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Scope and Mechanism of Carbohydrase Action. Stereospecific Hydration of 2,6-Anhydro-1-deoxy-D-*gluco*-hept-1-enitol Catalyzed by α- and β-Glucosidases and an Inverting Exo-α-glucanase[†]

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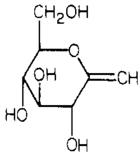
ABSTRACT: D-Glucosylases of several types have been found to catalyze specific hydration and glycosyl transfer reactions (the former predominating) when incubated with 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol. α-Glucosidases from *Candida tropicalis* and from rice convert this substrate into 1-deoxy-α-D-*gluco*-heptulose. The β-glucosidase from sweet almonds and an exo-1,6-glucanase (glucodextranase) from *Arthrobacter globiformis* convert the substrate to 1-deoxy-β-D-*gluco*-heptulose. The anomeric form of the sugar was identified by following ^{1}H NMR spectra of enzyme–enitol digests. The C-1 methyl group protons of the α and β anomers resonated at 1.45 and 1.40 ppm, respectively. Nonenzymatic anomerization of the β to the α form of the sugar was noted and, at equilibrium, ^{13}C and hydroxyl ^{1}H NMR spectra showed the presence of only the α anomer. The present work provides the first demonstration of the ability of an "inverting" glucanase to create specific anomeric configuration de novo from a substrate lacking α or β configuration. Indeed, the actions of glucodextranase on this enolic glycosyl donor lead not only to 1-deoxy-β-D-*gluco*-heptulose but also to a 1-deoxy-α-D-*gluco*-heptulosyl transfer product when enitol is the acceptor. These results reveal a catalytic mechanism that is able to convert a substrate to products of either configuration, depending on the type of acceptor. A mechanism is proposed for the stereospecific hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol by each enzyme, involving an incipient glycosyl carbonium ion and assuming the presence at the active site of two carboxyl groups arranged to account for catalysis of reactions with appropriate glycosidic substrates. These observations provide strong evidence for the functional flexibility of the catalytic groups of glycosylases.

The study of enzymatically catalyzed glycosylation reactions that occur without glycosidic bond cleavage has in recent years provided new insight into the catalytic capabilities and mechanism of a number of well-known carbohydrases, including α-amylases (Hehre et al., 1969, 1971, 1973; Okada et al., 1979), β-galactosidase (Lehmann & Schröter, 1972; Lehmann & Zeiger, 1977; Brockhaus & Lehmann, 1977), α- and β-glucosidases (Lehmann & Schröter, 1972; Hehre, 1974; Hehre et al., 1977), and β-amylase (Hehre et al., 1969, 1973, 1979; Genghof et al., 1978). A further opportunity to apply this experimental approach has now arisen due to the recent addition of a new type of compound to the list of nonglycosidic structures (free sugars, glycosyl fluorides, glycals) that yield a glycosyl residue upon protonation and thus have the potential (Hehre et al., 1969, 1973) to serve as glycosyl donors for appropriate enzymes. The new type of structure having these characteristics was introduced with Brockhaus & Lehmann's (1977) demonstration that 2,6-anhydro-1-deoxy-D-*galacto*-hept-1-enitol is an effective substrate for β-galactosidase. For reasons given below, we envisioned that the D-gluco homologue might serve as a glycosyl donor for various types of D-glucosyl mobilizing enzymes and, thereby, perhaps lead to new fundamental information on their catalytic mechanism(s).

In the present study, various α- and β-glucosidases and exo-α-glucanases were examined for the ability to catalyze the conversion of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (I)[1]



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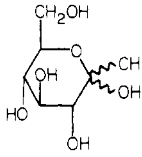

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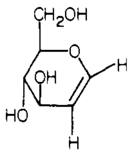

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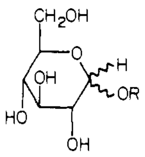

IV

to 1-deoxy-D-*gluco*-heptulose (II). Previously, the α-glucosidase of *Candida tropicalis* and the β-glucosidase of sweet almonds had been found to catalyze opposite stereospecific trans hydration of D-glucal (III) with the formation of α- or β-2-deoxy-D-glucose, respectively (Hehre et al., 1977). It

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[1] This compound, unambigously designated as an enolic sugar alcohol, gives rise to a 1-deoxy-D-*gluco*-heptulosyl carbonium ion when protonated at the double bond. This relationship is noted here as it is central to the present work, yet may not be immediately apparent from the formal name or from the term, enitol, used interchangeably in the present paper.

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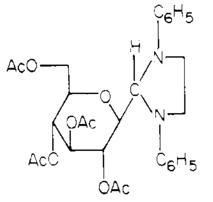
seemed likely that 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (I) might also be a substrate for these enzymes, since it has four hydroxyl groups which can be aligned with those of α- (or β-) D-glucosidic substrates (IV) to provide effective binding to both enzymes; also, an exocyclic enolic bond which can be positioned (for protonation by enzyme) in an orientation exceptionally close to those of the glycosylic bonds of α- and β-D-glucosidic substrates (IV). These same structural features would also favor the utilizability of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (I) by still other types of D-glucosylases. In particular, we were encouraged to examine this compound as a glycosyl donor for enzymes that catalyze the hydrolysis of α-glucans with the formation of D-glucose of inverted (β-) configuration. Up to the present, productive glycosylation reactions with substrates lacking α- or β-anomeric configuration have been reported only with glycosidases whose reactions with conventional (glycosidic) substrates proceed with retention of configuration. It was not known whether a carbohydrase, whose reactions have been found to lead invariably to inversion of configuration, would act on a substrate lacking an asymmetric anomeric carbon atom and, if so, which de novo configuration(s) might be created, and by what mechanism(s).

2,6-Anhydro-1-deoxy-D-*gluco*-hept-1-enitol (I) has, in fact, proved to be an effective substrate for several α- and β-glucosidases as well as for an exo-α-glucanase from *Arthrobacter globiformis* I-42, an enzyme shown by Sawai et al. (1976) and Ohya et al. (1978) to cleave β-D-glucose from the nonreducing ends of dextran chains. The present paper describes the reactions of these different D-glucosylases with I. The anomeric form of the product II has been examined in each case, since its configuration, which is created de novo, is a key to the reaction mechanisms used by the enzymes in catalyzing hydration of the common substrate. As will be made clear, the formation of 1-deoxy-D-*gluco*-heptulose is not always wholly the result of direct hydration of the enitol. In some instances, reactions of formation and subsequent hydrolysis of glycosyl transfer products are also catalyzed, and one of these indirect paths leading to 1-deoxy-D-*gluco*-heptulose will be illustrated. A more complete description of the glycosyl transfer products and of their synthesis by different D-glucosylases acting on 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol will be reported separately.

