Acknowledgments.—The authors are indebted to Dr. Peter Lim and staff for the chromatograms and optical rotations as well as the interpretation of the infrared spectra; and to Mr. O. P. Crews, Jr., and staff for large-scale preparations of intermediates.

MENLO PARK, CALIF.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE MOUNT SINAI HOSPITAL, AND FROM THE LABORATORY OF MARINE BIOCHEMISTRY AND ECOLOGY, N. Y. ZOOLOGICAL SOCIETY]

Holothurin. I. The Isolation, Properties and Sugar Components of Holothurin A[1]

BY J. D. CHANLEY, R. LEDEEN, J. WAX, R. F. NIGRELLI AND HARRY SOBOTKA

RECEIVED MAY 5, 1959

The isolation, properties and sugar components of holothurin A, the toxic principle of *Actinopyga agassizi*, a sea-cucumber found in the Bahama Islands are described. Elementary analysis of holothurin A leads to the empirical formula $C_{50-2}H_{81-5}O_{25-6}SNa$. The hydrolysis products derived from holothurin A suggest that it is a mixture of several glycosides, each of which contains a steroid aglycone of *ca.* 26–28 carbon and 4–5 oxygen atoms, one molecule each of four different sugars and one molecule of sulfuric acid as the sodium salt. The separation and identification of the monoses derived from holothurin A has been achieved. They were D-glucose, D-xylose, D-glucomethylose (quinovose) and 3-O-methylglucose. The neurotoxic, hemolytic and cancerostatic properties of holothurin A are referred to.

Some species of sea-cucumbers (class Holothuroidea) contain a poisonous principle. The ecological significance of this poison is not clear. It has been suggested that it protects these animals against predators. Sea-cucumbers of various species are eaten in various parts of the world, but occasional poisoning by this article of food is presumably not related to the toxin under discussion, which is ineffective by oral ingestion. A peculiar gland, found in this species and named after the zoologist Cuvier, is particularly rich in poison, which is also found in the blackish slimy integument. We have studied the chemical composition of the toxic substance from Cuvier's gland of *Actinopyga agassizi*, a holothuria found in the Bahama Islands.[2] In two preliminary notes[3,4] we reported that the toxic material holothurin, obtained from the water extract of Cuvier's gland, appeared to consist of a few steroid aglycones, bound individually to 4 molecules of monosaccharides. Further purification of holothurin and analysis of its hydrolytic products, as described below, is in accord with this view. This would place holothurin, as the substance has been designated,[2] into the class of cardiac glycosides or steroid saponins such as have hitherto been found in plants, especially monocotyledons, only. It shares with the plant drugs its saponin-like character and neurotoxic and hemolytic properties, which will be described elsewhere.[5,6] But, besides, holothurin contains one molecule of sulfuric acid, bound in ester linkage, which suggests a relationship with steroid alcohols, *e.g.*, scymnol and ranol in the bile of the most primitive vertebrates.[7]

Some of the holothurin glycosides resemble digitonin and other saponins in forming a complex with cholesterol. The glycosides which enter into such combination comprise over 60% of the total glycosides; they may be recovered from the complex by treatment with pyridine. Our experiments do not favor the assumption of a 1:1 complex with cholesterol, such as is formed by plant saponins; the complex is richer in cholesterol and the electrostatic situation at the sulfate ester site may be assumed to influence the composition of the adduct. We have designated the cholesterol precipitable fraction as holothurin A.

Holothurin A showed no absorption in the ultraviolet region. The infrared spectrum showed bands at 5.72 and 6.14 μ indicative of a five or six-membered ring lactone and one double bond. The absence of a positive Legal test in conjunction with the ultraviolet spectrum excluded the presence of an α,β-unsaturated lactone. Hydrogenation of the double bond failed under a variety of conditions. Elementary analysis of holothurin A leads to the formula $C_{50-2}H_{81-5}O_{25-6}SNa$. Methoxyl determination indicated the presence of one such group in holothurin A. Acid hydrolysis of the neutral non-reducing holothurin A yielded water insoluble aglycones, sulfuric acid and water-soluble reducing sugars. At least 4 individual steroid aglycones are obtainable from the hydrolysis of holothurin A. The elementary analysis and molecular weight determination[8] of these aglycones show all to have between 26 and 28 carbons and 4–5 oxygen atoms, while none of the aglycones contained a methoxyl group. The observed rotation of the total sugar mixture, obtained from the hydrolysis of holothurin A, has been found to be the same as that which would be calculated for an equimolecular mixture of the four sugars isolated and described below. These observations are consistent with the hypothesis that

