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The Molecular Constitution of an Insoluble Polysaccharide from Yeast, *Saccharomyces cerevisiae*

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Several of the polysaccharide constituents of yeast, including glycogen, yeast gum, and several water insoluble compounds, have been described by a number of investigators.[1-8] The chemical structure of one of these constituents, mannan, was studied in detail by Haworth, Hirst and Isherwood.[8] These authors showed that the yeast mannan differs fundamentally in structure from the mannan of the ivory-nut[9] and that synthesized from glucose by *Penicillium Charlesii G. Smith*. Zechmeister and Toth[4] studied the insoluble yeast polysaccharide which was first described by Salkowski[3] and referred to as "yeast cellulose." They found that although this difficultly hydrolyzable polysaccharide could be hydrolyzed, like cellulose, with 40 or 42% hydrochloric acid, it did not give the characteristic cellulose tests. No blue coloration could be obtained with a solution of iodine in potassium iodide and treatment with strong acid; it was insoluble in ammoniacal copper oxide solution (Schweitzer's reagent) and it did not yield cellobiose when treated with acetolyzing reagents. The results of these authors also indicated a rather unusual 1,3-glucosidic linkage between the anhydroglucose units in the chain. Most of the naturally occurring common polysaccharides such as cellulose, starch, glycogen, etc., possess the 1,4 linkage.

In the present investigation the structure of this polysaccharide was studied to determine: (a) type of glucosidic linkage involved (α or β); (b) whether we are dealing with a short chain polymer, such as starch, or glycogen, or with a linear polymer, such as cellulose; (c) magnitude of molecular size.

(1) A. Harden and W. J. Young, *J. Chem. Soc.*, 1928 (1912).
(2) E. Salkowski, *Ber.*, **27**, 3325 (1894).
(3) E. Salkowski, *Z. physiol. Chem.*, **92**, 75 (1914).
(4) L. Zechmeister and G. Toth, *Biochem. Z.*, **270**, 309 (1934).
(5) M. G. Sevag, C. Cattaneo and L. Maiweg, *Ann.*, **519**, 111 (1935).
(6) L. Zechmeister and G. Toth, *Biochem. Z.*, **284**, 133 (1936).
(7) R. A. McAnally and I. Smedley-Maclean, *Biochem. J.*, **31**, 72 (1937).
(8) W. N. Haworth, E. L. Hirst and F. A. Isherwood, *J. Chem. Soc.*, 784 (1937).
(9) F. Klages, *Ann.*, **509**, 159 (1934).

Experimental

Isolation of Polysaccharide.—2700 Grams of compressed baker's yeast grown on a grain mash was digested in two portions in 4-liter flasks with 2 liters each of 3% sodium hydroxide by heating on a boiling water-bath for four hours. The dark brown alkaline digest was allowed to remain at room temperature for one day, the supernatant liquid was then decanted and 2 liters of fresh 3% sodium hydroxide was added to each flask. The flasks with the contents were shaken, placed for two hours on a boiling water-bath and then allowed to cool overnight. The alkaline supernatant liquid, which again became dark brown in color, was decanted, the residue acidified with about 800 cc. of concentrated hydrochloric acid and 2 liters of 3% hydrochloric acid added to each flask. The flasks with the contents were digested for several hours on a water-bath, cooled, the supernatant liquid decanted and the digestion with 3% hydrochloric acid repeated. The final acid digest was decanted, the residue washed with distilled water, centrifuged, resuspended in water and again centrifuged. The residue was then washed well with boiling water, centrifuged, and the combined residues from the two flasks suspended in 1 liter of alcohol, and stored at room temperature for several days. The brownish-red alcohol solution was centrifuged off, the residue resuspended in alcohol and then filtered by suction, washed with ether and dried at 70° *in vacuo* for four hours. It dried to a horny hard brownish mass; when ground to a fine powder in a ball mill a grayish-white power was obtained. The yield was 64 g.; since the moisture content of the compressed yeast used averages about 70%, the yield on dry weight basis is 7.9%.

The polysaccharide did not reduce Fehling solution, was insoluble in water, and in dilute acid and alkali. It could, however, be hydrolyzed with fuming hydrochloric acid. Its ash content was 0.2% and nitrogen 0.56%. The nitrogen content of the acetylated polysaccharide was 0.052% and the methylated derivative 0.004%. This indicates that the nitrogen in the polysaccharide is an impurity and not an integral part of the molecule.

