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SUGARS IN THE ACID HYDROLYZATES OF POLYSACCHARIDES PART III. ISOLATION OF KOJIBIOSE (2-O- -D-GLUCOPYRANOSYL-D-GLUCOSE) AND , -TREHALOSE FROM HYDROL

SUGARS IN THE ACID HYDROLYZATES OF POLYSACCHARIDES

PART III. ISOLATION OF KOJIBIOSE (2-0-α-D-GLUCOPYRANOSYL-D-GLUCOSE) AND β, β-TREHALOSE FROM HYDROL*

By

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Hydrol is the residual syrup obtained after crystallization of D-glucose from acid hydrolyzate of starch. This is recognized as a complex mixture containing D-glucose and its related oligosaccharides. The latter arises mainly from the acid-catalyzed "reversion" of D-glucose and partly from incomplete hydrolysis of starch. The random combination of the hemiacetal function of α- or β-D-glucopyranosyl residue with one of the five available hydroxyl groups in the second D-glucopyranose can produce 11 isomers of glucose-disaccharide. Seven of these (α,α-trehalose (1), nigerose (2), maltose (1), isomaltose (3), sophorose (2), cellobiose (4), and gentiobiose (5)) have previously been identified in hydrol. 5-0-β-D-Glucopyranosyl-D-glucose, one of the two possible disaccharides resulting from the substitution of a D-glucopyranosyl residue on the hydroxyl group at carbon-5 of D-glucofuranose, has also been reported as a constituent of hydrol (6).

The present report deals with the isolation of kojibiose and β,β-trehalose as their crystalline acetates from hydrol. At the same time with this isolation, Matsuda (7) succeeded in chemical synthesis of 1 : 2-α-glucosidic linkage from 1, 3, 4, 6-tetra-O-acetyl-α-D-glucose and 2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl bromide in the presence of mercuric cyanide and obtained crystalline α- and β-kojibiose acetates.

The name "kojibiose" was first applied by Aso *et al.* (8) to an unfermentable sugar occurring in Saké or Koji extract, and was believed to be a disaccharide having hitherto unknown 1 : 2-α-glucosidic linkage. Later, Shibasaki reported

on the existence of this sugar in a mixture of oligosaccharides synthesized from maltose or glucose by *Schizosaccharomyces pombe* (9-20). Another enzymic synthesis of 1 : 2-α-linkage was reached by Barker *et al.* (11), who showed that *Betacoccus arabinosaceous*, when grown on a medium of lactose-sucrose or cellobiose-sucrose system, produced trisaccharides of "branched" type containing 1 : 2-α-linkage. Haq and Whelan (12) also synthesized a 1 : 2-α-linked glucose-disaccharide by heating 1, 2-anhydro-3, 4, 6-tri-O-acetyl-α-D-glucopyranose alone.
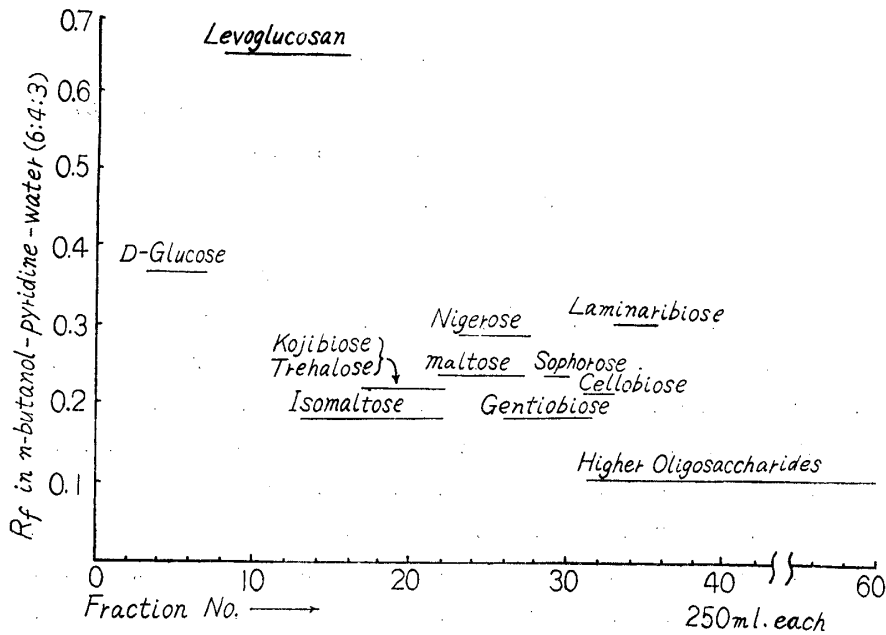