(1) Supported by Grant C-5097 from the National Science Foundation and by the Office of Naval Research under Contract NONR-2266. The material in this paper was presented in part before the Division of Carbohydrate Chemistry at the ACS meeting in Chicago, September 1958.

(2) R. F. Nigrelli and P. A. Zahl, *Proc. Soc. Exp. Biol. Med.*, **81**, 379 (1952).

(3) (a) R. F. Nigrelli, J. D. Chanley, S. Kohn and H. Sobotka, *Zoologica*, **40**, 47 (1955); (b) J. D. Chanley, S. Kohn, R. F. Nigrelli and H. Sobotka, *ibid.*, **40**, 99 (1955).

(4) *Cf.* T. Yamanouchi, *Publ. Seto Marine Biol. Lab.* 4 (Parts 2–3), 183 (1955).

(5) S. L. Friess, F. G. S. Standaert, E. R. Whitcomb, R. F. Nigrelli, J. D. Chanley and H. Sobotka, *J. Pharm. Exp. Therap.*, in press (1959).

(6) St. Carson and R. F. Nigrelli, in preparation.

(7) G. D. Haslewood, *Physiol. Rev.*, **35**, 178 (1955).

(8) To be reported in a separate communication.

holothurin A is a mixture of several closely related sulfate ester glycosides each of which contains a steroid aglycone of *ca.* 26–28 carbons and 4–5 oxygen atoms, one molecule each of four different sugars and one molecule of sulfur acid as the sodium salt.

Analyses of Sugar Mixture.—A qualitative investigation of the sugar mixture revealed that at least one hexose and one pentose (positive anthrone and orcinol tests) were present. The absence of ketoses was ascertained by the negative Dische carbazole–cysteine reaction[9] and the non-reduction of Benedict's reagent when the reaction was carried out at 55° for 15 min. Analysis of the sugar mixture showed it to contain 4% methoxyl. The reducing capacity of the mixture was substantially lowered after incubation with yeast. The presence of glucose was thus indicated. The anthrone reaction, carried out according to Yemm and Willis,[10] indicated that a methylpentose was present. These observations suggested that at least 3 and possibly 4 different aldehydic sugars were present in the mixture. When the sugar mixture was subjected to paper chromatography (butanol–ethanol–water) a separation into but three components resulted with R_F values of 0.25, 0.35 and 0.46, corresponding to glucose, xylose and possibly rhamnose (0.48). A paper chromatogram after fermentation with yeast showed but two components of R_F 0.35 and 0.46. The presence of glucose was thus confirmed. The sugar mixture was chromatographed on a Dowex-1-borate column and the elution pattern (see Experimental) established that the pentose was xylose and that rhamnose was absent, while the presence of glucose was again confirmed. The unknown sugar(s) (R_F 0.46), eluted first from the borate column, gave a positive anthrone as well as a positive Dische test[11] for a methyl pentose. When the total sugar mixture was chromatographed on paper using phenol–water as the developing solvent, four components were thereby resolved with R_F values of 0.37, 0.45, 0.59 and 0.71. The first two values corresponded to glucose and xylose. Likewise the unknown sugar mixture obtained from the borate chromatography and rechromatographed on paper (phenol–water) gave only the two components of R_F 0.59 and 0.71. Elution from the paper of the two fast running sugars and assay (see Exp.) established that the slower moving component was a methylpentose, while the fastest moving sugar was a hexose. Paper chromatography of this mixture, employing butanol–pyridine–water as the developing solvent, gave only one spot with R_F 0.61. A comparison of the reported R_F values for the aldehydic methylpentoses both with the butanol–pyridine–water[12] and the phenol–water system[13] and that obtained in this work eliminated all but two methylpentoses from consideration. They were gulomethylose (antiarose) and glucomethylose. The nature of the O-methyl sugar as well as the methylpentose could now be deduced by exclusion. The total sugar mixture had a specific rotation $[\alpha]^{27}D$ +38°. At this point it must be noted that among monomethyl-O-hexoses only 3-O-methylhexoses have as yet been found in nature.[14] If the reasonable assumption was made that the sugars were present in equimolar quantities (see borate chromatography in Exp.) then, if the methylpentose was the naturally occurring gulomethylose of $[\alpha]^{25}D$ + 38, the most likely candidate for the methylated sugar would be 3-O-methyl-galactose($[\alpha]^{25}D$ + 108). However, the R_G value of 3-O-methylgalactose (butanol–ethanol–water)[14] is much lower than that found for the sugar mixture containing the methylpentose and the O-methylhexose. On the other hand if the methylpentose was glucomethylose, then the most probable methylated sugar would be 3-O-methylglucose, since its specific rotation + 55.5° and its R_F (reported to be the same as glucomethylose in butanol–ethanol–water)[14] is consistent with all the findings for the total sugar mixture. This latter hypothesis seemed more likely. Confirmation of these deductions was achieved by chromatography of the total sugar mixture on cellulose. The four sugars were isolated, crystallized and identified by melting point, paper chromatography, rotation and analysis. They were glucose, xylose, glucomethylose and 3-O-methylglucose.

