[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Acid Reversion Products from D-Glucose

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By employing successive carbon and silicate chromatography, the disaccharides gentiobiose, isomaltose, cellobiose, maltose, sophorose and β,β-trehalose have been isolated, as their crystalline octaacetates, in an acid reversion mixture from D-glucose. There was also obtained levoglucosan triacetate and a previously unreported disaccharide octaacetate of unknown structure.

It has been known for many years that D-glucose undergoes condensation to form polymeric material when treated with mineral acids. Because the reaction occurs concurrently with the hydrolysis of starch under like conditions, it is designated "reversion." Musculus[3] in 1872 noted the formation of dextrin-like material by the action of acid on D-glucose. In spite of the profusion of literature on the subject, only a limited advance has been made toward a definitive analysis of reversion mixtures. Fischer[4] first isolated a disaccharide, from an acid reversion mixture, which he characterized by means of its crystalline osazone and named isomaltose. Gentiobiose,[5-8] isomaltose[6-8] and maltose[8] have been isolated or detected in acid reversion mixtures of D-glucose or in starch hydrolyzates (hydrols) which are known to have undergone marked reversion. It now appears to be agreed[8,9] that the reaction is a condensation of D-glucose with the elimination of water to produce di-, tri- and higher saccharides. The ease with which these products hydrolyze to form D-glucose demonstrates the presence of disaccharide units containing D-glycosyl linkages extending between one D-glucose molecule and one of the five available hydroxyl groups of a second. If one considers the possibility of random combination of α- and β-pyranosyl and furanosyl groups with these five hydroxyl positions and indefinite further polymerization, the number of predictable products becomes astronomical. A significant amount of D-glucose is lost in the acid reversion reaction by decomposition to form 5-(hydroxymethyl)-2-furaldehyde[10] and other products. We report evidence herein which indicates that a further amount of D-glucose is converted to anhydrides such as levoglucosan.

In the present study we have chosen conditions of concentration of D-glucose and acid, temperature and time of heating which would tend to produce practical working quantities of reversion materials containing a high proportion of disaccharides but a minimal quantity of higher polymers. By chromatography, first of the free sugars on carbon[11] and then of the acetylated fractions on Magnesol,[12] we have been able to isolate gentiobiose, isomaltose, maltose, cellobiose, sophorose and β,β-trehalose as their octaacetates and a previously unreported reducing disaccharide octaacetate, herein designated γ-acetate, of m.p. 155–157° (cor.) and $[\alpha]D$ +78° (chloroform). If this substance can be assumed to possess only pyranosyl rings it is by elimination through known compounds 2- or 3-*O*-α-D-glucopyranosyl-β-D-glucose octaacetate. The acetylation conditions employed by us (sodium acetate and acetic anhydride) leads to an equilibrium mixture in which the β-D-anomer predominates but in instances some of the α-D-form was isolated. All of the known disaccharide derivatives were isolated as their crystalline octaacetates and were identified by melting point, mixed melting point, optical rotation and X-ray powder diffraction pattern. The substances isolated are shown in Table I. From the data therein shown it can be seen that six of the possible eleven pyranose disaccharide combinations were found and that the 6-linked derivatives predominated. The latter finding probably reflects the greater steric availability of the terminal primary hydroxyl group in the D-glucopyranose unit. These two predominating 6-linked disaccharides, isomaltose and gentiobiose, were present in nearly equal amount, as previously noted.[6]

TABLE I

ACID REVERSION PRODUCTS FROM D-GLUCOSE (ISOLATED CHROMATOGRAPHICALLY AND IDENTIFIED AS CRYSTALLINE ACETATES)