Anal. Calcd. for $(C_6H_{10}O_5)_n$: C, 44.4; H, 6.2. Found: C, 44.5; H, 6.1.

Hydrolysis.—One gram of the polysaccharide was hydrolyzed with 50 cc. of 41% hydrochloric acid at 0° for twenty-four hours, diluted with 100 cc. of water and heated on the steam-bath for one hour. The solution was then cooled and diluted to 200 cc. The specific rotation, $[\alpha]_D$, of the hydrolyzed polysaccharide in this solution was +53.2°.[10] An osazone was isolated from the neutralized solution, which was identified as glucose osazone by its

(10) The specific rotation of the same concentration of glucose in 10% of hydrochloric acid solution was +55.0°. The rotation of an equilibrium mixture of α and β glucose in water is +52.5°.

melting point and shape of crystals. No mannose phenylhydrazone could be obtained. The solution also gave a negative test for the Seliwanoff reaction. Mannose and fructose were therefore not present in the hydrolysis product. The absence of pentose sugars and uronic acid was shown by the negative orcinol test and also by the fact that when the polysaccharide was distilled with 12% hydrochloric acid and the distillate treated with thiobarbituric acid no precipitate was formed. The reducing value determined on the neutralized solution by oxidation with ferricyanide and titration with ceric sulfate[11] was 92% calculated as glucose.

In order to determine the direction of mutarotation during hydrolysis of the polysaccharide, 1-g. portions of the material were placed in 3 flasks and dissolved with fuming hydrochloric acid at 0°. The specific rotations of the solutions were taken at intervals of one, two and five hours. Before taking the polarimetric readings, the solutions were diluted to 200 cc. and filtered. The specific rotations, $[\alpha]_D$ at the different intervals, were +0.47°, +1.02°, and +1.50°, respectively. The upward mutarotation during hydrolysis indicates the β-configuration of the glucose units in the polysaccharide.

Acetylation.—Two grams of the polysaccharide was suspended in 15 cc. of acetic acid containing a little chlorine. After thirty minutes at room temperature 20 cc. of acetic anhydride, through which sulfur dioxide had been bubbled, was added slowly and the mixture shaken for four hours. The mixture was then kept for four hours at 80° with occasional shaking. A small amount of insoluble material (apparently unacetylated) was filtered off and the filtrate poured into 500 cc. of ice water. The precipitated acetate was washed with water until free from acid, filtered and dried *in vacuo* at 80°.

Anal. Calcd. for $(C_6H_7O_5(CH_3CO)_3)_n$: CH_3CO, 44.8. Found: CH_3CO, 45.0. Specific rotation: $[\alpha]_D = -72°$ (in chloroform, $c = 1$).

Methylation.—The first stage of methylation was carried out in a medium of carbon tetrachloride as follows: 20 g. of the finely ground polysaccharide was treated with a mixture of 100 cc. of carbon tetrachloride and 72 cc. of methyl sulfate and vigorously stirred with a mechanical stirrer for fifteen minutes; 160 cc. of 30% sodium hydroxide was then added slowly over a period of half an hour; 370 cc. of 30% sodium hydroxide and 160 cc. of methyl sulfate were simultaneously admitted into the reaction flask, adding the methylating reagents from two dropping funnels in portions of 3.3 cc. of methyl sulfate and 7.5 cc. of sodium hydroxide every five minutes. At the end of this process the carbon tetrachloride was evaporated, and the mixture cooled and almost neutralized with sulfuric acid; 300 cc. of water was then added, the mixture heated to 100°, and the partially methylated polysaccharide separated and dried. It was then twice methylated in a medium of acetone by the method of Haworth, Hirst and Woolgar.[12] The methoxyl content, OCH_3, of the product was 35.5%. Further methylation of this product was carried out according to a modification of Muskat's method,[13] as follows. The partially methylated polysaccharide was dissolved in 200 cc. of dry anisole and the solution frozen by immersion of the reaction flask in a mixture of solid carbon dioxide and acetone at −50°. One hundred cc. of dry ammonia was condensed on the frozen mass and 3 g. of sodium dissolved in it and the temperature raised to −35°. After the anisole solution had melted, it was shaken well with the ammonia solution. The ammonia was then allowed to evaporate at room temperature, the last traces being removed by distilling off a portion of the anisole under reduced pressure. Fifteen cc. of methyl iodide was added and the solution refluxed overnight at 40°. The anisole was distilled off and the dry residue removed from the distilling flask by washing with about 500 cc. of boiling water. The methylated product, insoluble in hot water, was filtered on a hard filter paper when hot, and washed with boiling water. It was dried *in vacuo* and remethylated as before. The final product was dissolved in chloroform and precipitated in low-boiling petroleum ether.

