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Transglucosylation as a Probe of the Mechanism of Action of Mammalian Cytosolic β-Glucosidase*

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This study establishes that guinea pig liver cytosolic β-glucosidase generates a common glucosyl-enzyme intermediate from a variety of aryl β-D-glucoside substrates and that the intermediate can react with various acceptors to form distinct products at rates which are dependent on the structure, nucleophilicity, and concentration of the acceptor. Specifically, we demonstrate that water and linear alkanols will react with the glucosyl-enzyme intermediate to form D-glucose and alkyl-β-D-glucoside (*e.g.* octyl-β-D-glucoside), respectively. The rate of alcoholysis is 24-fold greater than the rate of hydrolysis of the glucosyl-enzyme intermediate and accounts for the increase in steady-state rate of substrate disappearance in the presence of alcohols. In addition, the substrate molecule itself (*e.g.* *p*-nitrophenyl-β-D-galactoside (pNP-Gal)) can serve as an acceptor in the transglycosylation reaction, thereby enabling the enzyme to synthesize disaccharide glycosides (*e.g.* pNP-β-Gal(6→1)β-Gal).

The transglycosylation data point to the presence of two hydrophobic subsites in the active site of the cytosolic β-glucosidase. These data support a model in which the cytosolic β-glucosidase binds an acceptor and a glycosyl donor simultaneously within its catalytic center and efficiently catalyzes the transfer of a sugar residue from the donor to the acceptor.

It has been demonstrated with a number of hydrolytic enzymes including chymotrypsin (1, 2), *Escherichia coli* β-galactosidase (3, 4), and serine esterase (5) that alcohols can function as cosubstrates and compete with water as the nucleophilic acceptor in the reactions catalyzed by these enzymes. In fact, it is well documented that many glycosidases catalyze transglycosylation reactions, namely the transfer of glycosyl residues from glycoside substrates to acceptors other than water (3, 6, 7). In a recent study of the broad specificity guinea pig liver cytosolic β-glucosidase (8), we presented evidence for the presence of a hydrophobic site on the enzyme which when occupied by linear alkanols (*e.g.* *n*-butyl alcohol) resulted in a 7-fold increase in the V_{max} for the β-glucosidase-catalyzed release of aglycone from 4-methylumbelliferyl-β-D-glucoside (4-MUGlc).[1] The concentration of linear alkanols required to elicit maximum stimulation of β-glucosidase activity was inversely proportional to the length of the alkyl chain of the alcohols. Moreover, our contention that this stimulatory potential is related to the hydrophobic nature of the carbon chain of the alcohols was reinforced by our observation that branched chain alcohols such as *sec*- and *tert*-butyl alcohol, which contain the same number of carbon atoms, but which exhibit lower octanol/water partitioning coefficients, were either ineffective or much less effective than their unbranched counterparts in increasing the V_{max} for the β-glucosidase-catalyzed cleavage of 4-MUGlc.

We have extended this earlier study to address the following questions: (i) how does a nonessential activator, like *n*-butyl alcohol increase the V_{max} for the β-glucosidase-catalyzed breakdown of an aryl glucoside such as 4-MUGlc? and (ii) how do the alcohols alter the substrate specificity of the enzyme? In the present investigation, we demonstrate that alcoholysis of the putative glucosyl-enzyme intermediate is much more rapid than the competing process of hydrolysis and that it forms the basis for the increased steady-state rate of substrate disappearance when alcohols are included in the β-glucosidase assay medium. We document the varying extent to which *n*-butyl alcohol activates the β-glucosidase-catalyzed hydrolysis of the β-D-glucose, β-D-galactose, β-D-xylose and α-L-arabinose derivatives of 4-methylumbelliferone and discuss the relevance of this finding with regard to the affinity of the enzyme for the four different substrates. By taking advantage of the phenomenon of transglycosylation, we have gained insight into several issues that are fundamental to understanding the mechanism of catalysis by the cytosolic β-glucosidase; specifically, (i) the rate-determining step in the enzyme-catalyzed hydrolysis of aryl glycosides, (ii) the nature of the catalytic intermediate(s) whose formation or breakdown may be rate limiting, and (iii) a reaction pathway describing the mechanism of action of the cytosolic β-glucosidase.