D-Glucose (100 g.) in 300 g. of 0.082 *N* HCl, 10 hr. at 98°

Common name	Definitive name[a]	Yield,[b] g.
Octaacetate of	Octaacetate of	
β,β-Trehalose	β-D-Glucopyranosyl β-D-glucopyranoside	0.072
β-Sophorose	2-*O*-β-D-Glucopyranosyl-β-D-glucopyranose	.17
β-Maltose	4-*O*-α-D-Glucopyranosyl-β-D-glucopyranose	.40
α-Cellobiose	4-*O*-β-D-Glucopyranosyl-α-D-glucopyranose	.069
β-Cellobiose	4-*O*-β-D-Glucopyranosyl-β-D-glucopyranose	.25
β-Isomaltose	6-*O*-α-D-Glucopyranosyl-β-D-glucopyranose	4.2
α-Gentiobiose	6-*O*-β-D-Glucopyranosyl-α-D-glucopyranose	0.063
β-Gentiobiose	6-*O*-β-D-Glucopyranosyl-β-D-glucopyranose	3.40
γ	?	0.22
Triacetate of	Triacetate of	
Levoglucosan	1,6-Anhydro-β-D-glucopyranose	0.3

[a] *Chem. Eng. News*, 31, 1776 (1953). [b] Basis 100 g. of initial D-glucose.

A small amount of levoglucosan (1,6-anhydro-β-D-glucopyranose) triacetate was isolated. The presence of some of this substance in an acid solution of D-glucose is predictable and is in accord with anhydride studies in other sugar structures.[13]

(1) Corn Industries Research Foundation Research Associate (A. T.) and Fellow (M. I.).

(2) Special Postdoctoral Research Fellow of the National Institutes of Health, United States Public Health Service.

(3) Musculus, *Bull. soc. chim. France*, [2] **18**, 66 (1872).

(4) E. Fischer, *Ber.*, **23**, 3687 (1890).

(5) H. Berlin, THIS JOURNAL, **48**, 2627 (1926).

(6) A. Thompson, M. L. Wolfrom and E. J. Quinn, *ibid.*, **75**, 3003 (1953).

(7) Edna M. Montgomery and F. B. Weakley, U. S. Patent 2,549,-840 (1951).

(8) W. R. Fetzer, E. K. Crosby, C. E. Engel and L. C. Kirst, *Ind. Eng. Chem.*, **45**, 1075 (1953).

(9) C. D. Hurd and S. N. Cantor, THIS JOURNAL, **60**, 2677 (1938).

(10) M. L. Wolfrom, R. D. Schuetz and L. F. Cavalieri, *ibid.*, **70**, 514 (1948).

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

(12) A hydrated magnesium acid silicate produced by the Westvaco Chemical Division of Food Machinery and Chemical Corp., South Charleston, W. Va.

(13) Laura C. Stewart and N. K. Richtmyer, *Abstracts Papers Am. Chem. Soc.*, **124**, 18D (1953).

Experimental

Preparation of Reversion Mixture.—A solution of 1500 g. of D-glucose in 4500 ml. of 0.082 *N* HCl was heated with stirring in a boiling water-bath (98°) for 10 hr., cooled and deacidified by passing through a column of Duolite A-4.[14]

Preliminary Fractionation on a Carbon Column.—An amount of the solution containing originally 100 g. of D-glucose was diluted to 2000 ml. and placed on a carbon (Darco G-60)[15]–Celite[16] (1:1 by wt.) column (475 × 105 mm., diam.) and washed with water (*ca.* 25,000 ml.) with suction until the effluent was negative to Benedict solution. The effluent, normally containing only D-glucose, was discarded. The developing solution was changed to 5% ethanol and the washing continued. The effluent portion (8,000 to 10,000 ml.) reacting positive to Benedict solution was collected. The developing solvent was then changed to 25% ethanol, the washing continued, and the effluent (6,000 ml.) collected until it was negative to Benedict solution. The 5% and 25% effluents were evaporated separately under reduced pressure to sirups which were dried by repeated evaporation of their methanol solutions under reduced pressure; yield from the 5% ethanol effluent, 6.5 g. (fraction I) and from the 25% ethanol effluent, 7.0 g. (fraction II).

