765. *The Polysaccharides of Baker's Yeast. Part V.*[1] *A Further Study of the Mannan.*

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The mannan, extracted from baker's yeast by autoclaving the cells in a neutral buffer, has been methylated, and the trimethylmannan completely hydrolysed with acid. The resulting mixture has been separated into 2,3,4,6-tetra-, 3,4-di-, 3,4,6-tri-, 2,4,6-tri-, and 2,3,4-tri-*O*-methyl-D-mannose, in the molar proportions 15 : 14 : 7 : 7 : 1.

Because partial acid hydrolysis of the mannan gives, predominantly, a series of α-1,6-linked oligosaccharides,[1] alternative fragmentation by acetolysis has been studied. This gave pure 2- and 6-*O*-α-D-mannopyranosyl D-mannose, and no trace of a 1,3-link was found by this method.

A unique structure cannot be ascribed to yeast mannan on the basis of these observations. From even the simplest picture of the repeating unit it is clear that the mannan is highly branched.

The structure of the mannan isolated from baker's yeast by extraction with alkali was investigated by Haworth, Hirst, and Isherwood [2] and subsequently by Haworth, Heath, and Peat,[3] using the method of methylation analysis. The difference in results in these two papers lay in the proportions of 2,3,4-tri-*O*-methylmannose shown to be present. The main evidence for its presence given in the first paper was that its oxidation with nitric acid yielded what was believed to be 2,3,4-tri-*O*-methylmannosaccharic acid, characterised as the crystalline amide. No other trimethylmannose was identified. This tri-*O*-methylmannosaccharamide was, however, later synthesised by a route which left no doubt as to its constitution [4] and shown not to be identical with that from the mannan. The trimethyl fraction prepared as before from methylated yeast mannan was therefore re-examined.[3] The result, supported by indisputable evidence, was unexpected. The principal components of the trimethyl fraction were 3,4,6- and 2,4,6-tri-*O*-methylmannose. There was indeed evidence that 2,3,4-tri-*O*-methylmannose was also present but it was at that time difficult to isolate and it was estimated to constitute not more than 10% and not less than 1% of the " trimethyl " fraction. The view that 1,6-links did not preponderate in the mannan derived support from the composition of tritylated mannan which showed that 67% of the mannose residues carried a trityl group. This is interpreted as meaning that in the original mannan at least two-thirds of the primary alcohol groups are not involved in the formation of 1,6-links.[5]

In 1955, Cifonelli and Smith [6] repeated the methylation analysis of alkali-extracted mannan and confirmed in every particular the results of Haworth, Heath, and Peat. In each case there were obtained 2,3,4,6-tetra-, 2,4,6-tri-, 3,4,6-tri, and 3,4-di-*O*-methyl-D-mannose in the molar proportions of 2 : 1 : 1 : 2 respectively, and, further, Cifonelli and Smith could detect no more than traces of 2,3,4-tri-*O*-methylmannose.

A different approach was made by Peat, Whelan, and Edwards,[1] namely, partial acid hydrolysis. In this case the extraction of the mannan from yeast by alkali was replaced by the milder method of autoclaving at 140° with a neutral buffer. The mannan so obtained was hydrolysed to 67% apparent conversion into mannose and a homologous series of oligosaccharides were isolated as the only recognisable hydrolytic products. The significant fact, in relationship to the earlier work, is that only 1,6-linkages were present in the homologous series. The disaccharide member of the series was proved to be identical

[1] Part IV, Peat, Whelan, and Edwards, *J.*, 1961, 29.
[2] Haworth, Hirst, and Isherwood, *J.*, 1937, 784.
[3] Haworth, Heath, and Peat, *J.*, 1941, 833.
[4] Haworth, Hirst, Isherwood, and J. K. N. Jones, *J.*, 1939, 1878.
[5] Lindstedt, *Arkiv Kemi, Min., Geol.*, 1945, *A*, **20**, No. 13.
[6] Cifonelli and Smith, *J. Amer. Chem. Soc.*, 1955, **77**, 5682.

with 6-*O*-α-D-mannopyranosyl-D-mannose, synthesised for the purpose. In addition these authors [1] provide evidence supporting the view that the 1,6-link has the α-configuration, contrary to earlier opinion.

