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The Structure of D-Glucosyl-D-xylose Synthesized by Maltose Phosphorylase[1]

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RECEIVED FEBRUARY 25, 1955

A crystalline reducing disaccharide consisting of D-glucose and D-xylose has been synthesized from β-D-glucose-1-phosphate and D-xylose by the agency of maltose phosphorylase from the organism *Neisseria meningitidis*. On hydrolysis of this disaccharide with acid downward mutarotation was observed, indicating that the two monosaccharide units are joined through an α-glycosidic bond. Complete methylation of the compound produced a methyl-hexa-*O*-methyl derivative, which on hydrolysis with acid gave rise to 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-xylose. On the basis of these data, the configuration of the new disaccharide is assumed to be 4-*o*-α-D-glucopyranosyl-D-xylopyranose.

It has previously been demonstrated[2-4] that the bacterium *Neisseria meningitidis* contains a phosphorylase which is capable of catalyzing the reversible reaction

$$\text{maltose} + \text{inorganic phosphate} \xrightleftharpoons{\text{maltose phosphorylase}} \beta\text{-D-glucose-1-phosphate} + \text{D-glucose}$$

Besides D-glucose, D-xylose was found to be the only sugar of a number of monosaccharides investigated that was capable of reacting in the reverse direction in the presence of this enzyme with β-D-glucose-1-phosphate to form a disaccharide

$$\beta\text{-D-glucose-1-phosphate} + \text{D-xylose} \xrightleftharpoons{\text{maltose phosphorylase}} \alpha\text{-D-glucosyl-D-xylose} + \text{inorganic phosphate}$$

The present investigation is concerned with the preparation and determination of the chemical properties, and the configuration of this disaccharide synthesized from β-D-glucose-1-phosphate and D-xylose by the enzyme from *N. meningitidis*.

The new disaccharide reduces Fehling and alkaline ferricyanide solutions. It mutarotates slightly downward, its specific rotation being $[\alpha]_D$ +97.5° after 9 minutes in solution, and +94.5° after 100 minutes. On hydrolysis with acid it produces one mole of D-glucose and one mole of D-xylose. Oxidation of the disaccharide with bromine[5] and subsequent hydrolysis of the oxidation product result in the formation of D-glucose and D-xylonic acid, indicating that the D-xylose constitutes the reducing moiety of the disaccharide. Treatment of the disaccharide with phenylhydrazine hydrochloride in the presence of sodium acetate produces a yellow colored osazone which is soluble in hot water but insoluble in cold.

Methylation of the disaccharide first with dimethyl sulfate and sodium hydroxide, and finally with methyl iodide and silver oxide produces a fully methylated methyl tetra-*O*-methyl-D-glucosyl-di-*O*-methyl-D-xyloside. On methanolysis of this methylated disaccharide and subsequent hydrolysis with acid, 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-xylose is obtained. Since D-xylofuranose has never been encountered in nature[6] it can be assumed that the D-xylose occurs in the disaccharide in the pyranose configuration. From these considerations it was concluded that the disaccharide is joined glycosidically through C-1 of D-glucose and C-4 of D-xylose and can be considered to be an analog of maltose. Its downward mutarotation indicates that the D-xylose moiety exists in the α-form. The decrease in specific rotation from +94.5 to +35.0° during the course of acid hydrolysis indicates that the two monosaccharide units are combined in the disaccharide through an α-glycosidic linkage. The most probable structure of the disaccharide is therefore 4-*O*-α-D-glucopyranosyl-α-D-xylopyranose, and its formula may be written as shown in Fig. 1.

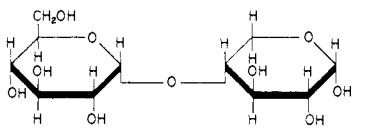

Fig. 1.—4-*O*-α-D-Glucopyranosyl-α-D-xylopyranose

Experimental

Reagents.—The maltose phosphorylase preparation was obtained from *N. meningitidis* bacteria as previously described.[7]

The barium salt of the β-D-glucose-1-phosphoric acid was synthesized according to the method of Reithel.[8] Before use, it was converted into the sodium salt by adding an equivalent amount of sodium sulfate to a solution of the barium salt.

