397. *The Structure of a Cell-wall Polysaccharide of Baker's Yeast.*

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The so-called yeast "glucan" has been methylated in an oxygen-free atmosphere. Analysis of the cleavage products on the partition chromatogram shows that the polysaccharide possesses a highly branched structure and that the average unit chain contains nine D-glucose radicals (confirmed by periodate oxidation). 2 : 3 : 4 : 6-Tetramethyl (1 mol.), 2 : 4 : 6-trimethyl (7 mols.), and 4 : 6-dimethyl glucose (1 mol.) were obtained crystalline. It is the first time that this dimethyl sugar has been obtained from material of natural origin after methylation.

Experimental losses were so low that we can claim to have obtained a nearly complete picture of the structure of this polysaccharide.

The cell-wall polysaccharide of baker's yeast has been examined by only a few workers since Salkowski (*Ber.*, 1894, **27**, 3325) first described it as "yeast cellulose." Salkowski obtained this insoluble carbohydrate by treatment of the yeast cell with 2% potassium hydroxide solution followed by autoclaving of the solid residue with water, and stated that it did not give a blue colour with iodine and yielded glucose and mannose after hydrolysis with dilute sulphuric acid. Zechmeister and Töth (*Biochem. Z.*, 1934, **270**, 309) treated the yeast with dilute alkali and then rendered the glycogen-like material (which is stained red-brown with iodine) soluble by boiling the product with 3% sulphuric acid. Essentially the same procedure was adopted by Hassid, Joslyn, and McCready (*J. Amer. Chem. Soc.*, 1941, **63**, 295), who used, however, hydrochloric acid in place of sulphuric acid. Both groups of investigators proved that the methylated polysaccharide yielded, as the main cleavage product, 2 : 4 : 6-trimethyl D-glucose, thus showing the presence of a 1 : 3-linkage between the component glucose radicals. Barry and Dillon (*Proc. Roy. Irish Acad.*, 1943, **49**, *B*, 177), by treatment of the polysaccharide with acid periodate, again obtained evidence of the 1 : 3-link since only a small amount of periodate underwent reduction. Furthermore, subsequent bromine oxidation of the periodate-treated material yielded an acid, isolated as its silver salt. From investigation of this substance Barry and Dillon deduced a chain-length of 28 glucose radicals for the yeast glucan. In no case was any evidence obtained whether or not the substance has a branched structure, nor was any "end-group" detected.

The method adopted in the present work was based on the earlier experiments mentioned above, but the "glycogen" component was successfully removed by exhaustion with warm 0·5N-acetic acid. This leaves the insoluble glucan in a non-reducing condition; we find that mineral acid leads to the formation of a reducing product at this stage. Throughout our work we avoided contact with mineral acids and during the methylation of the polysaccharide oxygen was excluded as long as the system was alkaline. We are confident that our material is in fact derived from the cell wall. The washed material isolated from the cytolysis of the yeast, when examined microscopically both in water and after staining with iodine, presented an appearance closely similar to that of intact yeast cells.

Methylation of yeast glucan is difficult. Zechmeister and Töth (*loc. cit.*) employed as starting-material an acetate made by the action of acetic anhydride and acetic and sulphuric acids. It seems to us that this procedure may lead to unpredictable degradation of the polysaccharide. Hassid *et al.* (*loc. cit.*) carried out direct methylation in presence first of carbon tetrachloride, then of acetone; they completed their etherification using sodium in liquid ammonia (Muskat, *J. Amer. Chem. Soc.*, 1934, **56**, 693, 2449). The use of liquid ammonia and sodium has been criticised by Freudenberg and Boppel (*Ber.*, 1940, **73**, 609) and by Schorigin (*J. Gen. Chem., Russia*, 1944, **14**, 825). Hassid *et al.* methanolysed their methylated material, by prolonged treatment with methanol containing 17% of hydrogen chloride, and distilled the resulting product in a high vacuum, recovering, from 5 g. of methylated polysaccharide, 3·689 g. of 2 : 4 : 6-trimethyl α- + β-methylglucosides. This corresponds with only 67·8% recovery. These authors further carried out viscosity measurements on the methylated polysaccharide applying Staudinger's $K_m = 10^{-3}$ for cellulose and obtained a value *ca.* 6500. As will be seen, we have now shown that yeast glucan possesses a highly branched structure; the application of the constant for the linear polysaccharide is therefore not valid. It may further be noted that Hassid *et al.* deduce, from reasonable evidence, the presence of β-linkages between the component radicals of yeast glucan but they represent the substance in their paper by a structural formula having links of the α-type.

