Rhizopus niveus glucoamylase and Arthrobacter globiformis glucodextranase, which catalyze the hydrolysis of starch and dextrans, respectively, to form D-glucose of inverted (β) configuration, were found to convert both α- and β-D-glucosyl fluoride to β-D-glucose and hydrogen fluoride. Each enzyme directly hydrolyzes α-D-glucosyl fluoride but utilizes the β-anomer in reactions that require 2 molecules of substrate and yield glucosyl transfer products which are then rapidly hydrolyzed to form β-D-glucose. Various D-glucopyranosyl compounds serve as acceptors for such reactions. Mixtures of β-D-glucosyl fluoride and methyl-α-D-glucopyranosyl-[1-14C]glucopyranoside and α-D-glucosyl fluoride (1→6)-α-D-glucopyranoside. Glucoamylase produced more of the α-maltoside; glucodextranase produced more of the α-isomaltoside. Thus, both exo-α-glucan hydrolyses" emerge as glucosylases that catalyze stereospecifically complementary hydrolytic and transglycosylative reactions with glucosyl donors of opposite configuration. These results not only provide a new view of the catalytic capabilities of these supposedly strict hydrolyses; they also furnish a basis for defining a detailed mechanism for catalysis. Present results, together with those of several recent studies obtained from this laboratory (especially similar findings obtained with β-amylase acting on α- and β-maltosyl fluoride (Hehre, E. J., Brewer, C. F., and Genghof, D. S. (1979) J. Biol. Chem. 254, 5942-5950), provide strong new evidence for the functional flexibility of the catalytic groups of carbohydrases.

The study of enzyme-catalyzed glycosylation reactions that occur without glycosidic bond cleavage has emerged as an effective means of gaining deeper understanding of the catalytic capabilities and mechanism of carbohydrases (1-8). For example, fresh insight was recently obtained into the functioning of β-amylase through the demonstration (7) that this classic exoglucanase utilizes both α- and β-maltosyl fluoride as substrates in reactions leading to the formation of β-maltose and hydrogen fluoride. β-Amylase was found to hydrolyze α-maltosyl fluoride directly, and evidence was obtained suggesting that it catalyzes maltosyl transfer from β-maltosyl fluoride to a second substrate molecule to form an α-1,4-linked higher saccharide, which is then rapidly hydrolyzed to yield β-maltose. Although the transfer product was not directly observed, the findings strongly suggested that β-amylase has a second (nonhydrolytic) mode of action in addition to its long known hydrolytic action and that the functional groups at the active site of the enzyme alternate in their catalytic roles to promote reactions which are not directly related by microscopical reversibility. Indeed, Hehre et al. (7) envision such functional flexibility as an attribute of the catalytic groups of glycosylases in general.

We find now that the ability to utilize both α- and β-anomeric forms of a substrate is not confined to β-amylase but is shared by two D-glucosyl mobilizing exo-α-glucanases, the glucoamylase of Rhizopus niveus and the glucodextranase of Arthrobacter globiformis. These enzymes catalyze reactions with α- and β-D-glucopyranosyl fluoride that are complementary to each other in the same sense as observed (7) with β-amylase acting on α- and β-maltosyl fluoride. Present findings with glucoamylase and glucodextranase, however, go beyond those obtained with β-amylase in an important respect. Glucosyl transfer products have been recovered and characterized in each case, thus providing unequivocal evidence of the ability of inverting exoglucanases to catalyze glycosylation reactions beyond hydrolysis and reversal (condensation) reactions.

R. niveus glucoamylase is representative of the extensively studied fungal glucoamylases which hydrolyze the terminal α-1,4- and α-1,6-glucosidic linkages of starch and related glucosaccharides (9-11) to produce β-D-glucose (12-15) and which similarly hydrolyze α-D-glucosyl fluoride (16-18). The R. niveus enzyme also catalyzes the condensation of glucose to form α-1,4- and α-1,6-linked glucosaccharides (19, 20), using β-D-glucose as the specific donor (20). Aside from catalyzing condensations, however, glucoamylases have been considered unable to effect glucosyl transfer reactions (11, 21-26). Likewise, A. globiformis glucodextranase (27, 28), which hydrolyzes the terminal α-1,6- and α-1,4-glucosidic linkages of dextrans and related glucosaccharides to form β-D-glucose, has shown no sign of glucosyl-transferring activity when acting on dextran (27).
Catalytic Flexibility of Glucoamylase and Glucodextranase

Based on the stereospecificity of the reactions catalyzed with α- and β-β-D-glucosyl fluoride, a detailed description is presented of the mechanism whereby these exoglucanases effect hydrolytic or glucosyl transfer reactions, respectively, with substrates of α- or β-form. The significance of the alignment of the present results with the concept that glycosidases and glycosyltransferases are interrelated glycosylases (catalysts of the interchange of a glycosyl residue and a proton) with functionally flexible catalytic groups is discussed.

EXPERIMENTAL PROCEDURES

Enzymes—Twice recrystallized, α-amylase-free glucoamylase from R. delemar (Megazyme Ltd., Dublin, Ireland) was used after ultracentrifugation and electrophoresis. Stock solutions were prepared by dissolving weighed amounts of the enzyme in ice-cold water or buffer. Purified glucodextranase from A. globiformis 142 (27, 28) catalyzed p-glucose release from dextran at the rate of 18 μmol/min/mg. Stock solutions were maintained in 0.05 M acetate buffer (pH 5.2) containing 0.005 M calcium chloride. Neither the glucoamylase (0.5 mg/ml) nor the glucodextranase (0.15 mg/ml) caused detectable release of glucose (glucose oxidase method) from 18 mm methyl, phenyl, or p-nitrophenyl α-D-glucosides (30 °C, 5 h). Cyclodextrin glycosyltransferase from Bacillus megaterium strain 5, free from hydrolitic activity for starch, assayed 17 units/mg based on the decline in I2-staining of soluble starch substrate (29). Dextrantransase from Leuconostoc mesenteroides B-512F (25 dextrantransase units/mg) was kindly supplied by Dr. Allene Jeanes. Purified Saccharomyces a-glucosidase and sweet almond β-glucosidase were purchased from Boehringer Mannheim Corp.