The striking features of this work [1] are the absence from the hydrolysate of 1,2- or 1,3-linked oligosaccharides and the presence of a uniform series of 1,6-linked oligosaccharides in the hydrolysate: however, when the hydrolysis of mannan was carried to a much lower degree of apparent conversion (29%), a second disaccharide, probably the α-1,2-disaccharide, was isolated in very small amount.[1] The experiments described in the present paper were designed to correlate the results of linkage analysis by methylation and by partial acid hydrolysis.

The mannan was extracted from baker's yeast by autoclaving the cells in a neutral buffer as previously described.[1] Methylation (to OMe, 43·7%) and complete hydrolysis gave a mixture of methylated mannoses which was fractionated on charcoal. Pure samples of (*a*) 2,3,4,6-tetra- and (*b*) 3,4-di-*O*-methyl-D-mannose were obtained. The intermediate fractions were mixtures which were subfractionated by paper chromatography and electrophoresis. In this way (*c*) 3,4,6-tri-*O*-methyl-D-mannose was obtained. There were indications of the presence of two other trimethylmannoses which could not be separated by these methods but were almost completely separable by the use of " Ultrasorb " (a highly active charcoal) and identified as (*d*) 2,4,6- and (*e*) 2,3,4-tri-*O*-methyl-D-mannose. The molar ratios of the products were (*a*) 15, (*b*) 14, (*c*) 7, (*d*) 7, (*e*) 1. The significant feature of this analysis is the proof of the presence of 2,3,4-tri-*O*-methylmannose in a proportion which suggests that one mannose residue in 44—45 is linked through positions 1 and 6 exclusively.

Since partial acid hydrolysis of yeast mannan gave, predominantly, a series of α-1,6-linked oligosaccharides,[1] alternative fragmentation by acetolysis was investigated. This method was applied to yeast mannan by J. K. N. Jones and Nicolson [7] who detected two disaccharides in the acetolysate. We found that, after acetolysis, deacetylation and chromatography on thick paper effected separation into five zones (*A*—*E*). The first zone was mannose; zone *B* a mixture of disaccharides; and zone *C* a mixture of trisaccharides. Zones *D* and *E* contained higher oligosaccharides.

Zone *B* contained two disaccharides which were partially separated on " Ultrasorb " into two pure specimens, *B*1 and *B*2. Each was identified as a disaccharide by electrophoresis in the presence of hydrogen sulphite ions (cf. Frahn and Mills [8]), and the constitution of each was established by periodate oxidation, before and after reduction with sodium borohydride (cf. Hough *et al.*[9]). Thus, sugar *B*1 showed R_F and M_G values identical with those of 2-*O*-α-D-mannopyranosyl-D-mannose, and $[\alpha]_D^{18}$ +48·0° (earlier values, +48·6°,[1] +40·0° [10]). When oxidised with periodate, it yielded, at pH 8 (condition for overoxidation), 1·8 mol. of formaldehyde; and the corresponding alcohol, formed by reduction, gave 0·96 mol. of formaldehyde at pH 8 and 1·01 mol. at pH 3·6. These values are close to those expected of a 1,2-linked disaccharide.[9] Similarly, sugar *B*2 had $[\alpha]_D^{18}$ +62·3° and R_F and M_G values identical with those of 6-*O*-α-D-mannopyranosyl-D-mannose. On oxidation, it gave only a trace of formaldehyde at pH 8 but its derived alcohol gave 0·94 mol. at pH 8 and 0·91 at pH 3·6. These results for *B*2 agree with those for a 1,6-linked disaccharide.[9]

The trisaccharide mixture (*C*) gave, on partial hydrolysis, the disaccharides *B*1 and *B*2, as did also the higher oligosaccharides in zones *D* and *E*. No trace of a 1,3-linkage was found in any of the fractions and, further, variations in the conditions of acetolysis made no difference to these results.

