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THE SYNTHESIS OF 1,6-ANHYDRO-β-D-GLUCOPYRANOSE AND D-GLUCOSYL OLIGOSACCHARIDES FROM MALTOSE BY A FUNGAL GLUCOSYLTRANSFERASE*

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ABSTRACT

The formation of 1,6-anhydro-β-D-glucopyranose and several D-glucosyl oligosaccharides has been observed during the action of a purified, fungal glucosyltransferase (EC 2.4.1.24) on maltose. Such products are synthesized by a transglucosylation mechanism involving the formation of a D-glucosyl–enzyme complex and the displacement of the D-glucosyl group by appropriate acceptor-substrates. The formation of the 1,6-anhydro bond is a novel type of transfer reaction and occurs by displacement of the enzyme from the D-glucosyl–enzyme complex by the proton of the primary hydroxyl group of the same glucosyl group. This reaction is characterized by inversion of configuration at the position of glucosidic bond-cleavage of the substrate. Synthesis of the D-glucosyl oligosaccharides occurs by displacement of the D-glucosyl groups from the enzyme by suitable acceptor-substrates. In these cases, the reactions are characterized by retention of configuration of the D-glucosidic bonds of the substrate. The list of oligosaccharides produced from maltose includes nigerose, kojibiose, isomaltose, maltotriose, panose, isomaltotriose, and 6-*O*-D-glucosyl-panose. The identity of these compounds has been established by methylation analysis and enzymic hydrolysis. D-Glucose is also a product of the reaction and arises from both the reducing and the non-reducing groups of maltose.

INTRODUCTION

Glucosyltransferases (EC 2.4.1.24) catalyze the transfer of glucosyl groups from many types of D-glucosyl donor-substrates to suitable acceptor substrates, resulting in the synthesis of new oligosaccharides[1]. Such reactions proceed by a transglucosylation mechanism[2]. On the basis of results of isotope studies and the known stereospecificity of enzymic reactions, it has been proposed that a D-glucosyl–enzyme complex is an intermediate in the reaction sequence and that the oligo-

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*Dedicated by J.H.P. and L.M.J. to Professor Dexter French on the occasion of his 60th birthday, with best wishes and much admiration.

saccharides are synthesized by the displacement of D-glucosyl groups from the complex by suitable acceptor-molecules[3,4]. D-Glucose and oligosaccharides function readily as displacement agents, leading to the synthesis of new oligosaccharides, but water and alcohols may also function as displacement agents leading to hydrolysis[1,4] or to the synthesis of alkyl glucosides[5-7]. In further comparative studies on glucoamylase and glycosyltransferase[8], we have now observed the synthesis of some new types of D-glucosyl derivatives, specifically 1,6-anhydro-β-D-glucopyranose, kojibiose, and maltotriose in addition to the oligosaccharides (isomaltose, panose, isomaltotriose, 6-*O*-D-glucosyl-panose and nigerose) previously characterized[3,9]. The new compounds have been isolated pure and their structures determined by methylation analysis and enzymic methods. The enzymic synthesis of 1,6-anhydro-β-D-glucopyranose is a novel type of transfer reaction involving the displacement of the enzyme from the D-glucosyl–enzyme complex by the proton of the primary alcoholic group of the same D-glucosyl group and net inversion of configuration at C-1 of this D-glucosyl group. A preliminary note on this aspect of the work has been published[10].

RESULTS AND DISCUSSION

A photograph of radioautogram strips, showing the types of D-glucosyl oligosaccharides that are produced from [^{14}C]maltose by the glucosyltransferase at different stages of enzymolysis, is reproduced in Fig. 1. The oligosaccharides are nigerose (3-*O*-α-D-glucopyranosyl-α-D-glucose), isomaltose (6-*O*-α-D-glucopyranosyl-α-D-glucose), maltotriose [*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-α-D-glucose], panose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-α-D-glucose], isomaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 6)-α-D-glucose], and 6-*O*-D-glucosyl-panose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-α-D-glucose]. Fig. 1 also shows that glucose is a reaction product of the enzyme and that two other compounds, labeled X and Y, are produced. Compounds X and Y have been isolated pure and X has been identified as 1,6-anhydro-α-D-glucopyranose (levoglucosan) and Y as kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucose) by standard chemical methods. The enzymic synthesis of the anhydro-D-glucose has not been previously observed and a mechanism proposed for synthesis of this compound is presented later.