Anal. Calcd. for $(C_6H_7O_2(OCH_3)_3)_n$: OCH_3, 45.6. Found: OCH_3, 45.0.

Specific rotation: $[\alpha]_D$ +4.5° (in chloroform, $c = 1$).

The specific viscosity, $\eta_{sp.}$, at 25° of a 0.4% solution of the methylated polysaccharide in *m*-cresol was 0.16. Applying Staudinger's formula with $K_m = 10^{-3}$, used by Carrington, Haworth, Hirst and Stacey[14] for methylated cellulose, a molecular weight of 8170 was obtained for the methylated derivative and 6500 for the polysaccharide.

Hydrolysis of Methylated Polysaccharide and Identification of Methylated Derivative.—Five grams of the methylated polysaccharide (OCH_3, 45.0%) was hydrolyzed with 120 g. of methanol, containing 17% of dry hydrogen chloride, for eighteen hours under a reflux condenser. The hot solution was then neutralized with lead carbonate, filtered when cold, and the filtrate evaporated to dryness. The residue was extracted with chloroform and the resulting solution, free of mineral matter, evaporated to dryness. The sirup was distilled at a pressure of 4×10^{-3} mm. from a flask fitted with a vacuum-jacketed fractionating column. The following fractions were obtained:

Fraction	Temp., °C.	Weight, g.	$n^{16}D$	OCH_3
I	145	0.847	1.4595	51.6
II	145–155	1.435	1.4595	52.2
III	155–165	1.297	1.4595	52.0
IV	165–170	0.110	1.4595	52.2

Employing the criteria of purity established by Hirst and Young[15] for mixtures of tetramethyl- and trimethylmethylglucosides, it is obvious from the indices of refraction and methoxyl contents of the four fractions that we are dealing with only one constituent, trimethylmethylglucoside (calculated OCH_3 content for $C_6H_8O_2(OCH_3)_4$, 52.6%), and that, apparently, no tetramethylmethylglucoside was present. Using the same fractionating column, mixtures of about 5 g. containing different proportions of pure tetramethylmethylglucoside and tri-

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(13) I. E. Muskat, THIS JOURNAL, **56**, 693, 2448 (1934).

(14) H. C. Carrington, W. N. Haworth, E. L. Hirst and M. Stacey, *J. Chem. Soc.*, 1902 (1939).

(15) E. L. Hirst, and G. T. Young, *ibid.*, 1247 (1938).

methylmethylglucoside could be separated quantitatively by the Hirst and Young method.

The sirupy non-reducing fractions were combined and hydrolyzed with 100 cc. of 6% sulfuric acid by boiling gently under a reflux condenser for twenty hours. The solution was neutralized with barium carbonate, filtered, and the filtrate evaporated *in vacuo*. The residue was extracted with boiling ether and filtered. Upon evaporation of the ether to a small volume, crystals readily formed. On recrystallization from ether, crystals with a melting point of 123° were obtained. The crystals did not form an osazone but reduced Fehling solution. The reducing value of the trimethylglucose with ferricyanide[11] was 68% of glucose.

Anal. Calcd. for $C_6H_9O_3(OCH_3)_3$: OCH_3, 41.9. Found: OCH_3, 41.7.

The specific rotation, $[\alpha]_D$, was +110° (in methanol, c = 1) and changed with acid catalysis to the equilibrium value of +70.0°; or +91.4° ⟶ +71.4° (in water, c = 1). The trimethylglucose did not undergo inversion of sign during condensation with methanol, containing 0.25% hydrogen chloride, a behavior regarded as characteristic for the 2,3,6-isomer.