Experimental Procedures

*Substrate Synthesis. 3,4,5,7-Tetra-O-acetyl-2,6-anhydro-*D-*glycero*-D-*gulo-heptose-N,N′-diphenylimidazolidine* (*V*).



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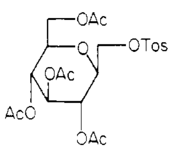

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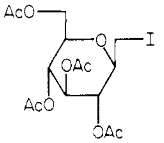

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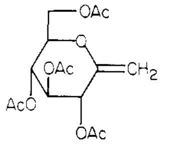

VIII

3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptulonitrile (Fuchs & Lehmann, 1975) (20 g) was added at once to a vigorously stirred mixture of Raney nickel (70 mL), sodium hypophosphite monohydrate (50 g), and *N*,*N′*-diphenylethylenediamine (22 g) in pyridine (150 mL) and acetic acid–water (1:1 v/v) (180 mL). Stirring was continued for 4 h (Albrecht et al., 1973). Nickel was filtered off, rinsed with CH_2Cl_2 (600 mL), and the combined filtrates were extracted with water (5 × 200 mL). After the mixture was dried over $MgSO_4$, the organic solution was evaporated under reduced pressure to yield a syrup, which on adding a small amount of ethanol gave pure V (18 g, 58%): mp 203 °C; $[\alpha]^{22}{}_{578}$ 26.2° (c 1.0, $CHCl_3$); $\nu_{max}{}^{KBr}$ 1730 cm^{-1} (OAc); NMR (90 MHz, $CDCl_3$) δ 1.87, 1.96, 2.00, and 2.1 (4 s, OAc), 3.5–3.65 (m, 5 H, –imidazolidine, H, H-2), 3.8–4.0 (m, 1 H, H-6), 4.15–4.25 (d, 2 H, H-7, H-7′), 4.98–5.05 (m, 3 H, H-3, H-4, H-5), 5.45 (s, 1 H, H-1), 6.68–6.8 (m, 6 H, Ph-H), 7.15–7.3 (m, 4 H, Ph-H).

Anal. Calcd for $C_{29}H_{34}N_2O_4$: C, 62.81; H, 6.18; N, 5.05. Found: C, 62.78; H, 6.28; N, 5.23.

*3,4,5,7-Tetra-O-acetyl-2,6-anhydro-1-O-tosyl-*D-*glycero*-D-*gulo-heptitol* (*VI*). V (20 g) was dissolved in CH_2Cl_2 (300 mL) and toluenesulfonic acid–H_2O (20 g) in acetone (200 mL) was added with stirring at 0 °C. After 1 h the precipitate was filtered off and washed with CH_2Cl_2 (200 mL), and the combined filtrates were extracted with aqueous 1% $NaHCO_3$ (200 mL) and then with water (2 × 200 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The solid residue was dissolved in glacial acetic acid (150 mL) and hydrogenated with Adams catalyst. After hydrogenation was complete, monitored by thin-layer chromatography (TLC) with benzene–methanol (4:1 v/v) as solvent, the catalyst was removed by filtration and the solution evaporated in vacuo to yield a syrupy residue. Without further purification, the product was tosylated in pyridine (100 mL) using tosyl chloride (8.1 g). After 24 h at room temperature, the reaction mixture was poured on ice water (200 mL) and extracted with $CHCl_3$ (400 mL); the organic layer was washed with water (500 mL) and dried over $MgSO_4$. Evaporation in vacuo yielded a residue which crystallized on adding 2-propanol. The crystalline material was recrystallized from ether to give pure VI (9.97 g, 53%): mp 143 °C; $[\alpha]^{22}{}_{578}$ –19.8° (c 1.0, $CHCl_3$); $\nu_{max}{}^{KBr}$ 1730 cm^{-1} (OAc); NMR (90 MHz, $CDCl_3$) δ 2.0–2.08 (3 s, 12 H, OAc), 2.45 (s, 3 H, Ph-CH_3), 3.6–3.8 (m, 2 H, H-1, H-1′), 4.0–4.2 (m, 4 H, H-6, H-7, H-7′, H-2), 4.8–5.2 (m, 3 H, H-3, H-4, H-5), 7.2–7.4, and 7.65–7.85 (2 d, 4 H, Ph).

Anal. Calcd for $C_{22}H_{28}O_{12}S$: C, 51.16; H, 5.46; S, 6.21. Found: C, 50.91; H, 5.46; S, 6.04.

*3,4,5,7-Tetra-O-acetyl-2,6-anhydro-1-deoxy-1-iodo-*D-*glycero*-D-*gulo-heptitol* (VII). A mixture of VI (10 g) and sodium iodide (4.45 g) in acetic anhydride (60 mL) was stirred at 125 °C for 30 min, then cooled and filtered, and the insoluble material was washed with acetone. The combined filtrates and washings were concentrated in vacuo to give a dark syrup. A solution of this syrup in chloroform (200 mL) was washed with aqueous 2% sodium hydrogen carbonate (2 × 200 mL) containing sodium bisulfite (2 × 100 mg) and then with water (200 mL), dried over $MgSO_4$, and concentrated. Crystallization and recrystallization from ether gave 7.77 g (85%) of pure VII: mp 132 °C; $[\alpha]^{22}{}_{578}$ –19.2° (c 1.0, chloroform); $\nu_{max}{}^{KBr}$ 1740 cm^{-1} (OAc); NMR (90 Hz, $CDCl_3$) δ 1.98, 2.00, 2.05, and 2.08 (4 s, 12 H, OAc), 3.00–3.85 (m, 4 H, H-1, H-1′, H-2, H-6), 4.2–4.3 (t, 2 H, H-7, H-7′), 4.8–5.25 (m, 3 H, H-3, H-4, H-5).

Anal. Calcd for $C_{15}H_{21}IO_9$: C, 38.15; H, 4.48. Found: C, 38.30; H, 4.47.