Fig. 1. Chromatography of Disaccharides of Hydrol on Carbon
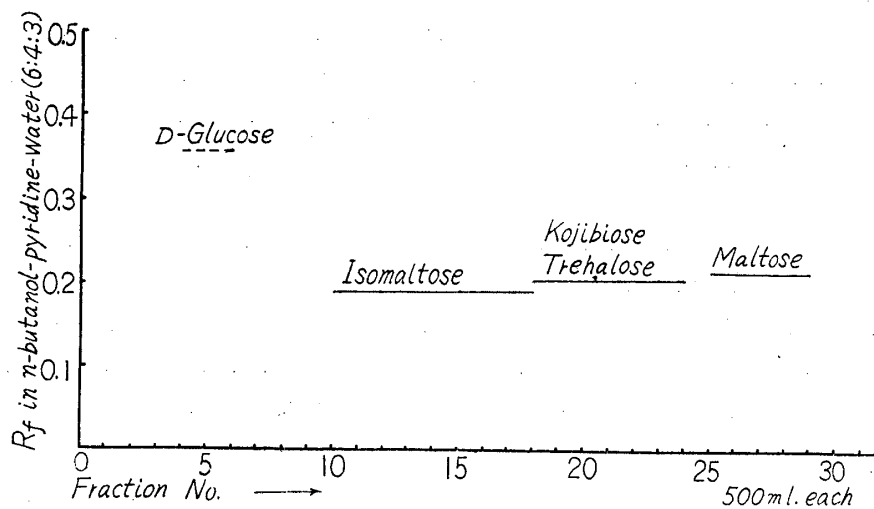Column : Hydrol sample, 250g. ; Column size, 460×120mm. diam.

Fig 2. Fractionation of Kojibiose-fraction on Carbon Column
in the Presence of Borate Buffer : Sugar sample, 18.0g. ;
Column size, 470×70 mm. diam.

In all these reports, however, no description of crystalline kojibiose in any form was given.

