Factors Determining Steric Course of Enzymic Glycosylation Reactions: Glycosyl Transfer Products Formed from 2,6-Anhydro-1-deoxy-D-*gluco*-hept-1-enitol by α-Glucosidases and an Inverting Exo-α-glucanase†

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ABSTRACT: Glycosyl transfer products were formed from 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (heptenitol) by purified α-glucosidases from *Candida tropicalis* and rice and by an inverting exo-α-glucanase (glucodextranase) from *Arthrobacter globiformis*. The products were structurally defined through ^1H and ^{13}C NMR (nuclear magnetic resonance) spectra of their crystalline per-*O*-acetates in comparison with those of authentic methyl 1-deoxy-α- and methyl 1-deoxy-β-D-*gluco*-heptuloside. 1-Deoxy-α-D-*gluco*-heptulosyl-(2→7)-heptenitol and 1-deoxy-α-D-*gluco*-heptulosyl-(2→7)-D-*gluco*-heptulose were produced by both the *Candida* α-glucosidase and the glucodextranase; 1-deoxy-α-D-*gluco*-heptulosyl-(2→5)- and 1-deoxy-α-D-*gluco*-heptulosyl-(2→7)-D-*gluco*-heptuloses by the rice α-glucosidase. These results, together with our earlier findings of sterospecific hydration of heptenitol catalyzed by the same enzymes [Hehre, E. J., Brewer, C. F., Uchiyama, T., Schlesselmann, P., & Lehmann, J. (1980) *Biochemistry 19*, 3557–3564], show the inadequacy of the long-accepted notion that carbohydrase-catalyzed reactions always lead to retention (or always lead to inversion) of substrate configuration. In particular, the finding that glucodextranase forms transfer products of α configuration and a hydration product of β configuration from the same substrate provides a clear example of the functioning of acceptors rather than donor substrates in selecting the steric course of reactions catalyzed by a glycosylase. The circumstances under which acceptor cosubstrates might be expected to show this significant effect are discussed. The opportunity presumably would exist whenever carbonium ion mediated reactions are catalyzed by glycosylases that provide oppositely oriented approaches of different acceptors to the catalytic center.

In a previous report (Hehre et al., 1980), D-glucosyl mobilizing enzymes of several different types were shown to catalyze specific hydration reactions with the prochiral glycosyl donor 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (heptenitol). α-Glucosidases from *Candida tropicalis* yeast and from rice were found to produce 1-deoxy-α-D-*gluco*-heptulose from this substrate; the β-glucosidase from sweet almonds and an inverting exo-α-glucanase from *Arthrobacter globiformis* produced 1-deoxy-β-D-*gluco*-heptulose. In addition, both α-glucosidases and the *Arthrobacter* glucodextranase were observed to catalyze the formation of saccharide transfer products from the heptenitol. This paper describes the structures and modes of formation of these transfer products. As will be discussed, a comparison of the results with those obtained previously for the hydrations catalyzed by the same enzymes provides unique information about the factors involved in determining the steric course of enzymic glycosylation reactions. Evidence that acceptor substrates are among these factors was reported earlier (Hehre et al., 1980), on the basis of the finding that glucodextranase acting on heptenitol leads to the formation of an α-D-*gluco*-heptulosylheptenitol transfer product while concurrently producing a hydration product of β configuration. More complete evidence relating to this point has now been obtained.

Traditionally, product configuration has been represented as being rigidly tied to that of the substrate, in that reactions catalyzed by any given carbohydrase have been assumed to either always result in retention or always result in inversion of donor substrate configuration. Studies with glycosidic substrates have provided a few contrary observations (Shibaoka et al., 1971, 1975; Suetsugu et al., 1971; Pazur et al., 1977, 1978), but the breakdown of this long-accepted generalization is most clearly shown in the glycosylation reactions which are catalyzed by various carbohydrases with glycals (Lehmann & Schröter, 1972; Hehre et al., 1977, 1981; Kanda et al., 1981) and other enolic glycosyl donors (Brockhaus & Lehmann, 1977; Hehre et al., 1980). Such actions do not conform to the usual notions of carbohydrase specificity. Instead, they support the concept that glycoside hydrolases and glycosyltransferases are glycosylases (Hehre et al., 1971, 1973) that thus may utilize, as productive substrates, compounds having no more than the ability to bind appropriately at the active site and to yield a glycosyl residue on protonation. Studies of hydration reactions catalyzed by α- and β-glucosidase with D-glucal or with heptenitol (Hehre et al., 1977, 1980) have, in fact, furnished unambiguous evidence for the ability of these glycosylases to control the anomeric configuration of reaction products without reference to that of the donor substrate. Gaining information on how this control is achieved is a major aim of this investigation of the structures of glycosyl transfer products synthesized from heptenitol by *C. tropicalis* α-glucosidase, rice α-glucosidase, and glucodextranase. In elucidation of these enzymically produced structures, authentic α-methyl and β-methyl 1-deoxy-D-*gluco*-heptulosides were employed as reference standards. Routes of chemical synthesis of these reference heptulosides are reported for the first time.

Experimental Procedures

General Methods. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Melting points are uncorrected. TLC[1] was performed on silica gel F_{254} (Merck); the

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solvent used for compounds with free OH groups was ethyl acetate–methanol (10:1 v/v) and for fully acetylated compounds was ethyl acetate–petroleum ether (bp 60–70 °C) (1:1 v/v). Detection was effected by charring with sulfuric acid. GLC was conducted in glass columns containing Chromosorb G coated with silicone rubber (SE-52, 3%), with nitrogen as carrier gas, and with flame-ionization detection. For column chromatography, Kieselgel 60 (Merck, 230–400 mesh) was used. Infrared spectra (KBr) were obtained with a Perkin-Elmer 137 spectrometer. ^{1}H NMR data ($CDCl_3$, internal Me_4Si) were recorded with Bruker WM 250 (250-MHz) and Bruker HX 360 (360-MHz) spectrometers, and ^{13}C NMR spectra were obtained with a Bruker HX 360, at 90.58 MHz.

Transfer Product Isolation. Saccharide products synthesized from 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (**4**) (Hehre et al., 1980) by purified *C. tropicalis* α-glucosidase (Sawai, 1967; Hehre et al., 1977), *A. globiformis* glucodextranase (Sawai et al., 1976; Ohya et al., 1978), and rice α-glucosidase (Makor Chemicals Inc., Jerusalem, Israel) were recovered from replicate 1-mL digests with each enzyme. Digests with *Candida* α-glucosidase (C, totaling 16 mL) comprised 270 mM substrate (**4**) and 0.3 mg/mL enzyme buffered with 0.05 M acetate at pH 5.15; incubation was at 30 °C for 5 h. Glucodextranase digests (GD, 20-mL total) contained 300 mM **4** and 1.0 mg/mL enzyme at pH 5.7; incubation was at 30 °C for 5 h. Rice α-glucosidase digests (R, 30 mL) comprised 360 mM **4** and 1.8 mg/mL enzyme at pH 5.5; incubation was at 30 °C for 2.25 h. Following incubation, each 1-mL digest was applied as a 20-cm band to prewashed Whatman No. 3 MM paper, along with adjacent D-glucose markers. Chromatography (descending) was with 1-butanol–ethanol–water (13:8:4 v/v/v) for 65–68 h. When dry, end guide strips were stained with silver nitrate (papers hung in air for 10 min following application of the NaOH reagent) to locate the reaction products (Hehre et al., 1980). Dry methanol was used to elute 1-deoxy-D-*gluco*-heptulose (R_{GLC} 1.47) and products migrating at R_{GLC} 1.25 (C_1 and GD_1), R_{GLC} 0.90 (R_2), and R_{GLC} 0.64 (C_3, R_3, and GD_3). Eluates of each product, accumulated from the replicate digests, were kept at −20 °C, protected from moisture. Each combined eluate was finally passed through a fine fritted glass filter, evaporated under vacuum at 30 °C, and dried under vacuum from absolute ethanol. The transfer products obtained from digests included the following: with *Candida* α-glucosidase, C_1 71 mg (8.5% of the available heptenitol substrate) and C_3 100 mg (12%); with glucodextranase, GD_1 116 mg (10%) and GD_3 59 mg (5%); with rice α-glucosidase, R_2 89 mg (4%) and R_3 40 mg (2%).