Since the trimethylglucose is reducing but does not form an osazone, it indicates that the hydroxyl on the second carbon atom must be substituted by one of the methyl groups. Also, since, from stereochemical considerations, the 1,3- and 1,6-rings in the glucose molecule are improbable, the possibility of a choice of the following 3 configurations with respect to position of the methoxyl groups remains: 2,3,4-, 2,3,6-, and 2,4,6-trimethylglucose. The 2,3,4-configuration may be eliminated on the ground that this isomer cannot be obtained in crystalline form.[16] The constants given for the 2,3,6-trimethylglucose[17,18] and those for 2,4,6-trimethylglucose[16] differ but slightly, and, as pointed out by Haworth and Sedgwick,[16] the essential difference is that the 2,4,6-isomer does not undergo inversion of sign during condensation with methanolic hydrogen chloride at room temperature. To further establish the fact that the trimethylglucose obtained by us was not the 2,3,6-derivative, the X-ray diffraction pattern of a pure sample of the latter was compared with the pattern of our isolated trimethyl derivative. Irvine and Hirst[17] found that the 2,3,6-trimethylglucose could be resolved into two crystalline modifications: fine needles, m. p. 114–115°, and short prisms, m. p. 92–93°. Since the melting points and the two crystalline forms were different, it was of interest to find out whether their diffraction patterns were also different. It was found possible to obtain by crystallization two products corresponding to those described by Irvine and Hirst, namely, fine needles, m. p. 117°, and short prisms, m. p. 93°. No difference could be observed in the two patterns of the two crystalline forms. If these substances had a fundamentally different structure, distinct differences in their patterns would have been evident. Since this was not the case, it is reasonable to assume that both crystalline modifications are essentially the same crystalline substance. Apparently the presence of small amounts of impurities which would not be evident in the X-ray patterns is capable of producing marked changes in the melting point.

As shown in Fig. 1, the pattern of the crystalline trimethylglucose derivative isolated from the methylated polysaccharide is distinctly different from the 2,3,6-trimethylglucose. This fact is added proof of the difference between the two isomers. The lines of the X-ray pattern of the 2,3,6-trimethylglucose are in agreement with the unit cell established by Cox, Goodwin and Wagstaff.[19]

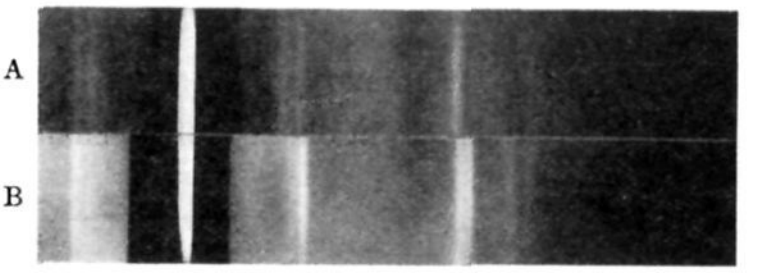
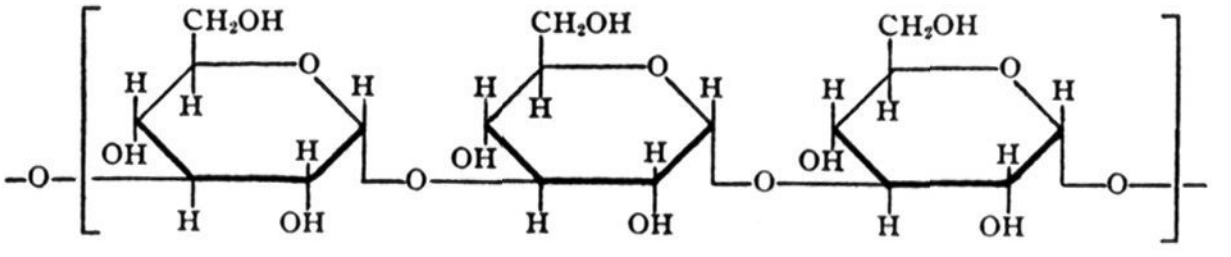


Fig. 1.—X-Ray diffraction of two isomers of trimethylglucose: A, 2,3,6-trimethylglucose; B, 2,4,6-trimethylglucose.

Discussion

The isolation of 2,4,6-trimethylglucose as the sole product of hydrolysis clearly indicates that the basic constituent of the yeast polysaccharide is a chain of glucopyranose units united by 1,3-glucosidic linkages. This yeast polysaccharide is of special interest in that it is the first of the dextrans investigated to have the glucopyranose units jointed by glucosidic linkages between the first and third carbon atoms. From the consideration of the optical rotations of the methylated and acetylated polysaccharides (methylated, $[\alpha]_D$ +4.5°; acetylated, $[\alpha]_D$ −72.0°), and also from the upward mutarotation during hydrolysis, it appears that the glucosidic linkages are mainly of the β-type. In accordance the following structural formula can be written for this polysaccharide.



Viscosity measurements of the methylated derivative indicate that this polysaccharide has a high molecular weight (about 6500). The apparent absence of an end-group (tetramethylglu-

(16) W. N. Haworth and W. G. Sedgwick, *J. Chem. Soc.*, 2573 (1926).