*3,4,5,7-Tetra-O-acetyl-2,6-anhydro-1-deoxy-*D-*gluco-hept-1-enitol* (*VIII*). VII (7.7 g) and AgF (9 g) were shaken vigorously in pyridine (25 mL) for 1 h; then the reaction mixture was stirred into ether (1 L) and again shaken for 1

h. The filtered mixture was washed with aqueous sodium thiosulfate (1%) (150 mL) and water (3 × 150 mL, dried ($MgSO_4$), and concentrated in vacuo to give an oil that crystallizes on adding a little ether. Recrystallization from ether gave VIII (4.5 g, 80%): mp 74 °C; $[\alpha]^{22}_{578}$ 54.0° (*c* 1.0, chloroform); ν_{max}^{KBr} 1730 (OAc), 1660 cm^{-1} (C=C); NMR (90 MHz, $CDCl_3$) δ 2.0–2.1 (3 s, 12 H, OAc), 3.65–3.9 (m, 1 H, H-5), 4.1–4.3 (m, 2 H, H-6, H-6′), 4.52 (t, 1 H, H-1), 4.76 (t, 1 H, H-1′) ($J_{1/1'}$ = 1.2 Hz), 5.0–5.4 (m, 3 H, H-2, H-3, H-4).

Anal. Calcd for $C_{15}H_{20}O_9$: C, 52.32; H, 5.86. Found: C, 52.15; H, 6.08.

Analytical Methods. All reactions involved in the preparation of derivatives V to VIII were monitored by TLC on silica gel F_{254} (E. Merck), using ether–petroleum ether (4:1 v/v) as solvent unless otherwise specified. Detection was effected by charring with sulfuric acid. Melting points are uncorrected. NMR spectra of V to VIII (internal standard, tetramethylsilane) were obtained with a Varian EM 390 (90 MHz) spectrometer; infrared spectra (KBr) were obtained with a Perkin-Elmer 137.

Enzymatic Studies. Enzymes. Highly purified α-glucosidase from *Candida tropicalis* var. *japonica*, which catalyzes the formation of α-D-glucose and α-D-glucosyl transfer products from maltose, dextrins, and α-D-glucosides (Sawai, 1958, 1960; Sawai & Hehre, 1962), was furnished by Professor Teruo Sawai. The enzyme had been purified by a multistage procedure (Sawai, 1967) and was free from β-glucosidase activity and from dextrinogenic action on starch. Purified rice α-glucosidase, purchased from Makor Chemicals, Inc., Jerusalem, Israel, catalyzed the hydrolysis of maltose at the rate of 55 μmol min^{-1} mg^{-1}. Crystalline sweet almond β-glucosidase (Boehringer Mannheim Corp., New York, NY) had a specific activity of 40 IU/mg as assayed with salicin. Highly purified glucodextranase from *Arthrobacter globiformis* I-42, an exo-α-1,6-glucanase that catalyzes the cleavage of β-D-glucose from the nonreducing ends of dextran chains (Sawai et al., 1976; Ohya et al., 1978), was the gift of Professor Sawai. It had a specific activity of 18 μmol min^{-1} mg^{-1} as assayed with dextran. *Saccharomyces* yeast α-glucosidase (maltase) (Boehringer Mannheim) and *Rhizopus niveus* glucoamylase (Seikagaku Kogyo, Ltd., Tokyo, Japan) were crystallized preparations of high specific activity.

Analytical Methods Used with Enzymatic Digest Components. Chromatography was carried out using Whatman No. 1 paper developed in descending manner with 1-butanol–ethanol–water (13:8:4 v/v). Staining was by a silver nitrate dipping method (Trevelyan et al., 1950), with papers hung in the air for 12 min following the alkaline reagent.

Quantitative determination of 1-deoxy-D-*gluco*-heptulose in solution, in the presence or absence of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol, was effected by a cuprimetric reduction procedure standardized concurrently with a reference sample of 1-deoxy-D-*gluco*-heptulose. Test samples (2.0 mL) were mixed with Somogyi (1952) reagent (2.0 mL); the mixtures were placed in a boiling water bath for 10.0 min and then cooled and treated with Nelson (1944) reagent (2.0 mL) and finally with distilled water (18 mL). Optical density measurements were made at 540 nm using a Gilford Microsample spectrophotometer. A linear relationship was found to exist between the optical density value and the quantity of sugar, in the range of 30 to 500 μg of 1-deoxy-D-*gluco*-heptulose.

Specific optical rotations were determined with a Rudolph and Sons Model 70 polarimeter and 2-dm tubes. The presence of methyl ketone was detected by treating 1 mL of a 1% solution of test sample with 0.4 mL of 4% I_2 – 6% KI and 0.2 mL of 1 N NaOH. Iodoform formation resulted in clouding with yellowish particles.

^1H and ^{13}C NMR Spectroscopy. Spectra at 23.5 kG were obtained using a Jeol PFT spectrometer interfaced with a Nicolet 1000 series computer. The instrument was operated in the pulse Fourier transform mode. ^{13}C NMR spectra were recorded in D_2O (99.7 atom % D) (Merck Sharpe and Dohme, Ltd., Pointe Claire-Dorval, Canada). Chemical-shift measurements were made at 25.15 MHz with respect to dioxane as an internal standard. Chemical shifts are reported with respect to tetramethylsilane, with dioxane at 67.4 ppm. ^1H NMR spectra were recorded in D_2O or in 0.05 M acetate-d_4–D_2O buffer of pD 5.3. The acetic-d_3 acid sodium salt (99+ atom % D) was purchased from Aldrich Chemical Corp. (Milwaukee, WI). Chemical-shift measurements were made with respect to 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal standard. Each spectrum consisted of 64 free induction decays using 2-s repetition times. The data were Fourier transformed.

Hydroxyl ^1H NMR spectra were recorded at 220 MHz using a Varian HR-220 spectrometer and Nicolet computer located at Rockefeller University. Spectra were recorded in 1:4 Me_2SO-d_6–acetone-d_6 (v/v) at ambient temperature, using Fourier transform techniques, and the data were treated for resolution enhancement using an optimum matched resolution filter. Tetramethylsilane was used as an internal standard.

*2,6-Anhydro-1-deoxy-*D*-gluco-hept-1-enitol* (*I*). 3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (VIII) (0.4 g) was deacetylated with 6 mL of 0.01 M sodium methoxide in dry methanol (25 °C, 3 h). The free enitol was stored at −20 °C as a stock solution in dry methanol. Known quantities were recovered, as needed, by solvent removal at 25 °C from measured volumes of the solution. On paper chromatography, the enitol (70 μg) produced a major spot, R_{GLC} 2.54, plus a just detectable spot that migrated at the same rate as 1-deoxy-D-*gluco*-heptulose (R_{GLC} 1.47). In tests with the Somogyi (1952) and Nelson (1944) reagents, 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol showed negligible reducing power ($<{}^1/_{30}$ of that of 1-deoxy-D-*gluco*-heptulose). It was essentially quantitatively hydrated by treatment with 0.02 N sulfuric acid (100 °C, 10 min); however, as judged by chromatography and analysis for reducing power, the enitol in solution at pH 5.2 was found to undergo little or no hydration in 2–3 h at 25 °C. The ^1H NMR spectrum of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol is described under Results.