Enzymic Hydrolysis of Transfer Products. Each product (C_1, GD_1, R_2, C_3, GD_3, R_3) was examined for susceptibility to enzymic hydrolysis. Test mixtures buffered with 0.05 M acetate at pH 5.4 contained 0.4 mg/mL saccharide product and either 0.3 mg/mL *Candida* α-glucosidase, 4 mg/mL rice α-glucosidase, 1.2 mg/mL glucodextranase, 1.7 mg/mL highly purified sweet almond β-glucosidase (40 IU/mg, Boehringer Mannheim, NY), or buffer alone. After incubation at 30 °C for 5 and 48 h, 80-μL samples were chromatographed on Whatman No. 1 paper (descending, 26 h) with 1-butanol–ethanol–water (13:8:4 v/v/v) and stained with silver nitrate.

Acetylation of Transfer Products C_1, GD_1, R_2, C_3, R_3, and GD_3. Each of the products (40 mg) was dissolved in pyridine–acetic anhydride (1:1 v/v) (5 mL), in the case of R_2, C_3, R_3, and GD_3 under addition of 4-(dimethylamino)pyridine (5 mg). After 24 h the reaction mixture was poured on ice (10 mL), extracted with chloroform (10 mL), and washed with a sodium bicarbonate solution (2 × 5 mL) and water (10 mL). The chloroform layer was dried over magnesium sulfate and evaporated to dryness under diminished pressure. The residue was purified by column chromatography (7 × 0.5 cm) with ether as solvent. The following products were obtained in crystalline form from ether: **1**, from C_1 and GD_1 (73%), 3,4,5,3′,4′,5′,7′-hepta-*O*-acetyl-1-deoxy-α-D-*gluco*-heptulopyranosyl-(2→7)-2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol; **2**, from C_3, R_3, and GD_3 (78%), 3,4,5,6,3′,4′,5′,7′-octa-*O*-acetyl-1-deoxy-α-D-*gluco*-heptulopyranosyl-(2→7)-1-deoxy-2-keto-D-*gluco*-heptulose; **3**, from R_2 (58%), 3,4,6,7,3′,4′,5′,7′-octa-*O*-acetyl-1-deoxy-α-D-*gluco*-heptulopyranosyl-(2→5)-1-deoxy-2-keto-D-*gluco*-heptulose.

Methyl 3,4,5,7-Tetra-O-acetyl-1-deoxy-α-D-gluco-heptulopyranoside (**6**). (Synthesis a) 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-D-*gluco-hept*-1-enitol (**5**), 1 mmol (344 mg), was dissolved in absolute methanol (5 mL). At −15 °C, a mixture of HCl in methanol [acetyl chloride (0.5 mL) and methanol (5 mL)] was added. After 1 h the reaction mixture was evaporated to dryness in vacuo at 0 °C and subsequently acetylated with a mixture of pyridine–acetic anhydride (1:1) (20 mL). After 15 h at room temperature the reaction solution was poured on ice (10 mL), extracted with chloroform (20 mL), washed with a sodium bicarbonate solution (10 mL) and water (10 mL), and dried over magnesium sulfate. After evaporation to dryness under diminished pressure, the colorless syrup crystallized from ether, yield 286 mg (76%). (Synthesis b) **5**, 1.033 g, was treated with absolute methanol (10 mL) and mercury(II) acetate (3 mmol, 956 mg). After 1 h, sodium cyanoborohydride (200 mg) and acetic acid (1 mL) were added. The solution was filtered off the black residue and extracted with a mixture of chloroform (30 mL) and water (10 mL), dried over magnesium sulfate, and evaporated in vacuo. **6** crystallized from ether with a yield of 767 mg (68%).

Methyl 3,4,5,7-Tetra-O-acetyl-1-deoxy-1-bromo-α-D-gluco-heptulopyranoside (**8**) *and Methyl 3,4,5,7-Tetra-O-acetyl-1-deoxy-1-bromo-β-D-gluco-heptulopyranoside* (**9**). **5**, 1.153 g, was treated with a 0.01 M methanolic sodium methoxide solution (10 mL). After 3 h the reaction mixture was deionized by filtration through a silica gel column (5.0 × 0.9 cm). Subsequently, the solution was treated with *N*-bromosuccinimide (660 mg). After 1.5 h, the reaction mixture was evaporated to dryness under diminished pressure and then acetylated by addition of pyridine (20 mL) and acetic anhydride (20 mL). After 24 h the reaction solution was poured on ice (20 mL), extracted with chloroform (30 mL), washed with a sodium bicarbonate solution (2 × 20 mL) and water (20 mL), dried over magnesium sulfate, and evaporated to dryness in vacuo. The products were separated by "flash" chromatography (Still et al., 1978) on a silica gel column (10 × 5 cm) and with ethyl acetate–petroleum ether (bp 60–70 °C) (1:2 v/v) as solvent. First **9** was obtained (23.1%) and then **8** (33.3%). Compound **8**: mp 96 °C; $[\alpha]_{578}^{22}$ +71.8° (*c* 1.0, chloroform; ^{1}H NMR (250 MHz) δ 2.04 and 2.10 (2 s, 12 H, OAc), 3.42 (s, OCH_3), 3.59 and 3.71 (2 d, H-1, H-1′), 3.89 (ddd, 1 H, H-6), 4.18 (dd, 1 H, H-7), 4.29 (dd, 1 H, H-7′), 5.17–5.27 (m, 2 H, H-3, H-4), 5.32–5.39 (dd, 1 H, H-5), $J_{1,1'}$ = 10.5 Hz, $J_{3,4}$ = 9 Hz, $J_{4,5}$ = 9 Hz, $J_{5,6}$ = 9 Hz, $J_{6,7}$ = 2.5 Hz, $J_{6,7'}$ = 4.5 Hz, $J_{7,7'}$ = 12 Hz. Anal. Calcd for $C_{16}H_{23}O_{10}Br$ (455.3): C, 42.21; H, 5.09. Found: C, 42.19; H, 5.13. Compound **9**: mp 103 °C; $[\alpha]_{578}^{22}$ +54° (*c* 1.0, chloroform); ^{1}H NMR (250 MHz) δ 2.00, 2.05, and 2.11 (3 s, 12 H, OAc), 3.39 (s, 3 H, OCH_3), 3.41 and 3.56 (dd, 2 H,

[1] Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance.

H-1, H-1′), 3.89 (ddd, H-6), 4.18–4.21 (m, 2 H, H-7, H-7′), 5.11 (dd, H-4), 5.36 (d, H-3), 5.45 (dd, H-5), $J_{1,1'}$ = 10.5 Hz; $J_{3,4}$ = 10 Hz, $J_{4,5}$ = 9 Hz, $J_{5,6}$ = 10 Hz, $J_{6,7}$ = 3 Hz, $J_{6,7'}$ = 4 Hz. Anal. Calcd for $C_{16}H_{23}O_{10}Br$ (455.3): C, 42.21; H, 5.09. Found: C, 42.33; H, 5.26.