Acetylation of Fraction II.—Acetylation of the amorphous material of fraction II from the carbon column was accomplished by heating 10 parts by weight of the carbohydrate and 5 parts of anhydrous and freshly fused sodium acetate with 70 parts of acetic anhydride at the boiling point until solution had taken place. The mixture was then allowed to cool and was poured with stirring into 500 parts of ice and water. After hydrolysis of the acetic anhydride, the acetate was removed by extraction with chloroform. The chloroform solution was washed with water, dried with anhydrous sodium sulfate, filtered and evaporated to a sirup. The yield of acetylated sirup was slightly less than twice the weight of the starting material.

Further Chromatography of Fraction I.—The above fraction I material from five columns (500 g. of D-glucose) was combined (35 g.) and run as before on the carbon column. The portion removed by 5% ethanol solution was concentrated to a sirup; yield 32.5 g. This material was acetylated by the procedure described above; yield 59 g. of sirup. This sirup was dissolved in ethanol, nucleated with β-isomaltose octaacetate and stirred mechanically. The crystalline product which separated was filtered and was recrystallized once from ethanol; yield[17] 21 g., m.p. 146–147° (cor.) unchanged on admixture with known β-isomaltose octaacetate, $[\alpha]^{25}D$ +96.3° (*c* 3.9, chloroform). These values are in agreement with the published[18] constants for β-isomaltose octaacetate; X-ray powder diffraction data: 9.98[19] −60,[20] 8.80–2, 7.73–1, 6.97–2, 6.27–100, 5.78–10, 5.33–50, 5.07–15, 5.81–5, 4.56–20, 4.35–15, 4.10–10, 3.93–10, 3.78–5, identical with those of a known specimen of β-isomaltose octaacetate.

The mother liquors from the above crystallization of β-isomaltose octaacetate were combined and concentrated to a sirup; yield 33.8 g. This sirup was dissolved in benzene and placed (5 g. per column) on Magnesol–Celite (5:1 by wt.) columns (250 × 75 mm. diam.) using 2500 ml. of benzene–*t*-butyl alcohol (100:1 by vol.) as the developer. After the column was extruded and streaked with indicator (1% $KMnO_4$ in 10% NaOH soln.) a zone appeared 15–70 mm. from the top of the column (other zones which appeared failed to produce crystalline material). This zone was removed, eluted with acetone, the acetone solution evaporated to a sirup and crystallized from ethanol; yield (from the combined runs) 360 mg., m.p. 176–177° cor. The crystals were recrystallized from ethanol; m.p. 180–182° cor. undepressed on admixture with known β,β-trehalose octaacetate, $[\alpha]^{31}D$ −17.2° (*c* 4.0, chloroform). These values are in agreement with published[21] data for β,β-trehalose octaacetate; X-ray powder diffraction data: 13.67[19] −10,[20] 11.41–70, 10.22–10, 9.07–30, 8.40–40, 7.71–1, 7.14–1, 6.68–1, 5.54–90, 5.29–30, 5.08–50, 4.76–5, 4.31–100, 4.02–1, 3.83–25, 3.64–15, 3.44–15, 3.31–20, 3.04–10, 2.89–15, identical with those of known β,β-trehalose octaacetate.

The combined effluents from the above columns were evaporated to a sirup; yield 13.0 g. This material was dissolved in benzene and placed on two columns (250 × 75 mm. diam.) of Magnesol–Celite (5:1 by wt.) and developed with 1600 ml. of benzene–*t*-butyl alcohol (200:1 by vol.). Two zones appeared. The upper zone contents were identified as β-isomaltose octaacetate. The lower zone, 110–180 mm. from the top, when removed as described above, yielded 1.83 g. of crystals of m.p. 103–105° cor. The crystals were recrystallized from ethanol; yield 1.5 g., m.p. 109–111° unchanged on admixture with a known sample of levoglucosan triacetate, $[\alpha]^{31}D$ −62.5° (*c* 4.0, chloroform). These constants are in agreement with published[9] values. The X-ray powder diffraction data obtained on the crystals were identical with those of known levoglucosan triacetate and were: 9.03[19] −20,[20] 7.85–45, 6.64–40, 6.40–30, 6.09–2, 5.72–5, 5.44–5, 5.06–50, 4.46–100, 3.90–30, 3.78–10, 3.67–10, 3.54–30.