We found that acetylation (pyridine and acetic anhydride) of the partly methylated material

considerably facilitated subsequent etherification. After twenty-six methylations the methoxyl content was 41·5% and was not appreciably raised (41·7%) by five further treatments. In each methylation the losses averaged 1·0—1·1%, the final yield being 55·6%. The methylated glucan was converted into its component radicals by boiling it with methanol containing 2% (w/w) of dry hydrogen chloride and then hydrolysing the resulting mixture of methylglucosides with N-hydrochloric acid at 95—100°. (It should be noted that acid hydrolysis showed that the unsubstituted polysaccharide was composed solely of glucose radicals; see Experimental section.) Analysis of the mixture of methylated glucoses thus obtained by means of Bell's silica–water partition column (*J.*, 1944, 473) gave three crystalline fractions in an aggregate yield of 89·9%. Previous work having shown (Bell, *loc. cit.*; *J.*, 1948, 992) that the recovery of methylated glucoses from the silica columns is of the order of 94% and that the loss on hydrolysis of methylated polysaccharides by N-hydrochloric acid is of the order of 6%, we are confident that we have accounted for *ca.* 100% of the original polysaccharide.

The three crystalline fractions were identified as 2 : 3 : 4 : 6-tetramethyl (1 mol.), 2 : 4 : 6-trimethyl (7 mols.), and 4 : 6-dimethyl D-glucose (1 mol.). Yeast glucan (which must be a polysaccharide of large molecular weight) thus consists of a highly branched structure. Each unit chain must have a mean structure based on about nine glucose radicals; the link between these radicals is of the 1 : 3-type and the inter-chain link is of 1 : 2-type. (It is of course possible that the inter-chain link is 1 : 3, with a 1 : 2-link in the chain itself.) Confirmatory evidence of the chain-length was obtained after oxidation by periodate according to Halsall, Hirst, and Jones (*J.*, 1947, 1399, 1427; *ibid.*, 1948, 27), a value for the chain-length of ten glucose radicals being found. From the small positive rotation (+3°) of the methylated glucan and the large negative rotation of the triacetyl glucan we agree with Hassid *et al.* that the majority of the glycosidic linkages must be of the β-type.

Experimental.

Separation of the Yeast Cell-wall.—Fresh "Encore" baker's yeast (6 kg.) was dispersed in 6% sodium hydroxide solution (4·8 l.), and the whole was heated to 60°, diluted to 40 l. with water, and allowed to cool to room temperature. The insoluble material was collected on the centrifuge, re-suspended in 3% sodium hydroxide solution (10 l.), and kept at room temperature for 3 hours. After dilution with water (20 l.) and again centrifugation, the sludge obtained was suspended in water (10 l.), heated to 80°, and strained through a G 1 Jena filter to remove large particles. After adjustment of the pH to 4·5 the solid was readily filtered off. It was once more digested at 80°, for 2 hours, with 3% sodium hydroxide solution (5 l.) and centrifuged. No carbohydrate was found in the solution (negative Molisch reaction). After adjustment of the pH to 4·5, the solid was collected on a filter and washed first with 2% aqueous acetic acid (10 l.), then with, in turn, ethanol, ether and light petroleum (b. p. 60—80°), 5 l. each. The solid was allowed to dry in the air. The yield of a white powder was 94 g. [Found: N (Kjeldahl), 0·9; P, 0·083%].