Experimental

Sun-dried Cuvier's glands of *Actinopyga agassizi* (100 g.) were refluxed with one liter of boiling benzene for 4 hr. with a water separator; 10 ml. of water was removed. The benzene which contained less than 1 g. of lipids was discarded. The benzene-insoluble residue was extracted with 600 and 200 ml. of hot water and centrifuged, leaving behind 6 g. of insoluble material of lipid nature. The aqueous extract (825 ml.) was diluted with twice its volume of ethanol (95%), kept two days at room temperature and filtered from insoluble material (2.5 g.). To the boiling solution (2.4 liters) a hot solution of 54 g. of cholesterol in 1600 ml. of ethanol (95 %) was added and the resulting mixture, containing 76% ethanol, was stirred and heated on the steam-bath for 90 min. After standing overnight at room temperature, the resulting adduct was collected by centrifugation and washed with ethanol–water (2:1); the combined supernatant and washings yielded 14 g. of cholesterol on evaporation. Extraction of the adduct with 1600 ml. of ether yielded additional uncombined cholesterol; yield of adduct 87 g.

The adduct was dissolved in 4 one-liter portions of dry pyridine at 45°; after standing a few hours at room temperature, the solution was poured into five times its volume of ether. The precipitated glycoside was centrifuged off and washed several times with ether; total yield 49 g. The treatment with pyridine and precipitation with ether was repeated to ensure complete decomposition of the complex; yield 40 g., $[\alpha]^{25}D$ 17.7 in water. The Liebermann–Burchard test for cholesterol was now negative and the material was completely soluble in water. The infrared spectrum of holothurin A, as a Nujol mull showed maxima at 5.72 and 6.14 μ and no absorption in the ultraviolet. No change in the absorption spectra, or optical rotation, $[\alpha]^{25}D$ −18.6°, was noted after four recrystallizations from methanol. The

(9) G. Ashwell, Colorimetric Analysis of Sugars in "Methods in Enzymology," Vol. III, Eds. Colowick and Kaplan, Academic Press, Inc., New York, N. Y., 1957, p. 76.

(10) E. W. Yemm and A. S. Willis, *Biochem. J.*, **57**, 508 (1954).

(11) *Cf.* ref. 9 esp. pp. 81–82.