Methyl 3,4,5,7-Tetra-O-acetyl-1-deoxy-1-iodo-α-D-gluco-heptulopyranoside (**10**). **5**, 110 mg, was deacetylated with 0.01 M methanolic sodium methoxide solution (5 mL). The reaction mixture was filtered through a silica gel column (5 × 0.5 cm) and silver acetate (58 mg) added to the solution. Subsequently, iodine in methanol (85 mg in 4 mL) was added dropwise over a period of 15 min. After 1 h the insoluble residue was filtered off and washed with methanol (5 mL) and the filtrate evaporated to dryness under diminished pressure. The product was purified by flash chromatography on a silica gel column (7 × 1.5 cm) and with ethyl acetate–methanol (10:1 v/v) as solvent, yield 100 mg. For identification, the product was acetylated in pyridine (5 mL) with acetic anhydride (5 mL). After 15 h the reaction solution was stirred onto ice (10 mL), extracted with chloroform (10 mL), washed with a sodium bicarbonate solution (10 mL) and then with water (10 mL), dried over magnesium sulfate, and evaporated to dryness in vacuo to yield crystalline **10**, 110 mg (69%): mp 91 °C; $[\alpha]_{578}^{22}$ + 59.8° (*c* 1.0, chloroform); ^{1}H NMR (250 MHz) δ 1.99, 2.04, and 2.09 (3 s, 12 H, OAc), 3.19 (d, 1 H, H-1), 3.35 (s, 3 H, OCH_3), 3.45 (d, 1 H, H-1′), 3.82 (ddd, H-6), 4.11–4.26 (m, H-7, H-7′), 5.12 (dd, 1 H, H-4), 5.35 (d, 1 H, H-3), 5.43 (dd, 1 H, H-5), $J_{1,1'}$ = 11.5 Hz, $J_{3,4}$ = 10 Hz, $J_{4,5}$ = 8.5 Hz, $J_{5,6}$ = 10 Hz, $J_{6,7}$ = 4 Hz, $J_{6,7'}$ = 3 Hz. Anal. Calcd for $C_{16}H_{23}O_{10}I$ (502.3): C, 38.26; H, 4.61. Found: C, 38.51; H, 4.47.

Dehalogenation of **8**, **9**, *and* **10**. The bromide or iodide (1 mmol) was dissolved in ethanol (10 mL) and boiled under reflux with triethylamine (250 μL) and Raney nickel (2.5 mL) for 1 h. Inorganic material was filtered off and the filtrate evaporated to dryness under diminished pressure. The syrupy residue was dissolved in chloroform (20 mL), washed with water (10 mL), and dried over magnesium sulfate. After evaporation to dryness in vacuo, the residue was taken up in ether, and petroleum ether (bp 60–70 °C) was added cautiously. At −20 °C colorless crystals were obtained in the case of **6** (73%) and in the case of **7** (71%).

3,4,5,6,7-Penta-O-acetyl-1-deoxy-2-keto-D-gluco-heptulose (*B*). 1-Deoxy-D-*gluco*-heptulose (200 mg) was dissolved in pyridine–acetic anhydride (1:1) (10 mL) and treated with 4-(dimethylamino)pyridine (20 mg). After 18 h the reaction mixture was worked up as usual. The resulting light brown syrup was taken up in a little ethyl acetate and submitted to column chromatography (10 × 1.5 cm) in ethyl acetate–petroleum ether (bp 60–70 °C) (1:2). Crystallization from ethyl acetate–petroleum ether (bp 60–70 °C) yielded colorless crystals, 279 mg (69%): mp 92 °C [lit. (Wolfrom et al., 1957) mp 91–92 °C]; $[\alpha]_{578}^{22}$ +6.7° (*c* 1.0, $CHCl_3$) [lit. (Wolfrom et al., 1957) $[\alpha]_{589}^{21}$ +7.0° (*c* 1.0, $CHCl_3$)].

3,4,5,7-Tetra-O-acetyl-2,6-anhydro-1-deoxy-D-gluco-[1-^{3}H]heptenitol. 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptose (100 mg) was dissolved in acetic acid (2 mL) and hydrogenated with Adams catalyst and tritium gas (1 Ci) (Amersham-Buchler). After 15 h the reaction was completed with hydrogen gas. The product tetra-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptitol was converted to [1-^{3}H]heptenitol tetra-*O*-acetate (**5**) as for the unlabeled compound (Hehre et al., 1980). [1-^{3}H]Heptenitol acetate was purified by cocrystallization with 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-deoxy-D-*gluco*-heptenitol, unlabeled material (50 mg), to give a specimen of 58 μCi/mmol (76 mg).

1-Deoxy-D-gluco-[1-^{3}H]heptulose. 1-^{3}H-Labeled heptenitol tetra-*O*-acetate (80 μmol, 0.058 μCi/μmol) was deacetylated in 1.6 mL of 0.015 M sodium methoxide in dry methanol (3 h, 25 °C). The [1-^{3}H]heptenitol, recovered by solvent evaporation under diminished pressure, was hydrated in 1.2 mL of 0.025 N sulfuric acid (pH 1.5, 100 °C, 10 min) and then neutralized to provide a 48 mM solution of 1-deoxy-D-*gluco*-[1-^{3}H]heptulose. For use, 0.25-mL aliquots (12 μmol) were dried under vacuum at 30 °C.

Enzymic Digests of Heptenitol in the Presence of 1-Deoxy-D-gluco-[1-^{3}H]heptulose. Paired reaction mixtures (0.4 mL) contained 120 μmol of heptenitol (**4**), 12 μmol of 1-deoxy-D-*gluco*-[1-^{3}H]heptulose, and either enzyme or boiled enzyme. Mixtures with *Candida* α-glucosidase (0.3 mg/mL) were incubated at pH 5.2, 30 °C, 5 h; those with rice α-glucosidase (1.8 mg/mL) at pH 5.5, 30 °C, 2.25 h; those with glucodextranase (1.0 mg/mL) at pH 5.7, 30 °C, 5 h. Following incubation, each digest was chromatographed as a 19-cm band on Whatman No. 3 MM paper (65 h, descending) in 1-butanol–ethanol–water (13:8:4 v/v/v). End guide strips were stained with silver nitrate to locate the positions of the D-*gluco*-heptulose and of individual saccharide products, which were then eluted with methanol. Control chromatograms were sectioned and eluted at the same levels as those of the test digests. Eluates of saccharides C_1, GD_1, C_3, R_3, GD_3, and R_2 were treated as follows. Known portions were dried under reduced pressure, hydrolyzed in 0.025 N sulfuric acid (100 °C, 10 min), neutralized, and analyzed for reducing power by a cuprimetric method (Hehre et al., 1980) standardized with 1-deoxy-D-*gluco*-heptulose. All values were adjusted to the original digest volume. In addition, known portions of the eluates were subjected to scintillation counting, on a Beckmann LS-230 counter. All counts were corrected for background and adjusted to the original digest volume.

Results

The formation of saccharide products as well as 1-deoxy-D-*gluco*-heptulose in digests of heptenitol with *C. tropicalis* and rice α-glucosidases, and with *A. globiformis* glucodextranase, was first recognized when stained chromatograms in each case showed the presence of products that migrated more slowly than the heptulose (R_{GLC} 1.47). Upon elution all of these products were found to be completely converted to the heptulose by mild acid treatment (0.025 N sulfuric acid, 100 °C, 10 min). Measurement of the quantity of reducing sugar thus derived from the eluted saccharides was then used to find, for each enzyme, the best reaction conditions for recovering these lesser components for detailed analysis. The digest conditions and isolation procedures are given under Experimental Procedures.

