[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY]

A Crystalline Mannotriose from the Enzymatic Hydrolysis of Guaran[1]

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By enzymatic hydrolysis of guaran there is obtained, in 7.5% yield, a crystalline trisaccharide, the structure of which is shown to be β-D-mannopyranosyl-(1 → 4)-β-D-mannopyranosyl-(1 → 4)-β-D-mannopyranose. There is also obtained in very low yield (*ca.* 0.5%) a crystalline disaccharide 6-α-D-galactopyranosyl-β-D-mannopyranose, identical with one of the disaccharides obtained from partial acid hydrolysis of guaran. Upon reduction of the trisaccharide with hydrogen and platinum the glycitol is obtained in quantitative yield and on acetylation is converted to the crystalline acetate in 70% yield.

Accumulated evidence on the structure of the polysaccharide, guaran,[2] indicates that the molecule is composed of a straight chain of D-mannopyranose units joined by β-D-1,4′-linkages and that on the average, every other D-mannopyranose unit bears a side chain composed of one D-galactopyranose residue connected by an α-D-1,6′-linkage. Periodate oxidation,[3-5] methylation data,[3,6,7] physical measurements on triacetate films,[8] X-ray investigations[9] and isolation of crystalline oligosaccharides from partial enzymatic[10] and acid[11,12] hydrolyses of the molecule confirm this proposed structure.

Through further investigation it has now become possible to isolate in crystalline condition two additional oligosaccharides from the enzymatic hydrolysate of guaran. These are shown to be 6-α-D-galactopyranosyl-β-D-mannopyranose, previously obtained by partial acid hydrolysis of guaran, and a new trisaccharide shown to be β-D-mannopyranosyl-(1→4)-β-D-mannopyranosyl-(1→4)-β-D-mannopyranose.

An enzyme preparation from germinated guar seeds[13] will partially hydrolyze an aqueous dispersion of guaran. Under optimum conditions[13] the hydrolysis terminates when 65% of the guaran is converted to monosaccharides. Separation of the hydrolysate into several fractions is possible by the chromatographic procedure of Whistler and Durso.[14] Approximately 65% of the hydrolysis products consist of monosaccharides and include most of the D-galactose from the polysaccharide. After removal of the monosaccharides from the chromatographic column with water, any mannobiose which may be present in the column is eluted with 2.5% ethanol. The galactomannose disaccharide is obtained in 0.5% yield on elution with 5% ethanol, and the mannotriose is obtained in 7.5% yield on elution with 7.5% ethanol.

The disaccharide is identified as the galactomannose previously isolated and characterized, by showing that the two sugars are alike in melting point, mixed melting point, X-ray pattern, optical rotation and flow rate on paper chromatography.

The mannotriose is a chain of 1,4′-linked β-D-mannopyranose units as evidenced by periodate oxidation data and the observation that partial acid hydrolysis produces only D-mannose and the previously identified mannobiose which contains a 1,4-β-D′-glycosidic linkage. Reduction of the crystalline mannotriose with hydrogen on platinum catalyst produces a glycitol which can be isolated as the crystalline dodecaacetate.

Isolation of these two oligosaccharides is further confirmatory evidence for the structure of guaran proposed earlier.[15]

Experimental

Enzyme Preparation.—Two kg. of guar seeds was germinated, extracted with water, centrifuged and the centrifugate fractionally precipitated with ammonium sulfate.[13] The fraction which was salted out between 20 and 30% ammonium sulfate was air-dried, ground in a mortar and stored at 5°.

Hydrolysis of Guaran.—Fifty grams of guaran (or guar flour) was homogenized with 2500 ml. of water in a Waring Blendor and the resulting suspension stirred at 95° for 8 hours to assure complete solution. After adjustment of the *p*H to 5.0 with 1 *N* hydrochloric acid, the solution was placed in a constant temperature bath at 35°. One gram of enzyme was added, and 1-ml. aliquots were withdrawn at intervals for determination of the reducing power by iodometric titration according to the procedure of Ingles and Israel.[16] Additional 1-g. portions of enzyme were added until the hydrolysis was approximately 65% complete,[10] which usually required about 70 hours, but the periods varied somewhat with the activity of the individual enzyme preparations. The hydrolysate was then neutralized with solid sodium bicarbonate and filtered prior to chromatography.