The hydrol now used was kindly supplied by Mr. H. Tsuyama of the Sanmatsu Industrial Co., and was separated from oxalic acid-hydrolyzate of sweet potato starch. Carbohydrate constituents of hydrol was fractionated by carbon
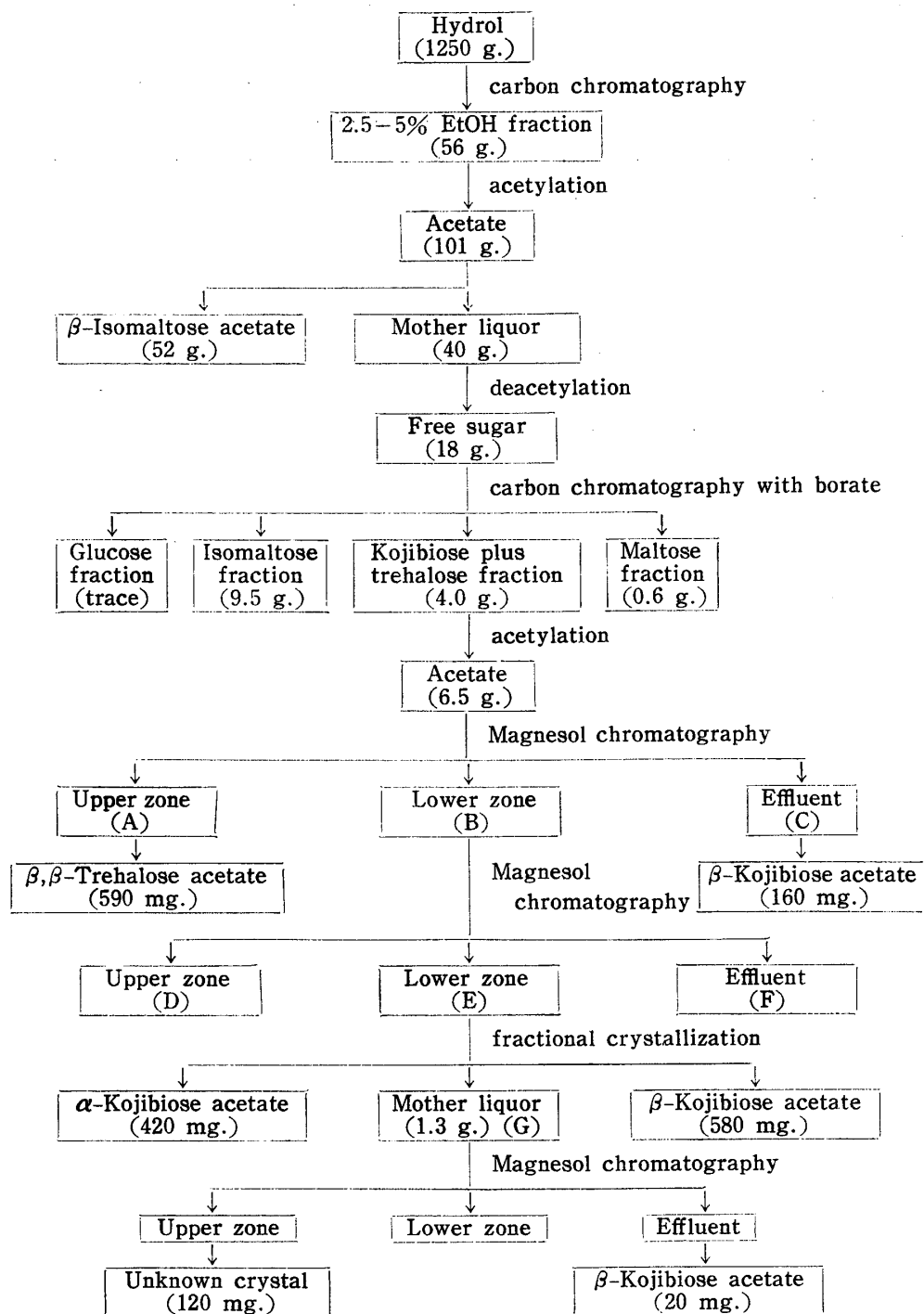