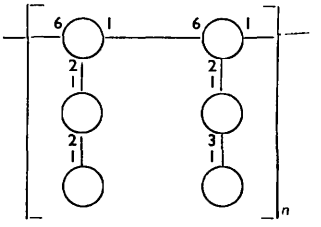
We have seen from the methylation analysis that 1,2-links (other than those involved

[7] J. K. N. Jones and Nicholson, *J.*, 1958, 27.
[8] Frahn and Mills, *Chem. and Ind.*, 1956, 1137.
[9] Hough, Woods, and Perry, *Chem. and Ind.*, 1957, 1100.
[10] Gorin and Perlin, *Canad. J. Chem.*, 1956, **34**, 1796.

in the branching) and 1,3-links are roughly equal in number so that the absence of 1,3-linkages from the acetolysate and of both 1,2- and 1,3-links from the partial acid hydrolysate [1] may be ascribed to relative stabilities of the linkages towards acid, in the order: 1,6 > 1,2 > 1,3. The question whether the oligosaccharides found in the acid hydrolysate of mannan could be artefacts formed by reversion synthesis from mannose has been examined in Part IV [1] and by J. K. N. Jones and Nicholson.[7] We also treated mannose with the acetolysing mixture but no reversion synthesis could be detected.

The information accumulated in this and the earlier papers does not lead to the postulation of a unique structure for yeast mannan. It is clear, however, that the molecule is very highly branched. Discrepancies between analysis by methylation and by partial acid hydrolysis are not due to heterogeneity of the mannan, since Northcote showed [11] by an electrophoretic method that alkali-extracted yeast mannan moved in the Tiselius apparatus as a single molecular species.

To arrive at a partial picture of the structure of mannan it will therefore be necessary to make certain assumptions, the most important of which is that the " core " or " nucleus " consists of a chain of mannose units formed by α-1,6-links. Each of these units carries a branch point and this branch linkage engages solely $C_{(2)}$ of each of the units of the main chain. Again, since the proportions of α-1,2- and α-1,3-links (excluding the branch points on the main chain) are the same, and equal to half the proportion of branch chain end-groups, the following picture emerges of the *simplest* repeating unit in yeast mannan: (α-mannopyranosyl residue represented by ○).



The origin of the 2,3,4-tri-*O*-methylmannose (one mole in 45) in the hydrolysate of methylated mannan remains obscure. It could be derived from an occasional mannose unit in the basic (1,6-linked) chain, which does not carry a side-chain. An alternative view would be that it constitutes the reducing end group of this basic chain, and this would give a more regular pattern for the structure. There is at present no experimental evidence to support either view.

It is of interest that two mannans from other sources appear closely to resemble yeast mannan in structure, if indeed they are not identical. These are (i) the main slime polysaccharide produced by *Saccharomyces rouxii* during the fermentation of glucose to arabitol (Gorin and Perlin [10]) and (ii) that produced during fermentation of glucose, sucrose, or lactose by *Bacillus polymyxa* (Ball and Adam [12]).

Experimental

General Methods.—The concentrations of all oligo- and poly-saccharide solutions were determined by acid hydrolysis to mannose as described in Part IV.[1] The methods of paper electrophoresis in borate buffer and of chromatography on charcoal–Celite columns have also been described,[13] but for the latter, either B.D.H. " activated charcoal " or " Ultrasorb " SC 120/240 (British Carbo-Norit Union) was used as adsorbent. For paper chromatography, the solvent systems butan-1-ol–ethanol–water (5 : 1 : 4 by vol.), ethyl methyl ketone saturated