*1-Deoxy-*D*-gluco-heptulose* (*II*) (*Reference Sample*). A solution containing 10.0 mg/mL of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol in 0.025 N sulfuric acid was heated (100 °C, 10 min), then cooled to 0 °C, neutralized, and brought to known volume. Chromatographic examination showed the presence of a single spot with R_{GLC} 1.47. The 1-deoxy-D-*gluco*-heptulose so prepared caused reduction of the Somogyi (1952) and Nelson (1944) reagents to the extent of ca. 75% of an equimolecular amount of D-glucose. For ^1H NMR spectroscopy (described under Results) a solution of the reference 1-deoxy-D-*gluco*-heptulose was brought to dryness (at 45 °C) in a RotoVap apparatus (Buchler Instruments, Inc., Fort Lee, NJ) and redissolved in D_2O to give a 28 mM solution.

Results

*Utilization of 2,6-Anhydro-1-deoxy-*D*-gluco-hept-1-enitol by Different *D*-Glucosylases: Identity of the Main Product.*

Table I: Enzymatic Hydration of 2,6-Anhydro-1-deoxy-D-*gluco*-hept-1-enitol

		digests (pH 5.2) incubated 15 min at 30 °C		
enzyme	mg/mL	reducing sugar formed, calcd as 1-deoxy-D-*gluco*-heptulose[a] (mM)	extent of hydration of 70 mM substrate (%)	app sp act. (μmol min^{-1} mg^{-1})
C. tropicalis α-glucosidase	0.08	15.8	23	13.2
rice α-glucosidase	1.0	15.0	21	1.0
A. globiformis glucodextranase	1.5	13.5	19	0.6
sweet almond β-glucosidase	2.0	10.4	15	0.35

[a] Corrected for 2.1 mM of apparent 1-deoxy-D-*gluco*-heptulose present in the concurrently incubated enitol-buffer control.

A number of D-glucosyl mobilizing enzymes of different types were examined for the ability to attack 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol. Among the enzymes tested, *Saccharomyces* yeast maltase and *Rhizopus glucoamylase* appeared to lack this ability. However, several other D-glucosylases were found to effectively utilize the compound. The following results, for example, were obtained upon incubating (at 30 °C for 15 min) mixtures of 70 mM enitol with 0.08 mg/mL of *Candida tropicalis* α-glucosidase, 1 mg/mL of rice α-glucosidase, 1.5 mg/mL of *Arthrobacter globiformis* glucodextranase, or 2 mg/mL of sweet almond β-glucosidase in 0.05 M acetate buffer (pH 5.2). On paper chromatography, each digest was distinguished from an incubated enitol–buffer control by the presence of a prominent, strongly reducing spot having the same migration value (R_{GLC} 1.47) as authentic 1-deoxy-D-*gluco*-heptulose, and by an enitol spot (R_{GLC} 2.54) smaller than the control. The digests with the two α-glucosidases and with the glucodextranase also showed several weakly stained spots which migrated more slowly than 1-deoxy-D-*gluco*-heptulose and which have proved to be transfer products.[2] Analysis of the digests by the Somogyi (1952) method showed (Table I) that appreciable levels of reducing sugar (10–16 mM, calculated as 1-deoxy-D-*gluco*-heptulose) has been produced during the brief (15 min) incubation with the enzymes in the concentrations used.

Evidence that this is, indeed, 1-deoxy-D-*gluco*-heptulose was obtained using samples of the reducing sugar (150–250 mg) recovered by preparative chromatography from digests of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol with three of the enzymes (*Candida* and rice α-glucosidases, and *Arthrobacter* glucodextranase).[2] Each sample was found to be indistinguishable from the authentic sugar in chromatographic behavior, in reducing power as measured by the Somogyi (1952) method, and in giving a positive iodoform test for methyl ketone (cf. Nelkin, 1972). Information on the structural form and anomeric configuration of the isolated (equilibrated) 1-deoxy-D-*gluco*-heptulose was obtained as follows. ^{1}H-decoupled ^{13}C NMR spectra of the sugar samples from the digests with the three enzymes proved to be superimposable. Each showed seven resonance peaks (Figure 1), signifying that equilibrated 1-deoxy-D-*gluco*-heptulose comprises essentially a single anomeric form. The optical rotations of the three isolated sugar samples were found to center at $[\alpha]^{23}_{D}$ +72° (c 2, water), unchanged on standing, a reasonable indication that each 1-deoxy-D-*gluco*-heptulose isolate is of α configuration since the closely related D-*gluco*-heptulose (known to be the α anomer) has a similar rotation, $[\alpha]^{20}_{D}$ +67.5° (Austin, 1930; Schaffer & Isbell, 1962).

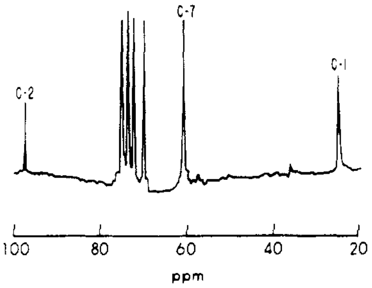

FIGURE 1: Proton-decoupled, Fourier transform ^{13}C NMR spectrum of 1-deoxy-D-*gluco*-heptulose (0.6 M in D_2O) isolated from a digest of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol with *C. tropicalis* α-glucosidase. Undecoupled spectrum allowed unequivocal assignment of three of the seven resonances: C-1 (quartet) 25.36 ppm, C-2 (singlet) 98.23 ppm, C-7 (triplet) 61.40 ppm, relative to tetrametylsilane.

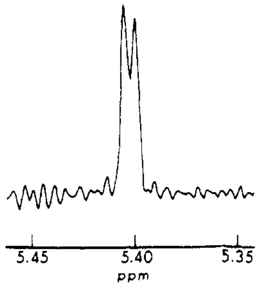

FIGURE 2: Fourier transform 220-MHz ^{1}H NMR spectrum of the 2-hydroxyl proton (5.40 ppm, $J_{OH\text{-}2,H\text{-}3}$ = 1.2 Hz) of a sample of 1-deoxy-D-*gluco*-heptulose (0.1 M in 20% $Me_2SO\text{-}d_6$ and 80% acetone-d_6) isolated from a digest of enitol with rice α-glucosidase. The chemical shift is relative to tetramethylsilane.