α-D-Glucopyranosyl Fluoride—Crystalline tetra-O-acetyl-α-D-glucopyranosyl fluoride, [α]D +89.1° (c, 3 in chloroform) (Ref. 30), was prepared from the direct fluorination of D-glucose pentaacetate with liquid anhydrous hydrogen fluoride at −98 °C (31, 32). On deacetylation with 0.5 M sodium methoxide in methanol (4 °C, 3 h), 11.4 g of the tritacetate yielded 5.2 g of crystalline α-D-glucopyranosyl fluoride. After two recrystallizations from methanol, this had [α]D +79.6° (c, 1.5 in water) (Ref. 32), [α]D +97.6° (water): gave −96% of the theoretical amounts of fluoride and glucose on hydrolysis by 0.2 N sulfuric acid (100 °C, 10 min) but was accompanied by <2% of free fluoride. To remove the latter, 400 mg of the α-D-glucopyranosyl fluoride were chromatographed on a column (1.6 × 30 cm) of dry Silica Gel 60 (E. Merck), using absolute ethanol/ethyl acetate (2:3) as solvent. The pure product (217 mg), crystallized from methanol, had <0.1% free fluoride ion. 19F NMR spectra, recorded in 0.1 M acetate-d3/D2O buffer of pD 5.5, showed a chemical shift of −152.96 ppm (relative to external trifluoromethane in acetone-d6) and J(H-F) 53.4 Hz, J(α-F) 28.6 Hz, in agreement with the values reported by Hall et al. (33). 1H NMR spectra are described under "Results." 1H- and 13C-NMR Spectra—Tetra-O-acetyl-β-D-glucopyranosyl fluoride was synthesized from tetra-O-acetyl-α-D-glucopyranosyl bromide (Fischer Chemical Co.) and silver fluoride (Ventron Corp., Danvers, MA) by an established method (34). After recrystallization from ether, this had [α]D +22.0° (c, 1.1 in chloroform) (Ref. 34), [α]D +21.9° (chloroform) and m.p. 83-84°C (Ref. 35, 86°C; Ref. 36, 87-89°C; Ref. 37, 89°C): it gave a single spot (Rf 0.44) on thin layer chromatography (ether-petroleum ether 1:3). 19F NMR spectra, recorded in acetone-d6, showed a chemical shift of −141.03 ppm relative to trifluoromethane (Ref. 33, Rf 141.0 ppm).

C14H20OF
Calculated: C 48.00, H 5.47, F 5.42
Found: C 48.16, H 5.42, F 5.29

Pure anhydrous β-D-glucopyranosyl fluoride was prepared by deacetylating 0.3 g (860-μmol) samples of the tritacetate with 3.0 ml of 0.015 M sodium methoxide in dry methanol (0 °C, 3 h). On completion of the reaction (25) 30 dextrantransase units/mg of Silica Gel 60 under vacuum at 25 °C, then added to a dry packed column of the same absorbent and developed with absolute ethanol/ethyl acetate (25). Fractions (3 ml) providing a single spot of Rf 0.64 on thin layer chromatography were pooled, dried at 25 °C in a Rotovap apparatus (Buchler Instruments, Fort Lee, NJ), and redisolved in 0.1 M acetate-d3/D2O buffer of pD 5.3, showed a chemical shift of −146.45 ppm relative to external trifluoromethane in acetone-d6 with J(H-F) 53.7 Hz and Jα-F 12.2 Hz. These values agree closely with those reported by Hall et al. (33). 1H NMR spectra are described under "Results." Stock solutions of β-D-glucosyl fluoride in methanol, kept at −20 °C and protected from moisture, remained stable for weeks. As required, the compound was obtained by drying measured volumes of such solutions in a Rotovap apparatus (25 °C) immediately before use. The compound is labile in aqueous solutions, the rate of spontaneous release of fluoride ion in 0.05 M acetate buffers between pH 4.5 and 5.6 was found to be 0.2%/min at 30 °C and 0.013%/min at 0 °C. Methyl-α- β-glucopyranoside—Commercial preparations were found to contain small amounts of glucose and of an impurity migrating on paper chromatograms as methyl-α-isomaltooligosaccharide (Rf 0.78). Preparations free from the latter impurity were obtained by chromatography on a column (2.8 × 15 cm) comprising a mixture of 50 g each of Dacco 60-activated carbon and Celite 535 (Johns-Manville Corp.). The column was washed with water and charged with 10 g of methyl-α-glucopyranoside (Pfanstiehl) dissolved in 30 ml of water. Development was with water, with fractions assayed for total sugar (37) and for purity (by paper chromatography, 3.5-mg samples). The final product (4.8 g) contained a trace of glucose as the only impurity visible chromatographically (0.5% by glucose oxidase assay). The absence of contamination by methyl-α-maltoside, which migrates at the same rate as glucose, was shown by the failure of material eluted at the glucose position to yield methyl-α-glucopyranoside (Rf 1.35) after treatment with glucosaminase.

Methyl-α- β-glucopyranoside—A stock solution in methanol was prepared from methyl-α-[1-13C]glucopyranoside (184 mCi/mmol, purified by paper chromatography; Amersham Corp.) mixed with sufficient purified, unlabeled methyl-α-glucopyranoside to contain 25 μmol of glucosyl/mg and 0.2 μCi/μmol of glucose. Purity of the label was checked by chromatography a 1.2-μl sample and eluting materials corresponding to methyl-α-glucopyranoside (Rf 1.35), glucose or methyl-α-maltoside (Rf 1.5), and methyl-α-isomaltooligosaccharide (Rf 2.0). The absence of these eluates by scintillation counting after rechromatography indicated the presence of radioactive impurities amounting to 0.015% of total counts in the Rf 1.0 region and 0.03% in the Rf 0.78 region. For experimental use, required amounts of the 13C-labeled methyl-α-glucopyranoside were obtained by evaporating known volumes of the stock solution under vacuum.

Methyl α- and β-maltosides—Methyl-α-maltoside was synthesized enzymatically as described by Pazur et al. (38). A mixture containing 300 mg of cyclohexaamylose (Hayashibara Co., Okayama, Japan), 300 mg of purified methyl-α-glucopyranoside, and 1 mg (17 units) of Maltopexium cyclodextrin glycosyltransferase (Pfizer, New York) was incubated at 37 °C for 100 h. The digest was applied (19-cm streak) on five sheets of Whatman No.1 paper and chromatographed for 22 h. Material migrating as methyl-α-maltoside (Rf 1.6) was collected after filtration and solvent evaporation; the product was dried to constant weight in a vacuum oven at 35 °C. Yield was 118 mg; [α]D +318° (c, 0.45) (Ref. 39, 19°). On chromatography, the product gave a single spot which was indistinguishable in migration rate and slow reduction of silver nitrate from a sample of the pure compound kindly supplied by Dr. John Pazur. 1H NMR spectra recorded at 25 and 50 °C showed H-1 (unsubstituted p-glucosyl residue) 5.38 ppm, J3, 2 = 3 Hz, H-1 "reducing" (α-glucosyl residue) 4.81 ppm, J3, 2 = 3.5 Hz (resonance obscured by HOD in the spectrum at 25 °C); and −OCH3 3.42 ppm.