EXPERIMENTAL PROCEDURES

Materials

Cytosolic β-glucosidase was purified to homogeneity, as described elsewhere (9), from frozen guinea pig livers purchased from Pel Freez, Rogers, AR; the final preparation exhibited a specific activity of 506,000 units/mg protein in the standard β-glucosidase assay described below.

4-Methylumbelliferyl-β-D-galactoside (4-MUGal), -β-D-xyloside, and -α-L-arabinoside, *p*-nitrophenyl-β-D-gentiobioside (pNP-gentiobioside), *p*-nitrophenyl-β-D-cellobioside (pNP-cellobioside), *n*-butyl alcohol, and *n*-octanol were purchased from Sigma. Benzyl alcohol was purchased from Aldrich. L-Picein (*p*-acetophenyl-β-D-glucoside) was obtained from Pfaltz and Bauer, Waterbury, CT. Trisil reagent for preparing trimethylsilane derivatives of saccharides was purchased from Pierce Chemical Co. *m*-Nitrophenyl-β-D-glucoside was a gift from Dr. L. D. Byers, Tulane University, New Orleans, LA. *p*-Nitrophenyl-β-D-sophoroside and *p*-nitrophenyl-β-D-laminaribioside

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[1] The abbreviations used are: 4-MUGlc, 4-methylumbelliferyl-β-D-glucoside; 4-MUGal, 4-methylumbelliferyl-β-D-galactoside; pNP, *p*-nitrophenol; HPLC, high performance liquid chromatography.

were provided by Dr. I. J. Goldstein, Michigan State University, Ann Arbor, MI.

Methods

The Standard β-Glucosidase Assay—Unless otherwise specified, the standard assay contained 5 mM substrate in 0.2 M sodium citrate buffer, pH 6.0, and enzyme (1–5 ng) in a final volume of 0.1 ml. Assays were initiated by addition of the enzyme and incubations were carried out at 37 °C for 15–30 min. With fluorogenic substrates (*e.g.* 4-MUGlc, 4-MUGal) the assays were terminated by addition of 2.9 ml of 0.3 M ammonia-glycine buffer, pH 10.5, and release of the fluorescent product, 4-MU, was monitored using a Turner fluorometer as described elsewhere (10). One unit of enzyme activity corresponds to the release of 1 nmol of product in 1 h.

The spectrophotometric assays which were carried out with the various phenyl-β-D-glucosides deserve individual mention. The release of phenol from phenyl-β-D-glucoside was monitored at 287 nm, following termination of the incubation by addition of 0.9 ml of 0.1 M NaOH (11). The production of *p*-acetophenol in the enzyme-catalyzed hydrolysis of *p*-acetophenyl-β-D-glucoside was calculated based on the absorbance at 330 nm after termination of the reaction with 0.9 ml of 15% (w/v) sodium carbonate (7). The release of the various nitrophenols from their respective β-D-glucosides was estimated based on their absorbance at 400 nm; the molar absorptivity coefficients used to calculate enzyme activities were 2,600, 21,830, 18,300, and 2,300 M^{-1} cm^{-1} for phenol, *p*-acetophenol, *p*-nitrophenol, and *m*-nitrophenol, respectively.

Determination of D-Glucose—To measure D-glucose release in β-glucosidase assays we used three coupling enzymes: hexokinase, glucose-6-phosphate dehydrogenase, and diaphorase. Following the initial β-glucosidase incubation, the assay tubes were boiled for 5 min to inactivate the cytosolic β-glucosidase. A master mix (0.68 ml) consisting of 0.56 ml of 10 mM $MgCl_2$, 0.1 ml of 0.5 M Tris buffer, pH 8.0, 10 μl of 10 mM ATP, and 10 μl of 10 mM $NADP^+$ was added prior to the addition of a mixture of hexokinase (2 units) and glucose-6-phosphate dehydrogenase (4 units). After incubation for 45 min at 37 °C, the tubes were boiled for 5 min. A master mix (0.4 ml) comprised of 0.1 ml of 10 mM *p*-iodonitrotetrazolium violet and 0.3 ml of 1.0 M sodium phosphate buffer, pH 7.5, was added and incubated for 10 min with diaphorase (5 units). The tubes were plunged into ice to terminate the enzymatic reaction and the amount of *p*-iodonitrotetrazolium violet-formazan formed was then quantitated based on the absorbance at 492 nm (12). The amount of D-glucose released by the β-glucosidase was calculated from a D-glucose standard curve.

