fraction IV of Figure 9 is shown in Figure 10A, and there is only one inflection point. Fraction IV appeared homogeneous in the ultracentrifuge. It gave a sharp symmetrical boundary and the plot of log $C$ against $X^2$ was essentially linear. The molecular weight, determined by sedimentation equilibrium, was $4.5 \pm 0.3 \times 10^4$, using $\bar{V}$ 0.618 of sucrose. The molecular weight of the original *K. brevis* mannan preparation (monoesterified phosphate) was $3.6 \times 10^4$. Thus, it is evident that cleavage of the diesterified phosphate groups does not result in a significant decrease in molecular size.

Treatment with 1 N HCl for 15 min at 100° cleaved the diesterified phosphate, as shown by the titration curve B in Figure 10. Alkaline degradation of the diester linkages also occurred. After 1 hr at 100° in 1 N NaOH, all phosphate had been converted into the monoester form. The titration curve is shown in Figure 10C. The products of alkaline degradation were passed through Sephadex G-25 and G-50 columns. In neither case was small molecular weight carbohydrate material observed; all carbohydrate was excluded from both columns. This eliminates the possibility of having small units ($M_3$-P, for example) linked to the mannan through a phosphate bridge, although this type of structure is common for exocellular phosphomannans (Jeanes and Watson, 1962).

α-Mannosidase digestion, similar to that described for the original mannan preparation, was carried out for mannan fractions II–IV in Figure 9. Approximately half of the sugar was released from each fraction (Table III) and the products gave an elution pattern on Sephadex G-25 identical with that obtained in the original incubation (Figure 7). Mannose to phosphorus ratios for the high molecular weight residue from α-mannosidase digestion are given in Table III. In every mannan fraction, as with the original preparation, there is a concentration of phosphorus into the residue. This suggests that the phosphorus is attached to the mannose units that make up the 1→6-linked backbone.

We have not determined the nature of the second linkage in the diesterified phosphomannan. Our present results indicate that it is not between small oligosaccharides nor large mannan molecules. We find considerable amounts of protein in the mannan fractions from DEAE Sephadex chromatography (Figure 9), 19% (w/w) of fraction II, 9% of fraction III, and 2.5% of fraction IV, as determined by the Lowry method using bovine albumin as standard. This was not removed by Dowex 50 ($H^+$) treatment.

*Mannan from C. albicans (792).* The neutral products from long-term acetolysis of *C. albicans* mannan are shown in Figure 1E, and the molar ratios are given in Table I. Paper chromatography (solvent systems A and B) showed the di- and trisaccharide fractions to have $R_F$ values identical with $M_2$ and $M_3$. Methylation of these two oligosaccharides confirmed that they have 1→2 linkages. Paper chromatography of the tetra-, penta- and hexasaccharide fractions (solvent system A) showed that these products had slightly higher $R_F$ values than the $M_4$, $M_5$, and $M_6$ isolated from bakers' yeast mannan (Stewart *et al.*, 1968). Methylation of *C. albicans* mannan indicates a high proportion of 1→2-linked mannose units (Figure 2C), and we expect that the tetra-, penta-, and hexasaccharides obtained from this mannan also contain predominantly 1→2 linkages. Since they are stable to long-term acetolysis, they cannot contain 1→6 linkages.

*Mannan from C. stellatoidea.* The Sephadex G-25 elution pattern of products from long-term acetolysis of *C. stellatoidea* mannan is shown in Figure 1F. It differs from *C. albicans* mannan in the ratios of penta- and hexasaccharides and in the accumulation of stable high molecular weight products (Table I). Further acetolysis for 13 hr at 40° of the high molecular weight products (peak VII, Figure 1F) and of the penta- and hexasaccharides (peaks V and VI, Figure 1F) resulted in no further degradation.

Methylation studies on this mannan (Figure 2D) had shown that it contained a high proportion of 1→2 link-

ages. Methylation studies on each of the acetolysis products gave the following results; the disaccharide was found to be 1→2-linked $M_2$; the trisaccharide fraction gave a ratio of 3:1 for 1→2- to 1→3-linked mannose units, indicating the presence of $M_2'$ as well as $M_3$; the tetrasaccharide gave methyl 3,4,6-tri-*O*-methylmannoside as the major trimethyl ether, showing this to be exclusively a 1→2-linked tetrasaccharide, isomeric with $M_4$ of structure III; the pentasaccharide gave methyl 3,4,6- and methyl 2,4,6-tri-*O*-methylmannoside in a ratio of 3:1 indicating that ratio of 1→2 and 1→3 linkages in this pentasaccharide; the hexasaccharide fraction had a ratio of three 1→2 linkages to two 1→3 linkages. None of the saccharides contained a 1→6 linkage. Insufficient material was available for a more complete structural analysis.

## Discussion

Acetolysis of yeast mannan cleaves selectively the 1→6 linkages in the polysaccharide, giving almost quantitative yields of mannose plus small oligosaccharides (di-, tri-, and tetrasaccharides) containing 1→2 and 1→3 linkages. These products can be separated by gel permeation chromatography and the ratios appear to correspond to the ratios of these units that preexist in 1→6 linkage in the mannan polymer. The ratio of these products differs from one strain of yeast to another and appears to be highly characteristic of the organism. Thus, whereas several *Saccharomyces* strains give comparable amounts of mannose, mannobiose, mannotriose, and mannotetraose, *K. brevis* yields no mannotetraose and *Candida* yields very large amounts of mannose along with the very unusual acetolysis-stable penta- and hexasaccharides. It is probable that this technique of comparing mannan structure can be of some value in classification of yeasts and for making structural comparisons for the purpose of identifying the immunochemical determinants of yeast mannans.

Nearly all yeast cell wall mannans contain some phosphate which, in the case of *K. brevis*, appears to be attached to the 3 or 4 position of the mannose units in the 1→6-linked backbone. We conclude this from the fact that acetolysis yields mannobiose and mannotriose phosphates in which the phosphate is attached to the reducing sugar unit that originates from cleavage of 1→6 linkages. This phosphate is diesterified in the original mannan, and we do not know the nature of the second group. Since there is a variable but significant amount of protein in the mannan preparations, the linkage could be to an amino acid in this protein. Such a structure could also link the invertase–mannan complex of Neumann and Lampen (1967).

Yeast strains, whose mannans yield only $M_2$ and $M_3$ on acetolysis, appear to lack the ability to synthesize the $\alpha$-1→3 linkage which is found at the end of the $M_4$ component. It is possible that these strains are characterized by the inability to make the enzyme required to form this linkage. The recent evidence that mannan may be synthesized by the polymerization of oligosaccharides linked to a lipid carrier (Lennarz, 1967) raises the interesting possibility that the 1→2- and 1→3-linked di-, tri-, and tetrasaccharides are the units that are first constructed on the carrier and that the polymerization reaction involves the formation of the 1→6-linked backbone. If this is so, then the differences in the various mannans could reflect in part the genetic controls operating at the level of synthesis of these small oligosaccharides.

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