[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Maltotetraose and Crystalline Pentadecaacetylmaltotetraitol[1]

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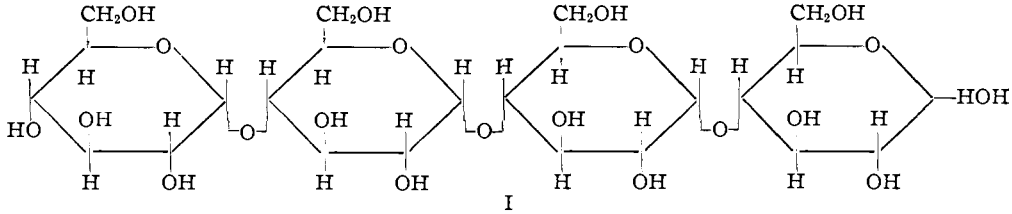
A tetrasaccharide, isolated from corn sirup by column chromatography on charcoal and cellulose, was characterized as maltotetraose by oxidations with hypoiodite and with periodate as well as by hydrolysis with β-amylase. Hydrogenation to maltotetraitol followed by acetylation yielded a crystalline pentadeca-*O*-acetyl derivative.

In recent years charcoal[2] and cellulose[3] column chromatography have become available for the separation of complex mixtures of sugars. From corn sirup, several members of the maltose homologous series have been isolated[4]: namely, maltose, maltotriose, maltotetraose and maltopentaose. The structure of maltose,[5] and of maltotriose[6] has been determined previously. Here is described the structure of the next homolog, maltotetraose.



I

Maltotetraose constitutes about 7% of the dry solids[4] of a 42 D.E. (dextrose equivalent) corn sirup. A concentrate, obtained by charcoal-column chromatography, can be refined further by cellulose-column chromatography to yield a product $[\alpha]^{25}D$ +165.5° (*c* 5.0, water), which, to date, has not crystallized. From molecular rotations[7,8] the specific optical rotation for maltotetraose has been calculated to be $[\alpha]D$ +166°. Upon acid hydrolysis a fourfold increase in iodine reducing power is observed, demonstrating that the material is a tetrasaccharide. In periodate oxidations the tetrasaccharide consumes 7.1 mole equivalents of periodate and produces 3.1 mole equivalents of formic acid as well as 1.3 mole equivalents of formaldehyde. To determine whether glucopyranose residues are linked 1 → 4 or glucofuranose residues are linked 1 → 5 the specificity of enzymic hydrolysis is employed. β-Amylase is known[9] to hydrolyze an α-(1 → 4)-glucosidic linkage where there are at least three adjacent, like bonds.[10] Maltotriose is not hydrolyzed.[10,11] However, the tetrasaccharide fraction is hydrolyzed with crystalline[12] sweet potato β-amylase to give an 87% yield of maltose. The pyranose rings in the isolated maltose exclude the 1 → 5 linkage, hence maltotetraose is comprised of four D-glucopyranose residues linked by three α-(1 → 4) bonds, and has the structure *O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose (I).

Additional evidence for this structure is the observation that maltotetraonic acid, prepared by bromine oxidation of maltotetraose, is hydrolyzed by α-amylase to maltose and maltobionic acid. This hydrolysis, as that of maltotetraose, demonstrates that the original molecule contained three adjacent α-(1 → 4) linkages between glucopyranose residues.

Maltotetraose, by low pressure hydrogenation, is reduced to maltotetraitol which can be isolated as a crystalline pentadecaacetate, m.p. 113–113.5°, $[\alpha]^{25}D$ +52.6° (*c* 0.49, chloroform).

Attempts to crystallize the 1-phenylflavazole[13] have been unsuccessful. However, with maltotriose there is obtained a yellow crystalline material, m.p. 180–182°, $[\alpha]^{25}D$ +87.0° (*c* 0.5, pyridine). This product constitutes the second crystalline derivative described for maltotriose, the first being hendecaacetyl-β-maltotriose.[6]

Experimental

Isolation of Maltotetraose.—Corn sirup[14] was depleted of lower molecular weight components by elution from a charcoal column[4] successively with water, 3.5% and 7.5% aqueous ethanol. A fraction containing most of the tetrasaccharide was then eluted with 15% aqueous ethanol and the eluate was evaporated to dryness at 50° under reduced pressure. A composite of similar fractions was further refined chromatographically[3] by elution from a column of cellulose[15] with a solution of ethyl acetate–pyridine–water (10:4:3 v./v.). The eluate was divided into fractions by means of an automatic fraction collector.[16] To screen the fractions for carbohydrate 1.0-ml. aliquots were mixed vigorously with 1.0 ml. of 1% orcinol in 100% sulfuric acid. As little as 2 μg. of D-glucose or of oligosaccharide was readily apparent from the red to brown color of the reaction mixture.