Preparation of Disaccharide.—β-D-Glucose-1-phosphate was allowed to react with a sixfold excess of D-xylose in the presence of the enzyme preparation from *N. meningitidis*. When the liberation of inorganic phosphate ceased, the reaction mixture was lyophilized. The disaccharide was prepared in five small scale batches, involving a total of about 6 g. of the barium salt of the phosphate ester. The material from the various lyophilized digests was combined, dissolved in water and deionized by passage through ion-exchange columns, Dowex 50, followed by Duolite A-4. The disaccharide was separated from the excess of D-xylose in the neutral effluent by Whistler and Durso's[9] charcoal column chromatographic method. The solution (200 ml.) containing the sugars was transferred to a 5-cm. diameter column, packed with a mixture of equal parts (by weight) of charcoal (Darco G-60) and Hyflo Supercel. The bed volume of the column was approximately 500 ml. By passing distilled water through the column the D-xylose was eluted leaving the disaccharide adsorbed on the column.

(1) This work was supported in part by a research contract with the United States Atomic Energy Commission and by a research grant (G-4074) from the National Institutes of Health, U. S. Public Health Service.

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After about 2 liters of water had been passed through the column, pentose could no longer be detected in the effluent by the Mejbaum orcinol test.[10] The adsorbed disaccharide was eluted from the column with 15% ethanol. After approximately 1.5 liters of the alcoholic eluate was collected the amount of disaccharide remaining on the column was negligible.

The alcoholic solution was concentrated by vacuum distillation, filtered and reduced to a sirup by further concentration in a vacuum desiccator. Crystallization of the sirup was induced by the addition of hot absolute ethanol and trituration of the mixture. When crystallization was complete the clear supernatant solution was decanted and the crystalline material was washed by decantation with cold absolute ethanol. After washing with ether the product was dried in the air and placed in a vacuum oven at 40° for 20 hours. The yield of crystalline disaccharide was 0.83 g.

Properties of the Disaccharide.—The crystalline disaccharide was soluble in water, it reduced Fehling and alkaline ferricyanide solutions, and when subjected to paper chromatographic analysis it migrated as a single compound in water saturated phenol and in a mixture of butanol (52%), acetic acid (13%) and water (35%). The compound was readily detected on the paper chromatograms when sprayed with solutions of either *p*-anisidine hydrochloride or *m*-phenylenediamine hydrochloride. The color produced by these reagents when the papers were heated was identical with that observed when known pentoses were chromatographed. In all of the solvents used the R_f of the compound was practically identical with that of maltose.

When 20 mg. of the disaccharide was dissolved in 1 ml. of water, heated on the steam-bath for half an hour with 1 ml. of solution containing 0.1 g. of phenylhydrazine hydrochloride, 0.15 g. of sodium acetate and a drop of a saturated solution of sodium bisulfite, and the solution allowed to cool, a yellow crystalline osazone formed. When examined under the microscope this osazone had an appearance similar to that of glucosazone.[11]

Analysis of the osazone for nitrogen gave a value of 11.2% (calculated for $C_{23}H_{30}N_4O_8$, 11.4%).

Oxidation of 10 mg. of the disaccharide with bromine in a benzoate buffered aqueous medium[5] resulted in the production of an aldobionic acid. This acid was isolated chromatographically according to the method previously described,[4] and then hydrolyzed with 1 *N* hydrochloric acid for 2.5 hours at 100°. Chromatographic analysis of the products showed D-glucose and D-xylonic acid. These data indicate that the reducing moiety in the disaccharide is D-xylose.

When the compound was dissolved in water and the solution examined in a polariscope, a slight downward mutarotation was observed, the initial specific rotation in water (*c* 1.88) being $[\alpha]_D$ +97.5°, and reaching a constant value of +94.5° after 100 minutes. Hydrolysis of the disaccharide with 1.0 *N* hydrochloric acid for 2.5 hours at 100°, followed by paper chromatographic separation of the products resulted in glucose and xylose as the sole products. The decrease in specific rotation from +94.5 to +35.0° during the course of hydrolysis indicates that the two monosaccharides are combined in the disaccharide through an α-glycosidic linkage. The disaccharide did not possess a sharp melting point. The crystals began to sinter at 58° and completely melted at 78°. C and H analyses of the compound fit the empirical formula $C_{11}H_{20}O_{10}\cdot 2H_2O$, indicating that the compound contains two molecules of water of crystallization.

Anal. Calcd. for $C_{11}H_{20}O_{10}\cdot 2H_2O$: C, 37.85; H, 6.95. Found: C, 37.68; H, 6.81.