Further Chromatography of Fraction II.—The acetylated fraction II (14.0 g. of sirup) from the carbon column was dissolved in ethanol and the β-gentiobiose octaacetate allowed to crystallize. The crystalline acetate was recrystallized from ethanol; yield 2.5 g. (see below). The combined mother liquors were evaporated to a sirup; yield 11.5 g. This material was dissolved in benzene and placed on Magnesol–Celite (5:1 by wt.) columns (250 × 75 mm. diam.) in 5-g. portions and developed with 3000 ml. of benzene–*t*-butyl alcohol (100:1 by vol.). When the column was streaked with indicator, four zones appeared which were numbered from the bottom to the top of the column.

Zone I, located 185–220 mm. from the column top, was eluted with acetone, evaporated to a sirup and crystallized from ethanol; yield 400 mg., m.p. 155–158° cor. The material was recrystallized from ethanol; m.p. 159–161° cor., $[\alpha]^{25}D$ +60.6° (*c* 3.8, chloroform). These data are in substantial agreement with published values for β-maltose octaacetate. X-Ray powder diffraction data on the crystals were identical with those of known β-maltose octaacetate and were: 12.96[19] −40,[20] 11.12–100, 8.88–5, 7.59–5, 6.97–5, 5.94–2, 5.47–90, 5.09–25, 4.79–15, 4.60–15, 4.39–15, 4.13–20, 3.93–20, 3.78–25, 3.63–5, 3.49–10.

Zone II, located 105–155 mm. from the column top, was eluted with acetone, evaporated to a sirup and crystallized from ethanol; yield 950 mg., m.p. 140–170°. A portion of this material (550 mg.) was rechromatographed on a Magnesol–Celite column (200 × 42 mm. diam.) using 4000 ml. of benzene–*t*-butyl alcohol (200:1 by vol.) as developer. Two zones were located near the bottom of the column. The two zones were eluted separately with acetone, evaporated to a sirup and this crystallized from ethanol.

The lower zone above yielded 100 mg. of shining prismatic needles; m.p. 186–188° cor. Upon further purification by crystallization from ethanol, the constants were: m.p. 191–192° cor., $[\alpha]^{25}D$ −3.8° (*c* 3.5, chloroform). These data are in agreement with the published values for β-sophorose[22–24] octaacetate. The X-ray diffraction data on the crystals were identical with those of a known specimen of β-sophorose octaacetate[25] and were: 10.43[19]–5,[20] 8.93–25, 7.52–100, 6.14–10, 5.52–50, 4.84–50, 4.44–40, 4.18–2, 4.00–2, 3.80–2, 3.73–2, 3.57–25.

The upper zone above produced 130 mg. of crystals, m.p. 155–157° cor., $[\alpha]^{25}D$ +78° (*c* 3.0, chloroform); X-ray powder diffraction data: 11.83[19]–100,[20] 10.02–15, 7.21–2, 6.26–2, 5.72–90, 5.32–70, 4.96–5, 4.74–30, 4.45–80, 4.23–10, 4.10–10, 3.94–5, 3.84–5, 3.47–15, 3.33–10, 3.26–10, 3.09–70.

Anal. Calcd. for $C_{12}H_{14}O_{11}(CH_3CO)_8$: C, 49.55; H, 5.64; mol. wt., 678. Found: C, 49.61; H, 5.66; mol. wt. (Rast), 675.

(14) A product of the Chemical Process Co., Redwood City, Calif.

(15) A product of the Darco Department, Atlas Powder Co., New York, N. Y.

(16) A siliceous filter-aid produced by the Johns–Manville Co., New York, N. Y.

(17) See Table I for yields based on 100 g. of initial D-glucose.