Unequivocal evidence that 1-deoxy-D-*gluco*-heptulose in the equilibrated state has the α configuration was obtained from the 220-MHz ^{1}H NMR spectrum of a deionized sample of the sugar recovered from a digest of enitol with rice α-glucosidase. The spectrum was recorded at room temperature with a 0.1 M solution of the sugar in 20% $Me_2SO\text{-}d_6$ and 80% acetone-d_6, under which conditions the resonances of the hydroxyl protons are revealed. The results show that all five hydroxyl group resonances are present between 3.7 and 5.4 ppm, with the furthest downfield resonance assignable to the anomeric 2-hydroxyl proton. Figure 2 shows the spectrum of the 2-hydroxyl proton, which is split into a doublet, $J_{OH\text{-}2,H\text{-}3}$ = 1.2 Hz, centered at 5.40 ppm. The J value of 1.2 Hz is diagnostic of the presence of an axial anomeric hydroxyl proton and a vicinal axial proton (Jochims et al., 1967) and shows that the 1-deoxy-D-*gluco*-heptulose is of α-pyranose form and C1 conformation. The rest of the spectrum is consistent with this structure, with the C-1 (methyl group) resonance located at 1.36 ppm relative to tetramethylsilane.

*^{1}H NMR Study of the Enzymatically Catalyzed Conversion of 2,6-Anhydro-1-deoxy-*D*-gluco-hept-1-enitol to 1-Deoxy-*D-

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[2] These less abundant components have been isolated (along with the reducing sugar) from large-scale digests prepard with the three enzymes. Studies in progress show that all of these components are 1-deoxy-D-*gluco*-heptulosyl transfer products arising from 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol as the glycosyl donor (unpublished results).

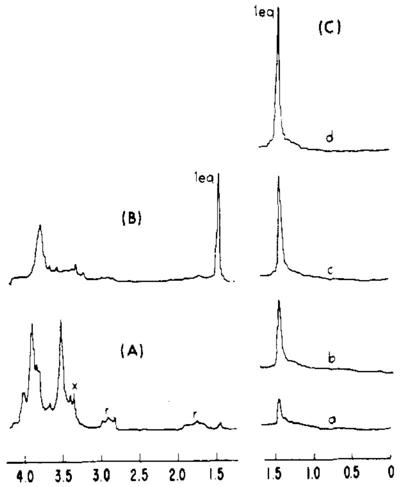

FIGURE 3: ^{1}H NMR Fourier transform spectra at 100 MHz, with resonances in parts per million relative to 3-(trimethylsilyl)-propanesulfonic acid sodium salt. (A) 2,6-Anhydro-1-deoxy-D-*gluco*-hept-1-enitol (56 mM in D_2O); r, resonance associated with the innernal reference standard; x, artifact. (B) 1-Deoxy-D-*gluco*-heptulose (28 mM in D_2O) prepared from 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol by mild acid treatment; 1eq, resonance peak of H-1, H-1′, and H-1″ protons of the equatorial methyl group of 1-deoxy-α-D-*gluco*-heptulose. (C) H-1 and H-1′ resonances of 1-deoxy-D-*gluco*-heptulose formed in a digest of 70 mM 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol with 0.22 mg/mL of *C. tropicalis* α-glucosidase in 0.05 M acetate-d_4–D_2O buffer (pD 5.3) at 21 °C; a, 2–4 min; b, 13–15 min; c, 28–30 min; d, 210 min. Line broadening of the methyl resonance occurs due to deuterium incorporation at this position in the course of the hydration reaction.

gluco-heptulose. To understand the reaction(s) whereby α- and β-glucosidases and glucodextranase lead to the formation of 1-deoxy-D-*gluco*-heptulose from enitol, a study was undertaken to determine the anomeric configuration of the sugar as it is formed in digests with the individual enzymes. ^{1}H NMR spectroscopy was used to detect and differentiate between the equatorial and axial C-1 (methyl group) protons of the 1-deoxy-D-*gluco*-heptulose in the digests. Control spectra were recorded with solutions in D_2O of 56 mM 2,6-anhydro-1-D-*gluco*-hept-1-enitol and of 28 mM 1-deoxy-D-*gluco*-heptulose (prepared by acid hydration of the enitol). The enitol spectrum (Figure 3A) shows a complex of proton resonances between 3.4 and 4.0 ppm, with large peaks at 3.50 and 3.88 ppm. The small signal at 1.45 ppm is assigned, for reasons given below, to the C-1 protons from a trace amount of 1-deoxy-α-D-*gluco*-heptulose. The spectrum of the equilibrated 1-deoxy-D-*gluco*-heptulose (Figure 3B) lacks the resonances at 3.50 and 3.88 ppm, but shows peaks at 3.78 and 1.45 ppm. The latter is assigned to the H-1, H-1′, and H-1″ protons of the methyl group bound equatorially to the anomeric carbon atom (C-2) of 1-deoxy-α-D-*gluco*-heptulose. The upfield location and high intensity of the resonance are characteristic of methyl group protons; the α configuration of the equilibrated sugar had been shown by experiments already described.

For ^{1}H NMR study of the hydration of enitol by several D-glucosylases, the substrate and individual enzymes were treated to exchange their labile hydrogens for deuterium atoms. Samples (42 μmol) of the enitol were dissolved in 5 mL of methyl alcohol-d_1 and recovered, just before use, by removing solvent at 25 °C in a vacuum evaporator. Each enzyme was exhaustively dialyzed (24 h, 6 °C) vs. 0.05 M acetate-d_4–D_2O buffer of pD 5.3. In the case of glucodextranase, 0.005 M $CaCl_2$ was incorporated in the buffer to stabilize the enzyme (Sawai et al., 1976).[3] At time zero, test mixtures were prepared by adding 0.60 mL of buffered, deuterium-labeled enzyme (0.12 mg of *C. tropicalis* α-glucosidase; 1.2 mg of rice α-glucosidase; 2.3 mg of glucodextranase; 3.0 mg of β-glucosidase), or 0.60 mL of the pD 5.3 buffer without enzyme, to 42 μmol of the deuterium-exchanged 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol. Thus, the concentration of each enzyme was ca. 2.5 times that employed in the experiment of Table I, so that substrate utilization would be essentially complete by 45 min. Each test mixture was transferred to a 5-mm NMR tube, and Fourier-transformed ^{1}H NMR spectra were recorded at intervals during 3+ h at 26 ± 1 °C. The upfield (0.0 to 1.6 ppm) regions of the resulting spectra are shown in Figure 3C for the digest with *C. tropicalis* α-glucosidase, and in Figure 4 for the digests with β-glucosidase and with glucodextranase.