Rate of Hydrolysis of the Disaccharide.—The hydrolysis of a 1.06% solution of the D-glucosyl-D-xylose in 6 *N* hydrochloric acid was followed by observing the change of rotation at 50°. The course of hydrolysis of this disaccharide was represented by a logarithmic curve, indicating the first order of reaction. The velocity constant *K* of the reaction was 4.30×10^{-3}. The velocity constant *K* of maltose determined under similar conditions was 1.28×10^{-2}, and that of 3-*O*-α-glucopyranosyl-L-arabinose was 4.37×10^{-3}.[12] This shows that the rate of hydrolysis of the D-glucosyl-D-xylose is almost identical with that of 3-*O*-α-glucopyranosyl-L-arabinose, but only about one-third that of maltose.

Methylation of the D-Glucosyl-D-xylose.—In order to protect the reducing group, the first stage of methylation was carried out in the cold according to the method of Isbell and Frush[13] as follows. A 0.70-g. portion of the disaccharide was dissolved in 4 ml. of water in a 50-ml. three-necked flask equipped with a mechanical stirrer. The flask was placed in an ice-bath, 0.09 ml. dimethyl sulfate, 0.05 ml. of 32% sodium hydroxide and a drop of phenolphthalein were introduced into the flask and stirred for 30 minutes. From then on 0.06-ml. aliquots of dimethyl sulfate were added at approximately 30-minute intervals, and from time to time 0.05-ml. portions of 32% sodium hydroxide were added in order to keep the reaction medium just alkaline to the indicator. After 6 hours, when a total of 0.60 ml. of dimethyl sulfate and 0.47 ml. of alkali had been added, the reaction mixture was left at room temperature overnight.

Methylation was continued the next day without regard to *p*H control by addition of portions of 0.7 ml. of dimethyl sulfate and 1 ml. of 32% sodium hydroxide at room temperature every 10 minutes until 4.2 ml. of dimethyl sulfate and 6.6 ml. of alkali had been added. The temperature was then raised to 50° and 4.2 ml. of dimethyl sulfate and 9.8 ml. of alkali were added over a period of 1 hour. Finally the temperature was raised to 70° and the same quantities of reagents were added as those at 50°. The flask was then heated on a steam-bath for 45 minutes, cooled to room temperature, the contents transferred to a separatory funnel and extracted six times with 10-ml. portions of chloroform. The extracts were concentrated on the steam-bath under a jet of air, and the final traces of the solvent were removed under vacuum. The yield of the methylated product was 0.78 g.

The partially methylated product was dissolved in acetone and returned to the reaction flask. The temperature of the solution was raised to 55° while stirring vigorously and portions of 0.7 ml. of dimethyl sulfate and 2.0 ml. of 32% sodium hydroxide were added at 10-minute intervals until a total of 8.4 ml. of dimethyl sulfate and 24 ml. of alkali had been added. After heating for half an hour at 100° with the addition of water to dissolve the sodium sulfate, the solution was cooled to room temperature and thoroughly extracted with chloroform. The extract was dried with a mixture of anhydrous sodium and magnesium sulfate, filtered and concentrated to dryness, whereupon a semi-crystalline product was obtained. Analysis of this sirup for its methoxyl content, OCH_3, gave a value of 50.0%. The calculated OCH_3 value for methyl tetra-*O*-methyl-D-glucosyl-di-*O*-methyl-D-xyloside, $C_{11}H_{13}O_3(OCH_3)_7$ is 52.93%.

The incompletely methylated disaccharide was further methylated by the use of Purdie's reagents as follows: the sirup (0.72 g.) was dissolved in 30 g. of methyl iodide in a three-necked flask equipped with a reflux condenser and a mercury seal stirrer. The methyl iodide was brought to a boil and 5 g. of silver oxide were added to the refluxing mixture in the course of a 2.5-hour period. After all the silver oxide had been added, refluxing of the methyl iodide was continued for 2 more hours. The mixture was diluted with chloroform and filtered. Removal of the solvent by concentration *in vacuo* yielded a residue weighing 0.68 g.

The material was dissolved in chloroform and transferred to a small still containing glass wool. The chloroform in the still was removed by heating on a steam-bath after which the still was connected to a vacuum line. The product distilled as a light yellow oil at 20 μ pressure and a bath temperature of 195°. The yield of the distilled sirup was 0.57 g., amounting to 84% of the initial charge in the still. The methoxyl content of this sirup, OCH_3, was 52.00% (calculated value for $C_{11}H_{13}O_3(OCH_3)_7$, 52.93%.

