Experimental

Isolation of C^{14}-Labeled Cotton Seed Oil.—The seeds from ten matured cotton bolls which were treated with 33.0 μc. of D-glucose-6-C^{14} (150 mg.) as described in our previous communication,[9] were separated from the radioactive cellulose and ground in a mortar to break the hulls. The ground product (8.49 g.) was then extracted with 250 ml. of benzene in a Soxhlet extractor for three days according to the method of Koo and King.[14] The extract was evaporated and the remaining oil was washed with a 10% solution of sodium carbonate and then with water. The dark brown product was further purified[15] by adsorption on a column containing a mixture of 10 g. of Florex XXX[16] and 1 g. of decolorizing carbon, and was subsequently eluted with 200 ml. of benzene. Evaporation of the solvent furnished 2.01 g. of bright yellow cotton seed oil.

Conversion of the C^{14}-Labeled Cotton Seed Oil to Glycerol and Fatty Acids.—A portion of C^{14}-labeled cotton seed oil (1.119 g.) was saponified with 15 ml. of 0.5 *N* alcoholic potassium hydroxide according to the A. O. A. C. Official Method.[17] The reaction mixture was then treated with 1 ml. of 6 *N* sulfuric acid and evaporated. After removal of the alcohol, the residue was boiled briefly with 20 ml. of 0.2 *N* sulfuric acid and the liberated fatty acids were extracted with 40 ml. of ether. The extract was washed with three 10-ml. portions of water and evaporated to dryness; yield 1.020 g. The combined aqueous layer and the washings were neutralized and evaporated to a dry residue which was extracted with three 20-ml. portions of absolute alcohol. Removal of the alcohol by evaporation under reduced pressure furnished crude glycerol; yield 123 mg.

Conversion of Cotton Seed Oil and Fatty Acids to Barium Carbonate.—A sample of the oil (*ca.* 12 mg.) was weighed in a cup and oxidized with 0.5 g. of potassium iodate and 25 ml. of the Van Slyke–Folch reagent, by heating with a very small flame for 15 min. The resulting carbon dioxide was swept out with a current of nitrogen and was absorbed in a solution of half-saturated barium hydroxide containing 2% barium chloride. The precipitate of barium carbonate was filtered on a fritted glass crucible and thoroughly washed with water, while avoiding any unnecessary exposure to the atmosphere. It was then dried and weighed. The barium carbonate obtained in this manner from 12.5 mg. of the C^{14}-labeled cotton seed oil and 13.7 mg. of the fatty acids amounted to 157.2 and 175 mg., respectively. Under the employed conditions the amount of blank was negligible and a sample of commercial cotton seed oil containing 79.2% carbon provided a 98.3% yield of barium carbonate.

Periodate Oxidation of Glycerol.—The above isolated glycerol (123 mg.) was dissolved in 5 ml. of 0.5 *M* sodium phosphate buffer at *p*H 5.8 and oxidized with a solution of 0.65 g. of sodium metaperiodate in 10 ml. of water. The formaldehyde and formic acid produced were recovered as barium carbonate (349.6 and 174.6 mg., respectively) according to the method described by Eisenberg.[12]

Counting Methods.—The samples of barium carbonate were counted at infinite thickness, using a preflush flow counter[18] connected to a scaler,[19] and compared with a standard sample.[20] The samples were counted long enough to reduce the random counting error to ±2%.

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(20) Obtained from Tracerlab, Inc., Boston 10, Mass.

COLUMBUS 10, OHIO

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH BRANCH[1]]

Crystalline Methyl α-Isomaltoside and its Homologs Obtained by Synthetic Action of Dextransucrase

BY R. W. JONES, ALLENE JEANES, C. S. STRINGER AND H. M. TSUCHIYA

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A new series of reference compounds, methyl α-isomaltoside (methyl 6-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) and its homologs, have been synthesized by the action of the enzyme NRRL B-512F[2] dextransucrase. The synthesis mixture was fractionated by chromatography on a carbon–Celite column. The products (D.P. 2 through 5) were isolated as pure crystalline compounds. They were identified and characterized by periodate oxidation, alkoxyl analyses, R_f values, melting points, optical rotations and X-ray analyses.