(12) We have found the following R_F values for glucose, rhamnose and fucose: 0.38, 0.61 and 0.51, respectively, in substantial agreement with the literature values. For summary of R_F values, *cf.* Ch. Tamm in "Progress i.th. Chem. of Org. Nat. Prod.," Vol. XIV (Ed. Zechmeister), Springer, Vienna, 1957, p. 104.

(13) M. A. Jermyn and F. A. Isherwood, *Biochem. J.*, **44**, 402 (1950); S. M. Partridge and R. G. Westall, *ibid.*, **42**, 238 (1950).

(14) E. L. Hirst, L. Hough and G. K. N. Jones, *J. Chem. Soc.*, 323 (1951); *cf.* E. L. Hirst and G. K. N. Jones, *Disc. Faraday Soc.*, **7**, 268 (1949).

analytical values for carbon, hydrogen, sulfur and sodium were not significantly changed on repeated crystallization. However, after one recrystallization from methanol, the value for methoxyl increased from 2.7 to 3.5% indicating that holothurin A retained some methanol of crystallization. It was this material which was used for neurophysiological,[5] cancerostatic[15] and hemolytic[6] experiments.

Elementary Composition.—The analytical data on four times recrystallized specimens, dried at 79° *in vacuo*, were: C, 52.27; H, 7.33; S, 2.77; Na, 1.99; CH_3O, 3.53. Figures for Na and S give a molecular weight of 1155. On the basis of these molecular weights the composition of holothurin should be $C_{50-52}H_{81-85}O_{25-26}SNa$.

Formation of the Free Acid.—One gram of the sodium salt of the saponin was dissolved in 25 ml. of water passed through a mixed bed column of MB-3 resin (20 cm. × 1 cm. diam.), in the cold room (4°). Lyophilization of the eluate yielded the saponin in 93% yield as the free acid. This is soluble in water and methanol but insoluble in ether, benzene and acetone. Its acid equivalent was 1050, somewhat low in comparison with the analytical data; evidently some of the sulfuric acid had been split off. Repeated passage through MB-3 resulted in loss of H_2SO_4 as evidenced by increase of the acid equivalent.

Analytical Methods.—The anthrone reagent was prepared according to Roe[16] and the reaction carried out in the following manner. One-ml. aliquots of sugar solution (20–120 μg.) was mixed with 10 ml. of the anthrone reagent and the mixture heated in a boiling water-bath. Samples, withdrawn after 3 and 15 min., were cooled and allowed to stand in the dark (20-30 min.) and their optical density, O.D., determined at 620 mμ, employing either a Beckman or Coleman spectrophotometer. For hexoses the O.D. readings, as reported,[5] increased with heating time while the reverse is true for methylpentoses *e.g.*, glucose (80 μg.) O.D. 0.057 (3 min.) to 0.280 (15 min.); rhamnose (80 μg.) O.D. 0.391 (3 min.) to 0.078 (15 min.). The orcinol reaction for pentose was carried out according to Meijbaum.[17] The O.D. determined at 665 mμ employing a Coleman, Beckman of Klett (660 filter) spectrophotometer. The methylpentoses were assayed according to Dische[11] by noting the increase in O.D. from 396 to 427 mμ. Reducing capacity of the sugar solutions were run according to the modified Folin–Wu method[18] or Nelson method.[19] Standard sugars were assayed concomitantly with the unknown sugars in all the aforementioned reactions.

Paper Chromatography.—Whatman No. 1 paper was used and the following solvent systems were employed: (A) aqueous phenol (phenol, 100 g., $+H_2O$, 40 ml.); (B) butanol–ethanol–water, mixed in the proportion 5:1:4 by volume (the top layer used); (C) butanol–ethanol–water (buffered) 11:3:6 by volume; the water (100 ml.) contained glacial acetic acid (1 ml.), concd. ammonium hydroxide (1 ml.) and boric acid (1 g.); (D) butanol–pyridine–water 6:4:3 by volume. The chromatograms were run *ca.* 17 to 18 hr. at 25–28° in the descending manner. Known sugars were spotted alongside the unknowns to give reference points. After completion of the run, the papers were dried at room temperature in an air draft and sprayed with either of the following reagents: (1) aniline–phthalate [aniline (0.93 g.) and phthalic acid (1.66 g.)] dissolved in butanol (100 ml.) saturated with water; (2) *p*-anisidine hydrochloride (1% in ethanol); (3) *m*-phenylenediamine (0.2 *M* in 76% ethanol). Color was developed by heating the sprayed paper at 100–110° for *ca.* 10 min.