Protein Determination—The Coomassie dye-binding method of Bradford (13) was used to estimate protein concentration; bovine serum albumin served as the standard.

Reversed-phase High Pressure Liquid Chromatography (HPLC) Analyses—The analysis of disaccharide transglycosylation products generated from *p*-nitrophenyl-β-D-glucoside (pNPGlc) or *p*-nitrophenyl-β-D-galactoside (pNPGal) was performed using reversed-phase HPLC. β-Glucosidase incubations were terminated by addition of 10 μl of 50% (v/v) trifluoroacetic acid. The samples were subjected to HPLC (Waters 600E gradient controller, model 484 Variable Absorbance Detector) on a reversed-phase column (Delta Pak C_{18}, 8 × 100 mm, Waters, Milford, MA). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (A) and 0.1% (v/v) trifluoroacetic acid in 95% acetonitrile (B). The elution gradient was programed as follows: 5 min of solvent A, 20 min of linear gradient from 0%-20% solvent B, 8 min of a linear gradient from 20–80% solvent B, and, finally, a 2-min linear gradient from 80–100% solvent B. The column was then washed with 100% solvent B for 2 min. The flow rate was 1.0 ml/min, and the eluent was monitored at 310 nm. The detector output was plotted on a Kipp and Zonen plotter, and the data were simultaneously acquired and quantitated on a Pharmacia LKB 200 fraction collector, capable of data integration.

Gas-Liquid Chromatographic Analyses—The β-glucosidase assay was performed in "reactivials" in the presence of 2 mM *n*-octanol and then dried *in vacuo*. The dried residue was dissolved in 20 μl of dimethylformamide. Trisil reagent (20 μl) was then added to convert the substrates and products to the respective trimethylsilane derivatives. After 30 min at room temperature, the reactivials were centrifuged for 2 min in a IEC clinical centrifuge. One μl of the clear supernatant was then subjected to gas-liquid chromatography on a DB-1 column (0.55 × 1500 mm) in a Hewlett Packard 5690A gas-liquid chromatograph system. The gradient was programed as follows: 1 min at 150 °C, 20 min of linear gradient from 150 °C to 250 °C, and a final 3 min at 250 °C. The helium flow rate through the column was 16.3 ml/min. The detector response was collected on the data integrator and the peak areas simultaneously quantitated.

RESULTS

Effect of Alcohols on the Rate of Cytosolic β-Glucosidase-catalyzed Hydrolysis of Glucoside Substrates—Based on an analogy to other glycosidases (4, 11, 14, 15), Fig. 1, *Scheme I*, was constructed to describe the probable reaction pathway for the hydrolysis of aryl β-D-glucosides by the guinea pig liver cytosolic β-glucosidase. The first step, defined by the equilibrium constant $K_S = k_{-1}/k_1$, involves formation of the ES Michaelis complex. The second step involves the expulsion of the aryl aglycone with the concomitant formation of the glucosyl-enzyme intermediate (*Enz-Glc*) and is represented by the rate constant k_2. The deglucosylation rate constant k_3 specifies the rate of the reaction of the glucosyl-enzyme intermediate with water to produce D-glucose and regenerate the enzyme. When hydrolytic enzymes (*e.g.* glycosidases, esterases) are studied in the presence of alcohols, the possibility of alcoholysis of intermediates must be considered. As is depicted in Fig. 1, *Scheme I*, water is not a unique reactant for the solvolytic step; nucleophilic attack of the glucosyl-enzyme intermediate by an alcohol ROH could lead to formation of the transglucosylation product ROGlc (k_4, *Scheme I*).

Having demonstrated previously (8) that normal alcohols stimulate cytosolic β-glucosidase activity 7-fold, we wanted to inquire into the mechanistic basis behind this phenomenon. If the alcohol functions as a nucleophilic acceptor of glucose moieties, then one would expect to find a disparity between the rates of production of D-glucose and aglycone.