Fig. 3. Isolation of Kojibiose and β,β-Trehalose from Hydrol

column chromatography (13) using water and aqueous ethanol as elut'on solvents. Kojibiose was eluted together with other disaccharides (isomaltose, trehalose, and maltose) in 2.5-percent ethanol fractions (Fig. 1). This sugar mixture was further chromatographed on a carbon column in the presence of borate buffer (14) and was separated into isomaltose, kojibiose plus trehalose, and maltose fractions (Fig. 2). After acetylation, kojibiose plus trehalose fraction was subjected to Magnesol column chromatography (15) and separated into two zones on the column (Fig. 3). The upper zone produced a crystalline β. β-trehalose octaacetate. However, this sugar could not be detected by paper chromatography through the above course of separation since it had the same Rf value as that of kojibiose and was extremely weak in its sensitivity to the spray reagents under the conditions stated in the Experimental section. The lower zone, after rechromatography on Magnesol column and subsequent fractional crystallization, produced two kinds of crystalline acetates distinguishable from the known octaacetates of other glucose-disaccharides; m. p. 166°C. $[\alpha]_D + 149.8°$ (α-kojibiose octaacetate), and m. p. 118°C, $[\alpha]_D + 111.5°$ (β-kojibiose octaacetate). Their infrared absorption spectra are shown in Figs. 4 and 5. On admixture with the
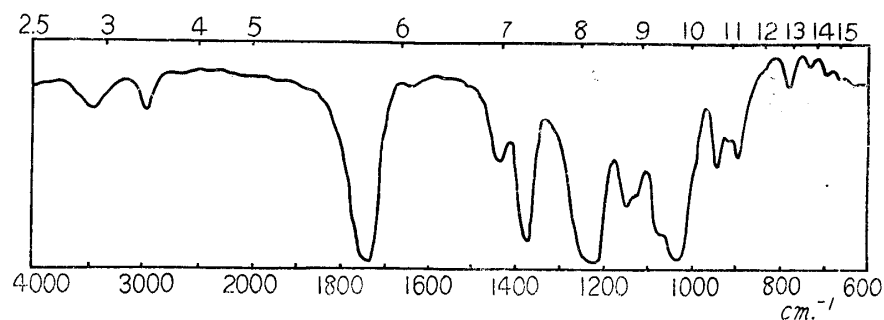

Fig. 4. Infrared Absorption Spectrum of α-Kojibiose Octaacetate (KBr disk).
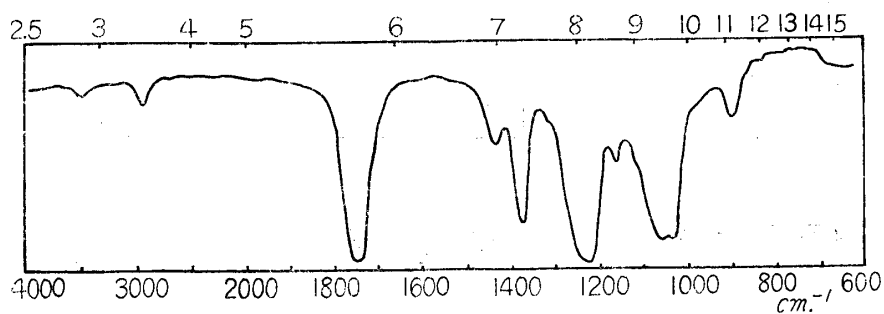

Fig. 5. Infrared Absorption Spectrum of β-Kojibiose Octaacetate (KBr disk).

samples synthesized by Matsuda, both acetates showed no depression of the melting point.

The designation that the above two acetates are respectively α- and β-octaacetate of kojibiose was made on the basis of their specific optical rotation and the following properties of their free sugars, which were consistent with the structure of 2-0-α-D-glucopyranosyl-D-glucose. (i) After deacetylation, the acetate of m. p. 166°C gave sugar A and the acetate of m. p. 118°C, sugar B. Acid hydrolysis of both sugars yielded only glucose as the product detectable on paper. (ii) Their mobilities on paper ionophoresis in sodium hydrogensulfite (16)corresponded to that expected for a glucose-disaccharide. This was also confirmed by oxidation with alkaline hypoiodite (17). (iii) The presence of α-glucosidic linkage in the sugars was indicated by their high specific optical rotation (sugar A, $[\alpha]_D + 135.8°$ and sugar B, $[\alpha]_D + 139.7°$), their resistance to almond β-glucosidase, and the presence of an infrared absorption at 842cm^{-1} (α-linkage) (18) and the absence of absorption around 890cm^{-1} (β-linkage) (18) (Fig. 6). (iv) The low mobilities
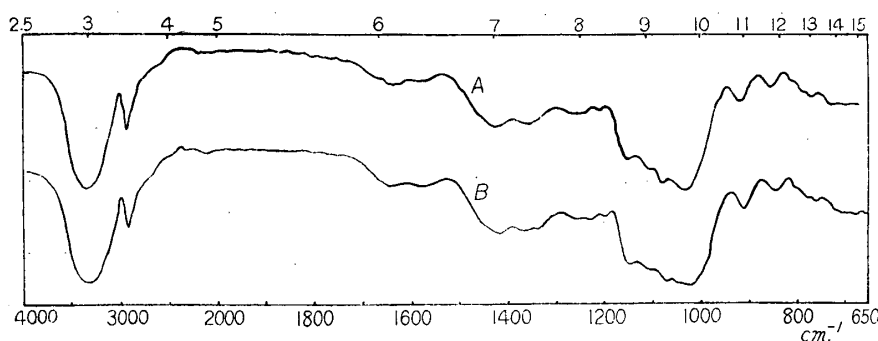