Crystalline methyl-β-isomaltoside, [α]D +79.3° (Ref. 39, 19°), was synthesized chemically as previously described (7). 1H NMR spectra recorded at 25 and 50 °C showed H-1′ ( unsubstituted p-glucosyl residue) 5.39 ppm, J3, 2 = 3 Hz, H-1′ "reducing" (α-glucosyl residue) 4.81 ppm, J3, 2 = 3.5 Hz (resonance obscured by HOD in the spectrum at 25 °C). 13C-NMR spectra were described under "Results."
RESULTS

In preliminary experiments, the glucoamylase of R. niterus and the glucoextranase of A. globiformis were found to catalyze the release of glucose (observed chromatographically) and of fluoride ion (detected with a fluoride ion electrode) from both α- and β-D-glucosyl fluoride. To learn whether the reactions with the α- and β-anomer are distinguishable kinetically, the reaction between rate of fluoride release and substrate concentration was examined. Two series of 0.60-ml digests at pH 5.6 (one for each anomer) were prepared for each enzyme. Glucoamylase digests comprised 4.8 to 40 mM α-D-glucosyl fluoride and 1 µg/ml of enzyme; 5 to 40 mM β-D-glucosyl fluoride and 0.5 mg/ml of enzyme. Glucoextranase digests comprised 3.6 to 36 mM α-D-glucosyl fluoride and 5.6 µg/ml of enzyme; 0.6 to 6 mM β-D-glucosyl fluoride and 0.17 mg/ml of enzyme.

Specific initial rates of enzymatically catalyzed fluoride release were calculated after correction for spontaneous hydrolysis of α- or β-D-glucosyl fluoride (0.5 and 3.5%, respectively) in the controls incubated without enzyme.

The results (Fig. 1) show that, with both enzymes, a great difference exists between the reactions on the two anomers. With α-D-glucosyl fluoride, plots of the velocity of fluoride release, v, as a function of substrate concentration, S, have the hyperbolic form usually associated with hydrolytic reactions; linear Lineweaver-Burk relationships obtain. Least squares calculations show K_{m} 25 mM and V_{max} 55.6 µmol of F⁻/min/mg for glucoamylase and K_{m} 39 mM and V_{max} 14.5 µmol of F⁻/min/mg for glucoextranase.

NMR spectra recorded at 25 and 50 °C showed, for the α-isomaltoside, H-1′ (unsubstituted α- glucosyl residue) 4.95 ppm, J_{1,2} ~3 Hz; H-1 (reducing α-glucosyl residue) 4.82 ppm, J_{1,2} ~3.0 Hz (resonance obscured by HOD in the spectrum at 25 °C); —OC₂ 3.42 ppm. They gave, for the P-isomaltoside, H-1′ 4.94 ppm, J_{1,2} ~3.3 Hz; H-1 4.40 ppm, J_{1,2} 7.7 Hz; —OCH₃ 3.55 ppm.

Additional Carbohydrates—Pure β-maltoxy1 fluoride was synthesized as recently described (7). D-Glucose, maltose, and L-glyceryl-a-D-glucopyranoside (41) were laboratory preparations, free from acetylated compounds. Spots were visualized by the sulfuric acid-char method. Paper chromatography was performed in the descending manner with Whatman No. 1 or 3MM paper and 1-butanol:pyridine:water (6:4:3) as developer. Staining was by a silver nitrate dipping technique (42), with papers hung in the air for 12-15 min after application of the developer. Staining was by a silver nitrate dipping technique (42), with papers hung in the air for 12-15 min after application of the developer.
fluoride, in contrast, plots of reaction velocity versus substrate concentration (Fig. 1c) and of \( v^{-1} \) versus \( S^{-1} \) (Fig. 1d) show upward concave curvature suggestive of the need, as in the case of \( \beta \)-amylase acting upon \( \beta \)-maltosyl fluoride (7), for 2 molecules of substrate to be bound to enzyme before reaction can occur. Glucodextranase is substantially more active than glucoamylase in catalyzing the release of fluoride from \( \beta \)-d-glucosyl fluoride. At low substrate concentrations, both enzymes act more rapidly on the \( \alpha \)- than on the \( \beta \)-anomer, e.g., at 6 mM, \( \alpha \)-d-glucosyl fluoride was attacked 2700 times faster than \( \beta \)-d-glucosyl fluoride by glucoamylase (11 times faster than glucodextranase).

As a control on the unusual kinetic findings with the \( \beta \)-anomer, initial rates of fluoride release were determined for a series of digests comprising 6 to 60 mM \( \beta \)-d-glucosyl fluoride and 1 \( \mu \)g/ml of purified sweet almond \( \beta \)-glucosidase (assaying 40 units/mg with salicin) incubated (pH 5.6) at 30 °C for 12 min. In this case, a plot of \( v \) versus \( S \) was hyperbolic and that of \( v^{-1} \) versus \( S^{-1} \) was linear, allowing calculation of \( k_\text{cat} \) at 53 mM and \( V_{\text{max}} \) at 263 pmol of F\(^{-} \)/min/mg. Since the range of fluoride ion concentrations in the \( \beta \)-glucosidase digests was comparable to that found with the exo-\( \alpha \)-glucanases, it is evident that the unusual kinetic order found with the latter (Fig. 1, c and d) is specific for glucodextranase and glucodextranase and is not an artifact attributable to an accelerated breakdown of \( \beta \)-d-glucosyl fluoride on exposure to higher concentrations of hydrogen fluoride.

Enhancement of Enzymic Utilization of \( \beta \)-d-Glucosyl Fluoride by \( \beta \)-Glucopyranosyl Compounds—The possibility that the reactions catalyzed by glucoamylase and glucodextranase with \( \beta \)-d-glucosyl fluoride might require 2 molecules of substrate, one functioning as a glucosyl donor and the other as a glucosyl acceptor, was examined by testing the ability of various glycosides and sugars to increase the rate of these reactions by serving as supplementary acceptors.

Individual test mixtures (0.60 ml, pH 5.2) containing 11 mM \( \beta \)-d-glucosyl fluoride, 1 mg/ml of glucodextranase or buffer, and 25 or 100 mM potential acceptor (or containing 6 mM \( \beta \)-d-glucosyl fluoride, 0.25 mg/ml of glucodextranase or buffer, and 25 or 100 mM potential acceptor) were set up at 2-min intervals. After incubation (30 °C, 14 min) each mixture was treated with 1.80 ml of TISAB buffer, and the fluoride ion concentration was immediately measured. Since all tests were made with freshly prepared solutions of \( \beta \)-d-glucosyl fluoride (kept at 0 °C less than 15 min) and since experimental runs were limited to six test mixtures and two controls (\( \beta \)-d-glucosyl fluoride plus enzyme; \( \beta \)-d-glucosyl fluoride plus buffer), substrate degradation was kept small (3.8%) and uniform throughout. Control values from one experimental run to the next were in excellent agreement, within 3% of one another.