(1) Journal Paper No. 761 of the Purdue University Agricultural Experiment Station.

(2) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(3) L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, 2511 (1949).

(4) R. L. Whistler and J. L. Hickson, *Anal. Chem.*, in press.

(5) W. N. Haworth and S. Peat, *J. Chem. Soc.*, 3094 (1926).

(6) M. L. Wolfrom, L. W. Georges, A. Thompson and I. L. Miller, THIS JOURNAL, **71**, 2873 (1949).

(7) K. Freudenberg, K. Friedrich and I. Bumann, *Ann.*, **494**, 41 (1932).

(8) R. W. Kerr, "The Chemistry and Industry of Starch," 2nd Ed., The Academic Press, Inc., New York, N. Y., 1950, p. 174.

(9) R. H. Hopkins, *Advances in Enzymol.*, **6**, 389 (1946).

(10) K. Myrbäck and G. Nycander, *Biochem. Z.*, **311**, 234 (1942).

(11) D. French, M. L. Levine, J. H. Pazur and E. Norberg, THIS JOURNAL, **72**, 1746 (1950).

(12) A. K. Balls, R. R. Thompson and M. K. Walden, *J. Biol. Chem.*, **163**, 571 (1946).

(13) G. Neumullor, *Arkiv. Kemi, Mineral., Geol.*, **A21**, No. 19, 13 pp. (1946).

(14) Corn Sirup, Unmixed, 42 D.E., Corn Products Refining Co., Argo, Ill.

(15) Whatman Cellulose Powder, Standard Grade, W. and R. Balston, Ltd., England.

(16) J. L. Hickson and R. L. Whistler, *Anal. Chem.*, **25**, 1425 (1953)

Appropriate eluate fractions were evaporated to dryness and the nature of the oligosaccharide verified by paper chromatography. A composite of the purer fractions was again subjected to chromatographic purification, after which no evidence of contaminating sugars was discernible on a paper chromatogram. Treatment with activated carbon in 25% aqueous ethanol and evaporation to dryness yielded a white amorphous powder $[\alpha]^{25}D$ +165.5° (*c* 5.0, water).

Iodine Reducing Power.[17]—Except for the use of 78.3-mg. samples and a reaction time of 30 minutes in the dark, the method was that previously described. When maltotetraose was hydrolyzed at reflux for 6 hours with 1 *N* sulfuric acid the reducing power was found to increase by a factor of 3.74. Under the same conditions maltose increased in reducing power by a factor of 1.90.

Periodate Oxidation.—In a 25-ml. volumetric flask, a 200-mg. sample of tetrasaccharide in 10.00 ml. of 0.3 *M* sodium metaperiodate was diluted to 25.00 ml. with water and the solution held at room temperature in the dark. At intervals 1.00-ml. portions were withdrawn to follow the progress of the oxidation, by estimation of periodate consumption as well as formic acid and formaldehyde production. An apparent end-point was reached in 5 hours (Table I).

TABLE I

PERIODATE OXIDATION OF MALTOTETRAOSE

Determination	Mole equivalents of substance Calcd.	Found
Periodate consumed	7	7.05
Formic acid produced	3	3.05
Formaldehyde produced	1	1.32

(a) **Formic Acid Produced.**—With 1.00 ml. of the oxidation mixture 1.0 ml. of ethylene glycol was mixed and let stand for 30 min. in the dark. Formic acid was titrated with 0.02 *N* sodium hydroxide to a methyl red end-point.

(b) **Total Oxidant Present.**—Another 1.00-ml. portion was treated with 5.0 ml. of 0.05 *N* hydrochloric acid and 1.0 ml. of 20% potassium iodide and the liberated iodine was titrated immediately with 0.1 *M* sodium thiosulfate to give the sum of the periodate and iodate present.