Table I lists data on the radioactivities and percent yield of the foregoing products in a mixture of ^{14}C-labeled maltose and glucosyltransferase incubated for 24 h. It may be noted from Table I as well as Fig. 1 that compounds having α-D-(1 → 6) linkages predominate in the reaction mixtures. Evidently, the primary hydroxyl group of the acceptor molecule is the favored point for attachment of the D-glucosyl group, and the resulting (1 → 6)-α-D-glucosidic linkage is relatively stable to further disproportionation by the enzyme. Isomaltose and panose are the oligosaccharides present in highest amounts in the 24-h digest, and give rise to 26% of the radioactivity. Other oligosaccharides comprise 7% and 1,6-anhydro-β-D-glucose gives 2%; D-glucose gives 65% of the activity. The appearance of maltotriose in digests of short

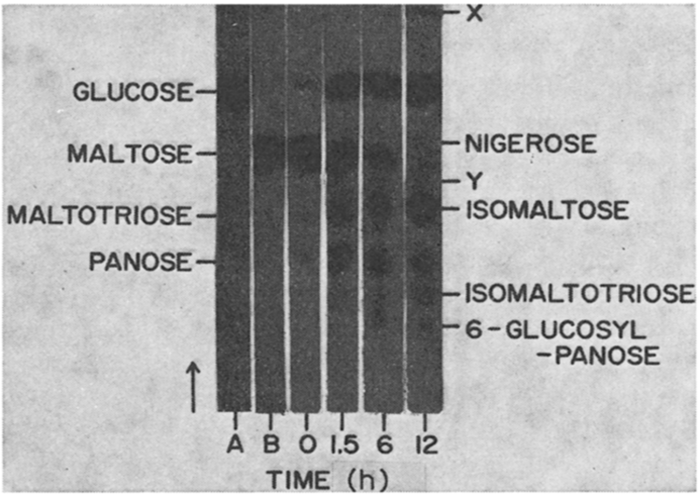

Fig. 1. A photograph of radioautograms of compounds incubated with glucosyltransferase: 0, 1.5, 6, 12 = [^{14}C]maltose incubated with enzyme for the times indicated, A = D-[^{14}C]glucose incubated with enzyme for 24 h, and B = [^{14}C]maltose devoid of enzyme and maintained for 24 h.

TABLE I

RADIOACTIVITIES (COUNTS.MIN^{-1}) OF PRODUCTS IN A 50-μl SAMPLE OF THE 24-H DIGEST OF [^{14}C]MALTOSE WITH GLUCOSYLTRANSFERASE

Compound	R$_F$ *value*[a]	*Radioactivity*	*Yield* (%)
Compound X	0.93	280	2
D-Glucose	0.75	9,700	65
Nigerose	0.60	160	1
Maltose	0.57	340	2
Compound Y	0.52	190	1
Isomaltose	0.47	2,400	16
Maltotriose	0.43	30	
Panose	0.38	1,600	10
Isomaltotriose	0.27	260	2
6-*O*-D-Glucosyl-panose	0.19	160	1

[a] An apparent *R*$_F$ value, defined as distance that the compound migrated divided by the height of the paper for 3 ascents of 6:4:3 (v/v) butyl alcohol–pyridine–water.

incubation (Fig. 1) is surprising, as the transfer of D-glucosyl groups to O-4 of the acceptor molecule has not been previously observed. The formation of this compound probably reflects concentration effects. Thus, when maltose is present in high concentration, transfers to O-4 of the nonreducing group of maltose, as well as to O-6, occur to yield maltotriose and panose. These products, as well as D-glucose, are present in the 0-h sample and are evidently produced during the time interval required to inactivate the enzyme in the 0-h sample. When the maltose is depleted, this acceptor substrate is no longer available, and the two trisaccharides cannot be synthesized. As oligosaccharides having α-D-(1 → 4)-linked terminal groups are disproportionated at a higher rate than those having α-D-(1 → 6) terminal groups, maltotriose disappears rapidly from the mixture but panose persists.