As illustrated in Fig. 2, a variety of compounds having an unsubstituted \( \beta \)-glucopyranosyl residue acted to increase the rate of C-F bond cleavage of \( \beta \)-d-glucosyl fluoride by both enzymes. In contrast, methyl-\( \alpha \)-d-mannoside and the \( \beta \)-nitrophenyl-\( \beta \)-d-galactosides tested depressed the rate for both enzymes. With glucoamylase, a 2- to 5-fold rate enhancement was observed with 12 of the 13 \( \beta \)-glucopyranosyl compounds examined; with glucodextranase, a 1.4- to 4.5-fold rate enhancement was found with 10 of the 13. None of the compounds tested had any effect on the release of fluoride from \( \beta \)-d-glucosyl fluoride in the absence of enzyme. Although the data do not permit precise comparison of the effects of individual compounds for the two enzymes, large differences were noted in several cases. \( \beta \)-Nitrophenyl \( \alpha \)-d-glucoside and sucrose had a large potentiating effect for glucoamylase but none for glucodextranase; \( \beta \)-nitrophenyl \( \beta \)-d-glucoside enhanced \( \beta \)-d-glucosyl fluoride utilization by glucoamylase but strongly inhibited the reaction catalyzed by glucodextranase. On the other hand, maltose and (to a lesser extent) \( \beta \)-d-glucose showed larger effects with glucodextranase than with glucoamylase. Overall, the results strongly suggest that \( \alpha \)- or \( \beta \)-d-glucopyranosyl compounds of various kinds serve as \( \beta \)-glucosyl acceptor substrates for both glucoamylase and glucodextranase when these enzymes act upon \( \beta \)-d-glucosyl fluoride.

Configuration of the Glucose Formed from \( \alpha \)- and \( \beta \)-d-Glucosyl Fluoride by the Actions of Glucoamylase and Glucodextranase—To further characterize the reactions catalyzed by the inverting exo-\( \alpha \)-glucanases with \( \alpha \)- and \( \beta \)-d-glucosyl fluoride, a study was made of the configuration of the glucose produced in each case, using \(^1\)H NMR spectroscopy. Reference spectra showed that the resonances of the anomeric protons of \( \alpha \)- and \( \beta \)-d-glucose are readily distinguished from...
the proton signals of either α- or β-D-glucosyl fluoride. ¹H NMR spectra of the latter compounds at 100 and 220 MHz, reported for the first time, showed each to be anomerically pure although the H-1 resonances have an unusual appearance in each case. At 100 MHz, the anomeric proton of α-D-glucosyl fluoride (Fig. 3A) appears as a doublet of doublets centered at 5.69 ppm with J₁,2 ~ 3 Hz and J₁,₃ 53.1 Hz; the doublet at 5.95 ppm, however, shows a slightly smaller J₁,₂ coupling constant than that at 5.42 ppm. The anomeric proton of β-D-glucosyl fluoride at 100 MHz (Fig. 3B) gives rise to a doublet of doublets centered at 5.23 ppm with J₁,₂ ~ 7 Hz and J₁,₃ 53.1 Hz; here, the multiplet at 5.49 ppm appears to be three resonances while that at 4.96 ppm is the expected doublet with J₁,₂ 6.9 Hz. These anomalous differences in the J splitting patterns are eliminated at a higher field. Thus, at 220 MHz, the anomeric proton of the α-anomer (Fig. 3C) produces a doublet of doublets centered at 5.69 ppm with symmetrical J₁,₂ values of 2.7 Hz and J₁,₃ of 53.1 Hz. Likewise, the anomeric proton of the β-anomer (Fig. 3D) shows a nearly symmetrical pair of doublets centered at 5.23 ppm with J₁,₂ 6.9 Hz and J₁,₃ 53.1 Hz, but with some evidence of resonance density toward the center of the downfield doublet. These results suggest that the different transition levels in the downfield multiplet of H-1 for both anomers at 100 MHz is due to a frequency-dependent kinetic exchange process, possibly involving solvent. The J₁,₂ and J₁,₃ coupling constants for both α- and β-D-glucosyl fluoride agree with those reported for the corresponding peracetates in CDC₅ (33). These findings are fully consistent with the structure of each compound.

For examination of the actions of glucoamylase and glucodextranase on α- and β-D-glucosyl fluoride, the enzymes were exhaustively dialyzed at 6°C versus 0.1 M acetic acid/deuterium oxide buffer of pH 5.3 to exchange their labile ¹H for ²H atoms. Replicate samples of each substrate were recovered from solution in methanol immediately before use by vacuum evaporation of solvent at 25°C. At separate zero times, mixtures were prepared by adding 0.60 ml of dialyzed glucoamylase (48 μg), dialyzed glucodextranase (210 μg), or buffer to 24 μmol of α-D-glucosyl fluoride. Similar mixtures were prepared by adding 0.60 ml of dialyzed glucoamylase (4 mg), glucodextranase (1 mg), or buffer to 30 μmol of β-D-glucosyl fluoride. Each mixture was transferred to a 5-mm NMR tube, and ¹H NMR spectra were recorded at intervals during incubation at 21°C. Each spectrum consisted of 64 free induction decays, using 3-s repetition times. The data were Fourier transformed.

As shown in Fig. 4, both glucoamylase and glucodextranase catalyze reactions leading to β-D-glucopyranose when either α- or β-D-glucosyl fluoride is the substrate. A-1, A-2, and A-3 represent the ¹H NMR spectra of mixtures of 40 mM α-D-glucosyl fluoride incubated at 21°C with buffer (22-25 min), glucoamylase (8-11 min), or glucodextranase (22-25 min), respectively. It is evident that the resonance of the anomeric proton of the substrate, centered at 5.69 ppm, is diminished in the enzymic digests (A-2, A-3) compared with the buffer control (A-1). Spectra A-2 and A-3 are further distinguished from the control by the presence of a large doublet at 4.65 ppm (J₁,2 7.7 Hz) assignable to the axial anomeric proton of β-D-glucopyranose. A just detectable doublet at 5.24 ppm (J₁,2 3.3 Hz) is also seen in spectrum A-3 of the α-D-glucosyl fluoride/glucodextranase digest incubated 22-25 min. This marks the equatorial anomeric proton of α-D-glucopyranose and the beginning of nonenzymic anomerization of the enzymically formed β-anomer. A reference spectrum of equilibrated glucose (Fig. 4B) illustrates the relative intensities of the 1 equatorial and 1 axial resonances at full anomerization.