Digests of heptenitol with *C. tropicalis* α-glucosidase yielded products C_1 (71 mg) and C_3 (100 mg), with rice α-glucosidase R_2 (98 mg) and R_3 (40 mg), and with glucodextranase GD_1 (116 mg) and GD_3 (59 mg). Preliminary investigations indicated C_1 and GD_1 to be heptulosylheptenitols indistinguishable from each other in chromatographic mobility (R_{GLC} 1.26) and in showing <5% of the reducing power of 1-deoxy-D-*gluco*-heptulose (Hehre et al., 1980) and infrared absorption (>C=CH_2 band at 1660 cm^{-1}), indicative of an intact heptenitol moiety.[2] On the other hand, R_2, C_3, R_3, and

[2] The presence of a heptenitol terminal was also indicated by the observation that C_1 and GD_1 were converted by gentle acid treatment (0.01 N sulfuric acid, 40 °C, 7 min) to a saccharide product with the same reducing power and chromatographic mobility (R_{GLC} 0.61) as those of C_3 and GD_3.

Table I: Properties of Crystalline Acetylated Derivatives of Transfer Products 1-3 and of Authentic Methyl 1-Deoxy-D-*gluco*-heptulosides 6 and 7

transfer product or glycoside	acetylated derivative	mp (°C)	$[\alpha]_{578}^{22}$ [a] (deg)	$[M]_{578}^{22}$ (deg)	formula (mol wt)	calcd	found
$C_1 = GD_1$	1	146	+99.4	644	$C_{28}H_{40}O_{17}$ (648.6)	C, 51.85; H, 6.21	C, 51.34; H, 6.21
$C_3 = R_3 = GD_3$	2	119	+62.4	441	$C_{30}H_{42}O_{19}$ (706.67)	C, 50.99; H, 5.99	C, 50.91; H, 5.93
R_2	3	113	+45.1	319	$C_{30}H_{42}O_{19}$ (706.67)	C, 50.99; H, 5.99	C, 51.18; H, 6.21
methyl 1-deoxy-α-D-*gluco*-heptuloside	6	156	+99.8	375	$C_{16}H_{24}O_{10}$ (376.4)	C, 51.06; H, 6.43	C, 51.00; H, 6.52
methyl 1-deoxy-β-D-*gluco*-heptuloside	7	83	+28.4	106	$C_{16}H_{24}O_{10}$ (376.4)	C, 51.06; H, 6.43	C, 51.16; H, 6.44

[a] 1, 6, and 7 (*c* 1.0, chloroform); 2 and 3 (*c* 0.75, chloroform).

Chart I

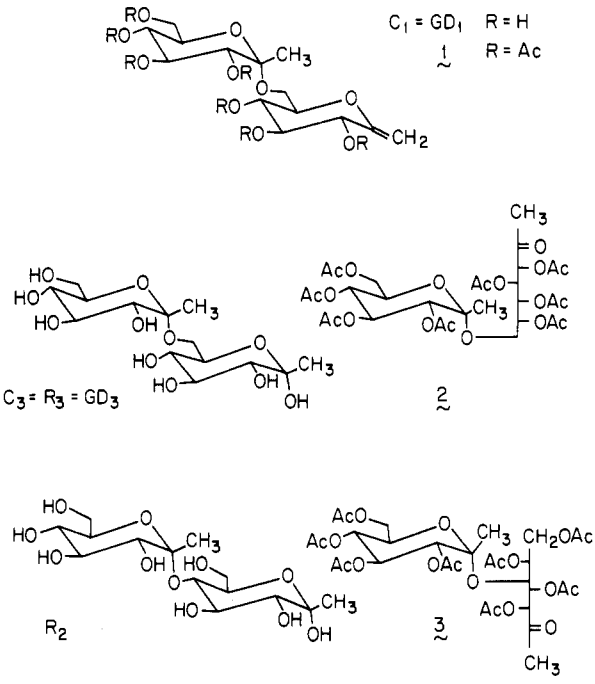


Chart II

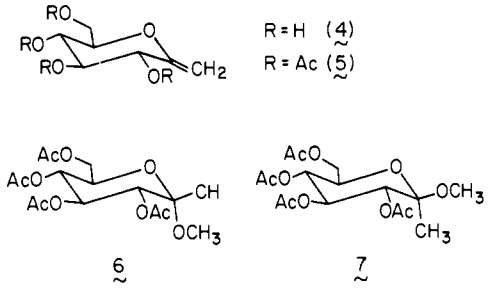
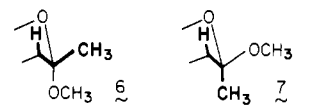


Chart III



GD_3 were found to have a reducing end group and appeared to be heptulosylheptuloses. C_3, R_3, and GD_3 showed 47–56% of the reducing power of 1-deoxy-D-*gluco*-heptulose and were further indistinguishable from each other chromatographically (R_{GLC} 0.61). R_2 differed from them in migrating more rapidly (R_{GLC} 0.86) and in showing less reducing power (31% relative to that of the heptulose).

On acetylation with acetic anhydride and pyridine, C_1 and GD_1 gave identical crystalline hepta-*O*-acetates (**1**): mp 146 °C; $[\alpha]_{578}^{22}$ +99.4° (Table I). Complete acetylation of C_3, R_3, GD_3, and R_2 could only be achieved under more drastic conditions [in the presence of 4-(dimethylamino)pyridine]. In all of these compounds, the reducing end moiety was acetylated in the open-chain, free keto form. The crystalline octa-*O*-acetyl derivatives of C_3, R_3, and GD_3 (**2**), with mp 119 °C and $[\alpha]_{578}^{22}$ +62.4°, were identical; they differed from the one obtained from R_2 and designated (**3**): mp 113 °C; $[\alpha]_{578}^{22}$ +45.1°. Further results, providing detailed evidence for the assigned structures of **1**, **2**, and **3**, are discussed below (Chart I).

The anomeric configuration of the nonreducing (glyconic) heptulosyl residue of the products synthesized from heptenitol by the three enzymes was initially examined by testing each product (C_1, GD_1, R_2, C_3, R_3, and GD_3) for susceptibility to hydrolysis by enzymes known to attack D-glucosidic substrates of only α (or only β) configuration. Test mixtures contained 0.4 mg/mL saccharide and either *Candida* α-glucosidase, rice α-glucosidase, glucodextranase, sweet almond β-glucosidase, or buffer without enzyme. Chromatograms (80-μL samples) made after 5-h incubation at 30 °C showed all products completely converted to 1-deoxy-D-*gluco*-heptulose (R_{GLC} 1.47) by the glucodextranase, partly converted to the heptulose by the *Candida* and rice α-glucosidases, and unaffected by the β-glucosidase. By 48 h, all of the products were completely hydrolyzed by the α-glucosidases as well as by glucodextranase; all remained unaffected by the β-glucosidase. These results suggest that all of the saccharides recovered from the digests of heptenitol with α-glucosidases and glucodextrase are 1-deoxy-α-D-*gluco*-heptulosyl compounds.

The second method used to determine the configuration of the glycosidically linked residue of the enzymically formed products was comparison of the ^1H and ^{13}C NMR spectra of their acetylated derivatives **1**, **2**, and **3** with the spectra of authentic reference compounds of known anomeric configuration. The following reactions were used to synthesize the reference compounds, starting with heptenitol (**4**) or its per-*O*-acetate (**5**).