Column Chromatography.—The Dowex-1 borate column was prepared and developed according to Khym and Zill[20,21]; the cellulose column was prepared and eluted according to Putnam.[22]

Hydrolysis of Holothurin A. Preparation of Sugar Mixture.—Heating holothurin A in 3 *N* hydrochloric acid on the steam-bath for 3.5 hr. leads to complete hydrolysis. In this time the maximum value for reducing sugars (Nelson's reagent) was attained. Shorter or milder treatment yields mixtures of products of partial hydrolysis, while further heating resulted in decolorization and decomposition of the sugars. The presence of sulfuric acid in the hydrolysate was attested, after removal of the aglycones, by precipitation of barium sulfate. In a typical run holothurin A (10 g.) dissolved in 3 *N* HCl (800 ml). was stirred and heated at 90–100° for 3.5 hr. The aglycone was separated by centrifugation and washed a few times with small portions of water (20 ml.). With vigorous stirring the combined mother liquor and washing were neutralized by the careful addition of freshly prepared silver carbonate. The precipitated silver chloride was removed by filtration and the sugar solution passed through an Amberlite column MB-3. The column was washed with water (150 ml.) and the combined eluates after treatment with Celite were evaporated in a flash evaporator to *ca.* 150 ml., the temperature of the bath never surpassing 40°. The sugar solution was again treated with Celite and taken to dryness by lyophilization. The resulting white amorphous solid was stored *in vacuo* over P_2O_5. Ten grams of the sugar mixture was obtained from 40 g. of holothurin A; $[\alpha]^{25}D$ +37.9° (concd. 11.2 mg. ml.), constant after 3 hr.; initial $[\alpha]^{25}D$ specific rotation $[\alpha]^{27}D$ +44°.

Calcd. for an equimolar mixture of glucose, xylose, glucomethylose and 3-O-methylglucose $[\alpha]^{27}D$ +40°.

Anal. Calcd. for: OCH_3, 4.5. Found: OCH_3, 4.23.

The R_F values for the components of this sugar mixture with solvent system A were 0.38, 0.45, 0.59, 0.71; found for glucose, xylose and 3-O-methylglucose[23]: 0.38, 0.45 and 0.71; for solvent system B and C the following R_F's were usually noted, 0.25, 0.35 and 0.46. The R_F's in this solvent system were subject to absolute variations. This was probably due to temperature changes. However, the changes in R_F of the sugar mixture were always of the same magnitude as the concomitantly run individual known sugars or mixtures of the latter. In some runs the hydrolysis mixture with solvent system B and or C gave spots varying in R_F from 0.12–0.17; 0.23–0.25; and 0.30–0.39; found for glucose, xylose, rhamnose 0.12–0.17; 0.23–0.25; 0.30–0.40, respectively.

Samples of sugar mixtures obtained from the glycoside prior to finding the optimum conditions of hydrolysis gave the same four spots with solvent system A but usually assayed below in pentose. However, when the orcinol reaction was performed on the unhydrolyzed glycosides the proportion of pentose present based on a molecular weight[2] of 1150 was consistent with the postulate of an equimolar mixture of four separate sugars. The anthrone reaction carried out as described above gave the same O.D. reading at 620 mμ after being heated 3 or 15 minutes. For example, a sugar hydrolysate containing 120 μg./ml. (calculated on reducing capacity by Nelsons' method, using glucose as standard) gave O.D. 0.200 (3 min.) and 0.213 (15 min.). Just such behavior would be anticipated from a mixture containing a hexose and methylpentose. Equivalent aliquots of a total sugar hydrolysate were assayed by the Folin–Wu method before and after fermentation with yeast. O.D. at 420 mμ was 0.574 (before) to 0.408 (after). Paper chromatography using solvent system C gave three spots (R_F 0.12 0.19, 0.30), while after fermentation only two spots were observed; the one corresponding to glucose (R_F 0.12) was now absent. Water extracts of parallel areas (not sprayed) gave a positive orcinol test for the sugar corresponding to R_F 0.19 and positive anthrone test for the sugars corresponding to R_F 0.12 (before fermentation). These experiments established the presence of glucose in the mixture.