The structures of the oligosaccharides synthesized from maltose have been determined by methylation analysis[11]. The methylation products were identified by g.l.c. and m.s.[12]. The configuration of the linkages in these compounds was established by their susceptibilities to hydrolysis by glucoamylase (EC 3.2.1.3), an enzyme that is specific for α-D linkages[13]. As glucoamylase hydrolyzes α-D-(1 → 6), α-D-(1 → 4), α-D-(1 → 3), and α-D-(1 → 2) linkages at different rates[14], structural information on the sequence of the linkages was also obtained from such experiments.

Some of the evidence for the structure of the enzymically produced 1,6-anhydro-β-D-glucopyranose is shown in Fig. 2. The triacetate (A) of authentic 1,6-anhydro-β-D-glucopyranose and that (B) of the enzymically produced compound have identical retention times on g.l.c. The derivatives obtained on methylation analysis of authentic levoglucosan (C) and the enzymically produced compound (D) also possess identical

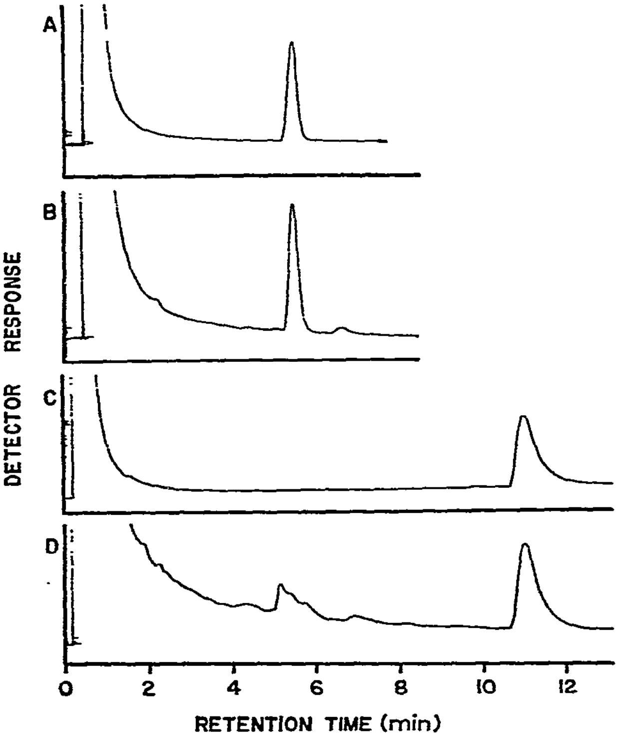

Fig. 2. A photograph of the g.l.c. patterns for the acetates and the methylated alditol acetates of 1,6-anhydro-β-D-glucopyranose and the enzyme-produced compound X: A = peracetate of 1,6-anhydro-β-D-glucopyranose, B = peracetate of compound X, C = 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol from 1,6-anhydro-β-D-glucopyranose, D = the methylated alditol acetate from compound X.

retention times on g.l.c. (Fig. 2). The derivative from both compounds was identified by mass spectrometry to be a 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol. Minor impurities extracted from the paper during preparation of the enzymically produced compound are responsible for the small amount of material having a retention time of approximately 5 min in this preparation. On acid hydrolysis, the compound is converted into D-glucose, which was identified by its R_F value on paper chromatograms and by the D-glucose oxidase reaction[15]. N.m.r. spectra of the enzymically produced compound and of authentic 1,6-anhydro-β-D-glucopyranose were identical, and established that the configuration of the anhydro linkage is β-D in the former product.

Whereas 1,6-anhydro-β-D-glucopyranose (levoglucosan) has been prepared from D-glucose and starch by pyrolytic reactions[16] and from aromatic D-glucosides by treatment with alkali[17], the enzymic synthesis of this compound has not been previously observed. A preliminary note[18] was published many years ago on the isolation of levoglucosan from an enzymic hydrolyzate of waxy starch, but neither the origin of this compound nor its mechanism of synthesis were established.