Fig. 2, *panel A*, depicts the gradual increase in the steady-state rate of *p*-nitrophenol production when increasing amounts of *n*-butyl alcohol were included in a β-glucosidase assay medium in which pNPGlc served as substrate. In the presence of 0.2 M *n*-butyl alcohol, an 8-fold increase in *p*-nitrophenol release from pNPGlc was observed. However, when D-glucose production was determined it was observed that the rate of D-glucose release decreased as the concentration of *n*-butyl alcohol increased. The latter observation is consistent with the hypothesis that the alcohol competes with water as a nucleophilic acceptor. The *dotted line* in Fig. 2 indicates the rate of formation of the transglucosylation product, alkyl-β-D-glucoside, in the pNPGlc-β-glucosidase incubation. The alkyl β-D-glucoside data were obtained by calcu-

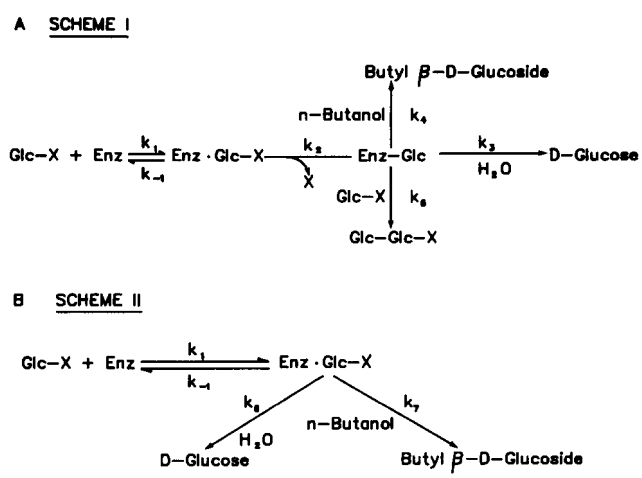

FIG. 1. **The two possible reaction pathways for the breakdown of aryl β-D-glucosides by the cytosolic β-glucosidase.**

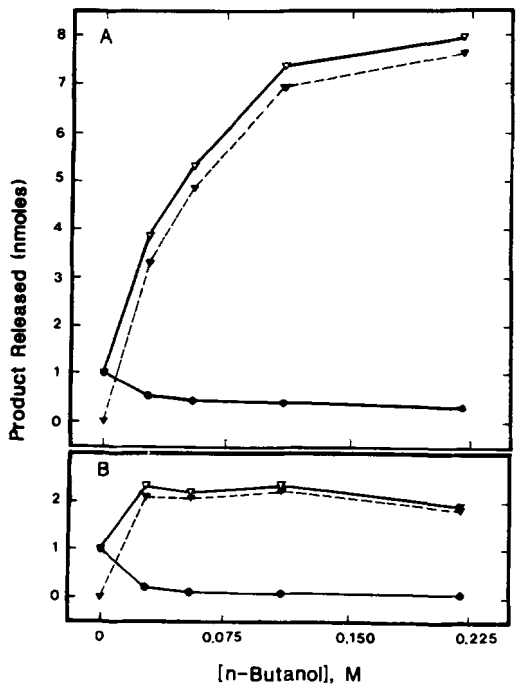

FIG. 2. **Effect of *n*-butyl alcohol on the β-glucosidase-catalyzed hydrolysis of pNPGlc and phenyl-β-D-glucoside.** The relative amounts (nanomoles) of pNP (▽) and D-glucose (●) released in 15 min *versus* concentration of *n*-butyl alcohol in the assay medium for two substrates, pNPGlc (*panel A*) and phenyl-β-D-glucoside (*panel B*). The *dotted line* refers to the estimated amount of butyl-β-D-glucoside (▼) formed due to transglucosylation.

lating the difference between the expected (assuming equimolar release of *p*-nitrophenol and D-glucose) and the actual amounts of D-glucose released. It is noteworthy that identical results were obtained when 4-MUGlc replaced pNPGlc as the substrate in the β-glucosidase assay and when the rates of aglycone and D-glucose release were estimated in the presence of increasing concentrations of *n*-propyl alcohol, *n*-butyl alcohol, or *n*-pentanol (data not shown). These data obtained with pNPGlc (Fig. 2, *panel A*) are consistent with the hypothesis that k_3 is the rate-determining step in the β-glucosidase-catalyzed hydrolysis of pNPGlc.