Synthesis of α and β Methyl 1-Deoxy-D-gluco-heptulosides (**6** *and* **7**). The acid-catalyzed addition of methanol to heptenitol per-*O*-acetate (**5**) was found to give an anomeric mixture (detected by GLC) of the methyl glycosides, 95% α (**6**) and 5% β (**7**) (Chart II). Methoxy mercuriation of **5** and methoxy iodination of **4** with subsequent acetylation, yielded acetylated intermediates that, on reduction, gave the α anomer (**6**) exclusively. In contrast, methoxy bromination of **4** yielded after per-O-acetylation the methyl 1-deoxy-1-bromo-D-*gluco*-heptuloside derivatives **8** and **9**, which could be separated by column chromatography on silica gel and crystallized; **8** and **9** were obtained in a ratio of 1:1.25. The pure 1-bromo derivatives were idividually subjected to reductive dehalogenation and yielded **6** and **7**, respectively.

Assignments of the α- and β-anomeric configuration to **6** and **7**, respectively, were carried out by comparing their ^{13}C–^1H coupling constants (Wehrli & Wirthlin, 1976; H. Fritz et al., unpublished results). The $^3J_{^{13}C\text{-}1\text{-}H\text{-}3}$ of **6** is 1.6 Hz, indicating a synclinal arrangement, whereas **7** shows a ^3J of 2.5 Hz, which is representative of an antiperiplanar arrangement

Table II: ^{1}H NMR Spectra (360 MHz) Recorded for **1**, A,[a] 2, B,[b] **3**, **6**, and **7** in $CDCl_3$

(A) Chemical Shifts (ppm) Relative to Tetramethylsilane Internal Standard

	1	A[a]	2	B[b]	3		6	7
H′-1	*1.34 (s)*		*1.39 (s)*		*1.53 (s)*	H-1	*1.35 (s)*	*1.47 (s)*
H′-3	5.00 (d)		5.02 (d)		5.01 (d)	H-3	5.01 (d)	5.14 (d)
H′-4	5.49 (dd)		5.38 (dd)		5.38 (dd)	H-4	5.46 (dd)	5.23 (dd)
H′-5	5.09 (dd)		5.08 (dd)		5.10 (dd)	H-5	5.08 (dd)	5.11 (dd)
H′-6	4.27 (ddd)		3.89 (ddd)		4.15 (ddd)	H-6	3.82 (ddd)	3.82 (ddd)
H′-7	4.10 (dd)		4.09 (dd)		4.07 (dd)	H-7	4.10 (dd)	4.15 (dd)
H′-7′	4.17 (dd)		4.23 (dd)		4.24 (dd)	H-7′	4.23 (dd)	4.22 (dd)
H-1	4.56 (dd)	4.57 (dd)	*c*	*c*	*c*	OCH_3	3.28 (s)	3.35 (s)
H-1′	4.89 (dd)	4.85 (dd)	*c*	*c*	*c*			
H-3	5.42 (ddd)	5.47 (ddd)	5.41 (d)	5.26 (d)	5.46 (d)			
H-4	5.18 (dd)	5.16 (dd)	5.76 (dd)	5.62 (dd)	5.47 (dd)			
H-5	5.08 (dd)	5.24 (dd)	5.46 (dd)	5.47 (dd)	4.33 (dd)			
H-6	3.92 (ddd)	3.84 (dd)	5.06 (ddd)	5.07 (ddd)	5.31 (ddd)			
H-7	3.48 (dd)	4.20 (dd)	3.49 (dd)	4.13 (dd)	4.28 (dd)			
H-7′	3.62 (dd)	4.30 (dd)	3.73 (dd)	4.34 (dd)	4.49 (dd)			
OAc	1.99–2.16 (5 s)	2.04–2.13 (4 s)	1.96–2.27 (8 s)	2.05–2.20 (4 s)	1.47–2.22 (9 s)	OAc	1.98–2.095 (4 s)	2.01–2.09 (4 s)

(B) Coupling Constants $^1J_{H-H}$ (Hz)

J	1	A	2	B	3	*J*	8	7
H′-3–H′-4	10		10		10	H-3–H-4	10	~9
H′-4–H′-5	9		9.5		9.5	H-4–H-5	9.5	~9
H′-5–H′-6	10		10		10	H-5–H-6	10	~9
H′-6–H′-7	2		2		2	H-6–H-7	2.5	2.5
H′-6–H′-7′	5		4		4	H-6–H-7′	5	4
H′-7–H′-7′	12		12		12	H-7–H-7′	12.5	12
H-1–H-1′	1.5	1.5						
H-1–H-3	1.5	1.5						
H-1′–H-3	1.5	1.5						
H-3–H-4	9	8.5	4	3.8	4			
H-4–H-5	8	9	4	6	~4			
H-5–H-6	10	9.8	4	5	~4			
H-6–H-7	3	2.5	4	3.5	4			
H-6–H-7′	8	4	7	5	7			
H-7–H-7′	10	12.5	12	12	12			

[a] 3,4,5,7-Tetra-*O*-acetylheptenitol (**5**). [b] 3,4,5,6,7-Penta-*O*-acetyl-1-deoxy-2-keto-D-*gluco*-heptulose. [c] In the area of the acetyl proton resonances H-1 is represented by a singlet. Its position cannot clearly be determined.

Table III: ^{13}C NMR Spectra (90.58 MHz) Recorded for **1**, **2**, 6, and 7 in $CDCl_3$[a]

	1	2		6	7
C′-1	20.34	20.35	C-1	19.49	17.98
C′-2	99.47	99.89	C-2	99.53	100.28
C′-3	*73.75*	*73.34*	C-3	*73.86*	*70.30*
C′-4	71.17	70.83	C-4	71.24	72.63
C′-5	68.93	69.21	C-5	69.03	68.93
C′-6	*68.48*	*68.78*	C-6	*68.54*	*70.43*
C′-7	62.19	62.08	C-7	62.28	62.48
C-1	96.49	27.05	OCH_3	48.24	48.99
C-2	152.84	201.30			
C-3	69.18	75.66			
C-4	73.16	68.86			
C-5	69.71	70.62			
C-6	77.22	68.78			
C-7	61.49	58.97			
C=O	169.25–170.64	169.44–170.60	C=O	169.51–170.57	169.22–170.63
CH_3–C=O	20.63–20.71	20.58–20.71	CH_3–C=O		

[a] Chemical shifts (ppm) relative to tetramethylsilane internal standard. Insufficient amount of compound **3** was available for a proper ^{13}C NMR investigation.

(Chart III). The same assignments were provided by the ^{13}C chemical shifts of C-6 (68.54 ppm for **6**; 70.43 ppm for **7**) since the ^{13}C resonance of this particular ring carbon atom in a glycoside is at lower field when the aglycon is axial than when it is equatorial; e.g., the ^{13}C chemical shift of C-5 is 67.41 ppm for methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside and 71.71 ppm for the β anomer (Gagnaire et al., 1976; H. Fritz et al., unpublished results). Polarimetric measurements, likewise, indicated **6** ($[\alpha]^{22}_{578}$ +99.8°) to be the α anomer and **7** ($[\alpha]^{22}_{578}$ +28.4°) the β anomer (Table I).