Methanolysis and Hydrolysis of the Methylated Disaccharide and Separation of the 2,3,4,6-Tetra-*O*-methyl-D-glucose and Di-*O*-methyl-D-xylose.—The methylated material (0.54 g.) was taken up in 20 ml. of 1 *N* methanolic hydrochloric acid and placed in a bomb tube. The tube was sealed and kept at 100° for 3 hours. It was then

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cooled, opened and the methanol removed by a jet of air while the tube was immersed in a 50° water-bath. A similar amount (20 ml.) of 1 *N* aqueous hydrochloric acid was introduced into the tube. The tube was sealed and heated again for 3 hours to hydrolyze the glycosidic methyl groups. At the end of that period the tube was cooled, opened and the contents passed through a Duolite A-4 anion-exchange column. The neutral effluent was concentrated to a sirup under reduced pressure and transferred to a separatory funnel, using approximately 20 ml. of hot water (in 2-ml. portions) and about the same amount of chloroform. The chloroform phase was drawn off, and the water was extracted five times with 10-ml. portions of chloroform.

Evaporation of the chloroform extract yielded a crystalline residue. This material was recrystallized from ether to which sufficient petroleum had been added to cause incipient turbidity. The product (0.24 g.) was identified as 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose.

Anal. Calcd. for $C_6H_8O_2(OCH_3)_4$: OCH_3, 52.6. Found: OCH_3, 51.6; specific rotation $[\alpha]D$ +95° → +81° (in water, *c* 2); melting point, 88°.

An aliquot of the aqueous phase was analyzed qualitatively by paper chromatography, using water saturated methyl ethyl ketone as a solvent.[14] This fraction showed the presence of traces of tetra-*O*-methylglucose and some slower moving impurity. The predominating compound, however, was one that migrated with the same R_f as 2,3-di-methyl-D-xylose. In order to purify the di-*O*-methyl-D-xylose derivative, the aqueous fraction was concentrated to an anhydrous sirup (0.199 g.), dissolved in methanol and adsorbed on five 20 mm. diameter discs of Whatman No. 3 filter paper. The methanol on the discs was evaporated by irradiation with an infrared lamp. The discs were then placed at the top of a powdered cellulose chromatography column wet with a water saturated mixture of heptane and butanol (7:3, by volume).[15]

The cellulose column was prepared by a gravity pack of an acetone slurry of Standard grade Whatman No. 1 powdered cellulose in a 2.3 × 60 cm. chromatography tube fitted with a precision ground stopcock so that the rate of flow could be controlled. The column bed, 48.5 cm. in length, had a volume of approximately 200 ml. with a holdup volume of about 115 ml. solvent. The acetone on the column was washed out with the water saturated heptane–butanol mixture which was admitted by siphon through a stopper at the top of the chromatography tube. After about 200 ml. of effluent had been collected the solvent was drained to the level of the cellulose bed and the discs of filter paper which were impregnated with the methylated sugars were placed on top of the column. Five ml. of solvent was pipetted onto the discs and the stopcock at the bottom of the column was adjusted so as to give a flow rate of about one ml. per minute. Two more 5-ml. portions of solvent were added to the top of the column and allowed to drain to bed level, after which 20 ml. of solvent was added and the siphon delivery from the solvent reservoir was connected to the top of the tube. Fractions of approximately 5 ml. were collected by a fraction collecting apparatus similar to that described by Hough, *et al.*[16]

After about 650 ml. of eluate had been collected aliquots of 0.02 ml. of each fraction were spotted in series on sheets of filter paper and sprayed with a solution of *p*-anisidine hydrochloride (saturated solution in *n*-butanol).[16] After the paper had been heated, it was found that tubes Nos. 28 to 42 and 66 to 94 contained compounds that reacted with the indicator. Using an infrared lamp and an air jet to dry the spots, 0.04-ml. aliquots of each of these 42 tubes were placed 2 cm. apart on the starting-lines of filter paper sheet chromatograms, Whatman No. 1, 18.5 × 24 inches.