The action of the enzyme, dextransucrase from the organism *Leuconostoc mesenteroides* NRRL B-512F,[2] on sucrose has been used previously to obtain the high polymer dextran[3,4] and the disaccharides, leucrose[5] and isomaltulose.[6] In these syntheses, the glycosyl acceptors are believed to have been sucrose or D-glucose and D-fructose, respectively. A number of other carbohydrates may serve as glycosyl acceptors,[4,7] as has been demonstrated by means of paper chromatography. When maltose was used as alternate acceptor, the trisaccharide panose was prepared.[8]

This paper reports the use of methyl α-D-glucoside as alternate acceptor in the synthesis of an homologous series of sugar derivatives. Transfer of the

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(2) *Leuconostoc mesenteroides* NRRL B-512F is a substrain of NRRL B-512. The origin of the strain B-512 and the production of dextran from it were reported by Allene Jeanes, C. A. Wilham and J. C. Miers, *J. Biol. Chem.*, **176**, 603 (1948). In 1950, the B-512F substrain supplanted B-512 for all work at the Northern Regional Research Laboratory. Since that time, the dextran from this substrain has been designated inexactly as B-512 in numerous publications. However, in this article and hereafter, the correct designation of the substrain will be used. The dextrans from B-512 and B-512F appear to be identical.

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glucosyl moiety from sucrose, the donor substrate, to carbon six of methyl α-D-glucoside, the acceptor substrate, would yield methyl α-isomaltoside (methyl 6-*O*-α-D-glucopyranosyl α-D-glucopyranoside). The methyl isomaltoside thus formed, as well as products of subsequent enzymic action, may in turn act as acceptors with deposition of a glucosyl unit on their terminal carbon. In this manner, an homologous series of α-1,6′-linked glucosides is obtained. Unless controlled, the enzymic action would result in the formation of polymeric material. In this study, the conditions were established to give optimum yields of low molecular weight products. The glycosides, having degrees of polymerization (D.P.) 2–5, were fractionated on carbon–Celite[9] and cellulose columns, crystallized and characterized.

Heretofore when an homologous series has been produced by enzyme action and isolated, the products have been sugars rather than sugar derivatives and have resulted from depolymeric rather than synthetic enzyme action.[10] The enzymic synthesis reported here has the advantageous feature of yielding only one anomeric form, the α-configuration. Recently, Scott and Senti[11] have degraded B-512F dextran by methanolysis and separated the mixed α-β-anomeric methyl glycosides of D.P. 1-11 on a cellulose column. However, the products D.P. 2 through 6 were not crystalline. Methyl β-isomaltoside has been obtained as an amorphous solid by Wolfrom, *et al.*[12]

Experimental

Enzymic Production of the Methyl α-Isomaltoside Series.—The dextransucrase preparation employed was derived from *Leuconostoc mesenteroides* NRRL B-512F and was that used to study the factors influencing molecular weight distribution of enzymatically synthesized dextran.[4] The methyl α-D-glucoside was a commercial preparation with melting point of 161–163°. The sucrose was table grade cane sugar.

Test enzymic syntheses were conducted at 4° on 5-ml. mixtures containing various amounts of methyl α-D-glucoside and sucrose. The low reaction temperature was employed because it favors the synthesis of low molecular weight reaction products.[4] One ml. of prechilled dextransucrase preparation was added to 4.0 ml. of prechilled, buffered solutions of methyl α-D-glucoside and sucrose. The enzymic activity in the reaction mixtures was 30 dextransucrase units[3] per ml. The concentrations of the acceptor substrate in the test mixtures were 0.1, 0.2, 0.4 and 1.0 *M* and the level of sucrose was 0.3 *M*. The syntheses were allowed to proceed for 23 hr., at which time, as shown by past observations, all of the sucrose was utilized. An equal volume of methanol was then added to each reaction mixture to inactivate the enzyme and to precipitate any high molecular weight dextran formed. The amounts of glycosides in the supernatant liquors were determined by quantitative paper chromatography,[13] using 1-butanol–methyl Cellosolve–water (2:1:1) as solvent.

On the basis of these analytical data, the condition was selected under which optimum results were obtained, and one liter of a preparative enzymic reaction mixture was set up. It contained 0.4 mole of methyl α-D-glucoside, 0.3 mole of sucrose and 4×10^4 dextransucrase units. The preparative mixture had a *p*H of 5.2 and was 0.01 *M* with respect to acetate ion. The synthesis was conducted at 4° for 23 hr. at which time the reaction was complete as determined by reducing value measurement.[3] The mixture was steamed at 100° for 15 minutes to inactivate the enzyme.

Composition of Reaction Mixture.—In order to remove polymeric material, the preparative enzymic mixture was made to 50% (v./v.) methanol and centrifuged. The insoluble portion (about 2% of the total carbohydrate) contained nothing that moved on a paper chromatogram when 1-butanol–methyl Cellosolve–water (2:1:1) was employed as developing solvent. Using the same solvent and ammoniacal 10% silver nitrate as a spraying reagent, the methanol-soluble fraction was analyzed by quantitative chromatography.