Isolation of the Glucan.—Cell-wall material (20 g.) was heated to 75° with 0·5N-acetic acid (700 ml.); the pH of the mixture was *ca.* 2. At 75° the mixture was fluid, but on cooling to 40° it became gelatinous. On centrifugation the gelatinous solid separated, but retained about one-third of the fluid. It was then stirred with water (700 ml.) at 75—80° for an hour and centrifuged. This process was repeated 6 times with 2 l. of water each time. The final supernatant fluid gave no precipitate on addition of ethanol. The gelatinous solid was then treated with 2 volumes of ethanol, centrifuged, washed with more ethanol, and collected on a G 3 Jena filter, with, finally, washing with ether and ligroin, the bulk being thereby greatly decreased; after drying at 100°/0·01 mm., a cream-coloured solid was obtained (7·2 g., 30% of the original cell-wall substance). The glucan (20 g.) was homogenised to a thick paste with water (200 ml.). This was freeze-dried to give a light powder which was used for the subsequent experiments [Found: N (Kjeldahl), 0·42; P, 0·008%].

Identification of Glucose as the Sole Component Radical.—Glucan (2 g.) was dissolved in 72% (w/v) sulphuric acid at room temperature and kept for 240 hours, giving a light brown solution. This was added to water (1 l.) and the whole boiled under reflux for 15 hours. The almost colourless solution was neutralised with barium carbonate and filtered, and the filtrate concentrated to 150 ml. under reduced pressure at 100°. At the same time a control experiment was carried out on cotton cellulose. The hydrolysates were then examined on the paper chromatogram by the procedure of Jermyn and Isherwood (*Biochem. J.*, 1949, **44**, 402) using ethyl acetate–pyridine–water as solvents. On development of the chromatogram with ammoniacal silver nitrate a single spot was obtained from each experiment, corresponding exactly in position with a glucose control. Tests for pentoses and uronic acids were negative.

Acetylation of the Glucan.—This was done essentially by the method of Barnett (*J. Soc. Chem. Ind.*, 1921, **40**, 253). Freeze-dried polysaccharide (0·5 g.) was suspended in glacial acetic acid (5 ml.) through which chlorine had been passed for a few minutes. After the suspension had been kept for 30 minutes at room temperature, acetic anhydride (5 ml.), containing a little dissolved sulphur dioxide, was added and the whole allowed to react overnight. The mixture was then heated on the boiling water-bath for 2 hours, cooled, and filtered into ice and water (200 ml.). The acetate which separated was filtered off, washed with water, and dried at 80° in a high vacuum (yield, 0·34 g.). This was shaken with a little charcoal in chloroform and the solution filtered through barium carbonate to remove peptised charcoal. The clear chloroform filtrate was boiled with water to remove the organic solvent, and the solid residue

washed and dried as before, to give material (0·2 g.), $[\alpha]_D^{18}$ −62·3° (*c*, 0·8 in chloroform; *l*, 2) (Found: Ac, 44·6. Calc. for $C_{12}H_{16}O_8$: Ac, 44·8%). Hassid *et al.* give $[\alpha]_D$ −72° for material similarly prepared.

Methylation of Glucan.—The freeze-dried polysaccharide (10 g.), suspended in dioxan (200 ml.), was treated, at 40—50°, with methyl sulphate (500 ml.) and 30% sodium hydroxide solution (1 l.), the reagents being added in one-tenth portions at ten-minute intervals, with vigorous stirring, in an atmosphere of nitrogen. After being heated to 80—95°, the solution was acidified to pH 6—7 by addition of acetic acid and the whole distilled under reduced pressure to remove dioxan. The solid which separated was repeatedly washed with boiling water and was then given a second treatment as before. The solid product of the second methylation was dried at 0·01 mm. and then dissolved in pyridine (300 ml.), and acetic anhydride (150 ml.) added. Next morning the mixture was poured into water (2 l.), the product separating as a white solid. The whole aqueous suspension was then concentrated under reduced pressure to a volume of *ca.* 500 ml., heated to *ca.* 95°, and filtered. The solid was washed with boiling water; after being dried in a high vacuum, 10·2 g. were obtained. The material was then soluble in dioxan; it was further subjected to thirty-one methylations in dioxan solution under the conditions described above. The methoxyl content was determined from time to time [Found: OMe, (10 methylations) 36·8, (26 methylations) 41·5, (31 methylations) 41·7%]. The final yield was 7·0 g. (55·6%); the average loss per methylation was therefore 1·0—1·1% (Found: N, 0·03; P, 0·00%). The final product had $[\alpha]_D$ +3·3° (*c*, 1·3 in chloroform; *l*, 2).