Fig. 6. Infrared Absorption Spectra of Kojibiose (KBr disk)
A : prepared from α-kojibiose acetate.
B : prepared from β-kojibiose acetate.

of their borate complexes on paper ionophoresis (19) were readily differentiated from those of 1 : 3-and 1 : 6-linked disaccharides. Paper chromatography using n-butanol-pyridine-water (6 : 4 : 3) showed that the sugars could not be maltose (α-1 : 4). (v) Their reducing power toward the Shaffer-Hartmann reagent (20) was extremely low (about 15% of the theoretical value for a disaccharide). Sophorose (β-1 : 2) is also known to have a much lower reducing power than 1 : 3-, 1 : 4-, or 1 : 6-linked disaccharides (21). (vi) when sprayed with aniline reagent on paper, the sugars as well as sophorose gave brownish-red stains different from those of the other reducing glucose-disaccharides. Furthermore, neither was disclosed by alkaline triphenyltetrazolium chloride (22) which detects all reducing gluco-saccharides except those with 2-0-substituent at the reducing unit (23).

Acetylation of reducing sugars employing sodium acetate as a catalyst at a high temperature is known to produce on equilibrium mixture in which β-anomer acetate predominates. Normally, α-form is produced in too small an amount to

be isolated. In the case of kojibiose, however, such acetylation with subsequent Magnesol column chromatography resulted in the isolation of a considerable proportion of α-form (420mg) in addition to β-form (760mg). Similar results were obtained by Matsuda.

Experimental

General Methods.

Paper-chromatographic separations were made with n-butanol-pyridine-water (6 : 4 : 3 by vol.). The spray reagents used to locate sugars were aniline hydrogenphthalate (24), silver nitrate-sodium hydroxide (25), and alkaline triphenyltetrazolium chloride (22). Unless otherwise stated, the first of these spray reagents was used. Evaporation of sugar solutions was conducted under reduced pressure. When optical rotation of free sugars was measured, the concentration was determined by acid hydrolysis to glucose; the concentration of ester derivatives was measured by weighing. Melting points are uncorrected.

Acetylation of sugars was accomplished by heating 10 parts by weight of the carbohydrate and 5 parts of freshly fused anhydrous sodium acetate with 70 parts of acetic anhydride at 100-105°C for 2.5hr. The reaction mixture was then poured into 500 parts of ice and water with vigorous stirring to hydrolyze the excess acetic anhydride, and the syrupy insoluble material was extracted with chloroform. The chloroform solution was washed with sodium carbonate solution and water, and evaporated to a syrup. The syrupy acetate was dried by distillation with methanol under reduced pressure. Deacetylation of sugar acetates was carried out by dissolving the acetate in 0.05N methanolic sodium methoxide (10 ml per g of the acetate) and keeping the solution at 0-5°C over night, after which the mixture was diluted with water (50 *ml* per g of the acetate), treated with Amberlite IR-120 (H^+ form) and IR-4B (OH^- form), and evaporated to a syrup.