(c) **Unreacted Periodate.**—To a third 1.00-ml. portion were added 10 ml. of saturated sodium bicarbonate, 10.00 ml. of 0.1 *N* sodium arsenite and 1.0 ml. of 20% potassium iodide. After 15 min. the excess arsenite, back-titrated with 0.1 *N* iodine, was equivalent to the unreduced periodate. The difference between the total oxidant and the unreacted periodate was an estimate of the periodate consumed.

(d) **Formaldehyde Produced.**[18]—Since periodate oxidation in acid media may yield low results because of a failure of the formate ester to saponify,[19] samples were oxidized in solutions buffered with bicarbonate[18] or phosphate.[20]

β-Amylolysis of Maltotetraose.[21]—A solution of 0.67 g of maltotetraose in 16 ml. of water, 1.6 ml. of acetate buffer[22] (*p*H 4.8) and 0.5 mg. of crystalline sweet potato β-amylase,[12] was diluted to 25.0 ml. with water, the solution covered with toluene and held at 37° for 93 hours. After deionization with Amberlite resins[23] IR-100H and IR-4B, the hydrolyzate was evaporated to dryness under reduced pressure and chromatographed on a cellulose column (2.5 × 46 cm.) with ethyl acetate–pyridine–water (10:4:3 v./v.). The orcinol–sulfuric acid test indicated four fractions of which the first eluted (less than 10 mg.) was not carbohydrate, the second (0.57 g., 87%) was maltose (by chromatographic comparison), the third (40 mg.) was tetrasaccharide and the fourth (20 mg.) was unidentified.

The maltose fraction was acetylated with 0.25 g. of freshly-fused sodium acetate and 4.0 ml. of acetic anhydride at 130° until solution was complete (about 25 min.), the mixture poured into 50 ml. of ice and water, stirred vigorously for 30 min. and extracted with chloroform. The chloroform extract was washed free of acetic acid and evaporated to dryness; crude yield 0.62 g. From 99.5% ethanol the octa-acetyl-β-maltose was obtained as a white, crystalline product, m.p. 158–158.5° (uncor.), unchanged upon admixture with an authentic sample. X-Ray diffraction patterns of the prepared and authentic acetates were identical.

β-Amylolysis of Maltotetraonic Acid.—To 3.33 g. of maltotetraose (0.005 mole) and 2.85 g. of barium benzoate (0.006 mole) in 37 ml. of water at room temperature were added 0.31 ml. of bromine (0.0075 mole),[24] the flask stoppered and kept in the dark for two days. Benzoic acid was filtered off and the filtrate aerated until the bromine had been removed. The residual solution was acidified with hydrochloric acid, heated on a steam-bath, and filtered. Silver carbonate was added to a *p*H of 5, the silver chloride filtered off, and the residual silver removed on Amberlite IR-120H. The solution was extracted with chloroform to remove the last traces of benzoic acid and the raffinate evaporated to dryness under reduced pressure to yield a sirup, 2.80 g. or 82% of theory, $[\alpha]^{27}D$ +146.2° (*c* 2.09, water).

An 0.68-g. portion of this sirup was hydrolyzed by β-amylase as previously described. From a cellulose column, using as a solvent ethyl acetate–acetic acid–water (9:2:2 v./v.), seven fractions were obtained as shown in Table II.

TABLE II

CHROMATOGRAPHY OF HYDROLYZED MALTOTETRAONIC ACID

Fraction	Yield, mg.	Substance by paper chromatography
A	...	Non-reducing, not identified
B	...	Non-reducing, not identified
C	30	Glucose
D	120	Maltobionic acid (34%)
E	200	Maltose (60%)
F	40	Maltotetraonic acid
G	140	Not identified

To 120 mg. of fraction D, Table II, in 1.43 ml. of water was added 220 mg. of brucine.[25] The mixture was heated on a steam-bath to produce a clear solution which, on evaporation to dryness, yielded 320 mg. of sirup. This sirup was dissolved in 0.35 ml. of water, 99.5% ethanol was added to produce a cloudy solution from which, on standing a week, separated fine needles of brucine. The mother liquid was evaporated to a sirup, which after decolorization with activated carbon deposited white crystals, m.p. 155–157° dec. (literature value 155–157° dec.[25]).