The chromatograms were developed with water-saturated methyl ethyl ketone,[14] sprayed with *p*-anisidine hydrochloride solution and heated. The observed R_f values showed that the first series of tubes, 28 to 42, contained a small amount of tetra-*O*-methyl-D-glucose, whereas the second series, 66 to 94, contained predominantly di-*O*-methyl-D-xylose. The first three tubes in this series appeared to be contaminated with a faster moving component and the last three tubes with a slower moving component. Tubes 69 to 91 (about 110 ml.) were therefore combined and concentrated by vacuum distillation. The sirupy residue was dissolved in water, treated with charcoal, filtered into a tared beaker and dried in a vacuum desiccator. Attempts to crystallize this sirup (0.122 g.) were not successful. Its methoxyl content, OCH_3, was 34.5% (calculated for $C_5H_8O_3(OCH_3)_2$, 34.8%. The specific rotation (*c* 4.89) of the sirup in water was $[\alpha]D$ +21.3°.

The specific rotation of 2,3-di-*O*-methyl-D-xylose is given in the literature[6,15,17] as $[\alpha]D$ +70° → +23°. That of the 2,4-di-methyl-D-xylose is recorded as $[\alpha]D$ −30° → +22°.[18] It therefore appears that the two di-*O*-methyl-D-xylose derivatives cannot be distinguished on the basis of their final equilibrium rotations. However, they can be differentiated by the specific rotations of their dimethyl anilides.

The possibility that the di-*O*-methyl-D-xylose derived from the methylated disaccharide is the 3,4-derivative can be excluded on the basis that the original disaccharide forms an osazone, showing that C-2 position in D-xylose is free and on treatment with dimethyl sulfate must be methylated. The 2,5-di-*O*-methyl-D-xylose derivative can be eliminated by its higher specific rotation $[\alpha]D$ +46° in water.[19]

Identification of 2,3-Di-*O*-methyl-D-xylose by its Anilide Derivative.—A 0.095-g. sample of the di-*O*-methyl-D-xylose was dissolved in 5 ml. of ethanol, treated with 0.050 g. of aniline and refluxed for 6 hours. The ethanol was distilled off and the residue dissolved in a minimum amount of ethyl acetate. This solution containing the reaction mixture was pipetted onto a small charcoal column (1 × 6 cm.) packed by gravity with an ether suspension of Darco-G-60. The reaction mixture was then washed through with benzene, and the eluate collected manually in 1-ml. fractions in test-tubes. As soon as a fraction was obtained the solvent was evaporated by a stream of air. Upon evaporation of the solvent white crystalline residues were deposited in the No. 4 to 24 test-tubes. The melting points of the various crystalline fractions increased progressively from 130 to 136°. The crystalline fractions were dissolved in ethyl acetate, combined, filtered and concentrated to a small volume. Petroleum ether was added to the solution to the point of incipient turbidity and the solution was placed at −10° overnight. The resultant crystalline clusters of needles were centrifuged, washed by decantation with cold ethyl ether, and dried. A yield of 0.064 g. of di-*O*-methyl-D-xylose anilide was obtained.

Anal. Calcd. for $C_{13}H_{19}O_4N$: C, 61.64; H, 7.56; N, 5.53; OCH_3, 24.50. Found: C, 61.77; H, 7.65; N, 5.51; OCH_3, 24.48.

The specific rotation of the anilide in ethyl acetate (*c* 1.99) was $[\alpha]D$ +188°; it mutarotated downward in ethyl acetate containing 4% acetic acid in the course of 130 minutes reaching a final value of +65°. The specific rotation of the anilide in dioxane after 30 minutes was $[\alpha]D$ +168°, and came to a final value of +76° after 25 hours. The specific rotation given by Hampton, *et al.*,[17] for 2,3-di-*O*-methyl-D-xylose anilide is $[\alpha]D$ +185° → +65°; melting point, 146°. The specific rotation for 2,4-di-*O*-methyl-D-xylose anilide reported by Barker, *et al.*,[18] is −82° in dioxane; melting point 170°.

The melting point of the di-*O*-methyl-D-xylose anilide derived from the disaccharide was found to be 136°. Similarly, when the anilide of authentic 2,3-di-*O*-methyl-D-xylose was prepared and isolated from commercial xylan according to the procedure of Hampton, *et al.*,[17] it also melted at the same temperature of 136°. When this authentic anilide was mixed with the anilide prepared from the di-*O*-methyl-D-xylose derived from the methylated disaccharide, no depression of melting point could be observed. This anilide is therefore the 2,3-di-*O*-methyl-D-xylose derivative.

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