Fractionation of Reaction Mixture.—Fractionation was carried out on a column 8.0 × 33 cm. containing 330 g. of Norit SG and 240 g. of Celite 501. The carbon–Celite mixture was pretreated with 5% ethanol, packed in the column and conditioned with 5% ethanol in the manner previously described.[10] A portion of the reaction mixture containing 33 g. of glucose equivalents, as measured by quantitative anthrone analysis,[13] in 150 ml. of 5% ethanol, was added to the column dripping under gravity. The rate of flow was gradually increased to 800–900 ml./hr. by applying vacuum. The fractionation was followed by semi-quantitative anthrone color test.[14] The ethanol concentration was increased when it appeared that the main portion of any component had been eluted. The percentages of ethanol used were 5, 7, 8, 10 and were then increased by 0.5% increments to 15.5%. As will be discussed in a later section, in addition to the glycoside series a second group of homologs constituting the isomaltose series of reducing sugars was present in small quantity in the reaction mixture. D-Fructose, methyl α-D-glucoside, the slight trace of sucrose and the main leucrose fraction were displaced in the order indicated by 5% ethanol. The main methyl α-isomaltoside was eluted by 7%, isomaltotriose by 8%, methyl α-isomaltotrioside by 10%, isomaltotetraose by 11%, methyl α-isomaltotetraoside by 13%, isomaltopentaose by 13.5% and both methyl α-isomaltopentaoside and isomaltohexaose by 14–15% ethanol. The tailings of each main glycoside fraction were discarded because they were contaminated with components of the reducing sugar series. The glycoside fractions (D.P. 2–4) containing the least amounts of impurities were rechromatographed on carbon–Celite. The impure methyl α-isomaltopentaoside was purified on a cellulose column using 1-butanol–methyl Cellosolve–water (2:1:1) as solvent.

Demineralization and Crystallization of Glycosides.—The effluent from the carbon–Celite column contained soluble silicate. This was removed by repeatedly dissolving the glycoside in water, filtering and taking to dryness. Other inorganic matter was removed from the D.P. 2 fraction by Dowex 50 and Duolite A-4 ion exchange resins, and from the other glycosides by crystallization of the carbohydrate.

The glycosides were crystallized by dissolving the amorphous powder in hot water, adding hot ethanol or methanol and cooling slowly. The D.P. 2 glycoside did not crystallize from methanol; it is very soluble in cold absolute methanol. Initial crystallization of D.P. 4 and 5 was induced by cooling the aqueous alcoholic solution in Dry Ice until a precipitate formed and warming to dissolve the solid. This cycle was repeated until adequate seed crystals formed.

Identification of the Isomaltose Series.—A member of the isomaltose series of D.P. = *n* was found in the tailings of each glycoside of D.P. = *n* − 1 from the fractionation on carbon-Celite columns. Isomaltotriose and isomaltotetraose were purified by large-scale paper chromatography. Isomaltohexaose was purified on a cellulose column. It was not practical to purify the isomaltopentaose, because it was present in very small amount with a great quantity of D.P. 4 glycoside. Under the conditions used, the first member of the series, isomaltose, would be expected to have been eluted from the carbon along with leucrose. A paper chromatogram of the fraction afforded evidence of two components, but the minor constituent was not identified.

These substances, which moved on paper at the same rate as members of the isomaltose series, were proved to be re-

(9) The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

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TABLE I

PHYSICAL CONSTANTS OF THE CRYSTALLINE GLYCOSIDES

D.P. of component	Crystallizing solvent (aqueous)	Alkoxy analyses: Alkoxyl, % Found	Alkoxy analyses: Alkoxyl, % Theory	Mole alcohol per mole glycoside	M.p., °C.	$[\alpha]^{25}D$ (c 2, water)[a]	R_f[b]	Crystal form
1							0.49	
2	EtOH	15.9	15.4	1.07	110–111	+177.4	.33	Large, clear prisms
3	EtOH	...	...	0.0[c]	232	182.3	.21	Flat hexagonal plates
3	MeOH	11.7	11.3	1.08	140–146	185.4		Large prisms
4	EtOH	...	...	1.0[c]	162–167	186.7	.13	Clusters of needles
4	MeOH	8.90	8.71	1.04	192–193	188.6		Clusters of needles
5	EtOH	4.55	4.54	0.25	161–164	188.7	.07	Submicroscopic
5	MeOH							Microscopic needles