Our kinetic analyses revealed that the k_{cat} value obtained with phenyl-β-D-glucoside is only 40% that of the k_{cat} for pNPGlc (data not shown). If k_3 is the same for different aryl-β-D-glucoside substrates, it is reasonable to conclude then that $k_2 < k_3$ and that k_2 must be at least partially rate limiting in the cytosolic β-glucosidase-catalyzed hydrolysis of phenyl-β-D-glucoside. As is shown in Fig. 2, *panel B*, when phenyl-β-D-glucoside served as the β-glucosidase substrate, the presence of *n*-butyl alcohol caused only a 2-fold increase in the rate of phenol release. However, a steady decline in the rate of D-glucose release with increasing concentrations of *n*-butyl alcohol in the assay medium was observed. This observation indicates that *n*-butyl alcohol competes effectively with water in the solvolysis of the glucosyl-enzyme intermediate. These results obtained when phenyl-β-D-glucoside served as the β-glucosidase substrate are in agreement with the outcomes expected for a substrate for which $k_2 < k_3$.

n-Butyl Alcohol Binds to the Cytosolic β-Glucosidase—The acceleration in the rate of enzyme-catalyzed reactions caused by cosolvents such as alcohols may be due to either (i) their direct participation as cosubstrates in the reaction, or (ii) solvent effects on the structure of the enzyme. If organic solvents such as dioxane or tetrahydrofuran fail to cause the rate enhancement elicited by alcohols, as is the case with the cytosolic β-glucosidase, it is unlikely that the increase in rate is due to a general solvent effect on protein structure.

Fig. 2 depicts the leveling off of the rates of alcoholysis and hydrolysis of the putative glucosyl-enzyme intermediate as the concentration of *n*-butyl alcohol in the β-glucosidase assay is increased from 0.0 to 0.2 M. This observation can be accounted for by the postulation of a saturable hydrophobic site on the cytosolic β-glucosidase to which *n*-butyl alcohol can bind. In fact, treatment of the velocity of butyl-β-D-glucoside formation *versus* *n*-butyl alcohol concentration data by Lineweaver-Burk analysis yielded a perfectly linear double-reciprocal plot (data not shown); thus *n*-butyl alcohol displays Michaelis-Menten saturation kinetics in the cytosolic β-glucosidase-catalyzed alcoholysis of pNPGlc.

This result is also consistent with data contained in our earlier report (8) wherein we had interpreted the increased nucleophilic reactivity with increasing chain length of the alkyl moiety of the linear alkanols as being reflective of the binding of the alkanols to a hydrophobic site on the cytosolic β-glucosidase. It is important to recognize that even though rate enhancement by alcohols in enzyme-catalyzed reactions could be attributed to alcoholysis, one need not invoke binding of the alcohol to a site on the enzyme; there are several instances where the evidence lends only bare support for interaction of an alcohol molecule with the enzyme (3, 16).

Partitioning of an Enzyme-Glucose Intermediate between Two Acceptors—The second procedure used to inquire if a common intermediate is formed during the hydrolysis of different glucoside substrates by the same enzyme involves measuring product ratios (*e.g.* butyl-β-D-glucoside/D-glucose) with the various substrates and a fixed concentration of two acceptors (*e.g.* *n*-butyl alcohol and water).

The alkyl-β-D-glucoside/aglycone product ratios obtained with four different β-glucosidase substrates are summarized in Table I. These ratios were obtained by estimating the amount of aglycone and D-glucose produced in a β-glucosidase incubation that contained 0.2 M *n*-butyl alcohol. It is evident that differences in the pK_a of the aglycone moiety had no effect on the ratio of the alcoholysis/hydrolysis products generated in the β-glucosidase-catalyzed breakdown of these various phenyl glucosides in the presence of 0.2 M *n*-butyl alcohol. This finding indicates that the breakdown of all of these substrates proceeds through a common glucosyl-enzyme intermediate. Furthermore, the invariant butyl-β-D-glucoside/aglycone ratio (0.96) obtained with four β-D-glucosides (Table I) indicates that only 4% of the glucosyl-intermediate proceeds through the hydrolysis pathway to form D-glucose, while 96% of the intermediate reacts with *n*-butyl alcohol to form butyl-β-D-glucoside. Hence, we conclude that the rate of alcoholysis (k_4) is 24-fold higher than the rate of hydrolysis (k_3) of the common glucosyl-enzyme intermediate.