Preliminary Fractionation on Carbon-Celite Column.

Hydrol (250 g; ca 85% solid) was diluted with 2 *l* of water, adjusted to pH 6.5 with sodium hydroxide solution, and introduced onto the top of a column (500×120 mm diam.) of carbon (Shirasagi) Celite 545 (1 : 1 by wt.). The column was first developed by passage of water (25 *l*) at a rate of about 1 *l*/hr. under slight suction. The developer was then changed to aqueous ethanol solutions in successive concentrations of 2.5% (10 *l*), 5% (20 *l*), 10% (20 *l*), 15% (20 *l*) and 30% (20 *l*). The effluent was collected in 2.5 *l* fractions and examined for carbohydrate content with the Molisch reagent. The Molisch-positive fractions were separetely evaporated to a syrup and analyzed for sugar composition by paper chromatography with the reagents of aniline and silver nitrate. The elution behavior of sugar constituents from the column is shown in Fig. 1.

Isolation and identification of laminaribiose is reported in the following paper.

Four additional carbon-chromatographic separations of hydrol on the same scale as above were made; total hydrol used was thus 1250 g. Fractions No. 17-22 from five columns were combined and evaporated to a syrup, which was further dried by distillation with methanol under reduced pressure; yield 56 g. The material was acetylated (amorphous acetate, 101 g) and crystallized from ethanol; yield, 52 g, m. p. 142-144°C. The crystals were recrystallized from the same solvent; m. p. 144-145°C, unchanged on admixture with a known β-isomaltose octaacetate.

Further Fractionation of Kojibiose Fraction on Carbon Column in the Presence of Borate Buffer.

The mother liquor from the above crystallization of β-isomaltose acetate was deacetylated to a free sugar; yield, 16 g. The material was then separated on a carbon-Celite column (470×70 mm diam.) (Fig. 2). In this case, the developer was a borate buffer (boric acid, 7.45g/l and sodium hydroxide, 4.00 g/l; pH obtained, 10.0) containing ethanol in increasing concentrations (at an interval of 0.5%) from zero to 6.0 percent and 2 l of the buffer at each concentration of ethanol was used. The effluent was collected in 500 ml fractions, freed from sodium ion by treatment with Amberlite IR-120 (H⁺), and evaporated to dryness. The residue was dissolved in methanol and then distilled under reduced pressure to remove boric acid as its volatile ester, the procedure being repeated three times. The product was extracted with hot methanol and the composition was analyzed by paper chromatography.

Fractions No. 4-6 contained a trace of glucose which was identified on paper. Fractions No. 10-17 contained a substance migrating with isomaltose on paper; 9.5 g. The derived β-octaacetate had m.p. and mixed m.p. 145~146°C. Fractions No. 19-24 contained a substance having Rf 0.22 in n-butanol-pyridine-water (6 : 4 : 3); yield, 4.0 g. This was acetylated but it failed to produce any crystalline material, and then the technique of Magnesol column chromatography was attempted as will be described below. Fractions No. 25—29 contained a substance migrating with maltose on paper; yield, 0.6 g. Identification was accomplished on preparation of its β-octaacetate; m.p. and mixed m.p. 158°C.

Magnesol Column Chromatography.

The sugar acetate (6.5 g) of the above fractions No. 19—24 was dissolved in benzene, the solution was placed (6.5/2 g per column) on columns (260×40 mm diam.) of Magnesol-Celite (5 : 1 by wt.) and developed with 3 l of benzene-t-butanol (100 : 1 by vol.) under suction (Fig. 3). When the developed column was extruded and streaked with an indicator (1% potassium permanganate in 10% sodium hydroxide solution), two zones appeared. After the streaking of the indicator was scraped out, the upper zone (A), located 15—90 mm from the top of the column, was cut and extracted with acetone. The acetone solution

was evaporated to a syrup and the material was crystallized from ethanol; yield (from two columns), 590 mg, m.p. 173—174°C. The crystals were purified by recrystallization; m.p. 177°C unchanged on admixture with a known specimen of β,β-trehalose octaacetate, $[\alpha]_D^{15}$—17.3° (c 3.0 in chloroform) (Found: C, 49.72; H, 5.52. Calcd. for $C_{12}H_{14}O_{11}$ $(CH_3CO)_8$: C, 49.55; H, 5.64%).