Spectra C-1, C-2, and C-3 are, respectively, of mixtures of 50 mM β-D-glucosyl fluoride incubated with buffer for 52-55 min, with glucoamylase for 32-35 min, or with glucodextranase for 52-55 min. Resonance of the anomeric proton of the substrate, centered at 5.23 ppm, is much diminished in the enzymic digest; in addition, their spectra (C-2 and C-3) show a large new resonance doublet at 4.65 ppm (J₁,2 7.7 Hz) assignable to the 1 axial proton of β-D-glucopyranose and also a low intensity doublet at 5.24 ppm (J₁,2 3.3 Hz) referable to a small amount of α-D-glucose formed by partial anomerization of the enzymatically formed β-D-glucopyranose. These results provide clear evidence of the anomeric purity of the substrates and of the formation of β-D-glucose from each substrate by the actions of the glucoamylase and glucodextranase preparations.¹

¹ In an experiment directly controlling against the possibility that the production of β-D-glucose might be due to a trace of β-glucosidase accompanying the exoglucanase preparations, 50 mM salicin was incubated (30°C, 60 min) with glucoamylase (6.67 mg/ml) and separately with glucodextranase (1.67 mg/ml) in 0.05 M acetate buffer of pH 5.2. No glucose was detected in either digest, although the method (glucose oxidase) used would have revealed as little as 0.04% hydrolysis.
portable capable of catalyzing only reactions involving water as a cosubstrate or product (11, 21-27).

Glucodextranase

&i/pmol) as acceptor, the amounts of these presumed trans-
measured by scintillation counting of eluates from chromato-
grams of test and appropriate control mixtures.* Data gath-
effect of this apparent acceptor on the utilization of
glycerol in different digests could be accurately
recovered as methyl-o-maltoside and methyl-a-maltoside.

The conditions finally used with each exoglucanase, to-
gether with the transfer product yields, are given in Table I.

products were recovered as methyl-o-maltoside and methyl-a-maltoside.

Digest components

| Enzyme          | β-D-Glucosyl fluoride | Methyl-a-D-['4C]glucoside | Glucose | RRED | RREL |
|-----------------|-----------------------|---------------------------|---------|------|------|
| Glucoamylase    | 768                   | 1152                      | 376     | 5.33 | 2.18 |
| Glucodextranase | 216                   | 432                       | 107     | 1.08 | 16.46|

* Calculated from dry weight of material eluted at RRED 1.0, less
  weight of the 14C-labeled RREL 1.0 glucoside component.

* Each product was assumed to contain one 14C-labeled glucose residue.
  Yields were calculated as counts per minute of product +
  pmol/μmol of methyl α-D-['4C]glucoside used.

* Separated from glucose by carbon-celite column chromatography
  of material eluted at RREL 1.0.

* These products contain a radioactive impurity carried by the
  methyl-α-D-['4C]glucoside 0.03% (of its counts). Thus the equivalent of 0.35 pmol (18%)
  of the glucoamylase product and 0.13 pmol (0.8%)
  of the glucodextranase product represents accompanying impurity.

TABLE I

| Products isolated from digests of β-D-glucosyl fluoride and methyl-
  α-D-['4C]glucoside with glucoamylase and glucodextranase |
|----------------------------------------------------------|

Glucoamylase digests (8 × 1.2 ml) were 80 mM β-D-glucosyl fluoride, 120 mM methyl-α-D-['4C]glucoside, 2 mg/ml of enzyme, 0.1 M acetate buffer, pH 5.0; incubation was at 30 °C for 30 min. Glucodextranase digests (0 × 1.0 ml) were 40 mM β-D-glucosyl fluoride, 80 mM methyl-

α-D-['4C]glucoside, 0.4 mg/ml of enzyme, 0.05 M acetate buffer, pH 5.2, containing 0.005 M CaCl2; incubation was at 30 °C for 90 min. Each 0.0 ml of glucoamylase digest (0.0 ml of glucodextranase digest) was chromatographed as a 17-cm band on Whatman No. 3MM paper.

Products isolated from digests of p-D-glucosyl fluoride and methyl-α-D-
['4C]glucoside. A, spectrum recorded in D2O at 29 and 50 °C, in comparison with those of methyl-α- and β-maltoside and of methyl-α- and β-isomaltoside. Spectra of the RREL 1.0 isolate from either the glucoamylase or glucodextranase digest were found to be indistinguishable from those of methyl-α-malto-
side. The downfield region of each had the appearance shown

in Fig. 5 (A and B) for the glucodextranase RREL 1.0 product. The doublet at 5.38 ppm, J1,2 = 3 Hz is assignable to the equatorial anomeric proton of an unsubstituted α-1,4-linked (or α-1,3-linked) β-glucopyranosyl residue. Were this residue α-1,2- or
  or-1,6-linked, the doublet would have appeared at higher field,
  as reported for a-kojibiose or a-isomaltose (45, 46) and found
  for methyl-α-isomaltoside in the present study. If the residue
  was of β-configuration, the anomeric proton would not only
  have resonated at higher field but shown a larger coupling constant (J1,2 = 7–8 Hz) as in sophorose, laminaribiose, cellobiose, gentiobiose, and their methylglycosides (45, 46). Reso-

nance of the anomeric portion of the α-D-glucopyranosyl moiety linked to the methyl group appears as a doublet at 4.81 ppm, J1,2 = 7 Hz, in spectra recorded at 50 °C (Fig. 5B); it is hidden by the HOD signal in spectra recorded at 25 °C. The methyl group resonance, present as a sharp singlet at 3.48 ppm, is not illustrated. These findings are consistent with the methyl-α-maltoside structure of RREL 1.0 glycoside synthesized by glucoamylase and by glucodextranase from β-D-glucosyl

on No. 1 paper. As indicated in Table I, calculations based on
the measured radioactivity (assuming each product to contain a single glucose[14C] residue) show that the glucoamylase

digest provided 3.33 μmol of RREL 1.0 product and 2.16 μmol of
RREL 0.78 product. The order of yields was reversed for the
glucodextranase digest, from which 16.46 μmol of RREL 0.78
product and 1.08 μmol of that having RREL 1.0 were obtained.

Since only about one-half of the available β-D-glucosyl flu-
ride was utilized in each digest, the two glucoamylase products represent ~2% of the substrate utilized; the glucodextranase products represent ~16%.