(18) M. L. Wolfrom, L. W. Georges and I. L. Miller, This Journal, **71**, 125 (1949).

(19) Interplanar spacing, Å., CuKα radiation.

(20) Relative intensity as percentage strongest line, estimated visually.

(21) H. H. Schlubach and W. Schetelig, *Z. physiol. Chem.*, **213**, 87 (1932).

(22) K. Freudenberg and K. Soff, *Ber.*, **69**, 1245 (1936).

(23) K. Freudenberg, H. Knauber and F. Cramer, *Chem. Ber.*, **84**, 144 (1951).

(24) J. Rabaté, *Bull. soc. chim. France*, [5] **7**, 565 (1940).

(25) We are indebted to Dr. F. Cramer of Heidelberg Universität for an authentic sample of β-sophorose octaacetate.

This substance has not to our knowledge been previously reported and is herein designated as γ-acetate.

Zone III above, located 60–105 mm. from the column top, was eluted with acetone, evaporated to a sirup and crystallized from ethanol; yield 810 mg. (total yield 3.40 g., including product crystallized directly), m.p. 191–193° cor., $[\alpha]^{25}D$ −5.5° (*c* 3.9, chloroform). These data are in agreement with those published for β-gentiobiose octaacetate. X-Ray powder diffraction data, identical with those of known β-gentiobiose octaacetate, were: 14.03[19]–10,[20] 11.91–10, 10.05–100, 8.96–30, 7.14–25, 5.61–1, 5.48–15, 4.98–20, 4.79–20, 4.45–25, 4.27–25, 4.00–1, 3.78–30, 3.53–20, 3.41–10.

After several days, an additional crop of crystals separated from the mother liquor material of the above zone III; yield 60 mg., m.p. 211–215° cor. Upon recrystallization from ethanol, the constants were: m.p. 227–230° cor., $[\alpha]^{31}D$ +35.3° (*c* 1.7, chloroform). Although these constants are in poor agreement with published values (229.5° and +42°),[26] indicating the presence of impurities, the X-ray powder diffraction data are identical with those of a known sample of α-cellobiose octaacetate: 11.48[19]–15,[20] 10.37–10, 8.71–10, 8.35–2, 7.02–1, 5.41–100, 5.04–50, 4.74–15, 4.38–15, 4.27–20, 4.02–15, 3.77–15, 3.59–10, 3.44–5, 3.29–10. This substance is thus identified as α-cellobiose octaacetate.

(26) C. S. Hudson and J. M. Johnson, THIS JOURNAL, **37**, 1276 (1915).

Zone IV, 15–60 mm. from the top of the column, was recovered and crystallized in a similar manner; yield 550 mg., m.p. 184–188° cor. After further purification by Magnesol chromatography, as described above, the constants were: m.p. 191–192° cor., $[\alpha]^{25}D$ +51.0° (*c* 4, chloroform). X-Ray powder diffraction data were identical with those of a known sample of α-gentiobiose octaacetate; 11.85[19]–1,[20] 10.05–90, 8.67–100, 7.69–1, 7.08–10, 6.73–1, 5.51–1, 5.44–5, 5.12–25, 4.93–25, 4.73–10, 4.43–20, 4.32–5, 3.92–25.

β-Cellobiose Octaacetate.—Fraction II material from the carbon column of a second run yielded 15 g. of acetylated substance. This sirup was dissolved in benzene and chromatographed on three columns (250 × 75 mm., diam.) of Magnesol–Celite (5:1 by wt.) using 4000 ml. of benzene–*t*-butyl alcohol as developer. The second zone from the bottom, 120–145 mm. from the top of the column, yielded 250 mg. of material, m.p. 160–165°. This material was thrice crystallized from ethanol; yield 50 mg., m.p. 197–199° cor. unchanged on admixture with a known sample of β-cellobiose octaacetate, $[\alpha]^{25}D$ −15.7° (*c* 4.0, chloroform).[26] X-Ray powder diffraction data, identical with those of a known sample of β-cellobiose octaacetate, were: 15.75[19]–15,[20] 12.23–50, 9.43–100, 8.02–2, 7.36–5, 5.40–50, 5.11–10, 4.34–40, 4.65–15, 4.42–10, 4.21–25, 4.12–25, 3.91–20.