Cleavage of the Methylated Glucan.—The material (1·4 g.) was refluxed with methanol (50 ml.) containing 2% (w/w) of hydrogen chloride for 18 hours, after which time it had all dissolved. After removal of the hydrogen chloride by treatment with silver carbonate, and of solids by filtration, the resulting solution was evaporated under reduced pressure. The residual syrup, dissolved in N-hydrochloric acid, was heated, under reflux, on the boiling water-bath for a further 18 hours. The solution, freed from hydrochloric acid by excess of silver carbonate in the customary manner, was examined on the paper chromatogram with *n*-butanol–water as solvent (Hirst, Hough, and Jones, *J.*, 1949, 928) and aniline phthalate for development. Spots corresponding to tetra-, tri-, and di-methyl glucoses were found, with R_F values of 0·76, 0·6, and 0·4 respectively. It should be noted that hydrolysis by hydrochloric acid in greater strength than N. should be avoided; in some experiments when stronger acid was employed, the tetramethyl glucose fraction was found to contain a non-crystalline component, indistinguishable on the chromatogram from 2 : 3 : 4 : 6-tetramethyl glucose. As this appeared to be a non-reducing material when examined by hypoiodite oxidation (Macleod and Robison, *Biochem. J.*, 1929, **23**, 517), we consider that it is probably a trehalose-like substance (or substances) formed by condensation of two molecules of the trimethyl sugar. Confirmation of this view was obtained by hydrolytic treatment (as described above for methylated glucan) which resolved the substance into trimethyl glucose, as shown by chromatography and hypoiodite reduction.

Separation of the Cleavage Products.—The hydrolysate obtained above was concentrated under reduced pressure to 10 ml. in presence of a little barium carbonate. The methylated sugars were then subjected to chromatographic separation by Bell's method (*J.*, 1944, 473) using a 10-g. column of silica. (The silica was prepared according to Tristram, *Biochem. J.*, 1946, **40**, 721.) Three fractions, all of which crystallised spontaneously, were obtained as follows: Me_4, 0·1594 g.; Me_3, 0·9854 g.; Me_2, 0·1139 g. The molecular ratios corresponded to 1 : 7 : 1, and the agregate yield was 89·9% of theory.

Identification of the Individual Separated Fractions.—(a) *The tetramethyl fraction.* A sample of the whole fraction (*i.e.*, crystals and syrup) had $[\alpha]_D^{18}$ +90° falling to +83° (*c*, 0·6 in water; *l*, 2). An authentic specimen of 2 : 3 : 4 : 6-tetramethyl glucose examined under the same conditions had $[\alpha]_D^{18}$ +93° falling to +83°. The crystals, after being drained on a tile, melted at 93°. Mixed with authentic material (m. p. 95°) this figure was not appreciably depressed. On the paper chromatogram (solvents: butanol–water) the whole fraction showed a single spot of R_F 0·76, identical with a control sample of 2 : 3 : 4 : 6-tetramethyl glucose (Found: OMe, 52·1. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%).

(b) *The trimethyl fraction.* This after recrystallisation from ether had $[\alpha]_D^{18}$ +84° falling to +70° (*c*, 1·2 in water; *l*, 2). Lake and Peat (*J.*, 1938, 1417) give $[\alpha]_D$ (in water) +98·2° falling to +74·8°, and Hassid *et al.* (*loc. cit.*) give +91·4° falling to +71·4°. The crystals melted at 120° and, on admixture, did not depress the m. p. of authentic 2 : 4 : 6-trimethylglucose (m. p. 115°) (Found: OMe, 41·8. Calc. for $C_9H_{18}O_6$: OMe, 41·2%).