Separation of Sugar Mixture by Dowex-1 Borate Chromatography.[24]—One ml. of a solution of the sugar mixture containing 30 mg., diluted with 9 ml. of 0.01 *M* sodium tetraborate, was placed on a Dowex-1 borate column (11 cm. × 1

(15) (a) R. F. Nigrelli, *Zoologica*, **37**, 89 (1952); (b) T. D. Sullivan, K. T. Ladue and R. F. Nigrelli, *Disc. Faraday Soc.*, 40 (1955).

(16) J. H. Roe, *J. Biol. Chem.*, **212**, 325 (1955).

(17) W. Meijbaum, *Z. physiol. Chem.*, **258**, 117 (1938). In both the anthrone and orcinol reactions a considerable variability was noted in the optical density readings from day to day using standard solution of known sugars. However, good proportionality was observed between the O.D. and concentration of sugar in any particular set.

(18) P. B. Hawk, W. H. Summerson and B. L. Oser, "Practical Physiological Chemistry," 12th Ed., The Blakiston Co., Philadelphia, Pa., 1947, p. 520.

(19) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).

(20) J. X. Khym and P. Zill, THIS JOURNAL, **74**, 2090 (1952); L. P. Zill, J. X. Khym and G. M. Cheniae, *ibid.*, **75**, 1339 (1953).

(21) J. O. Lampen, *J. Biol. Chem.*, **204**, 999 (1953).

(22) E. W. Putnam, Column Chromatography of Sugars, in "Methods in Enzymology," eds. Colowick and Caplan, Academic Press, Inc., New York, N. Y., 1957, pp. 59–61.

(23) The 3-O-methylglucose was kindly supplied by Dr. H. G. Fletcher, Jr., National Institutes of Health, Bethesda, Md.

(24) We wish to thank Dr. Milton Tabachnick for carrying out this experiment.

cm. diam.) and eluted with varying concentrations of sodium tetraborate. Individual fractions of *ca.* 10 ml. were collected and assayed by the orcinol and anthrone reaction. The rate of elution was approximately 40 ml. per hour. The results are summarized in Table I.

TABLE I

CHROMATOGRAPHY OF SUGAR MIXTURE ON DOWEX-1 BORATE COLUMN

Vol. fraction, ml.	Na tetraborate (molar concn.)	Sugars	Anthrone	Orcinol
0–45	0.005	.....	..	..
45–115	.01	.....	..	..
115–480	.015	.....	..	..
480–790	.015	Glumethylose + 3-O-methylglucose (710–740)[a]	+	..
790–940	.015	.....	..	..
940–1340	.02	.....	..	..
1340–1560	.02	Xylose (1365–1395)	..	+
1560–1650	.02	.....	..	..
1650–1850	.02	Glucose (1800–1840)	+	..
1850–1865	.02	.....	..	..
1865–2095	.03	.....	..	..
2095–2320	.1	.....	..	..

[a] Figures in parentheses indicate the peak range of elution.