The lower zone (B) (3.7 g from two columns), 160—260 mm from the top of the column, produced no crystalline material and was rechromatographed (3.7/2 g per column) on Magnesol-Celite (5 : 1 by wt.) columns (265 × 35 mm diam.), using 1.4 l of the same solvent. Two zones appeared. The upper zone (D), near the column top, produced no crystalline material. The lower zone (E), 50—200 mm from the top, produced two kinds of crystals when treated as described above. The two acetates were separated from each other by fractional crystallization based on the difference of their solubility in warm ethanol and purified by repeated recrystallization. One of them, less soluble in warm ethanol, was an acetate of m.p. 166°C and $[\alpha]_D^{18}$+149.8° (c 2.1 in chloroform); yield, 420 mg (from two columns) (Found: C, 49.79; H, 5.85. Calcd. for $C_{12}H_{14}O_{11}$ $(CH_3CO)_8$: C, 49.55; H, 5.64%). The other was an acetate of m.p. 118°C and $[\alpha]_D^{18}$+111.5° (c 2.0 in chloroform); yield: 580 mg (from two columns) (Found: C, 49.40; H, 5.56. Calcd. for $C_{12}H_{14}O_{11}$ $(CH_3CO)_8$; C, 49.55; H, 5.64%). These two acetates were distinguished by their constants from the previously reported octaacetates of glucose-disaccharides. The properties of their free sugars were in conformity with 1 : 2-α-linked glucose-disaccharides (see the section of Properties and Structure of Kojibiose). From their specific optical rotations as shown above, therefore, it was concluded that the acetate of m.p. 166°C is α-kojibiose octaacetate and the acetate of m.p. 118°C, β-kojibiose octaacetate.

In the first Magnesol column chromatography, the effluents (C) from two columns were combined and evaporated to a syrup which produced crystalline β-kojibiose acetate from ethanol; yield 160 mg, m.p. and mixed m.p. 118°C.

The mother liquor (G) remaining after the above fractional crystallization of α- and β-kojibiose acetates was evaporated to a syrup (1.3 g) and further chromatographed on a column (200×35 mm diam.) of Magnesol-Cetile (5 : 1 by wt.) with 1.3 l of benzene-t-butanol (100 : 1 by vol.). Two zones appeared. The upper zone, 20—60 mm from the top of the column, was treated in the same way as above and crystallized from ethanol; yield, 120 mg, m.p. 152—153°C. After several recrystallizations, the constants were: m.p. 156—157°C, $[\alpha]_D^{21}$+184° (c 0.25 in chloroform). These values are not in accord with those of the known α- and β-octaacetates of glucose-disaccharides. On admixture with α-kojibiose octaacetate, β-maltose octaacetate and β-laminaribiose octaacetate, the substance showed depression of the melting point. Further examination should be made for its characterization. The lower zone yielded no crystalline material. The effluent form the column yielded β-kojibiose octaacetate; yield, 20 mg, m.p.

and mixed m.p. 117—118°C.

Properties and Structure of Kojibiose.