Anomeric Configuration of Glyconic Moiety of **1**, **2**, *and* **3** *on the Basis of NMR Spectra.* Tables II and III present, respectively, the ^{1}H and ^{13}C NMR data obtained for the acetylated derivatives **1**, **2**, and **3** of the enzymically produced transfer products and for the methyl α- and β-heptuloside tetraacetate standards **6** and **7**. As evident from the ^{1}H NMR chemical shifts (italic in Table IIA), the H′-1 resonance of **1**, at 1.34 ppm, corresponds to that of the H-1 of the methyl α-glycoside, **6**. In **2** and **3** the H′-1 signal is at lower field. Obviously, the acetyl groups close to the C-1 methyl group

Table IV: Specific Radioactivity of Saccharides Recovered from Enzymic Digests of Heptenitol in the Presence of 1-Deoxy-D-*gluco*-[1-^{3}H]heptulose

saccharide		found			calcd, saccharide with	
		cpm[a]	μg[a]	cpm/μg	one residue labeled (cpm/μg)[b]	two residues labeled (cpm/μg)[c]
heptulosylheptenitol	C_1	250	198	1	32-85	
	GD_1	2750	335	8	23-89	
heptulosylheptuloses	C_3	25 600	554	46	32-85	64-170
	R_3	1800	55	33	31-90	62-180
	GD_3	23 800	816	29	23-89	46-178
	R_2	14 200	274	52	31-90	62-180

[a] Corrected for small values found with eluates from corresponding sections of chromatograms of incubated control mixtures with boiled enzyme. [b] Range between 0.5 of the specific radioactivity of the heptulose separated from incubated test digests and 0.5 of that of heptulose recovered from incubated controls with boiled enzyme. [c] Range between the specific activity of heptulose recovered from incubated test digests and that of heptulose from incubated controls.

in these partly open-chain derivatives cause a significant downfield shift in the H′-1 resonance compared with the signals in the methyl glycosides or in **1**. The H′-1 resonance of **2** is downfield of the H-1 resonance of the methyl α-glycoside **6** only can be correlated with it. The H′-1 resonance of **3** is downfield of the H-1 resonances of both **6** and **7** and, thus, is not able to be specifically correlated with one vs. the other. However, the H′-3, H′-4, and H′-7 signals of **3** correspond to the H-3, H-4, and H-7 resonances of the methyl α-glycoside, **6**, rather than to those of the β-glycoside, **7**.

Further information on configuration is provided by the ^{13}C NMR spectra (Table III, italic figures). The ^{13}C chemical shifts of C-3 and C-6 in the anomeric heptulosides show significant dependence on the anomeric configuration (H. Fritz et al., unpublished results) and can, by themselves, be used for structural assignment in **1** and **2**. In both compounds the C′-3 and C′-6 chemical shifts correspond to those of the methyl α-glycoside **6** and not to the β-glycoside **7**. On the basis of the above specific findings and the strong overall agreement between the corresponding ^{1}H and ^{13}C NMR signals in the acetylated transfer products **1**, **2**, and **3**, and in the methyl tetra-*O*-acetyl-1-deoxy-α-D-*gluco*-heptuloside **6**, we regard **1**, **2**, and **3** as all being α anomers.

Position of Attachment of the 1-Deoxy-α-D-gluco-heptulosyl Moiety. Significant differences in ^{1}H chemical shifts in the aglyconic part of the peracetylated saccharide products **1**, **2**, and **3** indicate the points of attachment. As shown by the underlined figures in Table II, the H-7 and H-7′ resonances of compound **1** appear at appreciably higher field than those of tetra-*O*-acetylheptenitol (A). Likewise, the H-7 and H-7′ resonances of **2** show this relationship in comparison to those of penta-*O*-acetyl-1-deoxy-2-keto-D-*gluco*-heptulose (B). Similarly, the H-5 resonance of compound **3** appears at appreciably higher field than H-5 of penta-*O*-acetyl-1-deoxy-2-keto-D-*gluco*-heptulose. These results show **1** and **2** to be 2→7 linked and **3** to be 2→5 linked.

Mode of Formation of Saccharides. Experiments were carried out to learn whether the saccharides produced in enzymic digests of heptenitol arise directly from the latter as glycosyl donor or are formed indirectly from its hydration product (heptulose) by reactions representing reversals of hydrolysis. Mixtures of *Candida* α-glucosidase, rice α-glucosidase, or glucodextranase, corresponding to those yielding saccharide products from heptenitol, gave no sign of such products when examined chromatographically. Further experiments were made with paired mixtures containing heptenitol and enzyme (or boiled enzyme) in the concentrations used in isolating the saccharides, plus 1-deoxy-D-*gluco*-[1-^{3}H]heptulose at one-tenth the concentration of the heptenitol. After incubation, the mixtures were chromatographed, and identical sections of the test and control chromatograms, corresponding to heptulose and to each saccharide evident in stained guide strips of the former, were eluted. The heptulose content (following mild acid hydrolysis in the case of the saccharides) and radioactivity of each product were measured.

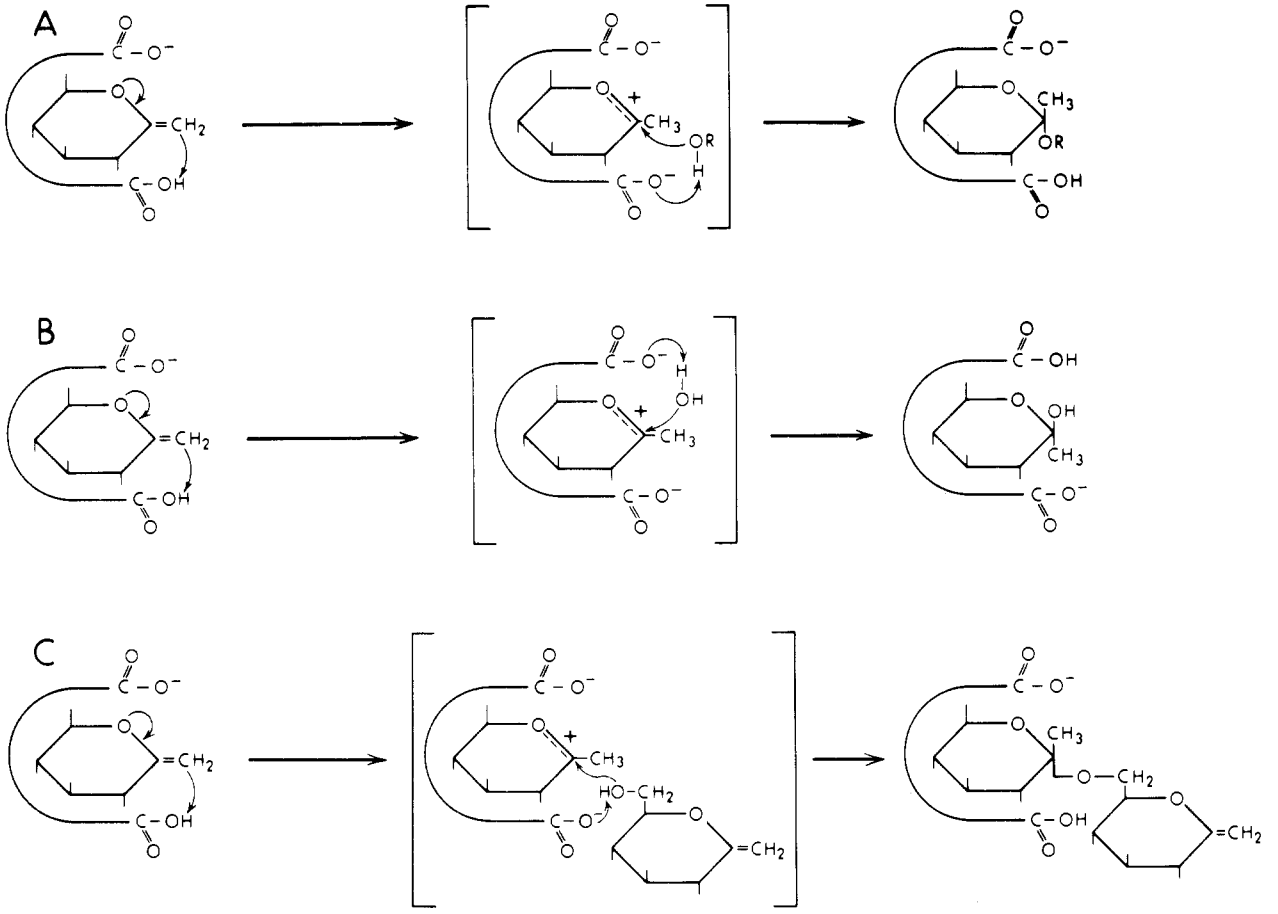
As shown in Table IV, the heptulose recovered from the mixtures with boiled enzyme showed a specific activity of 170–180 cpm/μg; heptulose from the test mixtures had lower activity (46–64 cpm/μg) due to dilution with the sugar formed by the enzymic hydration of the unlabeled heptenitol. The very low specific activities of C_1 and GD_1 indicate that these products arise primarily by the transfer of the heptulosyl moiety from one heptenitol molecule to another. Similarly, the radioactivities found for C_3, R_3, GD_3, and R_2 all fall within the range expected if these heptulosylheptuloses were produced by heptulosyl transfer from the unlabeled heptenitol to a molecule of [1-^{3}H]heptulose. Although the findings do not exclude the presence of some proportion of heptulosylheptulose labeled in both moieties as expected for condensation products, they do indicate that the observed saccharides are primarily glycosyl transfer products with heptenitol as the donor.[3]