A diagrammatic representation of the mechanism proposed for the enzymic synthesis of 1,6-anhydro-β-D-glucopyranose from maltose is shown in Fig. 3. First, the maltose is bound at the active site of the enzyme by hydrogen bonding of the appropriate hydroxyl groups of two D-glucose residues of maltose. The D-glucose residues of maltose are shown in the $^4C_1$ (D) conformation, which is the most probable conformation for these groups[19,20]. Next, protonation of the D-glucosidic oxygen atom occurs by a proton from an amino acid residue at the active site of the enzyme, resulting in the release of D-glucose from the reducing moiety and a D-glucosyl cation from the non-reducing moiety of maltose.

As the oxocarbonium ion is held by ionic as well as by hydrogen bonds at the active site of the enzyme and the D-glucose is held by hydrogen bonds only, the latter dissociates from the complex, leaving a D-glucosyl cation attached to the enzyme as a reaction intermediate. This intermediate may disproportionate by several routes.

First, a dissociation of the D-glucosyl–enzyme complex may occur by displacement of the D-glucosyl group by acceptor substrates, resulting in the synthesis of oligosaccharides. D-Glucose, maltose, isomaltose, and other oligosaccharides function as acceptor substrates that most generally effect displacements by their primary alcoholic groups. However, displacement may occur by other hydroxyl groups of the substrate or with alcohols, resulting in the formation of such compounds as kojibiose, nigerose, maltotriose, or alkyl glucosides. As in other transglucosylation reactions[21,22], the configuration of the D-glucosidic linkage of the original substrate is retained in the disproportionation by this route.

Second, a dissociation of the glucosyl–enzyme complex may occur by the addition of water to the complex, resulting in hydrolysis of the maltose to D-glucose. Evidence for this route is the observation that 65% of the D-glucose residues of maltose appeared as free D-glucose in the 24-h digest (Table I). It is not yet known