Identification of Cytosolic β-Glucosidase-catalyzed Alcoholysis Products—We proceeded to identify the alcoholysis product and ascertain the stereochemical outcome of the β-glucosidase-catalyzed transfer of glucose from 4-MUGlc to alcohol. We employed gas-liquid chromatography to separate the tri-

TABLE I

Ratio of products in the presence of 0.2 M n-butyl alcohol

Substrate	Aglycone pK_a	Butyl-β-D-glucoside/aglycone
Phenyl-β-D-glucoside	10.0	0.958
p-Acetophenyl-β-D-glucoside	8.6	0.960
m-Nitrophenyl-β-D-glucoside	8.3	0.962
p-Nitrophenyl-β-D-glucoside	7.1	0.958

methylsilane derivatives of the substrates and the expected products generated in the β-glucosidase assay performed in the presence of 2 mM *n*-octanol. Owing to the availability of the α- and β-anomers of octyl glucoside, we elected to perform the analysis of the cytosolic β-glucosidase-catalyzed alcoholysis products using *n*-octanol. As shown in Fig. 3, a major peak at 17.4 min and a minor peak at 10.5 min were observed. These corresponded to authentic standards of octyl-β-D-glucoside and β-D-glucose, respectively. The small peak eluting at 15 min was not identified. It is noteworthy that the octyl-α-D-glucoside and α-D-glucose standards eluted at 16.2 and 8.7 min, respectively. In this experiment we determined the octyl-β-D-glucoside/β-D-glucose ratio to be 93:7, a result which indicated that alcoholysis of the glucosyl-enzyme intermediate was more rapid than its hydrolysis. This finding also supports the postulate that the discrepancy between the expected and observed D-glucose yields in the presence of alcohols in the cytosolic β-glucosidase-catalyzed breakdown of aryl-β-D-glucosides is due to the formation of the alcoholysis product, namely, alkyl-β-D-glucoside.

Cytosolic β-Glucosidase Catalyzes Transglucosylation to Sugar Glycosides—It is well documented that the increased reactivity of alcohols toward carbonyl-type carbon includes the alcoholic groups present in sugar molecules. If glycosyl oxocarbonium ion intermediates are generated in both the enzymatic and nonenzymatic breakdown of glycosides, appropriate sugar molecules might be expected to compete with water in the solvolysis step. Indeed, during nonenzymatic acid-catalyzed hydrolysis of aryl glycosides glycosyl cation intermediates are generated. Overend *et al.* (17) documented that such intermediates can be captured by 0.1 M glycosides. Also, several glycosidases have been proven to catalyze transglycosylation to sugar acceptors (18–20). It is widely appreciated that cellulolytic enzymes too can catalyze transfer of sugar moieties to various acceptors and thus contribute to the synthesis of an oligosaccharide chain. It is important to recognize that while exoglycosidases can transfer only monosaccharides to acceptors, endoglycosidases can catalyze transglucosylation of an oligosaccharide (18, 21).

Distler and Jourdian (19) demonstrated that the soluble, aryl hexosidase from bovine liver was capable of transglycosylation. We used reversed-phase HPLC to address the question of whether the guinea pig liver cytosolic β-glucosidase is capable of catalyzing similar transglucosylation reactions (Fig. 1, *Scheme I*). We used 10 mM pNPGlc as the substrate in the β-glucosidase assay, and subjected the assay mixture to chromatography on a C_{18} Deltapak column. The eluent was monitored at 310 nm. Three peaks with retention times of 27.5, 29.4, and 38.3 min were observed (Fig. 4, *panel A*). The

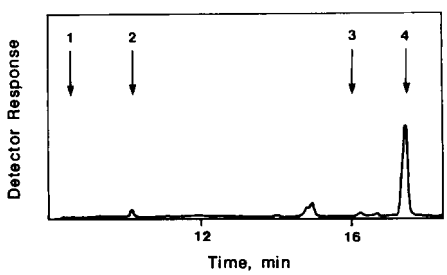

FIG. 3. **Identification of the β-glucosidase-catalyzed octanolysis products using gas-liquid chromatography.** The detector response was recorded on a Hewlett Packard 5690A integrator and the peaks identified on the basis of authentic standards. The *arrows 1–4* correspond to α-D-glucose, β-D-glucose, octyl-α-D-glucoside, and octyl-β-D-glucoside, which elute at 8.7, 10.5, 16.0, and 17.4 min, respectively.