Discussion

1-Deoxy-α-D-*gluco*-heptulosyl transfer products were shown to be formed from 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (heptenitol) by purified α-glucosidases of *C. tropicalis* and rice and by an inverting exo-α-glucanase (glucodextranase) from *Arthrobacter globiformis*. These results, coupled with our previous findings of stereospecific hydration of heptenitol by the same enzymes (Hehre et al., 1980) provide new insight into the catalytic capabilities of each and into the factors involved in determining the steric course of the reactions they catalyze. The occurrence of productive glycosylation reactions with heptenitol is, by itself, highly significant in that it contradicts the traditional assumption that carbohydrases have an absolute requirement for substrates of only α- (or only β-) anomeric configuration. The reactions with heptenitol, as with the enzymic utilization of other prochiral glycosyl donors (Lehmann & Schröter, 1972; Brockhaus & Lehmann, 1977; Hehre et al., 1977, 1981; Kanda et al., 1981), show the greater generality of a different concept of carbohydrase action that

[3] With glucodextranase, it is difficult to envision α-saccharide formation occuring from the heptenitol hydration product (1-deoxy-β-D-*gluco*-heptulose) as a glycosyl donor. The position of the axial 1-methyl group of this compound in the donor site would be expected to preclude proximate binding of the acceptor's hydroxyl group in an orientation leading to formation of an α-linked transfer product.

Scheme I



redefines glycoside hydrolases and glycosyltransferases as interrelated glycosylases, i.e., catalysts of the interchange of a glycosyl residue and a proton (Hehre et al., 1971, 1973). In order to undergo so simple a chemical change, a donor substrate would need no more than the ability to bind appropriately at an active site and to yield a glycosyl residue on protonation.

On the basis of the stereochemical nature of the products formed from heptenitol by the actions of *Candida* and rice α-glucosidases, and of glucodextranase, possible mechanisms for the hydration and glycosyl transfer reactions catalyzed by the enzymes are shown in Scheme I. We assume the catalytic functions of each enzyme to be served by two suitably disposed functional groups, possibly carboxyl groups, at the active center. One group would serve as a specific or general acid while the other, as a carboxylate anion, could act as a charge-stabilizing group or nucleophilic-specific base. Heptenitol (as a glycosyl donor) is envisioned as binding at the active site with its exocyclic enolic bond positioned between these groups. The first step in either hydration or glycosyl transfer by each enzyme (Scheme I) would involve protonation of the vinylic double bond and formation of a transient glycosyl carbonium ion–enzyme complex. Protonation by each enzyme could arise, as illustrated, from the same carboxyl group that protonates the glycosidic oxygen atom of α-D-glucosidic substrates. The possibility cannot be excluded that the opposite catalytic group may be the protonating source, as found in the *Candida* α-glucosidase-catalyzed hydration of D-glucal (Hehre et al., 1977) and in the glucodextranase-catalyzed utilization of β-D-glucosyl fluoride (Kitahata et al., 1981). However, H. Fritz et al. (unpublished results) have recently found that in the β-galactosidase-catalyzed utilization of a C_8 D-*galacto*-enitol, which resembles heptenitol in possessing an exocyclic double bond [but with a prochiral $=C(CH_3)D$ group in place of the symmetric $=CH_2$ of heptenitol], the direction of protonation is opposite that found with D-galactal (Lehmann & Zieger, 1977) and thus is the same as the direction of protonation for β-D-galactosides.

Scheme IA shows the proposed second glycosyl–proton interchange step in the reactions catalyzed by the α-glucosidases. Breakdown of the heptulose–enzyme complex in both hydration and glycosyl transfer reactions would be effected by a similarly directed attack of water or bound carbohydrate, at C-2, possibly with assistance (general base catalysis) from one of the carboxyl groups, to create a product of α configuration in each case. This mechanism is consistent with the observed formation of 1-deoxy-α-D-*gluco*-heptulose by both *Candida* and rice α-glucosidases (Hehre et al., 1980), of α-D-*gluco*-heptulosyl-(2→7)-heptenitol (C_1) and α-D-*gluco*-heptulosyl-(2→7)-D-*gluco*-heptulose (C_3) by *Candida* α-glucosidase, and of α-D-*gluco*-heptulosyl-(2→5)- and α-D-*gluco*-heptulosyl-(2→7)-D-gluco-heptuloses (R_2 and R_3) by rice α-glucosidase.

Parts B and C of Scheme I show, respectively, the proposed mechanism of breakdown of the complex in the reactions of hydration and glycosyl transfer catalyzed by glucodextranase. In hydration (Scheme IB) the intermediate would be subjected to stereospecific attack at C-2 by solvent to give the observed product, 1-deoxy-β-D-*gluco*-heptulose (Hehre et al., 1980). In the glycosyl transfer reactions (Scheme IC), C-2 would be subjected to an oppositely directed attack by the 7-hydroxyl group of a molecule of heptenitol (or of D-*gluco*-heptulose) bound at the enzyme's acceptor site in proximity to the catalytic groups, providing the observed α-D-*gluco*-heptulosyl-(2→7)-heptenitol (GD_1) and α-D-*gluco*-heptulosyl-(2→7)-D-

gluco-heptulose (GD_3) transfer products. The occurrence of these stereocomplementary and essentially irreversible reactions is strong evidence that the catalytic groups of glucodextranase are functionally flexible beyond the requirements of the principle of microscopic reversibility. Such functional flexibility, envisioned by Hehre et al. (1979) as an attribute of the catalytic groups of glycosylases in general, has, by now, been shown with a variety of glycosidases and glycanases (Rupley et al., 1969; Lehmann & Zieger, 1977; Hehre et al., 1977, 1979, 1980, 1981; Kitahata et al., 1981; Kanda et al., 1981).

The ability of glucodextranase to catalyze the formation of both 1-deoxy-β-D-*gluco*-heptulosyl and 1-deoxy-α-D-*gluco*-heptulosyl transfer products with heptenitol shows an underlying similarity with the recently discovered ability of this enzyme to produce β-D-glucose from α-D-glucosyl fluoride and α-D-glucosyl transfer products from β-D-glucosyl fluoride (Kitahata et al., 1981). In each case a product of β configuration is formed when the acceptor is water; one of α configuration when it is bound carbohydrate. This similarity in end result, obtained with both chiral and prochiral glycosyl donors, would indicate that glucodextranase is so structured as to limit the approach of water and carbohydrate acceptors to the catalytic center from opposing directions, possibly by means of a specific binding site for water oriented oppositely to that for carbohydrate acceptors.