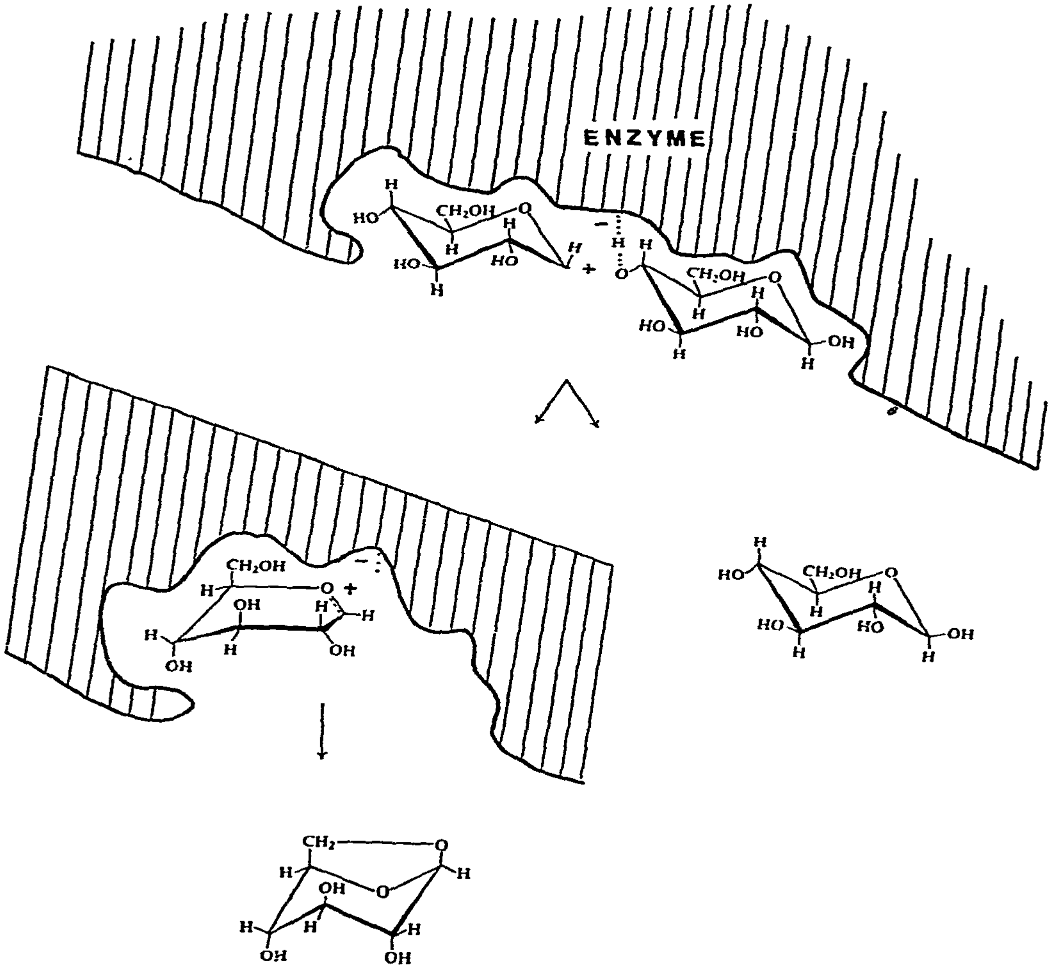

Fig. 3. Reaction mechanism proposed for the enzymic synthesis of 1,6-anhydro-β-D-glucopyranose from maltose.

whether displacement by this route occurs with or without inversion of configuration.

Third, a disproportionation involving a conformational change in the enzyme and in the glucosyl group may occur. It is suggested that this conformational change occurs in the enzyme, converting the D-glucosyl group from a $^4H_3$(D) into a $^3H_4$(D) conformer. In the $^3H_4$(D) conformer, the 6-hydroxyl group of the glucosyl group is located near the negative charge on the enzyme and the positive charge on the oxocarbonium ion. As a consequence, the proton from this hydroxyl group neutralizes the negative charge and the resulting incipient D-glucosyl di-ion is stabilized by formation of a 1,6-anhydro bridge having the β-D configuration. The product dissociates from the enzyme, thus effecting the synthesis of 1,6-anhydro-β-D-glucopyranose.

EXPERIMENTAL

Enzymes. — Glucosyltransferase (EC 2.4.1.24) was isolated from a commercial enzyme preparation obtained from *Aspergillus niger* and marketed under the trade name of DIAZYME (Miles Laboratories Inc., Elkhart, ID). DIAZYME is a light-brown powder containing high levels of glucoamylase and has been developed for use for the commercial production of D-glucose. Glucosyltransferase is an undesirable enzyme in such preparations as it markedly decreases the yields of D-glucose during enzymic conversion of starch[23]. At present, a mutant of *Aspergillus foetidus* is used to produce DIAZYME and this mutant produces little if any glucosyltransferase[23]. However, DIAZYME preparations from *Aspergillus niger* strains generally contain high levels of glucosyltransferase as well as glucoamylase[24]. Such preparations have been used to isolate glucosyltransferase by chromatography based on the use of DEAE-cellulose[25]. Criteria of purity of the glucosyltransferase included ultracentrifugation patterns and electrophoretic behavior[24], as well as ion-exchange chromatography. The details for isolating glucosyltransferase from DIAZYME have been published earlier[25]. The initial, u.v.-absorbing fractions eluted by buffer of pH 8 from the DEAE-cellulose column contained the transferase activity. These fractions were combined and dialyzed exhaustively against distilled water. U.v. measurements at 280 nm showed that the resulting solution contained 0.2% of protein. The enzyme sample converted maltose rapidly into D-glucose and new oligosaccharides, and was used in the experiments described in this report. D-Glucose was not liberated from cellobiose (4-*O*-β-D-glucopyranosyl-α-D-glucose), methyl β-D-glucoside, or *p*-nitrophenyl β-D-glucoside on incubation of 2% solutions of these substrates with an equal volume of enzyme, even on prolonged incubation. Evidently the preparation does not contain any β-D-glucosidase activity. The presence of glucoamylase in the preparations is unlikely as this enzyme binds with a high affinity-constant to this ion exchanger[24].

Glucoamylase (EC 3.2.1.3) was also isolated pure from the DIAZYME preparation by chromatography on DEAE-cellulose. This enzyme occurs in two isoenzyme forms[25], I and II, with the glucoamylase II eluting at pH 6 and glucoamylase I eluting at pH 4.5. Glucoamylase I was used in the present studies. Solutions of this enzyme were diluted with water to yield activities that effected hydrolysis of 50% of the substrate under study during a 6-h period. Analysis of the products from the substrates was performed by qualitative paper chromatography, as described in a later section.