Characterization of the Transfer Products—The separated radioactive products were examined by two methods. First, their 1H NMR spectra are recorded in D2O at 25 and 50 °C, in comparison with those of methyl-α- and β-maltoside and

of methyl-α- and β-isomaltoside. The downfield region of each had the appearance shown in Fig. 5 (A and B) for the glucodextranase RREL 1.0 product. The doublet at 5.38 ppm, J1,2 = 3 Hz is assignable to the equatorial anomeric proton of an unsubstituted α-1,4-linked (or α-1,3-linked) β-glucopyranosyl residue. Were this residue α-1,2- or
  or-1,6-linked, the doublet would have appeared at higher field,
  as reported for α-kojibiose or α-isomaltose (45, 46) and found
  for methyl-α-isomaltoside in the present study. If the residue
  was of β-configuration, the anomeric proton would not only
  have resonated at higher field but shown a larger coupling constant (J1,2 = 7–8 Hz) as in sophorose, laminaribiose, cellobiose, gentiobiose, and their methylglycosides (45, 46). Reso-

nance of the anomeric portion of the α-D-glucopyranosyl moiety linked to the methyl group appears as a doublet at 4.81 ppm, J1,2 = 7 Hz, in spectra recorded at 50 °C (Fig. 5B); it is hidden by the HOD signal in spectra recorded at 25 °C. The methyl group resonance, present as a sharp singlet at 3.48 ppm, is not illustrated. These findings are consistent with the methyl-α-maltoside structure of RREL 1.0 glycoside synthesized by glucoamylase and by glucodextranase from β-D-glucosyl

FIG. 5. Fourier transform 1H NMR spectra at 100 MHz of glycosides recovered from enzymic digests of β-D-glucosyl fluoride and methyl-α-D-['4C]glucoside. A, spectrum recorded at 25 °C of a 6 mM solution in D2O of the major (RREL 1.0) transfer product isolated from digests with glucoamylase. B, spectrum of the same solution recorded at 50 °C. C, spectrum recorded at 25 °C of a 6 mM solution in D2O of the major (RREL 0.78) transfer product recovered from a digest with glucodextranase. D, spectrum of the solution described in C, recorded at 50 °C. H1, resonance of the equatorial anomeric proton of the nonreducing, i.e. unsubstituted, β-glucopyranosyl residue. H1, resonance of the equatorial anomeric proton of the substituted β-glucopyranosyl residue glycosidically linked to the methyl group. Resonances assignable to the methyl group protons, present in all spectra in the 3.4-3.8 ppm region, are not illustrated. Chemical shift values (parts per million) are relative to 3-(trimethylsilyl)propanesulfonic acid.
flouride and methyl-α-D-glucoside; only one other structure, methyl-α-nigeroside, could account for the 1H NMR findings.

1H NMR spectra of the Rf 0.78 isolate from the glucodextranase digest and of that from the glucoamylase digest were essentially indistinguishable from spectra recorded with methyl-α-isomaltoside. In each case, the downfield region appeared as shown in Fig. 5 (C and D) for the glucodextranase Rf, 0.78 product. The doublet at 4.96 ppm, J1,2 3.3 Hz, is assignable to the equatorial anomeric proton of an unsubstituted α-1,6-linked d-glucopyranosyl moiety. Were these moieties α-1,2-, α-1,3-, or α-1,4-linked, the doublet would have appeared at lower field, as reported for a-kojibiose, α-nigerose, or α-maltose (45, 46) and found (above) for methyl-α-maltoside; if β linked, the resonance would have been at lower field and shown a larger coupling constant (J1,2 7-8 Hz), as with sophorose, laminariobiose, cellobiose, gentiobiase, and their methylglycosides (45, 46). The anomeric proton of the d-glucopyranosyl residue linked to the methyl group is seen at 4.81 ppm, J1,2 3 Hz, in spectra recorded at 50°C (Fig. 4D), the methyl group protons (not illustrate) are at 3.42 ppm. These results leave no doubt as to the methyl-α-isomaltoside structure of the Rf 0.78 products isolated from the glucodextranase and glucoamylase digests.

Further characterization of the radioactive products was obtained by measuring the release of methyl-α-D-glucoside [14C] following treatment with enzymes of defined specificity. Samples of each isolate (0.08 μmol, ~7600 cpm) were examined in 50-μl test mixtures buffered at pH 5.0. The Rf 0.78 products were tested with glucoamylase (1.7 mg/ml), sweet almond β-glucosidase (2 mg/ml), and buffer alone; the Rf 0.78 products were tested with glucodextranase (1.7 mg/ml), β-glucosidase (2 mg/ml), and buffer. Mixtures were incubated at 30°C for 2.5 h (4 h in the case of glucodextranase) and then chromatographed. Materials migrating at the rates of methyl-α-D-glucoside (Rf 1.37) and of the product under test (Rf 1.0 or 0.78) were eluted, and the radioactivity of each was measured. The counts in the two eluates in every chromatogram accounted for the total radioactivity (~7600 cpm).

As shown in Table I, the two 14C-labeled Rf 1.0 products were completely hydrolyzed to methyl-α-D-[14C]glucoside and unlabeled glucose by glucoamylase. Thus, whereas all counts were at Rf 1.0 in chromatograms of the products in buffer, >99% of the radioactivity appeared at Rf 1.37 following incubation with glucodextranase. Eluates at Rf 1.0 were essentially devoid of radioactivity, showing that the nonreducing terminal glucose moiety of each product was derived from the unlabeled β-D-glucosyl fluoride. An insignificant degree of hydrolysis was produced by the β-glucosidase preparation. The results indicate that both isolated Rf 1.0 products have the structure methyl-α-D-glucopyranosyl-(1 → 4)-α-D-[14C]-glucopyranoside. An α-1,3-interglucosidic linkage is improbable in view of the limited ability of glucoamylase to hydrolyze (23, 47) or form (18) such linkage.

Of the Rf 0.78 products, the one from the digest with glucoamylase was hydrolyzed by glucodextranase with the formation of methyl-α-D-[14C]glucoside having 82% of the radioactivity of the unhydrolyzed material; 18% was resistant and remained localized in the Rf 0.78 region (it represents an impurity as noted in Tables I and II). The glucose component of the hydrolysate was free of radioactivity.

The Process of Formation of the Glycosides—An important question remains with respect to the methyl-α-D-glucopyranosyl-α-D-[14C]glucopyranosides formed from β-D-glucosyl fluoride and methyl-α-D-[14C]glucoside by glucoamylase and glucodextranase. Do these products arise by d-glucosyl transfer directly from the β-D-glucosyl fluoride, or are they formed by condensation of the major reaction product (D-glucose) with the methylglucoside, i.e. by reactions representing reversals of hydrolysis? To resolve this question, mixtures (80 μl) containing β-D-glucosyl fluoride, methyl-α-D-[14C]glucoside, and glucoamylase (or glucodextranase) such as used for isolating the glycosides were compared with similar mixtures containing 40 mM equilibrated glucose in place of the β-D-glucosyl fluoride. Suitable methyl-α-D-[14C]glucoside/buffer controls were included. After incubation, 50 μl of each mixture was chromatographed; material at the levels of methyl-α-D-glucoside, methyl-α-maltoside, and methyl-α-isomaltoside were eluted and the radioactivity was measured.