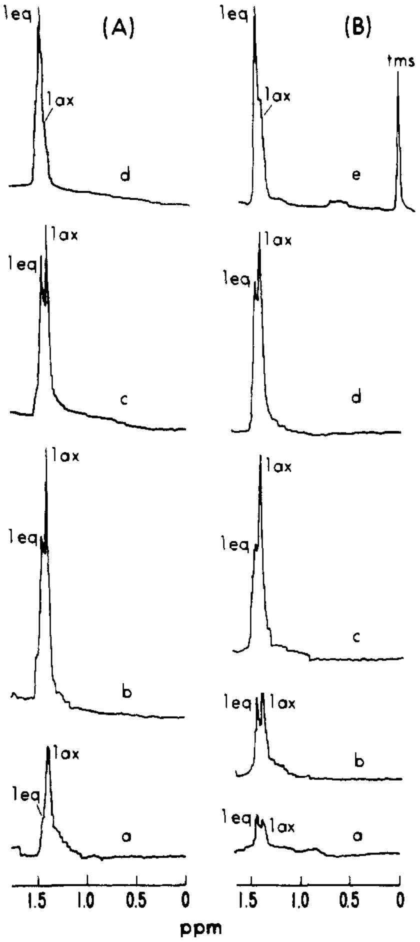

FIGURE 4: ^{1}H NMR spectra at 100 MHz showing H-1 and H-1′ resonances of 1-deoxy-D-*gluco*-heptulose formed in enzymatic digests of 70 mM 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol in 0.05 M acetate-d_4–D_2O buffer (pD 5.3), incubated at 21 °C. (A) Digest with 5.0 mg/mL sweet almond β-glucosidase; a, 13–15 min; b, 48–50 min; c, 90–92 min; d, 285 min. (B) Digest with 3.8 mg/mL *A. globiformis* "inverting" glucodextranase: a, 2–4 min; b, 12–14 min; c, 28–30 min; d, 90–92 min; e, 300 min (with 3-(trimethylsilyl)propanesulfonic acid sodium salt added). Line broadening of the methyl resonance occurs due to deuterium incorporation at this position in the course of the hydration reaction.

In the case of the *C. tropicalis* α-glucosidase–enitol digest, a single upfield resonance peak at 1.45 ppm is present in all spectra. The size of this peak greatly increases as incubation

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[3] The presence of calcium chloride did not influence the chemical shift of protons in control spectra of 1-deoxy-D-*gluco*-heptulose or enitol, or cause any detectable breakdown of the latter.

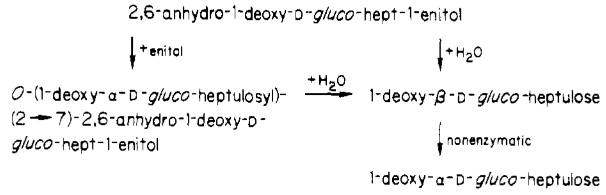
extends from 2–4 to 28–30 min (Figure 3C, spectra a–c) and is maximal and constant by 48–50 min (illustrated in spectrum d, recorded at 210 min). The signal at 1.45 ppm is that of the equatorial H-1 and H-1′ methyl group protons of 1-deoxy-α-D-*gluco*-heptulose; ^{2}H is incorporated into the H-1″ position. Although not illustrated, a similar set of spectra was obtained with the rice α-glucosidase–enitol digest. A control mixture of 70 mM enitol in the pD 5.3 buffer showed a smaller 1.45-ppm resonance signal than that seen with the *C. tropicalis* digest at 2–4 min; its size remained essentially unchanged during 3 h at 26 °C.

As shown in Figure 4A, the action of sweet almond β-glucosidase in hydrating 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol is quite different from that of the two α-glucosidases. Each of the recorded ^{1}H NMR spectra of the β-glucosidase digest shows two resonance signals in the methyl group region. Spectrum a (13–15 min incubation) shows a substantial resonance peak at 1.40 ppm, not seen in enitol–buffer control spectra, plus a small 1.45-ppm signal of about the same size as found in a concurrent enitol–buffer control The 1.40-ppm resonance, attributable to the axial methyl group protons of 1-deoxy-β-D-*gluco*-heptulose, thus marks the product that is formed from enitol by β-glucosidase action. This peak increases during the period of enzymatic activity and reaches maximal height after 48–50 min (spectrum b). Thereafter, it gradually diminishes, though it remains discernible at 285 min (spectrum d). In contrast, the peak at 1.45 ppm (especially its size relative to the 1.40-ppm peak) increases with time and continues to do so long after the enzymatic action on the enitol is expected to have reached completion. Only in spectrum d (285 min) is the 1.45-ppm signal predominant over that at 1.40 ppm. Both the delayed appearance of the 1.45-ppm resonance and its increase in the postenzymatic period signify that the accumulation of 1-deoxy-α-D-*gluco*-heptulose in the digest is due to slow anomerization of the enzymatically formed β anomer. From the ratio of the two peaks at 90 min, compared to that at 285 min, the conversion coefficient, k_D, was estimated at ~0.002 min^{-1}. This is comparable to the k_D values of other sugars, e.g., 0.0022 min^{-1} for D-glucose mutarotation in D_2O (Pacsu, 1933; Nicolle & Weisbuch, 1955). There is no evidence to suggest that any of the α anomer is formed directly from the enitol by the action of β-glucosidase.

The action of the inverting bacterial exo-α-1,6-glucanase (glucodextranase) on enitol resembles but is more complex than that of β-glucosidase. Each of the recorded ^{1}H NMR spectra (Figure 4B, a–e) shows resonance peaks at both 1.40 and 1.45 ppm. In this case it is clear that, early in incubation, both resonances reflect the presence of enzymatically formed products since the enitol sample used in this experiment was free from accompanying 1-deoxy-D-*gluco*-heptulose. That is, both resonances are present in spectra a and b of the digest (at 2–4 and 12–14 min), whereas neither resonance was detected in spectra of the concurrent enitol–buffer control.