[a] Calculated on dry weight basis. Solvent: 1-butanol–methyl Cellosolve–water (2:1:1). [c] Determined from optical rotation.

ducing sugars by use of the chromatographic spray reagent 2,3,5-triphenyltetrazolium chloride.[15,16] Their identity was established further from the R_f values of their partial acid hydrolysis products. Hydrolysis was carried out on a steam-bath for 3 hours in 0.4 *N* sulfuric acid. The acid was removed by neutralizing with saturated barium hydroxide and centrifuging. A paper chromatogram showed that each hydrolyzate contained D-glucose and the expected members of the isomaltose series. In addition, the isomaltotriose hydrolyzate contained a trace of reducing sugar apparently having a linkage other than α-1,6'. When neutralized hydrolyzates were chromatographed and sprayed with ammoniacal silver nitrate, an unidentified yellow spot of R_f near that of methyl α-D-glucoside appeared. On drying and heating, it became purple.

Analytical Determinations.—D.P. 2 samples were prepared for analysis by heating *in vacuo* (Abderhalden) at 79° and the other samples by heating at 137°. No loss of weight occurred. The crystals were not hygroscopic under normal atmospheric conditions. They did prove to be hygroscopic in 65% relative humidity at 22°.

Methoxyl content was determined by the Vieböck and Schwappach method.[17] These analyses showed the crystals to be alcoholates. The alcohol was so tightly bound that dissolving in water and taking to dryness in the Abderhalden at 79° did not eliminate all of it. Dry weights for all subsequent analyses were calculated assuming exactly 0, 0.25 or 1 mole of alcohol per mole of glycoside (see Table I).

Periodate oxidation analyses were carried out using a slight modification of the procedure of Rankin and Jeanes.[18] Fifty-milligram samples were weighed on a microbalance, and for the final aliquot 0.01 *N* iodine was used instead of the recommended 0.1 *N* iodine. Reaction appeared to be complete at 430 hr.

Paper chromatographic techniques used have been described elsewhere.[13,19]

Results

The amount of each component in the test and preparative reaction mixtures, determined by paper chromatography, is shown in Table II. Physical constants, melting points, optical rotation and R_f values of crystalline products are listed in Table I.

The purity and identity of the crystalline products were established by paper chromatography, periodate oxidation (Table III) and alkoxyl analysis (Table I).

Paper chromatography showed the D.P. 2, 3 and 5 glycosides to be pure. The D.P. 4 component contained a trace of isomaltopentaose. The R_f values, using 1-butanol–methyl Cellosolve–water (2:1:1) as a developing solvent, are given in Table I.

When the assigned D.P. is plotted against log $(1/R_f - 1)$, a straight line is obtained, thus indicating that the components are members of an homologous series.[19–21]

TABLE II

COMPOSITION OF 50% METHANOL-SOLUBLE FRACTION OF THE GLYCOSIDE MIXTURE[a]

Component	Test reaction mixture A: Moles Me-α-D-glucoside per mole sucrose 0.1	B: 0.2	C: 0.4	D: 1.0	Prep. mixt. 0.3 mole 0.4
	% of total, as glucose				
D.P. 1	11.8	15.5	27.5	51.0	26.0
Fructose and glucose	51.5	40.8	29.9	17.5	30.2
D.P. 2[b]	4.6	10.6	12.9	17.3	12.7
Leucrose	5.2	2.4	0.6	..	1.2
D.P. 3	8.8	11.4	13.5	10.1	13.9
D.P. 4	10.8	11.4	10.5	3.5	10.9
D.P. 5	7.2	7.8	5.1	0.5	5.1

[a] In the preparative fraction this constituted 98% of the total carbohydrate. About 2% of these solubles were of D.P. greater than 5. [b] Any trace of sucrose remaining in the reaction mixture would move on a chromatogram at the same rate as D.P. 2.

TABLE III

PERIODATE OXIDATION OF GLYCOSIDES

D.P.	Mole/mole glucoside: IO_4^- reduced	Mole/mole glucoside: HCOOH formed	Mole/A.G.U.: IO_4[a] reduced	Mole/A.G.U.: HCOOH[b] formed
1[c]	2.01	1.02	2.01	1.02
2	3.91	2.00	1.96	1.00
3	6.13	3.08	2.04	1.03
4	8.18	4.16	2.04	1.04
5	10.21	5.12	2.04	1.02

[a] Theory is 2.00. [b] Theory is 1.00. [c] Known sample used as a standard.

Periodate oxidation of methyl α-isomaltoside and its homologs should result in the reduction of two moles of periodate and the formation of one mole of formic acid for each anhydroglucose unit in the molecule. Table III shows that theoretical results were obtained.