Chromatography of Hydrolysate.—Five hundred ml. of filtered hydrolysate was chromatographed on a 98 × 300 mm. Darco:Celite (1:1) column.[11] Monosaccharides were eluted with 2 l. of water, the mannobiose was eluted with 2 l. of 2.5% ethanol and the galactomannose disaccharide was removed along with mannobiose and mannotriose by elution with 1 l. of 5% ethanol. This last fraction, after concentration to a sirup, was readsorbed on a fresh column and re-eluted as above. This purification was repeated five times in all, after which the 5% ethanol eluate contained chiefly galactomannose. This fraction was concentrated to a small volume, methanol and butanol (2:1) were added and the solution was seeded. Crystallization occurred in 24 hr. The melting point of the crystals was 200–201°, $[\alpha]^{25}D$ +120° (*c* 1.6 in water). A mixed melting point with the known compound showed no depression. X-Ray patterns of the new compound and the known reference disaccharide were identical.

Elution of the first chromatographic column with a second liter of 5% ethanol and 2 l. of 7.5% ethanol yielded a solution of a trisaccharide which was concentrated *in vacuo* almost to dryness. A small volume of absolute ethanol was added to the solution and, after clarification with charcoal, absolute ethanol was again added to incipient clouding, where-

(1) Journal Paper No. 603 of the Purdue University Agricultural Experiment Station.

(2) E. Heyne and R. L. Whistler, THIS JOURNAL, **70**, 2249 (1948).

(3) F. Ahmed and R. L. Whistler, *ibid.*, **72**, 2524 (1950).

(4) R. L. Whistler, T. K. Li and W. Dvonch, *ibid.*, **70**, 3144 (1948).

(5) J. F. Carson and W. D. Maclay, *ibid.*, **70**, 2220 (1948).

(6) C. M. Rafique and F. Smith, *ibid.*, **72**, 4634 (1950).

(7) J. W. Swanson, *ibid.*, **71**, 1510 (1949).

(8) C. L. Smart and R. L. Whistler, *J. Polymer Sci*, **4**, 87 (1949).

(9) K. J. Palmer and M. Ballantyne, THIS JOURNAL, **72**, 736 (1950).

(10) R. L. Whistler and J. F. Stein, *ibid.*, **73**, 4187 (1951).

(11) R. L. Whistler and D. F. Durso, *ibid.*, **73**, 4189 (1951).

(12) R. L. Whistler and D. F. Durso, *ibid.*, **74**, in press (1952).

(13) R. L. Whistler, W. H. Eoff and D. M. Doty, *ibid.*, **72**, 4938 (1950).

(14) R. L. Whistler and D. F. Durso, *ibid.*, **72**, 677 (1950).

(15) See, for example, reference 3.

(16) O. G. Ingles and G. C. Israel, *J. Chem. Soc.*, 810 (1948).

upon the solution was heated to boiling on the steam-bath. Crystallization began immediately, the trisaccharide crystallizing as small flat plates analyzing as the trihydrate; m.p. 137–137.5°, $[\alpha]^{25}D$ −24.7° → −23.3° (*c* 1.29 in water).

Anal. Calcd. for $C_{18}H_{32}O_{16}\cdot 3H_2O$: C, 38.7; H, 6.8. Found: C, 38.7; H, 6.8.

To further establish the presence of three molecules of water of crystallization, Karl Fischer moisture determinations[17] were made on the crystalline trisaccharide. The vessel in which the titrations were carried out consisted of a 50 × 16 mm. test-tube with platinum electrodes sealed in the bottom. The tube was closed by means of a rubber serum-bottle-stopper. The reagent and standard methanol solution were introduced into the tube through hypodermic needles fitted to the burets, which were inserted through the rubber stopper. An additional needle inserted through the stopper served as a vent. Approximately 5% excess of reagent was admitted to the tube containing the sample of approximately 40 mg. After complete solution of the trisaccharide in the reagent, excess reagent was back-titrated with standard water in methanol solution. The reagent was standardized against sodium acetate trihydrate.

Anal. Calcd. for $C_{18}H_{32}O_{16}\cdot 3H_2O$: H_2O, 9.7. Found: H_2O, 9.2.

Periodate Oxidation.—Periodate oxidation[18] was carried out on approximately 40-mg. samples of trisaccharide trihydrate. Periodate consumed and formic acid and formaldehyde[19] produced were determined.