The findings (Table III) show that methylmaltoisomalto- and methylisomalto-isomalto formation in digests of β-D-glucosyl fluoride and methyl-α-D-[14C]glucoside, with either of the exoglucoamylases, is not accounted for by condensation reactions with free glucose as the donor. The digests containing 40 mM glucose (a higher concentration than would be found in the

| Enzyme and substrates | Methyl maltoside | Methyl isomaltoside |
|-----------------------|------------------|---------------------|
| Glucoamylase          | 80, 16,702       | 37.5, 7,941         |
| Methylglucoside       | 120              |                     |
| Equilibrated glucose  | 40, 2,273        | 5, 942              |
| Methylisomaltoside    | 120              |                     |
| Glucoamylase          | 60, 2,766        | 6, 64,979           |
| Methylglucoside       | 80               |                     |
| Equilibrated glucose  | 40, 72           | 0.2, 2,700          |
| Methylisomaltoside    | 80               |                     |

* Based on counts per minute of material eluted at Rf 1.0 and Rf 0.78, corrected for traces of radioactivity in eluates from chromatograms of incubated methyl-α-D-[14C]glucoside/buffer control mixtures.

* Digests contained 2 mg/ml of enzyme in 0.1 M acetate buffer (pH 5.0); incubation at 30°C for 30 min.

* Based on counts per minute of material eluted at Rf 1.0 and Rf 0.78.

* Digests contained 0.4 mg/ml of enzyme in 0.05 M acetate buffer (pH 5.2) with 0.005 M CaCl2, incubation at 30°C for 30 min.

TABLE II

| Isolated glycoside | Radioactivity found as methyl-α-D-[14C]glucoside after treatment |
|--------------------|---------------------------------------------------------------|
| Glucoamylase       | 99.8, 4.4                                                     |
| Glucodextranase    | 99.2, 6.7                                                     |
| Glucoamylase       | 82.2, 1.2                                                     |
| Glucodextranase    | 99.5, 0.2                                                     |

* Authentic methyl-α-maltoside and methyl-α-isomaltoside migrate at Rf 1.0 and at Rf 0.78, respectively, in the chromatographic system employed.

* Incomplete hydrolysis appears due to the presence of an impurity of unknown composition, comprising 16% of the radioactivity of this isolate (see Table I, Footnote d).

TABLE III

| Digest components | Product yields from 50 l digest |
|-------------------|--------------------------------|
| Glucosyl donor used for methyl maltoside-isomaltoside formation in enzymic digests of β-D-glucosyl fluoride plus methyl-α-D-[14C]glucoside | Methyl maltoside | Methyl isomaltoside |
| Glucosyl donor used for methyl maltoside-isomaltoside formation in enzymic digests of β-D-glucosyl fluoride plus methyl-α-D-[14C]glucoside | Methyl maltoside | Methyl isomaltoside |
| Glucoamylase      | 80, 16,702          | 37.5, 7,941         |
| Methylglucoside   | 120                |                     |
| Equilibrated glucose | 40, 2,273        | 5, 942              |
| Methylisomaltoside| 120                |                     |
| Glucoamylase      | 60, 2,766          | 6, 64,979           |
| Methylglucoside   | 80                 |                     |
| Equilibrated glucose | 40, 72           | 0.2, 2,700          |
| Methylisomaltoside| 80                 |                     |
digests with β-D-glucosyl fluoride at any time during incubation) showed only a small fraction of the products found in the digest with β-D-glucosyl fluoride. Thus, these products are primarily formed by direct glucosyl transfer from β-D-glucosyl fluoride to methyl-α-D-[14C]glucose with inversion of configuration.

**DISCUSSION**

The ability of a carbohydrate to utilize both α- and β-anomeric forms of the same compound as glycosyl substrates, initially demonstrated with β-amylase acting on α- and β-maltosyl fluoride (7), is shown to be an attribute of glucoamylase and glucoexorotanase as well. Study of the reactions catalyzed with α- and β-D-glucosyl fluoride by these two “inverting exo-α-glucanases” reveals that each is the catalyst of two stereochemically complementary types of glycosylation reactions. Reactions with α-glucans, α-glucosaccharides, and α-D-glucosyl fluoride proceed with water as acceptor and yield β-D-glucose; those with β-D-glucose and β-D-glucosyl fluoride have β-D-glucopyranosyl compounds as acceptors and yield α-D-glucose transfer products. The capacity of glucoamylase and glucoexorotanase to catalyze reactions without water as a cosubstrate or product is evident both in the “substrate activation” kinetics and the rate enhancement by various β-glucopyranosyl compounds observed when β-D-glucosyl fluoride is the substrate. Unequivocal evidence is provided for each enzyme in the mechanism of glucosyl transfer products from β-D-glucosyl fluoride with methyl-α-D-[14C]glucose as acceptor. These products, methyl α-D-glucopyranosyl-(1 → 4)-α-D-[14C]glucopyranoside and methyl-α-D-glucopyranosyl-(1 → 6)-α-D-[14C]glucopyranoside, have the structures one would expect if the β-D-glucosyl fluoride were transferred to methyl-α-D-[14C]glucose with inversion of configuration in reactions catalyzed by these exoglucanases. Recovery of mostly the α-maltoside (5.3 pmol), plus some α-isomaltoside (2.2 pmol), from digests with glucoamylase is consistent with the specificity of this enzyme which effects rapid maltose synthesis and slower isomaltose synthesis from β-D-glucose (21) and which preferentially hydrolyzes α-1,4-glucosidic linkages but also slowly cleaves α-1,6-linkages (22, 23, 47, 48). Similarly, recovery of mainly the α-isomaltoside (16.5 pmol), plus a little α-maltoside (1.1 pmol), from the glucoexorotanase digests is consistent with this enzyme’s preferential hydrolysis of α-1,6-glucosidic linkages and slow cleavage of α-1,4-linkages (28). Various control experiments show that the isolated glycosides of various other substrates are illustrated in Scheme 1 which, for simplicity, is confined to certain reactions effected by glucoamylase. The hydrolysis or formation of α-1,6-glucosidic linkages by both enzymes, for example, would proceed respectively by type I or II reactions similar to those in Scheme 1. Evidence has been reported for the presence of a carboxyl group and a carboxyl anion in the active site of R. delemar glucoamylase (49), and these groups are considered essential for catalysis by the R. niveus enzyme as well (50). We have taken this as a reasonable model for the active site functional groups of both glucoamylase and glucoexorotanase, although no information is available on the structure of the latter.

Scheme IA (I) illustrates the mechanism envisioned for the hydrolysis of maltose by glucoamylase, in which the carboxyl group acts as a general acid and the carboxylate anion acts as a general base assisting the attack of a water molecule on the substrate. It is written as a concerted reaction for reasons given below. The condensation of β-D-glucopyranose to form the product (Scheme IA) for the hydrolysis of maltose is consistent with the kinetics of β-D-glucopyranose hydrolysis and with the mechanism of the R. niveus enzyme. The hydrolysis or formation of α-1,6-glucosidic linkages by both enzymes, for example, would proceed respectively by type I or II reactions similar to those in Scheme 1. Evidence has been reported for the presence of a carboxyl group and a carboxyl anion in the active site of R. delemar glucoamylase (49), and these groups are considered essential for catalysis by the R. niveus enzyme as well (50). We have taken this as a reasonable model for the active site functional groups of both glucoamylase and glucoexorotanase, although no information is available on the structure of the latter.