Maltotetraitol Pentadecaacetate.—To 1.33 g. of maltotetraose in 100 ml. of water and 0.75 g. of platinum oxide catalyst,[26] hydrogen was added to 50 p.s.i. pressure and the mixture agitated at room temperature for six days. At this time 0.22 g. of fresh catalyst was added and the hydrogenation continued for three days. The resulting solution showed no reducing power by Fehling test. After the catalyst was filtered off, the solution was evaporated at 50° under reduced pressure to produce a white amorphous powder; yield 1.45 g., $[\alpha]^{27}D$ +149.9° (*c* 2.09, water). The maltotetraitol did not crystallize.

One gram of maltotetraitol was acetylated with 0.40 g. of freshly fused sodium acetate and 7 ml. of acetic anhydride by the procedure given above. From the washed chloroform extract upon evaporation there was obtained a straw colored sirup; yield 1.74 g. (90%). To purify the product, 0.94 g. of the sirup in 30 ml. of benzene was chromatographed[27] on a 75 × 220 mm. column of Silene EF[28] (240 g.) and diatomaceous earth (100 g.) by development with four liters of a solution of *t*-butyl alcohol in benzene (1:35 v./v.). The product was recovered from the column eluate by evaporation to dryness; yield 1.07 g. of crude sirup. The crude

(17) R. L. Whistler and C. C. Tu, THIS JOURNAL, **74**, 3609 (1952).

(18) R. E. Reeves, THIS JOURNAL, **63**, 1476 (1941).

(19) P. Karrer and K. Pfaehler, *Helv. Chim. Acta*, **17**, 766 (1934).

(20) D. J. Bell, *J. Chem. Soc.*, 992 (1948).

(21) T. G. Halsall, E. L. Hirst, L. Hough and J. K. N. Jones, *ibid.*, 3200 (1949).

(22) P. Bernfeld and P. Gurtler, *Helv. Chim. Acta*, **31**, 106, 108 (1948).

(23) Products of Rohm and Haas, Inc., Philadelphia, Penna.

(24) C. S. Hudson and H. S. Isbell, THIS JOURNAL, **51**, 2225 (1929).

(25) F. H. Stodola and J. B. Lockwood, *J. Biol. Chem.*, **171**, 213 (1947).

(26) R. Adams, V. Voorhees and R. L. Shriner, "Organic Synthesis," H. Gilman, Editor, Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1932, p. 452; Coll. Vol. I, 1941, p. 463.

(27) A. Thompson and M. L. Wolfrom, THIS JOURNAL, **73**, 5849 (1951).

(28) A product of Columbia–Southern Chemical Division of Pittsburgh Plate Glass Co., Pittsburgh, Penna.

product in ethyl ether was decolorized with activated carbon. Evaporation of the ether yielded a crystalline product which was refined by recrystallization from ethyl ether–petroleum ether to produce crystalline pentadecaacetylmaltotetraitol, m.p. 113–113.5° (uncor.), $[\alpha]^{25}D$ +52.6° (*c* 0.49, chloroform).

Anal. Calcd. for $C_{54}H_{74}O_{36}$: C, 49.92; H, 5.74. Found: C, 49.87; H, 5.84.

3-[1′(*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl)-*d*-*erythro*-trihydroxypropyl]-1-phenylflavazole.[13]—To 2.52 g. (0.005 mole) of maltotriose in 50 ml. of water were added 0.55 g. of freshly crystallized *o*-phenylenediamine (0.005 mole), 2.7 g. of distilled phenylhydrazine (0.025 mole) and 4.2 g. of acetic acid (0.07 mole). The solution was refluxed under carbon dioxide for nine hours. On cooling, the product was obtained crystalline in a crude form; yield 0.4 g. (12%). Recrystallization from glacial acetic acid produced the flavazole as a yellow material, crystalline, as verified by X-ray diffraction studies, m.p. 180–182°, $[\alpha]^{25}D$ +87.0° (*c* 0.5, pyridine).

Anal. Calcd. for $C_{30}H_{36}O_{13}N_4$: N, 8.48. Found: N, 8.18.