With heptenitol as donor substrate, it is evident that the presence of either type of acceptor suffices by itself to select which of two sterically different reaction paths will be catalyzed by the enzyme. This result, the demonstration that acceptor molecules can function as sole external determinants of the anomeric configuration of products, contradicts the long-accepted notion that enzymic reaction products always have the same (or always have the opposite) configuration as the donor substrate. Since the donor in this instance is a prochiral compound, the questions that arise are whether it is possible for acceptors to have a "steering role" in reactions with chiral donor substrates or, more broadly, to define the circumstances under which acceptors may have a stereoselective function. With respect to the first question, Pazur et al. (1977, 1978) have shown the ability of an *Aspergillus niger* glucosyltransferase to convert maltose (with D-glucose as acceptor) to α-D-glucobiosyl transfer products, as well as to produce 1,6-anhydro-β-D-glucose (levoglucosan) from maltose in a reaction in which the glyconic D-glucosyl residue of the donor becomes a "self-acceptor", in a binding site oriented to the catalytic center differently from the usual acceptor sites. On the other hand, it is evident that, under certain circumstances, the donor rather than the acceptor substrate represents the external factor that limits the catalyzed reaction(s) to a particular steric course. Glucodextranase, for example, directly hydrolyzes α-D-glucosyl fluoride to form β-D-glucose but effects reactions leading to α-D-glucosyl transfer products when β-D-glucosyl fluoride is the donor substrate (Kitahata et al., 1981); however, though the appropriate acceptors are present, the enzyme appears unable to catalyze the direct hydrolysis of β-D-glucosyl fluoride (Kitahata et al., 1981) or to effect glucosyl transfer reactions from α-D-glucosyl fluoride or from dextran (Ohya et al., 1978). Apparently, the conditions required before acceptors of different types can lead to sterically different reactions from a given donor include not only some provision for opposing binding orientations for different acceptors but also a reaction mechanism allowing product configuration to be determined at a last step. The reactions catalyzed by glucodextranase with heptenitol are effected by such a mechanism (Scheme IB,C), and likewise, carbonium ion mediation has been proposed for the reactions catalyzed by *A. niger* glucosyltransferase with maltose (Pazur et al., 1978). In contrast, the glucodextranase-catalyzed reactions with α- and β-D-glucosyl fluoride are envisioned as proceeding by a concerted displacement mechanism (Kitahata et al., 1981). Here, the configuration of the glycosyl donor determines the steric course of any reaction that might be catalyzed with that donor.

It is to be noted that, by virtue of its catalytic group flexibility, glucodextranase has the ability to promote reactions with different substrates by different mechanisms. A close parallel is found in the case of sweet potato β-amylase, which catalyzes reactions with α- and β-maltosyl fluoride that appear to proceed by a concerted displacement mechanism (Hehre et al., 1979); but also reactions with maltal (Hehre et al., 1981) and maltenitol (M. Gäbelein et al., unpublished results) that appear to be carbonium ion mediated. The utilization of different glycosidic substrates by different mechanisms also has been reported for hen's egg lysozyme acting on various para-substituted di-*N*-acetyl-β-chitobiosides (Tsai et al., 1969) and for *Bacillus subtilis* α-amylase acting on various para-substituted phenyl α-maltosides (Shibaoka et al., 1971; Suetsugu et al., 1971). The present findings thus provide further support for the view (Kitahata et al., 1981) emphasizing that a glycosylase may act by more than one mechanism and that, although it has been customary to speak of "the mechanism" of a glycosylase, the latter appears to be a too narrow simplification.

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Kinetics of Cytochrome *b* Oxidation in Antimycin-Treated Submitochondrial Particles†

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ABSTRACT: It has been shown that in bovine heart submitochondrial particles, antimycin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) inhibit the oxidation of NADH, succinate, and reduced ubiquinone incompletely, the uninhibited rate being about 20–40 nmol of substrate oxidized min^{-1} (mg of protein)$^{-1}$. By contrast, rotenone, cyanide, BAL (2,3-dimercaptopropanol), and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole [Trumpower, B. L., & Haggerty, J. G. (1980) *J. Bioenerg. Biomembr. 12*, 151–164] caused essentially complete inhibition when added alone or after maximal inhibition by antimycin or HQNO. Having thus ascertained that the electron leak through the antimycin block appeared to follow the normal path through complex III (ubiquinol: cytochrome *c* oxidoreductase) and cytochrome oxidase, the reduction of the *b* cytochromes by substrates and their oxidation through the leak in the antimycin block by molecular oxygen were studied. It was shown that at normal electron flux from NADH and succinate, both cytochromes b_{562} and b_{566} were reduced in antimycin-treated submitochondrial particles. Their oxidation after substrate exhaustion was biphasic, however. At 565 minus 575 nm, 56% of the total reduced cytochrome *b* was oxidized through the leak in the antimycin block at a more rapid rate, while the remaining 44% was oxidized about 10 times slower. When electron flux from substrates to complex III was slowed down by the use of inhibitors or substrates at $\leq 0.1K_m$ concentration, then only reduced b_{562} accumulated in antimycin-treated particles. The oxidation of b_{562} after substrate exhaustion or inhibition of substrate oxidation by an appropriate inhibitor occurred at a rate comparable to that of the slower reoxidation phase described above. These results indicated, therefore, that cytochromes b_{566} and b_{562} are oxidized through the leak in the antimycin block at two different rates, the reoxidation rate of b_{566} being about 10 times faster than that of b_{562}. The implications of these findings on the kinetic relationship of these two cytochromes in the respiratory chain have been discussed.

The electron transport system of bovine heart mitochondria contains three spectroscopically distinct *b* cytochromes (Davis et al., 1973). A low potential cytochrome *b* (b_{560}) fractionates into the succinate:ubiquinone oxidoreductase complex (complex II). This cytochrome has been purified in a preparation composed of two polypeptides of M_r 13 500 and 15 000 (Hatefi & Galante, 1980). The role of cytochrome b_{560} is not clear, even though the purified preparation has been shown to recombine in a 1:1 molar ratio with succinate dehydrogenase and reconstitute a highly active succinate:ubiquinone oxidoreductase system (Hatefi & Galante, 1980). In addition, it has been shown that in complex II dithionite-reduced b_{560} is rapidly oxidized by fumarate via succinate dehydrogenase or by ubiquinone. The other two *b* cytochromes (b_{562} and b_{566}) occur in the ubiquinol:cytochrome *c* oxidoreductase complex (complex III).[1] Although involved in electron transfer from substrates to cytochromes c_1 + *c*, the precise roles of cytochromes b_{562} and b_{566} in electron transfer through complex III are not clear either. Cytochrome b_{562} is reduced when submitochondrial particles are treated with substrates, and is oxidized when the reduced particles are treated with oxygen. Reduction of b_{566} is not observed under these conditions and requires treatment of the particles with antimycin, which creates an oxidation–reduction "crossover" between the *b* and the *c* cytochromes. Even in the presence of antimycin, the reduction of b_{566} by substrates requires that cytochromes c_1 + *c* and the complex III iron–sulfur protein be oxidized (this effect is referred to as oxidant-induced extra reduction of

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[1] In addition to b_{562} and b_{566}, a cytochrome *b* like chromophore with an absorption peak at 558 nm at 77 K was identified in complex III by Davis et al. (1973). Recently, Briquet et al. (1981) have observed a similar component in yeast mitochondria and have designated it cytochrome b_{558}.

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