THE ANALYTICAL RESULTS OBTAINED IN THIS EXPERIMENT

	Moles HCO_2H per mole sugar	Moles HIO_4 per mole sugar	Moles HCHO per mole sugar
Calcd.:	3.0	6.0	1.0
Found:	2.9	5.9	1.1

Partial Acid Hydrolysis.—Sixty mg. of trisaccharide was dissolved in 2.0 ml. of 0.05 *N* hydrochloric acid and the solution heated at 100°. At intervals, aliquots to furnish 2 mg. of sugar were withdrawn, neutralized immediately with sodium bicarbonate solution and placed on a paper strip chromatogram. At zero time only the trisaccharide was present, and after 24 hr. only D-mannose could be detected. The rotation of the solution at 24 hr. was +13.5°. The rotation of a 3% solution of D-mannose in water was +14°. Aliquots taken between zero and 24 hours showed only 4-β-D-mannopyranosyl-D-mannopyranose, intact trisaccharide and D-mannose present. In another experiment, 3.0 g. of trisaccharide was dissolved in 100 ml. of 0.2 *N* hydrochloric acid, heated at 100° on the steam-bath for 3 hr., diluted to 2 l. with water and immediately adsorbed on a pre-wet 44 × 365 mm. column of Darco:Celite (1:1). The column was eluted with water, 2.5% ethanol and 7.5% ethanol. D-Mannose was determined in the water fraction by preparation of its phenylhydrazone.[2] Mannobiose was isolated from the 2.5% ethanol fraction and crystallized from a water:methanol:butanol mixture; m.p. 192–193°, $[\alpha]^{25}D$ −2.3 (final) (*c* 1.0 in water). A mixed melting point with the known disaccharide showed no depression. These constants are identical with those reported for 4-β-D-mannopyranosyl-β-D-mannopyranose.[10] The unhydrolyzed trisaccharide was isolated from the 7.5% ethanol fraction and crystallized from water:ethanol. The constants were identical with those of the unreacted sugar.

Rate of Hydrolysis.—In order to establish the fact that both bonds in the trisaccharide hydrolyze with similar rapidity, a rate curve for acid hydrolysis was determined. A sample, 400.3 mg. of trisaccharide, was dissolved in 20 ml. of 0.1 *N* hydrochloric acid and the solution heated at 100° for 24 hr. At intervals 1-ml. aliquots were withdrawn and the reducing power determined. The results obtained are presented in Table I.

TABLE I

	Time (hr.)	Reducing power (meq. × 10^2)	Ml. $Na_2S_2O_3$ blank = 36.30
1	(3 min.)	7.40	28.90
2	1.0	8.95	27.35
3	2.0	12.65	23.65
4	3.0	13.80	22.50
5	5.0	17.10	19.20
6	8.0	18.20	18.10
7	10.0	18.70	17.60
8	12.0	19.40	16.90
9	21.0	22.60	13.70

A plot of log $(a - x)$, where $(a - x)$ = ml. of $Na_2S_2O_3$, *versus* time gave a straight line, indicating that the initial rate of hydrolysis proceeds at the same rate at both bonds of the trisaccharide. The combination of the data presented here, *i.e.*, rate of hydrolysis, products of hydrolysis and periodate data, establishes the structure of the trisaccharide as β-D-mannopyranosyl-(1→4)-β-D-mannopyranosyl-(1→4)-β-D-mannopyranose.

Crystalline Derivative.—The trisaccharide was reduced to the glycitol with hydrogen and platinum catalyst at room temperature and 45 p.s.i.[20] Three grams of trisaccharide was dissolved in 100 ml. of water and 1.2 g. of platinum oxide added. The platinum oxide was prepared by fusion of chloroplatinic acid and sodium nitrate according to the procedure of Adams.[21] Hydrogen was admitted at 45 p.s.i. and the pressure kept constant for 5 days at room temperature with continuous shaking. After 5 days, the shaker was stopped, the platinum black allowed to settle and 1.5 ml. of solution was withdrawn for reducing power determination. Iodometric titration showed zero reducing power and the reaction with Fehling solution was negative. The remaining solution was concentrated to dryness *in vacuo*, yielding a dry, amorphous, extremely hygroscopic product; yield 3.0 g. (100%).

One gram of the glycitol was acetylated by heating for 2 hours at 100° with 20 ml. of acetic anhydride and 0.5 g. of fused sodium acetate. The mixture was then poured with vigorous stirring into 100 ml. of ice-water and stirred for 1 hour, whereupon the acetate crystallized. The mixture was extracted repeatedly with chloroform which was washed with water, saturated sodium bicarbonate and water, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a thick sirup. This sirup crystallized spontaneously; yield 2.0 g. After two recrystallizations from absolute ethanol the melting point was 113.5–114°, $[\alpha]^{25}D$ −21° (*c* 1.18 in chloroform).

Anal. Calcd. for $C_{42}H_{58}O_{28}$: C, 49.9; H, 5.7; CH_3CO, 51.1; mol. wt., 1010. Found: C, 50.0; H, 5.7; CH_3CO, 51.0; mol. wt. (Rast), 1071.

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