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LAFAYETTE, INDIANA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

A Crystalline Galactobiose from Acid Hydrolysis of Okra Mucilage[1]

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By acid hydrolysis of okra mucilage there is obtained a crystalline galactobiose which is shown to be 4-*O*-D-galactopyranosyl-D-galactopyranose. This is the first isolation of a crystalline galactobiose. Its occurrence as a hydrolytic fragment of okra mucilage suggests that 1 → 4′-glycosidic linkages are present between some of the D-galactose units in the polysaccharide.

Common okra, *Hibiscus esculentus*, which is widely grown in the southern United States for food, is known for the viscous, mucilagenous solutions which result when it is crushed and extracted with water. It is this mucilagenous property which led to its examination for blood volume expansion.[2]

Aqueous extraction of defatted okra yields a solution of a polysaccharide composed of D-galactose, D-galacturonic acid and L-rhamnose. In view of the clinical interest in the mucilagenous polysaccharide an examination of its structure has been undertaken in this Laboratory. Here is reported the isolation and characterization of a crystalline galactobiose obtained upon incomplete acid hydrolysis of okra mucilage. Evidence is also presented for the existence of two additional galactobioses from the same hydrolyzate.

The disaccharide is obtained by stopping the hydrolysis of okra mucilage at the appropriate stage of maximum yield. On neutralization of the hydrolyzate it is fractionated using charcoal chromatography.[3] After washing the column with water to remove monosaccharides, the disaccharides are eluted with 5% ethanol. This eluate, which contains a mixture of oligosaccharides, as shown by paper chromatography, is further fractionated on a column of cellulose[4] to yield three distinct neutral disaccharide components. Paper chromatographic analyses made during the acid hydrolysis of each of the three neutral disaccharides show D-galactobiose as the only product. Consequently, the assumption may be drawn that the three neutral disaccharides are galactobioses differing only in the manner in which the D-galactose units are linked to each other.

(1) Journal Paper No. 762 of the Purdue Agricultural Experiment Station, Lafayette, Indiana.

(2) H. B. Benjamin, H. K. Ihrig and D. A. Roth, *Rev. canad. biol.*, **10**, 215 (1951).

(3) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(4) L. Hough, J. K. N. Jones and W. H. Wadman, *Nature*, **162**, 448 (1948).

The disaccharide obtained in highest yield has been crystallized and its physical constants have been determined. On methylation and hydrolysis of the completely methylated product there is obtained 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose. Periodate oxidation of the unmethylated disaccharide in alkaline solution yields formaldehyde, which is evidence that the C-5 of the reducing moiety of the disaccharide cannot be involved in the glycosidic linkage. Thus, the galactobiose is 4-*O*-D-galactopyranosyl-D-galactopyranose. Its high positive rotation suggests an alpha configuration at the anomeric carbon involved in the linkage. This is the first reported isolation of an unsubstituted crystalline galactobiose. Since subjecting crystalline D-galactose to the same conditions as those used in the partial hydrolysis of the mucilage does not give rise to this disaccharide, the galactobiose must arise as a true fragment of the polysaccharide.

Experimental

Material.—Fresh okra pods were cut into small pieces and macerated with 95% ethanol in a Waring blendor. After filtration on a large büchner funnel the residue was washed twice with 95% ethanol and twice with acetone. The air-dried product was then extracted with water in a Waring blendor. Insolubles were removed in an International Centrifuge and were re-extracted with water. The mucilagenous extracts were combined and passed through a Sharples Supercentrifuge at 40,000 r.p.m. to remove the last traces of insoluble material. To the centrifugate was added an equal volume of 95% ethanol to precipitate the mucilage, which was filtered on cheese cloth and dried by washing in a Waring blendor once with 95% ethanol and twice with fresh portions of absolute ethanol. It was finally washed with ether and placed over calcium chloride in a vacuum desiccator. The yield was approximately 1.5% of the dry material estimated to be present in the fresh okra pods. That the mucilage is located in the pods and not in the seeds was shown by carefully separating the seeds from the pods and finding the mucilage could be extracted only from pods.

Hydrolysis of Okra Mucilage.—To 50 g. of okra mucilage homogeneously dispersed in 2 l. of water at 80° there was