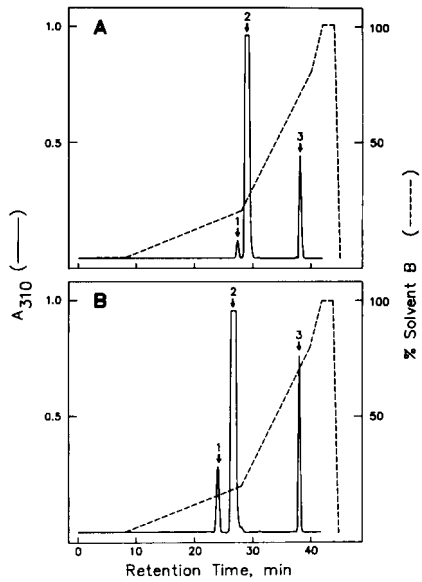

FIG. 4. **Demonstration of transglycosylation to sugar moieties in the substrates.** Reversed-phase HPLC elution profile of the assay mixture obtained after incubation of the enzyme with 10 mM pNPGlc (*panel A*) and 10 mM pNPGal (*panel B*). The absorbance at 310 nm recorded by the detector is plotted *versus* the retention time. In *panel A*, the *arrows* labeled *1–3*, correspond to the authentic pNP-gentiobioside (27.4 min), pNPGlc (29.5 min), and pNP (38.3 min) standards, respectively. In *panel B*, the *arrows* labeled *1–3*, correspond to pNPGalGal (24.5 min), pNPGal (27.5 min), and pNP (38.3 min), respectively.

peaks were identified on the basis of authentic standards to be pNP-β-D-gentiobioside, pNPGlc, and pNP, respectively. The disaccharide glycoside formed was about 14% that of the amount of pNP released. The reversed-phase HPLC separation procedure is capable of separating pNP-β-D-gentiobioside (β6→1), pNP-β-D-sophoroside (β2→1), pNP-β-D-laminaribioside (β3→1), and pNP-β-D-cellobioside (β4→1); these compounds eluted at retention times of 27.4, 29.2, 30.3, and 30.5 min, respectively. The disaccharide glycoside formed upon incubating the cytosolic β-glucosidase with 10 mM pNPGlc eluted at 27.4 min, the elution position of the pNP-β-D-gentiobioside standard. Similarly, when 10 mM pNPGal was used as the substrate we observed three peaks with retention times of 24.3, 27.4, and 38.3 min; the latter corresponded precisely to pNP-β-Gal(6→1)β-Gal, pNP-β-Gal, and pNP standards, respectively (Fig. 4, *panel B*). The amount of pNP-β-Gal(6→1)β-Gal disaccharide (pNPGalGal) formed was 60% of the amount of pNP generated.

We have found that transfer of the glucosyl residue from the glucosyl-enzyme intermediate to the sugar moiety of the glucoside substrate can be completely inhibited by the addition of an alkanol such as *n*-butyl alcohol to the incubation medium (data not shown). Hence, we have depicted the enzyme-catalyzed transfer of glucose to its own substrate (pNPGlc) as a simultaneously competing process to hydrolysis or alcoholysis toward alkanols (Fig. 1, *Scheme I*).

We were interested in knowing if the substrate-binding site was distinct from the acceptor site within the catalytic center of the cytosolic β-glucosidase. Since the amount of disaccharide glycoside formed was much greater when pNPGal served as the substrate we elected to perform a Michaelis-Menten type of analysis to determine the K_m values for pNPGal based on the velocity of pNP release and the rate of pNPGalGal disaccharide formation. We postulated that if the substrate-binding site is distinct from the acceptor site, then the K_m estimated based on the rate of formation of the transglyco-

sylation product, pNPGalGal, should be greater than the K_m for pNP formation. In an effort to verify this hypothesis, we varied the concentration of pNPGal in the β-glucosidase assay medium from 0.2 to 5 mM and determined the rates of formation of the aglycone, pNP, and the disaccharide product, pNP-β-Gal(6→1)β-Gal. The integration of peaks in the HPLC profiles allowed us to calculate the rates of formation of the aglycone and the disaccharide product. As is evident in Fig. 5, the velocity of formation of pNP and pNPGalGal both display typical hyperbolic saturation kinetics *versus* pNPGal (substrate) concentration and exhibit K_m values of 0.46 and 6.50 mM, respectively (Table II).