[11] Northcote, *Biochem. J.*, 1954, **58**, 353.
[12] Ball and Adams, *Canad. J. Chem.*, 1959, **37**, 1012.
[13] Peat, Whelan, and Roberts, *J.*, 1957, 3916.

with water, and ethyl acetate–acetic acid–water (3 : 1 : 3, by vol.) were used in addition to those already described.[13] Reducing-sugar zones were detected with either *p*-anisidine hydrochloride [14] or benzidine–trichloroacetic acid,[15] and non-reducing sugars with silver nitrate–sodium hydroxide.[16] Formaldehyde, liberated on periodate oxidation, was determined with chromotropic acid.[17]

Isolation of Yeast Mannan.—The mannan was isolated from baker's yeast (10 lb.) and purified as previously described [1] (yield 45 g.). It had $[\alpha]_D^{18}$ +87° and contained 94·4% as carbohydrate, as determined by complete hydrolysis to mannose. Examination of a complete hydrolysate by paper chromatography indicated that mannose was the only sugar present. The mannan acetate, prepared by Bell and Northcote's method,[18] had $[\alpha]_D^{18}$ +59·7° in $CHCl_3$.

Methylation of Mannan.—The mannan (10 g.) was partly methylated by the Haworth procedure and then by the Kuhn modification [19] of Purdie's method. The methylated mannan (yield 9·2 g.; OMe, 43·7%) was dissolved in chloroform (100 ml.) and fractionated by addition of light petroleum (b. p. 60—80°) into four fractions. These fractions ranged in $[\alpha]_D^{18}$ from +87·5° to +88° and methoxyl content from 43·7 to 44%. They were bulked for further study.

The methylated mannan (3·57 g.) was hydrolysed by the method of Haworth *et al.*[3] and the fragments were chromatographed on a charcoal (B.D.H. " activated ")–Celite column (85 × 5 cm.) with a gradient of 0—25% ethanol spread over 15 l. Fractions (200 ml. each) were collected and examined by paper chromatography of small portions. On the basis of this the fractions were bulked into groups and evaporated to dryness to give the following: (i) a chromatographically pure di-*O*-methylmannose (0·77 g.); (ii) a mixture of di- and tri-*O*-methylmannoses (0·37 g.); (iii) mixed tri-*O*-methylmannoses (0·71 g.); (iv) a mixture of tri- and tetra-*O*-methylmannoses (0·39 g.); (v) chromatographically pure tetra-*O*-methylmannose (0·74 g.); weight recovered, 3·0 g. Fractions (ii) and (iv) were further separated by chromatography on thick filter paper in the butan-1-ol–ethanol–water solvent, and the components added to the respective di-, tri-, or tetra-*O*-methylmannose fractions. The combined tri-*O*-methylmannose fractions were further separated by electrophoresis in borate buffer (0·2M; pH 10) into an electrophoretically mobile (T1) and non-mobile (T2) fraction. Fraction T1 behaved as a single component on paper chromatography and electrophoresis, but fraction T2 contained more than one component. Fraction T2 (0·54 g.) was therefore chromatographed on a column (80 × 2·2 cm.) of charcoal (" Ultrasorb ")–Celite with gradient elution from 0·6 to 2·5% aqueous ethyl methyl ketone (8 l.). Fractions (40 ml. each) were examined with the phenol–sulphuric acid reagent [20] and grouped as follows: eluents no. 122—160 gave a single component (fraction T3, 0·392 g.); eluents no. 171—190 gave a single component (fraction T4, 0·063 g.); eluents no. 161—170 gave a mixture of the above two components (0·029 g.).

*Identification of Methylmannoses.—Di-*O*-methylmannose.* This had R_F and M_G values identical with those of 3,4-di-*O*-methyl-D-mannose. When crystallised from acetone–ether, it had $[\alpha]_D^{18}$ +3·2° (equilibrium, *c.* 1·4 in water), m. p. 112·5° (not depressed on admixture with an authentic specimen), and OMe 26·9% (total isolated, 0·956 g.).