(17) J. C. Irvine and E. L. Hirst, *ibid.*, 1213 (1922).

(18) J. C. Irvine and I. M. A. Black, *ibid.*, 862 (1926).

(19) E. G. Cox, T. H. Goodwin and A. I. Wagstaff, *ibid.*, 1495 (1935).

cose) in the methylated polysaccharide can be explained by the assumption that either the chain exists in the form of a continuous loop or that the number of glucose units is too great to allow the isolation of tetramethylglucose under the conditions employed.

The authors are indebted to Mr. W. H. Dore for making the diffraction patterns of the trimethylglucoses and for his interest and suggestions during the course of this investigation. The authors also express their appreciation to the Acme Brewery of San Francisco for supplying the yeast used in these investigations.

Summary

The structure of an insoluble polysaccharide isolated from yeast (*Saccharomyces cerevisiae*) was studied. The low specific rotations of the acetylated and methylated derivatives, and the upward mutarotation during hydrolysis suggest that the glucosidic linkages of the anhydroglucose units are predominantly of the β-type.

On methylation and subsequent hydrolysis of the polysaccharide 2,4,6-trimethylglucose was obtained as the sole product of hydrolysis. No end-group (tetramethylglucose) could be detected. This suggests that the molecule is probably of the closed chain type.

The molecular weight of the polysaccharide determined by the Staudinger viscosity method was approximately 6500.

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Catalytic Dehydration of 4-Morpholineëthanol[1a]

BY HARRY W. BLOCK[1b] WITH J. PHILIP MASON

The dehydration of 4-morpholineëthanol, if analogous to the dehydration of ethanol, should yield 2,2′-dimorpholinodiethyl ether and N-vinylmorpholine. The work done by Meyer and Hopff[2] indicates that N-vinylmorpholine would probably be quite unstable. These investigators prepared dimethylvinylamine by the distillation of neurine chloride and diethylvinylamine by the distillation of β-diethylaminoethyltrimethylammonium hydroxide. In the former case they obtained a 3% yield and in the latter a 2% yield. Both products polymerized readily.

Campbell and Campbell[3] pyrolyzed the Grignard complex of 2-methyl-2-hydroxy-1-dimethylaminopropane and obtained the solid hydrochloride of 1-dimethylamino-2-methyl-1-propene which was described as very unstable, decomposing quickly in moist air. On hydrolysis it yielded isobutyraldehyde and dimethylamine. Phou-Ti Sou[4] dehydrated alcohols of the type R_2NCH_2-$C(OH)R_2'$ with phosphorus pentachloride in ether solution and obtained some $R_2NCH{=}CR_2'$ which hydrolyzed readily in an alkaline solution to form $R_2'CHCHO$.

The products which were identified from the catalytic dehydration of 4-morpholineëthanol were acetylene, morpholine, 1,2-dimorpholinoethane, 2,2′-dimorpholinodiethyl ether, and unreacted morpholineëthanol.

Since no fraction having the properties expected of N-vinylmorpholine was obtained, an attempt was made to prepare this compound by treating morpholinoethyl chloride with alcoholic potassium hydroxide solution. However, the only product isolated from this reaction was morpholinoethyl ethyl ether.

There are two mechanisms possible for the formation of acetylene, morpholine and 1,2-dimorpholinoethane from morpholineëthanol. One possibility is that N-vinylmorpholine may be formed as an intermediate which loses acetylene to yield morpholine. The morpholine could react with morpholineëthanol to form 1,2-dimorpholinoethane. This reaction between morpholine and morpholineëthanol was suggested by Dr. A. L. Wilson.[5]

A second mechanism, suggested by Dr. Wilson,[5] is that morpholineëthanol may decompose to form morpholine and either ethylene oxide or acetalde-

(1a) This name was suggested by Dr. E. J. Crane as preferable to β-4-morpholineëthanol used previously[12] by us.

(1b) Taken from the thesis submitted by Harry W. Block to the faculty of the Graduate School of Boston University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Meyer and Hopff, *Ber.*, **54B**, 2274 (1921).

(3) Campbell and Campbell, THIS JOURNAL, **60**, 1373 (1938).

(4) Phou-Ti Sou, *Bull. faculté sci. univ. franco-chinoise Peiping*, No. 5, 1–7 (1935); *C. A.*, **30**, 4463 (1936).

(5) Private communication.