Further proof of the structure of the sugar was obtained by the action of sodium metaperiodate solution; a control with pure 2 : 4 : 6-trimethyl glucose was carried out simultaneously. In neither case, after 25 hours, was any evidence of periodate reduction obtained.

The whole fraction, without recrystallisation, was examined on the paper chromatogram (solvents: butanol–water), the following R_F values being obtained: glucan material, 0·60; authentic 2 : 4 : 6-trimethyl glucose 0·60; 3 : 4 : 6-trimethyl glucose 0·635.

Oxidation by bromine water, followed by conversion of the resulting lactone into the corresponding amide (Lake and Peat, *loc. cit.*), yielded crystals, m. p. 98°, and these, mixed with authentic 2 : 4 : 6-trimethylgluconamide (m. p. 100°) melted at 100°. The amide gave a negative Weerman reaction (Haworth, Peat, and Whetstone, *J.*, 1938, 975).

(c) *The dimethyl fraction.* A sample of the whole fraction had $[\alpha]_D^{18}$ +87·5° (50 minutes) falling to +67·0° (18 hours) (*c*, 0·4 in water; *l*, 2). An authentic specimen of 4 : 6-dimethyl glucose had $[\alpha]_D^{18}$ +93·1° falling to +62·4°. Bell and Synge (*J.*, 1937, 1711) and Bell and Lorber (*J.*, 1940, 453) give respectively +63·6° and +65·7° for $[\alpha]_D$ at equilibrium.

After recrystallisation from ethyl acetate, needles were obtained, having m. p. 155°, not depressed after admixture with authentic 4 : 6-dimethyl glucose, m. p. 158°. On the paper chromatogram (butanol–water) the whole fraction gave a single spot of R_F 0·4, identical in its behaviour with authentic material.

On oxidation with sodium metaperiodate solution a sample of the entire fraction consumed exactly 2 moles of periodate, and yielded 2 moles of formic acid. No formaldehyde was detected by dimedon. Control experiments with authentic 4 : 6-dimethyl glucose, made at the same time, yielded identical results.

End-group Assay of the Glucan by Periodate Oxidation.—This was carried out essentially by the method of Halsall, Hirst, and Jones (*J.*, 1947, 1399, 1427). Since yeast glucan is completely insoluble in water, unlike the polysaccharides used by the above authors, a detailed description of our experiment is given.

To the freeze-dried glucan (493·3 mg.), suspended in water (120 ml.), potassium chloride (5 g.) was added, and the final volume noted. A 10-ml. sample was withdrawn to serve as a control during the subsequent titration. To this control sample, 50% aqueous ethylene glycol (2 ml.) was added. (The glycol was previously distilled over solid potassium hydroxide and then diluted with water, and the pH adjusted to 5.) The suspension was then made up to a volume of 15 ml. and 5-ml. aliquots were titrated, in a stream of carbon dioxide-free air, against 0·01N-sodium hydroxide (methyl-red).

To the main bulk of the original suspension was added sodium periodate solution (0·25M.; 15 ml.), and the whole agitated for 25 hours. The suspension was then allowed to settle and a 10-ml. sample of the supernatant fluid withdrawn and treated in the same way as the control. The whole procedure of agitation and withdrawal of samples at 24-hour intervals was repeated until the production of acid titratable to pH 5 had reached a maximum (280 hours). After this a slight decrease in the acid titre was noticed. The results were :

Time of sampling (hrs.)	24	96	168	240	280	300
Titre (0·01N-NaOH; ml.)	0·48	0·61	0·64	0·66	0·69	0·68

The value 0·69 ml. is equivalent to 12·65 mg. of formic acid and corresponds to a unit chain length of 10·2 glucose radicals.

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