The purified acetates of m.p. 166°C and 118°C were converted into free sugars A and B, respectively, by deacetylation with sodium methoxide. The evidence for the two sugars being both 2-0-α-D-glucopyranosyl-D-glucose are as follows : (i) Specific Optical Rotation. Sugar A has $[\alpha]_D^{15}+135.8°$ (equil.: c 0.53 in water) and sugar B $[\alpha]_D^{15}+139.7°$ (equil.: c 0.63 in water). (ii) Acid Hydrolysis. Complete hydrolysis of the sugars with N sulfuric acid in boiling water bath for 3 hr. produced only glucose which was identified on the paper chromatogram. (iii) Molecular Size. Paper ionophoresis of the sugars in 0.4 M sodium hydrogensulfite (16) under 8 V/cm for 2 hr. showd M_G 0.70 corresponding to a glucose-disaccharide. Under conditions in which glucose was oxidized stoichiometrically by iodine in alkaline solution (17), sugar A (0.665 mg) and sugar B (0.790 mg) consumed iodine equivalent to 0.365 mg and 0.444 mg of glucose, respectively. These iodine consumptions were equivalent to 95.9 and 93.7 per cent, respectively, of the expected theoretical value for a glucose-disaccharide. (iv) Treatment with Almond Emulsin. The sugars, when incubated with almond β-glucosidase at 30°C for 3 days according to the capillary method (26) using 0.5 per cent sugar and 1 per cent enzyme solution, were not hydrolyzed although the controls containing sophorose, cellobiose, and laminaribiose were completely hydrolyzed under similar conditions. (v) Infrared Spectra. The sugars were identical in the infrared spectra (KBr disk) which displayed the presence of a peak at 832 cm^{-1} (α-linkage) (18) and the absence of absorption around 890 cm^{-1} (β-linkage) (18). (vi) Paper Ionophoresis and Paper Chromagraphy. On paper ionophoresis in 0.05 M borate buffer (pH 9.8) (19) under 18 V/cm for 3 hr., the sugars moved as a single component with a low mobility (M_G 0.31) similar to those of sophorose (M_G 0.30), maltose (M_G 0.33), and cellobiose (M_G 0.27), whereas 1 : 3- and 1 : 6-linked disaccharides showed higher values (nigerose M_G 0.67, laminaribiose M_G 0.65, isomaltose M_G 0.68 and gentiobiose M_G 0.71). Paper chromatography of the sugars in n-butanol-pyridine-water (6 : 4 : 3) indicated that each moved as a single component with Rf 0.22 which was between those of maltose (0.26) and isomaltose (0.19). The Rf values of other disaccharides were sophorose 0.26, laminaribiose 0.31, nigerose 0.29, cellobiose 0.22 and gentiobiose 0.19. (vii) Copper Reducing Value. When estimated by the Shaffer-Hartmann method (20), sugar A (1.90 mg) had a reducing power equivalent to 0.15 mg of glucose. This corresponded to 15.2 per cent of the calculated value for a glucose-disaccharide. Those of sugar B (2.04mg) were 0.17 mg and 15.9 per cent respectively. (viii) Color Reactions with Aniline and Triphenyltetrazolium Chloride Spray. By aniline spray on paper, the sugars were detected as brownish red stains similar to that of sophorose. This reaction could be distinguished from those of other reducing glucose-disaccharides; 1:3- and 1:6-linked

disaccharides appeared as a deep-brown stain and 1 : 4-linked disacharides as a light-brown stain which changed into greenish brown during storage of the sprayed paper in the dark for a week or two. The sugars as well as sophorose were not detectable by alkaline triphenyltetrazolium chloride spray whereas nigerose, laminaribiose, maltose, cellobiose, isomaltose and gentiobiose readily with this reagent.

Summary

By employing successive carbon and silicate chromatography, kojibiose, a glucose-disaccharide having 1 : 2-α-glucosidic linkage, was isolated as its crystalline acetates (α-octaacetate; m.p. 166 °C, $[\alpha]_D + 149.8°$ and β-octaacetate; m.p. 118°C, $[\alpha]_D + 111.5°$) from a commercial hydrol. β,β-Trehalose was obtained as its crystalline octaacetate.

Acknowledgment

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