Carbohydrates. — Maltose of high purity was available in the laboratory and had been isolated from an enzymolyzate of starch with beta amylase[3]. Maltotriose, isomaltose, panose, and nigerose were also available and had been isolated from a digest of amylodextrin with salivary amylase[27] and from a digest of maltose with glucosyltransferase[3,9]. 1,6-Anhydro-β-D-glucopyranose[16] was provided by Dr. C. P. J. Glaudemans, National Institutes of Health, Bethesda, MD, 1,6-anhydro-β-D-glucofuranose triacetate[29] by Dr. F. Otey, Northern Regional Research Center, Peoria, IL, isomaltotriose[30] by Dr. H. Erbetova, Czechoslovak Academy of Sciences, Prague, Czechoslovakia, and kojibiose[31] by Dr. S. Okada, Osaka Municipal Technical Research Institute, Osaka, Japan. Methyl α-D-glucoside and methyl β-D-glucoside were synthesized from D-glucose by using methanolic hydrogen chloride. The glucosides were crystallized from ethyl alcohol and further purified to remove occluded D-glucose by chromatography on cellulose columns[32]. Cellobiose and *p*-nitrophenyl β-D-glucoside were purchased from commercial suppliers (Pfanstiehl Laboratories Inc., Waukegan, IL, and Pierce Chemical Co., Rockford, IL).

D-[1-^{14}C]Glucose and [4-^{14}C]maltose were purchased from an isotope supplier (ICN Pharmaceuticals, Inc., Irvine CA). Both labeled compounds were further purified by preparative paper-chromatography[27] to remove traces of labeled impurities. Radioautography[3] was employed for locating the positions of the compounds on the paper chromatograms. The labeled D-glucose and maltose were eluted from the paper strip, and evaporated to dryness by lyophilization.

Action of glucosyltransferase on [^{14}C]maltose. — Uniformly labeled pure maltose (1.6 × 10^6 c.p.m.) and 5 mg of non-labeled maltose were dissolved in 0.1 ml of water and mixed with 0.1 ml of 0.1M sodium acetate buffer (pH 3.5) and 0.2 ml of glucosyltransferase solution. Samples (5 μl) of the mixture were placed on Whatman No. 1 paper immediately after addition of the enzyme, and also after incubation for 1.5, 3, 6, 12, and 24 h. Similar experiments were performed by using comparable amounts of D-[^{14}C]glucose and non-labeled D-glucose in place of maltose, and a blank of labeled and non-labeled maltose in the acetate buffer at the foregoing concentration.

Experiments were also performed in the foregoing manner with comparable amounts of methyl β-D-glucoside, *p*-nitrophenyl β-D-glucoside, cellobiose, or starch as the substrate and glucosyltransferase as the enzyme. All mixtures were analyzed by paper chromatography in 6:4:3 (v/v) butyl alcohol–pyridine–water by three ascents of the solvent[27]. Radioautograms were prepared of the chromatogram by exposure to No Screen X-ray film for several days. A photograph of the radioautograph strips for some of the time intervals and for the controls of [^{14}C]maltose and D-[1-^{14}C]glucose is reproduced in Fig. 1. Radioactivities of the products in the digest were measured by placing a Geiger–Mueller end-window tube directly on the paper. The values for the products in a 24-h digest are recorded in Table I.

The glucosyltransferase did not hydrolyze the β-D-glucosides or cellobiose. However, the enzyme did liberate D-glucose from starch, as has been reported earlier[25]. The glucosyltransferase preparation was not contaminated with a β-D-glucosidase. That the preparation was free from glucoamylase has been discussed in an earlier section.

Isolation of the products of glucosyltransferase action on maltose. — Samples of non-labeled maltose (0.2 or 2 g) were employed as the substrate for preparation of reaction mixtures from which sufficient of the transfer products could be isolated for characterization studies. Duplicate samples (0.2 g) of maltose were dissolved in 2 ml of water and mixed with 0.2M acetate buffer (1 ml) of pH 3.5 and glucosyltransferase (2 ml). One sample was incubated for 6 h and the other for 24 h. At the end of these time-intervals, the enzyme was inactivated by heat and the digest concentrated to 1 ml by lyophilization. The products were isolated from the digest by preparative paper-chromatography[27] with 6:4:3 (v/v) butyl alcohol–pyridine–water. To obtain products of sufficient purity for structural analysis, the samples were subjected to a second and in some cases a third purification by preparative paper-chromatography.