Scheme IA (I) illustrates the mechanism envisioned for the hydrolysis of maltose by glucoamylase, in which the carboxyl group acts as a general acid and the carboxyl anion acts as a general base assisting the attack of a water molecule on the substrate. This is written as a concerted reaction for reasons given below. The condensation of β-D-glucopyranose to form maltose, catalyzed by R. niveus glucoamylase (29), is illustrated in Scheme IA (II). In this case, the C-4 hydroxy group of the donor substrate is displaced by the C-4 hydroxyl group of the acceptor, with the functional roles of the catalytic groups reversed as required by the principle of microscopic reversibility. In Scheme IB, a mechanism for the hydrolysis of α-D-glucosyl fluoride by glucoamylase or glucoexorotanase is presented which proceeds by a pathway similar to that in Scheme IA (I) for the hydrolysis of maltose. This is consistent with the kinetics of α-D-glucosyl fluoride hydrolysis and with the formation of β-D-glucose as the hydrolysis product with either enzyme.

Although 1H NMR spectra which record the conversion of β-D-glucosyl fluoride to β-D-glucose by glucoamylase and glucoexorotanase (Fig. 4C) show no sign of the presence of β-maltosyl or β-isomaltosyl fluoride, the failure of these expected intermediates to accumulate in steady state is readily accounted for. Glucoamylase, for example, catalyzed cleavage of the C-F bond of 40 mM β-D-glucosyl fluoride at the rate of 0.6645 μmol/min/mg (Fig. 1C) in a reaction presumed to yield mostly β-maltosyl fluoride. In other tests, the enzyme was found to catalyze the release of glucose (measured with glucose oxidase) from 2.4 and 0.24 mM β-maltosyl fluoride at rates of 0.79 and 0.15 μmol/min/mg, respectively. Thus, even a β-maltosyl fluoride level as low as 0.6% of that of the β-D-glucosyl fluoride could not be reached or sustained with hydrolysis occurring several times faster than synthesis.

Y. Tsujikaka, personal communication.
Catalytic Flexibility of Glucoamylase and Glucodextranase

Scheme IC shows the postulated mechanism for the formation of methyl-α-maltoside from β-D-glucosyl fluoride and methyl-α-D-glucoside. This is similar to the condensation mechanism shown in Scheme LA (II) for the formation of maltose from β-D-glucopyranosyl fluoride. Scheme IC is also consistent with the kinetics of β-D-glucosyl fluoride utilization which indicates a requirement for 2 molecules of substrate at the active site in order for reaction to occur. The fluorne atom of the donor molecule would be displaced by the C-4 hydroxyl of the second substrate molecule located at the acceptor site, forming β-maltosyl fluoride; this intermediate would rapidly hydrolyze to β-D-glucosyl fluoride and β-D-glucosyl fluoride along the pathway shown in Scheme LA (II). The requirement for 2 bound β-D-glucosyl fluoride molecules to effect C-P cleavage favors a concerted mechanism, as shown, rather than a stepwise mechanism in which the fluoride ion of the donor departs first, leaving a carbocation that is subsequently captured by the C-4 hydroxyl group of an incoming acceptor molecule. The apparent concerted displacement mechanism for β-D-glucosyl fluoride favors, although it does not prove, that the condensation of β-D-glucosyl to form maltose (20) also involves a concerted mechanism as in Scheme LA (II). From considerations of microscopic reversibility, the hydrolysis of maltose to give β-D-glucose would then also be concerted, as shown. However, it is also possible that maltose and other glycosidically linked substrates may undergo stepwise reactions with carbonium ion intermediates. Indeed, as noted below, there are positive indications that a given enzyme may not act on all substrates with the same reaction mechanism.

Regardless of the details of the proposed mechanisms, it is clear that, for both enzymes, the functional roles of the catalytic groups must be reversed not only in the hydrolysis-condensation reactions (Scheme LA) which are reversals of each other, but also in the stereospecifically different and essentially irreversible reactions with α- and β-D-glucosyl fluoride (Scheme I, B and C). In the case of glucodextranase, the apparent operation of a concerted mechanism illustrates a further capability arising out of the functional flexibility of the catalytic groups. Recent work (8) has shown that this enzyme catalyzes hydration and glycosyl transfer reactions with 2,6-anhydro-1-deoxy-D-glucopyranosyl-1-enol as a mechanistic which, in all probability, involves a carbonium ion intermediate. Thus, it would appear that glucodextranase is not limited to a single reaction mechanism with all substrates. Carbohydrases have often been characterized as operating by one particular mechanism, but this view may be too rigid. Dahlquist et al. (51), for example, concluded on the basis of the secondary isotope effect on the rate of hydrolysis of phenyl-β-D-[3H]glucoside by sweet almond β-glucosidase that, “the mechanism of this enzyme would seem to be a classic example of the displacement mechanism suggested by Koehler (1953).” However, the same enzyme catalyzes the hydration of D-glucal by a mechanism in which the direction of protonation of the substrate is opposite that assumed for β-D-glucosides and which appears to involve a carbonium ion intermediate (2). Similarly, β-galactosidase catalyzes reactions with 2-D-galactol and with β-D-galactosides that differ from each other in protonation direction and mechanism (3), and β-amylase, which appears to act upon α- and β-maltosyl fluoride by a concerted reaction (7), has been found to catalyze the hydration of maltal by a process that apparently is carbonium ion-mediated. The ability of these several enzymes to act on different substrates by different reaction mechanisms can be related to the functional flexibility of their catalytic groups. If such flexibility is an attribute of glycosylases in general (7), an increasing number of enzymes may be expected to be found capable of acting by more than one mechanism. Reactions requiring different mechanisms have, in fact, been reported for lysozyme (52) and for the saccharifying α-amylase of Bacillus subtilis (53-55).

Present findings, finally, support a concept of carboxydrase action that departs from traditional views in considering that glycoside hydrolases and glycosyltransferases form a class of interrelated glycosylases whose reactions effect a simple chemical change, the interchange of a glycosyl residue and a proton, glycosyl-X+H-X′ = glycosyl-X′+H-X (1, 18, 56). This concept informs us that a compound may need no more than the ability to be suitably aligned at the active site of an enzyme and the ability to yield a glycosyl residue on protonation in order to serve as a glycosyl donor. There are now many examples of enzymic glycosylation reactions catalyzed with substrates lacking a glycosidic bond and/or α- or β-anomeric configuration. The fresh insight into enzymic mechanisms which the study of such reactions is able to provide suggests that the above unifying concept will find increasing adoption as a guiding principle in place of present models.

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