The 1.40-ppm resonance peak, representing the axial H-1 and H-1′ methyl group protons of 1-deoxy-β-D-*gluco*-heptulose, increases both absolutely, and relative to the 1.45-ppm peak, during the first 30 min of incubation (spectra a–c). Thereafter, it diminishes relative to that at 1.45 ppm—notably between 90 and 300 min (spectra d and e) after enzyme-catalyzed reactions presumably have been completed. The final dominance of the 1.45-ppm resonance thus is the result of nonenzymatic anomerization of the β to the stable α form of 1-deoxy-D-*gluco*-heptulose. As noted above, however, the 1.45-ppm resonance seen in spectra recorded early in digestion cannot be attributed to such anomerization except perhaps in small part.

Scheme I



Discussion

A novel demonstration is presented of the capacity of D-glucosylases of several different types to catalyze the conversion of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol into 1-deoxy-D-*gluco*-heptulose, and to do so in individually stereospecific ways. These findings extend the variety of productive glycosylation reactions known to be catalyzed from substrates lacking α- or β-anomeric configuration and/or a glycosidic bond (see introductory statement for references), and illustrate anew how the study of such reactions can provide fresh insight into the catalytic mechanism of carbohydrases.

A key requirement for elucidating the reactions with enitol catalyzed by *Candida* and rice α-glucosidases, sweet almond β-glucosidase, and *Arthrobacter* glucodextranase was the ability to identify the anomeric form(s) of the 1-deoxy-D-*gluco*-heptulose produced in the individual reactions. Since the one published description of this sugar (Nelkin, 1972) carries no information on configuration, a reliable reference point was first established through studies of the sugar under equilibrium conditions. ^{13}C NMR spectra of isolates of the sugar showed them to comprise a single anomeric form. An indication that this is 1-deoxy-α-D-*gluco*-heptulose was found in the similarity between the specific optical rotation of the isolates, +72°, and of α-D-*gluco*-heptulose, +67.5°. Definitive proof was obtained using ^{1}H NMR spectroscopy under conditions where the solvent does not lead to rapid hydroxyl proton exchange. Jochims et al. (1967) had shown, with various pyranose sugars, that small (0.7–1.5 Hz) long-range couplings appear between and only between the protons of an axial hydroxyl group vicinal to an axial proton. Thus, D-*gluco*-heptulose was found to exist exclusively as the α anomer in C1 conformation with a $J_{\mathrm{OH\text{-}2,H\text{-}3}}$ coupling constant of 0.9 Hz. In a spectrum recorded under similar conditions, 1-deoxy-D-*gluco*-heptulose from a digest of enitol with rice α-glucosidase showed a similar small (1.2 Hz) coupling for the C-2 hydroxyl group. The assignment is unambiguous since the four remaining hydroxyl proton resonances of the sugar showed coupling to geminally bonded protons with splittings of 4–5 Hz. These results provide direct evidence that equilibrated 1-deoxy-D-*gluco*-heptulose exists as the α anomer. All substituents, as in the case of D-*gluco*-heptulose, are optimally oriented for stability of the α-pyranose in C1 conformation. The 1-methyl group, the 3-, 4-, and 5-hydroxyl groups, and the 7-primary carbinol group are all equatorial; any anomeric effect of the axial 2-hydroxyl group would further enhance the stability.

With this knowledge, the sole upfield resonance present in ^{1}H NMR spectra of equilibrated 1-deoxy-D-*gluco*-heptulose in D_2O (at 1.45 ppm relative to 3-(trimethylsilyl)propanesulfonic acid sodium salt) can with confidence be assigned to the equatorially oriented methyl group protons of the α anomer.[4] Using this assignment, ^{1}H NMR spectra of enzymatic digests of enitol revealed that 1-deoxy-α-D-*gluco*-heptulose is formed by the actions of *Candida* and rice α-glucosidases. In contrast, ^{1}H NMR spectra of the digest with sweet almond β-glucosidase showed the early formation of a resonance peak at 1.40 ppm, the later appearance of resonance at 1.45 ppm, and the final replacement of the 1.40 by the 1.45 peak after the enzymatic reaction had reached completion. These findings leave no doubt that 1-deoxy-β-D-*gluco*-heptulose is produced by the action of β-glucosidase on enitol, and that the α anomer is formed entirely as the result of slow nonenzymatic anomerization. These results with the α- and β-glucosidases show that the anomeric form of 1-deoxy-D-*gluco*-heptulose created by each enzyme is the same as that of its reaction products formed from D-glucosidic substrates. The actions of these enzymes in creating anomeric specificity without the guidance of substrate configuration are very similar to those reported recently for the *Candida* α-glucosidase and sweet almond β-glucosidase in catalyzing the stereospecific trans hydration of D-glucal to the corresponding α and β anomers of 2-deoxy-D-glucose, respectively (Hehre et al., 1977). They likewise parallel the actions of *Escherichia coli* β-galactosidase in effecting the formation of glyceryl 2-deoxy-β-D-galactoside from D-galactal and glycerol (Lehmann & Schröter, 1972; Lehmann & Zeiger, 1977) and of glyceryl 2,6-anhydro-1-deoxy-D-*galacto*-heptuloside from 1-deoxy-β-D-*galacto*-hept-1-enitol and glycerol (Brockhaus & Lehmann, 1977).

To define a possible mechanism for the specific hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol catalyzed by the α- and β-glucosidases, we assume that the catalytic functions of these enzymes are served by two suitably disposed functional groups at the active site, possibly two carboxyl groups. One group would function as a specific or general acid in conventional reactions by protonating the glycosidic oxygen atom of D-glucosidic substrates, while the other, as a carboxylate anion, could act as a charge-stabilizing group or nucleophilic-specific base. The enitol is envisioned as binding to each glucosidase with its exocyclic double bond positioned between the pair of carboxyl groups. The first catalytic step would involve protonation of the vinylic double bond, leading to a transient glycosyl carbonium ion. The present data do not indicate from which direction the protonation occurs. However, it could be from the same carboxyl group that protonates the glycosidic oxygen atom of D-glucosides. Or, as suggested by the results obtained with the specific trans hydration of D-glucal by *Candida* α-glucosidase and sweet almond β-glucosidase (Hehre et al., 1977), protonation of the hept-1-enitol by these enzymes could occur from the direction of the other carboxyl group previously believed to be restricted to catalytic functions associated with its anionic form. Experiments are underway to resolve this question. The second step of the proposed reaction would involve the selective attack of water (D_2O) or other acceptor molecule[2] on the glycosyl carbonium ion intermediate, possibly with assistance (general-base catalysis) from one of the carboxyl groups, to form the anomeric products. Thus, the α or β configuration of the end product in each reaction is believed to arise in the second step.