Duplicate samples (2 g) of maltose were dissolved in 20 ml of water. To one sample were added 10 ml of 0.2M sodium acetate buffer (pH 3.5) and 20 ml of glucosyltransferase, and to the other 10 ml of buffer and 20 ml of water. The samples were incubated for 24 h at room temperature. Analysis of the mixtures by paper chromatography showed that new compounds were produced in the enzymic digest, but not in the solution devoid of enzyme. The digests were lyophilized and the residues dissolved in 8 ml of water. Samples (0.1 ml) of the digests and authentic 1,6-anhydro-β-D-glucopyranose as a marker compound were placed on preparative paper-chromatograms. The chromatograms were developed in the foregoing solvent system. The marker strip was stained with silver nitrate reagent to locate the position of the 1,6-anhydro-D-glucose, and the areas of the chromatograms at the R_F value of 1,6-anhydroglucose were extracted with water. The two sets of samples, one from the enzymic digest and the other from the control, were combined separately and lyophilized. Each sample was dissolved in 1 ml of water and rechromatographed by the method just described, with 10 chromatograms on paper that had been washed with the solvent and with water. Approximately 10 mg of a compound characterized as 1,6-anhydro-β-D-glucopyranose, as described in the next section, was obtained from the enzymic digest. This compound was not obtained from the control mixture.

Characterization of 1,6-anhydro-β-D-glucopyranose. — The compound having an R_F value higher than that of D-glucose was identified as 1,6-anhydro-β-D-glucopyranose as follows. It migrated on paper chromatograms at a rate identical to that of 1,6-anhydro-β-D-glucopyranose (levoglucosan), R_F value 0.93 in 6:4:3 (v/v) butyl alcohol–pyridine–water and R_F 0.75 in 4:1:1 (v/v) butyl alcohol–ethyl alcohol–water. On the paper chromatograms, the two compounds reacted with silver nitrate reagent[33], but not with copper sulfate–molybdic acid reagent[34], indicating that a free reducing group was not present in either sample. Acid hydrolysis of the new compound (1 mg) or authentic 1,6-anhydro-β-D-glucopyranose in 0.1M hydrochloric (0.1 ml) acid for 2 h at 100°, showed conversion at comparable rates into D-glucose. The latter was identified by R_F values in several solvent systems and by the D-glucose oxidase spray-method[15].

Samples (0.5 mg) of the enzymically produced compound and 1,6-anhydro-β-D-glucopyranose were acetylated in 5 parts of dry pyridine and 5 parts of dry acetic anhydride for 15 h at room temperature. The solvent and reagent were completely removed by evaporation under nitrogen. G.l.c. of the products was performed on a column of OV-17 at 210°. The acetates of both compounds exhibited identical retention times, as shown in Fig. 2.

Samples (0.5 mg) of the enzymically produced compound and 1,6-anhydro-β-D-glucopyranose were methylated by the Hakomori method[11] and the resulting mixture was analyzed by g.l.c. and mass spectrometry[12]. The methylated alditol acetate derivatives of both compounds exhibited identical retention times on g.l.c. on a column of OV-225 at 170°, as shown in Fig. 2. The mass spectra of the derivatized compounds showed the following fragments in both samples: *m/e* 45 (20), 117 (100), 161 (40), 189 (30), and 233 (10). The values in parentheses denote relative abundance, with the most abundant fragment being assigned a value of 100. The foregoing data identify the methyl derivatives of both compounds as 1,5-6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol[12].