The results of alkoxyl analyses are given in Table I. As indicated there (footnote *c*), the alcohol content of two of the substances crystallized from ethanol has been determined by optical rotation. The optical rotation (dry weight basis) was calculated assuming the moles of solvent indicated (Table I).

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Since this rotational value agreed well with the rotation, on a dry weight basis, of the corresponding product crystallized from methanol and for which alcohol content had been measured, the moles of solvent in the product crystallized from ethanol were established.

Carbon and hydrogen analyses also were used to verify the presence of one mole of ethanol per mole of D.P. 2 glycoside. Calculated as a mono alcoholate, $C_{15}H_{30}O_{12}$: C, 44.77; H, 7.52. Found: C, 44.47, 44.53; H, 7.29, 7.52.

Discussion

The theoretically predictable result of enzymic synthesis was obtained. Over the concentration range tested for the acceptor, methyl α-D-glucoside, increase in the acceptor concentration raised the yield of the products of lower D.P. and decreased the amounts of those of higher molecular weight. The yield of each compound in the preparative synthesis mixture, which was set up under conditions essentially similar to test reaction C, agreed closely with that in the test mixture.

As might also have been anticipated, compounds other than methyl α-isomaltoside and its homologs were formed. In addition to methyl α-D-glucoside, at least two other acceptors, D-fructose and D-glucose, were present. Fructose would arise from the transfer of glucosyl units from sucrose to acceptors. Leucrose (5-*O*-α-D-glucopyranosyl-α-D-fructopyranose)[5] is a normal product of dextransucrase activity. It is believed to arise from the deposition of a glucosyl unit on D-fructose. Since D-fructose is a very poor glycosyl acceptor, an increase in the concentration of a better acceptor, methyl α-D-glucoside, should lower the yield of leucrose.[7] The experimental results given in Table II show this to be the case. The occurrence of small amounts of D-glucose in dextransucrase reactions has been noted previously.[7] Glucose might arise from the action of dextransucrase in transferring the glucosyl residue from sucrose or other donor substrates to water as an acceptor.[22] Transfer of glucosyl units from sucrose to glucose would result in the formation of the isomaltose series of reducing sugars. However, the members of this series were present in such small quantity that their presence was not detected until they were concentrated by fractionation on carbon.

In previous work on the isomaltose series, excellent results were obtained by pretreating the carbon–Celite column with 2.5% ethanol.[10] In this present work, a 5% ethanol pretreatment was used in order to minimize irreversible reactions between glycoside and carbon. However, observations made during this and other investigations[23] indicate that 2.5% ethanol pretreatment would have been more satisfactory than the 5% used.

Some general observations on the behavior of sugars and glycosides on chromatographic and ion exchange columns are of interest. There is a striking difference between the relative rates of movement of glycosides *vs.* sugars on paper and carbon. The glycosides move more rapidly on cellulose than do sugars (sugar of D.P. = n and glycosides of D.P. = $n + 1$ at the same rate), but the sugars are eluted from carbon at lower ethanol concentration than the corresponding glycoside (sugar of D.P. = n and glycoside of D.P. = $n - 1$ at about the same concentration). Similar observations have been reported previously.[24] A difference in the rate of washing from Duolite A-4 resin was also noted. Sugars may be washed from the resin quantitatively; but there is evidence that elution of the sugars is retarded, apparently by adsorption on the resin. However, our glycosides showed no evidence of adsorption since they passed through such a column without the slightest indication of retardation. A similar behavior was reported by Roseman[25] concerning Amberlite IRA-400 anion exchange resin. These observations suggest a possible method of purification of glycosides containing traces of unwanted reducing sugars.

Examination of all the crystalline glycosides by X-ray analysis confirmed observations obtained by other means regarding purity, crystallinity and solvent content of the compounds.[26] These data indicate that the crystal structure of the D.P. 3 glycoside crystallized from ethanol is strikingly different from the other glycosides. This, along with its very high, sharp melting point, is further confirmation that it contains no solvent while the other crystals do. The D.P. 4 glycoside crystallized from ethanol has a pattern identical with that from methanol, although they are solvates of different alcohols. The alcohol content of D.P. 5 from methanol has not been determined, but since its X-ray picture is identical with that of the D.P. 5 from ethanol (1/4 mole solvent) it is believed that it is also solvated. Much difficulty was encountered in crystallizing D.P. 5 from methanol; but when obtained, the crystals were larger and gave much sharper X-ray patterns than the D.P. 5 from ethanol.

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