In the case of glucodextranase acting upon 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol, ^{1}H NMR spectra showed the presence of both 1.40- and 1.45-ppm resonances of comparable intensity very early in digestion. Thereafter, during the period of enzymatic activity, the 1.40-ppm signal representing the C-1 protons of 1-deoxy-β-D-*gluco*-heptulose increased greatly relative to that at 1.45 ppm. The first mentioned finding might suggest that the enzyme catalyzes the hydration of enitol in two stereochemically opposite ways, to produce both anomers of 1-deoxy-D-*gluco*-heptulose. This cannot be rigorously excluded, but it appears unlikely since the ratio between the two products (and the two resonances) should then have remained relatively constant. The key to understanding the basis for the experimental findings comes from the following observations. Studies in progress (to be reported separately) show that one of the glycosyl transfer products formed from enitol in ca. 10% yield by the action of glucodextranase[2] arises early in digestion and has the structure, *O*-(1-deoxy-α-D-*gluco*-heptulosyl)-(2→7)-2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol. ^{1}H NMR spectra of the isolated compound in D_2O reveal, in the upfield region, a single peak at 1.46 ppm. It appears most likely that the small 1.45-ppm (±0.01 ppm) signal seen early in enitol–glucodextranase digests (Figure 4B, a) is that of the C-1 protons of the 1-deoxy-α-D-*gluco*-heptulosyl moiety of this transfer product (whose presence in the digest is thus accounted for) rather than of free 1-deoxy-α-D-*gluco*-heptulose. Moreover, part of the 1-deoxy-β-D-*gluco*-heptulose (1.40-ppm resonance) must arise from hydrolysis of the α-glycosidic linkage of this transfer product with inversion of configuration, a reaction readily catalyzed by the enzyme (unpublished experiments). Scheme I illustrates the reactions that we consider to occur in the enitol digest with glucodextranase.

The significance of these results with glucodextranase is that they provide the first demonstration of the ability of an "inverting" enzyme to catalyze glycosylation reactions in which specific anomeric configuration is created de novo from a glycosyl donor lacking α or β configuration. Until now, the ability to bring about such reactions has been reported only for glycosidases, whose reactions with conventional substrates provide configurational retention. That glucodextranase is able to create either α- or β-anomeric configuration in the products of the reaction with the enitol raises the fascinating question of what determines which particular configuration is to be created from a donor substrate lacking an asymmetric anomeric carbon atom. A clue is found in the unique complementariness between these findings with glucodextranase and recent results obtained with β-amylase (Hehre et al., 1979). Glucodextranase, which cleaves β-D-glucose from the ends of α-1,6-linked dextran chains, would appear to catalyze the formation of 1-deoxy-β-D-*gluco*-heptulose from enitol by both a direct hydration reaction and a two-step process comprising the formation of a 1-deoxy-α-D-*gluco*-heptulosyl transfer product (by a reaction requiring two molecules of substrate), followed by hydrolysis of this intermediate with configurational inversion. β-Amylase, which cleaves β-maltose from the ends of α-1,4-linked starch chains, catalyzes the formation of β-maltose from α-maltosyl fluoride by direct hydrolysis and also from β-maltosyl fluoride by a two-step process involving the formation of an α-maltosyl transfer product (by a reaction

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[4] Our findings that the equatorially oriented methyl group protons of the α anomer resonate at 1.45 ppm and those oriented axially (β anomer) resonate at 1.40 ppm are in accord with the well-established rule for D-pyranoses in C1 conformation that the equatorial anomeric proton of an α anomer resonates at lower field than the axial anomeric proton of the corresponding β form (Lemieux & Stevens, 1966). They also agree with published observations that the equatorially oriented methyl group protons of methyl β-D-glycopyranosides (of C1 conformation) appear at slightly lower fields (<0.2 ppm) than the axial methyl group protons of the corresponding methyl α-D-glycopyranosides (Perlin et al., 1970; Overend, 1972). The conclusion of James & Angyal (1972) and Angyal et al. (1976) that the C1 methyl group protons of 1-deoxy-α-D-fructopyranose resonate at a higher field (1.30 ppm) than those of the β anomer (1.46 ppm) might appear contrary to our findings, but would be rationalized with all of the above if both anomers of 1-deoxy-D-fructopyranose were of 1C conformation.

requiring two molecules of substrate), followed by hydrolysis of this intermediate with inversion. A catalytic mechanism is thus operative in both exo-α-glucanases that leads to a sugar of β-anomeric form when water is the acceptor, and to a glycosyl transfer product of the opposite (α) configuration when a second substrate molecule is the acceptor. The findings with glucodextranase reveal that the type of acceptor is the *only* external guide needed in determining which of the two complementary reactions is catalyzed. We envision that the initial action of glucodextranase on enitol is to protonate the exocyclic double bond to form a transient 1-deoxy-D-*gluco*-heptulosyl oxycarbonium ion–enzyme complex. Breakdown of this complex would then occur in either of two ways, each involving a different catalytic group (possibly carboxyl residues) at the active site. One reaction path, determined by the directed attack of water on the intermediate, would lead to the formation of 1-deoxy-β-D-*gluco*-heptulose and to protonation of a functional group of the enzyme, possibly by way of general-base catalysis. The second path, determined by an oppositely directed attack on C-2 by the 7-hydroxyl group of a second enitol molecule bound at the acceptor site, would lead to the formation of an α-2,7-linked glycosyl transfer product and to protonation of a different group of the enzyme by way of general-base catalysis. These results again emphasize the catalytic flexibility and reversible general-acid and -base properties of the active-site functional groups of glycosidases (Hehre et al., 1979).

In sum, glucodextranase as well as certain α- and β-glucosidases have been shown to catalyze specific glycosylation reactions with 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol, a compound that lacks α- or β-anomeric configuration or a glycosidic bond. The possiblity of obtaining such results is envisioned in the concept that glycoside hydrolases (EC 3.2) and glycosyl transferases (EC 2.4) are *glycosylases*, whose reactions effect a rather simple chemical change, the interchange of a glycosyl residue and a proton (Hehre et al., 1973). Thus, in order to serve as a donor substrate, a compound presumably would need no more than the ability to bind in a suitable alignment at the catalytic site of an enzyme and yield a glycosyl residue upon protonation. In catalyzing reactions with the enitol, in which specific anomeric configuration and specific glycosidic linkages are created de novo, each of the D-glucosylases studied has given evidence of having broader catalytic capabilities than required for their actions upon glycosidic substrates. The findings provide strong new evidence for the proposal (Hehre et al., 1979) that emphasizes the functional flexibility of the catalytic groups of glycosylases.

Acknowledgments

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