Samples (5 mg) of authentic and enzymically produced 1,6-anhydro-β-D-glucopyranose were evaporated from D_2O (4 × 1 ml) to effect complete exchange of the hydroxylic protons. The spectra of D_2O solutions of both samples were obtained with a JEOL PS-100 FT n.m.r. spectrometer, and the chemicals hifts (δ, p.p.m.) relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate were determined. Sixteen free-induction decays were accumulated in order to provide adequate signal-to-noise ratios. The spectra of the two samples were identical. Further, the spectra are highly characteristic of the structure of the compound and may be partially assigned by comparison with the spectrum of the corresponding triacetate. Thus, the resonances of H-1, H-5, and H-6a,6b occur at δ 5.49, 4.67, and 4.15, 3.80, respectively, and the corresponding signals for the triacetate ($CDCl_3$) are observed at 5.47, 4.63 and 4.12, 3.82, respectively. The signals of H-2, 3, and 4 move from 3.57 (1H) and 3.73 (2H) for the native compound to 4.62 (2H) and 4.80 (1H) in the spectrum of the triacetate. The $J_{6a,6b}$ coupling constant is 7.6 Hz and is characteristic of the interaction in the dioxolane ring; the value is 14 Hz for the analogous coupling in the triacetate of 1,6-anhydro-β-D-glucofuranose[29] in which the methylene group is located in a six-membered ring[35].

Characterization of the oligosaccharides. — The structural characterization of isomaltose, panose, and nigerose from digests of maltose with the glucosyltransferase has been reported earlier[3,9]. Confirmation of the structures was achieved in the present study by methylation analysis. Samples (~2 mg) of the purified oligosaccharides were methylated, and analyzed by g.l.c. and m.s. In each instance, the expected methylated derivatives were obtained from the oligosaccharides in the proper molar ratios.

Isomaltotriose and 6-*O*-D-glucosyl-panose had been identified only tentatively in earlier studies[3]. Methylation of these compounds followed by g.l.c. and m.s. revealed that the former yielded 1 mol of 2,3,4,6,-tetra-*O*-methyl-D-glucose and 2 mol of 2,3,4-tri-*O*-methyl-D-glucose, whereas the latter yielded 1 mol of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2 mol of 2,3,4-tri-*O*-methyl-D-glucose, and 1 mol of 2,3,6-tri-*O*-methyl-D-glucose. Enzymic tests were performed with these compounds with glucoamylase by incubating 0.1 ml of a 1% solution of the compounds with 0.1 ml of glucoamylase, and examining the digests for reducing sugars by paper chromatography. Isomaltotriose yielded D-glucose and isomaltose, and 6-*O*-D-glucosyl-panose yielded

D-glucose and panose in the initial stages of enzyme action. These products were identified by R_F values on paper chromatograms developed by 2 ascents of 6:4:3 (v/v) butyl alcohol–pyridine–water: glucose (0.56), maltose (0.38), isomaltose (0.28), panose (0.17), isomaltotriose (0.14), and 6-*O*-D-glucosyl-panose (0.07). Prolonged incubation with glucoamylase converted both oligosaccharides completely into D-glucose. On the basis of the foregoing results, the structures of these compounds are established to be *O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 6)-α-D-glucose for isomaltotriose and *O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-α-D-glucose for 6-*O*-D-glucosyl-panose.

Methylation analysis of the oligosaccharide that migrated on paper at an R_F value slightly below isomaltose yielded one mol of 2,3,4,6-tetra-*O*-methyl-D-glucose and two mol of 2,3,6-tri-*O*-methyl-D-glucose. The compound was hydrolyzed by glucoamylase initially to D-glucose and maltose, having R_F values of 0.57 and 0.38, respectively, and eventually completely to D-glucose. The R_F value of the new compound, after 2 ascents of the solvent, was 0.21, identical to the R_F value of authentic maltotriose[27]. These data established that the oligosaccharide is maltotriose, having the structure *O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-α-D-glucose.

Methylation analysis of the oligosaccharide preparation having an R_F value slightly below maltose yielded methyl derivatives indicating that the sample contained kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucose) and perhaps some other compound. The kojibiose was purified by preparative paper-chromatography[27] in 5:1:4 (v/v) butyl alcohol–acetic acid–water by ten ascents of the solvent. Methylation analysis of the compound having the same R_F value as a reference sample of kojibiose yielded 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose in equimolar ratios. The derivatives were identified, after reduction and subsequent acetylation, as the alditol acetates by g.l.c. and m.s. The enzymically produced compound was hydrolyzed by glucoamylase to D-glucose at the same rate as authentic kojibiose. The structure for this compound is, therefore, established to be that of 2-*O*-α-D-glucopyranosyl-α-D-glucose.

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