*Tri-*O*-methylmannoses.* (i) Fraction T1 (0·43 g.). This fraction had R_F 0·81 in butan-1-ol–ethanol–water and M_G 0·38 in 0·2M-borate buffer (pH 10). It did not crystallise initially but did so on being kept for several months in a desiccator. The crystals had m. p. 101°, $[\alpha]_D^{17}$ +9·8° (equilibrium, *c.* 0·7 in water) and OMe 40·6%, in good agreement with values reported for 3,4,6-tri-*O*-methyl-D-mannose. The anilide derivative had m. p. 141°. On oxidation with 0·3M-sodium metaperiodate (3 mol.), the sugar consumed [21,5] 0·92 mol. (22·5 hr.), 0·93 mol. (48 hr.) of oxidant (theor., 1 mol.).

(ii) Fraction T3 (0·39 g.). This did not crystallise but the syrup had R_F 0·85 in butan-1-ol–ethanol–water, $[\alpha]_D^{18}$ +15·2° (*c* 0·6 in water), and OMe, 38·0%. The crystalline anilide had m. p. 132·5°. On treatment with periodate under the above conditions, the sugar consumed no detectable amount of periodate. These values agree with those reported for 2,4,6-tri-*O*-methyl-D-mannose.[3,10]

[14] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[15] Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114.
[16] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[17] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.
[18] Bell and Northcote, *J.*, 1950, 1944.
[19] Kuhn, *Chem. Ber.*, 1955, **88**, 1492, 1537.
[20] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.
[21] Rappaport, Reifer, and Weinmann, *Mikrochim. Acta*, 1937, **1**, 290.

(iii) Fraction T4 (63 mg.). The syrup, which did not crystallise, had R_F 0·86 in butan-1-ol–ethanol–water; $[\alpha]_D^{18}$ +2·8° (in water), and OMe, 38·5%. On periodate oxidation as above, the sugar consumed 0·87 mol. of oxidant in 71 hr. When oxidised with sodium metaperiodate in presence of 1% of *p*-hydroxybenzaldehyde [17] it liberated 0·90 mol. of formaldehyde in 90 hr. These values are in agreement with those reported for 2,3,4-tri-*O*-methyl-D-mannose.[2,4]

Tetra-O-methylmannose (1·03 g.). This had R_F and M_G values identical with those of 2,3,4,6-tetra-*O*-methyl-D-mannose. It had $[\alpha]_D^{18}$ +4·2° (*c* 2·8 in water) and OMe 52·0%. The anilide derivative had m. p. 143°. These properties define the substance as 2,3,4,6-tetra-*O*-methyl-D-mannose.

Acetolysis of Yeast Mannan.[22]—Dry yeast mannan (5 g.) was slowly stirred into a cooled (0°) mixture of acetic anhydride (30 ml.), glacial acetic acid (30 ml.), and concentrated sulphuric acid (3 ml.). The mixture was shaken at 18° for 120 hr. and then kept at 2° for 10 hr. Thereafter the mixture was slowly stirred into ice-water (500 ml.) and brought to pH 4 with sodium carbonate. Sugar acetates were extracted from the mixture with chloroform (5 × 150 ml.) and the extracts washed with dilute sodium carbonate and finally with water. After being dried, the chloroform was removed at 30°, leaving a syrup, which was deacetylated by the barium methoxide method.

A portion of the sugar product (400 mg.) was fractionated by thick-paper chromatography in ethyl acetate–acetic acid–water (3 : 1 : 3, by vol.), with multiple development for two 24-hr. periods. Five sugar zones (*A* to *E*, in order of decreasing R_F values) were detected on the control strips and the zones corresponding to each were separately eluted with water to recover the sugars. On complete acid hydrolysis of a small portion (2 mg.) of each zone, only mannose was detected by chromatography.

Zone A. This had R_F and M_G values identical with those of a mannose control and it was not further examined.