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[CONTRIBUTION FROM THE RESEARCH AND DEVELOPMENT BRANCH, FITZSIMONS ARMY HOSPITAL]

Chromatographic Isolation of Polysaccharides from *Mycobacterium tuberculosis*

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A method for the isolation of polysaccharides is presented, making use of chromatographic adsorption. By using this procedure, 12 different polysaccharide fractions were isolated from the autolysate from a human strain of *Mycobacterium tuberculosis*. Additional polysaccharides, not present in the autolysate, were extracted from the tubercle bacilli, using an electrolytic current. These were isolated into nine different polysaccharide fractions by chromatography. Of the 21 distinct polysaccharide fractions obtained, 19 have the ability to bind antibodies *in vitro*.

Introduction

The isolation of pure and chemically unaltered polysaccharides from *Mycobacterium tuberculosis* is an important problem in immunology and serology. The methods used by previous workers were fractional precipitation methods.[1,2] However, these methods have yielded fractions containing varying small amounts of nitrogenous materials. In most cases these are believed to be protein and nucleic acid impurities that are adsorbed to the polysaccharides. The chromatographic separation excludes coprecipitation, overlapping solubility and incomplete recovery, all common to fractional precipitation methods, and is most likely to separate adsorbed impurities from the polysaccharides. Also, none of the eluants used in this paper are likely to chemically alter the polysaccharides.

In an effort to obtain polysaccharides from the tubercle bacillus, not present in the autolysate, a method for electrolytic extraction of the bacilli was devised. This method was chosen because it avoided the use of heat or chemicals that might alter the structure of the polysaccharides within the cells.

(1) F. B. Seibert, *Bibliotheca Tuberculosea, Separatum Fasc.*, **3**, (1950).

(2) M. Stacey and P. W. Kent, "Advances in Carbohydrate Chemistry," **3**, 311 (1948).

Experimental

Preparation of Autolysate.—A strain of *M. tuberculosis*, isolated in this Laboratory from a tuberculous patient, was grown on Long medium[3] for ten weeks at 37.5°. The bacilli were then killed by adding 90% phenol to form a 2% phenol suspension. The suspension was incubated for two additional weeks. The cells were removed by centrifugation and stored at 4°.

The supernatant was Seitz-filtered and concentrated from 900 to 150 ml. by vacuum distillation in the cold. It was electrodialyzed from a cellophane bag against continuously changing distilled water until no current was observed at 120 v. d.c. During the electrodialysis the *p*H dropped from 7.0 to 5.0, and a sizable precipitate accumulated, consisting mainly of proteins and nucleic acids. The supernatant contained a mixture of polysaccharides which in this paper is called the polysaccharide autolysate. This autolysate was concentrated to 5 ml. volume in preparation for chromatography. Sterile techniques were used.

Electrolytic Extraction of Cells.—The cells were washed thoroughly with distilled water and dried for two days by vacuum desiccation; the dry weight was 14.2 g. An aqueous suspension was then prepared by adding 135 ml. of distilled water. This was placed in a stoppered chamber containing two carbon electrodes and a sterile cotton escape vent for evolved gases; the apparatus was thermostated at 0°. A potential of 120 v. d.c. (higher potentials presently are under investigation) was applied for eight hours; the current rose to 350 ma. The cells were centrifuged from the supernatant and resuspended in 135 ml. of distilled water. The electrolysis was repeated four additional times, until the current dropped to 20 ma. The supernatants were combined, filtered, concentrated from 775 ml. to 150 ml. and electrodialyzed. Again a precipitate, mainly proteins and

(3) E. R. Long and F. B. Seibert, *Am. Rev. Tuberc.*, **13**, 393 (1926).