Based on the reducing capacity[19] 80% of the sugar mixture was recovered. The appearance of the pentose and the hexose eluted last coincided with that found for xylose and glucose in a mixture containing also rhamnose and is in agreement with the data reported.[20,21] The more easily eluted sugar mixture (ref. Table I, 489–790 ml.) did not correspond to rhamnose; it was passed through an Amberlite column (IR 120, H^+ form), evaporated in a flash evaporator to near dryness, one liter of absolute methanol added and the mixture again taken down to dryness. The addition of methanol and evaporation was repeated twice more and the final residue was taken up with 5 ml. of water. It gave a positive methylpentose[9] and anthrone reaction. Paper chromatography, using solvent system A, gave two spots with R_F 0.59 (glucomethylose) and 0.71 (3-O-methylglucose) while in the solvent system D only one spot was obtained, R_F 0.46. Elution with water from the paper of the separate areas showed the sugar with R_F 0.59 to be the methylpentose[9] (Dische test). The anthrone reaction carried out for 3 and 15 min. confirmed this conclusion; O. D. 0.39 (3 min.) to 0.086 (15 min.). The anthrone reaction on the extract (R_F 0.71) showed this sugar to be a hexose and obviously the methylated derivative; O.D. 0.022 (3 min.) to 0.078 (15 min.).

Cellulose Chromatography and Separation of the Four Sugars.—In a typical run, one gram of sugar mixture ($[\alpha]^{27}_D$ +38°) in 1.2 ml. of water was placed on a cellulose column (50 cm. × 3.8 cm. diam.) and eluted with butanol (saturated with water): over a 24–30 hr. period *ca.* 350 fractions (5 ml.) were collected. Samples from every other tube were spotted and chromatographed on paper, employing solvent system A. The sugar composition of each fraction was thus ascertained. Table II summarizes the results in a typical run. The fractions from several runs which contain but one sugar were combined and evaporated to dryness under reduced pressure. All rotations refer to water and were taken in a 10 cm. tube.

Glucomethylose.—Two recrystallizations of the appropriate residue from ethyl acetate gave the pure sugar; m.p. 146°; $[\alpha]^{26}_D$ +27.9 (concn. 9.66 mg./ml.) constant after 1 hr.; initial specific rotation + 75; reported[26] m.p. 146°; $[\alpha]^{20}_D$ +29.1.

Anal. Calcd. for $C_6H_{12}O_5$: C, 43.90; H, 7.32. Found: C, 44.00; H, 7.39.

Xylose.—Two recrystallizations of the appropriate residue from absolute ethanol gave pure compounds of m.p. 148–151° undepressed on admixture with an authentic sample; $[\alpha]^{27}_D$ +19.6 (*c* 15.0 mg./ml.) constant after 2 hr.; initial for specific rotation +50.

TABLE II

CHROMATOGRAPHY OF SUGAR MIXTURE ON CELLULOSE COLUMN

Volume fraction, ml.	Sugars
0–300	.....
300–450	Glucomethylose (0.59)[a]
450–650	Glucomethylose (0.59) + 3-O-methylglucose (0.71)
650–700	3-O-Methylglucose + xylose (0.45) + glucomethylose (trace)
700–750	Xylose
950–1000	Xylose (trace) + glucose (trace)
1000–1600	Glucose (0.38)
1600–1700	.....

[a] Figures in parentheses indicate R_F values in phenol–water.

Glucose.—Recrystallization of the appropriate residue from ethanol gave pure material of m.p. 147° undepressed on admixture with an authentic sample; $[\alpha]^{26}_D$ +52.4° (concn. 15.8 mg./ml.) constant after two hours.

3-O-Methylglucose.—The fractions containing only glucomethylose and 3-O-methylglucose were combined and evaporated to dryness under reduced pressure. The residue was rechromatographed on cellulose as above. The final eluates yielded fractions containing only one sugar with R_F = 0.71 (solvent system A). These fractions were worked up as above and recrystallization of the final residues from acetone gave the pure sugar; m.p. 162–167°; $[\alpha]^{30}_D$ +59.5° (concn. 4.2 mg./ml.) constant after 3 hr.; initial specific rotation +98°; reported[26] m.p. 161–168°; $[\alpha]^{25}_D$ +55.5.

Anal. Calcd. for $C_7H_{14}O_6$: C, 43.40; H, 7.27; OCH_3, 15.99. Found: C, 43.61; H, 7.11; OCH_3, 16.51.

NEW YORK, N.Y.

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(26) E. J. Bourne and S. Peat, *Advances in Carbohydrate Chem.*, **5**, 155 (1950).