Zone B. This migrated as an elongated spot on paper chromatography and was shown by paper electrophoresis in borate buffer to contain two components (M_G values 0·55 and 0·61). The mixture (*ca.* 70 mg.) was chromatographed on a column of " Ultrasorb "–Celite (55 × 2·5 cm.) with gradient elution by 0·25 ⟶ 8% aqueous ethanol in 20 l. Small portions (1 ml.) of each 100 ml. of eluent were examined with the phenol–sulphuric acid reagent.[20] Two incompletely separated peaks of concentration in the eluent were obtained and, by selection of peak fractions only, two chromatographically pure sugars, *B*1 (25 mg.) and *B*2 (10 mg.), were obtained.

Sugar *B*1 migrated as a disaccharide on electrophoresis in 0·4M-sodium hydrogen sulphite [8] (M_G 0·73; maltose and maltotriose controls had M_G values 0·73 and 0·40, respectively). It had R_F and M_G values identical with those of 2-*O*-α-D-mannopyranosyl-D-mannose. The disaccharide (1·4 mg.) was oxidised at 18° in a solution containing 0·3M-sodium metaperiodate (0·5 ml.), 1% *p*-hydroxybenzaldehyde (0·3 ml.), 0·25M-phosphate buffer (pH 8·0; 1 ml.), and water to 10 ml. Portions (2 ml. each) were removed at intervals for estimation of formaldehyde. A control digest, containing the reagents but no sugar, was similarly treated. Another portion of the disaccharide (1·4 mg.) in water (2 ml.) was reduced with 0·3% sodium borohydride solution (1 ml.) for 48 hr. at 20°. Sufficient 2N-sulphuric acid was then added to make the solution acid and, after 5 min., the pH was adjusted to 8. The reduced sugar was then oxidised as above. Another portion (1·5 mg.) was reduced and then oxidised in a similar manner but at pH 3·6 (0·1M-acetate buffer). The results (CH_2O liberated, mole/mole) were:

Time (hr.):	12·5	25·6	47·5
Sugar at pH 8	1·42	1·66	1·81
Sugar alcohol at pH 8	0·88	1·01	0·96
Sugar alcohol at pH 3·6	0·84	1·04	1·01

A further portion of the acetolysis mixture, when separated by the above method, gave a sugar identical with *B*1, and having $[\alpha]_D^{16}$ +48·0° (*c* 0·08 in water).

Sugar *B*2 also migrated as a disaccharide on electrophoresis in sodium hydrogen sulphite solution. Its R_F and M_G values were identical with those of 6-*O*-α-D-mannopyranosyl-D-mannose. The sugar (0·8 mg.) was oxidised with periodate at pH 8 as described above and

[22] Aspinall, Rashbrook, and Kessler, *J.*, 1958, 215.

the alcohol, derived by borohydride reduction, was also oxidised. Results (CH_2O liberated, mole/mole) were:

Time (hr.):	12·5	25·6	47·5
Sugar at pH 8	0·13	0·07	0·07
Sugar alcohol at pH 8	0·85	0·87	0·94
Sugar alcohol at pH 3·6	0·71	0·91	0·91

A further quantity of sugar *B*2, when obtained as above, had $[\alpha]_D^{16}$ +62·3° (*c* 0·06 in water).

Zones C, D, *and* E. On chromatography in the butan-1-ol–ethanol–water solvent, these had R_F values relative to that of galactose of 0·25, 0·15, and 0·05, respectively. On partial hydrolysis by 0·3N-sulphuric acid at 100° for 1 hr., zone *C* gave disaccharides identical in chromatographic and electrophoretic behaviour with sugars *B*1 and *B*2. Zones *D* and *E* also gave the same two disaccharides when similarly treated. No trace of any other disaccharides was found in the acetolysate.

Acetolysis of Mannose.—Dry D-mannose (5 g.) was treated under the same acetolysing conditions as for the yeast mannan, and the deacetylated products were examined chromatographically. Mannose was the only product detected.

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