Determination of K_I Values for the Interaction of Linear Alkanols and the Active Site of the Cytosolic β-Glucosidase—The β-glucosidase reaction is stimulated by low concentrations (0.05–0.2 M) of *n*-butyl alcohol (8). However, when the concentration of *n*-butyl alcohol in the assay medium was increased further (0.2–0.6 M) we observed that the K_m value for the substrate 4-MUGlc also increased (8). We therefore speculated that the interaction of a second molecule of *n*-butyl alcohol with the substrate aglycone-binding subsite in the active site of the cytosolic β-glucosidase formed the basis for the competitive inhibition observed at the higher alcohol concentration range. Having demonstrated the enzyme-catalyzed formation of alkyl β-D-glucosides in the presence of alcohols (Fig. 3), it is reasonable to regard the alcohol molecule as a cosubstrate in the β-glucosidase-catalyzed breakdown of aryl β-D-glucosides. It appears that *n*-butyl alcohol behaves as a substrate at low concentrations and as an inhibitor at high concentrations. In this report we explore the possibility of fitting the data to a structural model which would be compatible with the above-mentioned results. We postulate that, at low concentrations, *n*-butyl alcohol binds to a high affinity site B, whereas at higher concentrations it also occupies a second site A; the latter site A is presumably the hydrophobic binding subsite in the active center of the cytosolic β-glucosidase which interacts with the 4-MU or the pNP moieties of the substrates 4-MUGlc and pNPGlc, respectively.

The biphasic kinetic response observed in the presence of alcohols (8) could be rationalized as follows; at low concentrations, *n*-butyl alcohol binds to site B (Fig. 6) and acts as a nucleophilic competitor to water, thereby increasing the V_{max} owing to the 24-fold higher rate of alcoholysis relative to hydrolysis (Table I). In contrast, at higher concentrations, *n*-butyl alcohol competes with 4-MUGlc and increases the K_m for 4-MUGlc. Hence, a plot of K_m/V_{max} *versus* alcohol concentration would initially display a decrease but then increase owing to increased K_m values (see Fig. 5 in Ref. 8). A critical assumption in this model is that the affinity of *n*-butyl alcohol for site B is greater than its affinity for site A (*i.e.* $K_B < K_I$).

In the kinetic scheme under consideration (Fig. 6), the substrates A and B correspond to 4-MUGlc (or pNPGlc) and a linear alkanol, respectively. Since, in the presence of alcohols, the glucosyl-enzyme intermediate proceeds largely in the direction of alcoholysis we have assigned the products 4-MU (or pNP) and the respective alkyl-β-D-glucoside as P and Q, respectively. The kinetic analysis needed to validate this model requires a Lineweaver-Burk analysis of 1/v (where v represents the velocity of formation of alkyl-β-D-glucoside in the β-glucosidase-catalyzed alcoholysis of 4-MUGlc) *versus* 1/[4-MUGlc] at various concentrations of a particular alcohol. A replot of the slopes (K_m/V_{max} values) of these Lineweaver-Burk plots *versus* the concentration of alcohol is obtained, from which it is possible to calculate K_B and K_I values. The extrapolated 1/[alcohol]-axis intercept is equal to $-1/K_B\{1 + 1/\gamma\}$, where $\gamma = K_I/K_B$. If indeed the substrate B has a higher affinity for its own site B, relative to site A, then the value of γ must be greater than unity.

In our previous study (8) we had reported the slope values (K_m/V_{max}) from Lineweaver-Burk analyses of 1/v (where v represents the velocity of 4-MU formation) *versus* 1/[4-MUGlc] at various concentrations of ethanol, *n*-propyl alcohol, *n*-butyl alcohol, or *n*-pentanol. We subjected this data to the above-mentioned kinetic analysis in an effort to gather evidence for or against the kinetic scheme proposed in Fig. 6 for the interaction of alcohols with two different hydrophobic sites on the cytosolic β-glucosidase. This kinetic analysis assumes that 1/v, calculated on the basis of rates of formation of 4-MU, approximates 1/v for the formation of alkyl-β-D-glucoside. Our observation that even when a low concentration of an alcohol is included in the assay medium 96% of the glucosyl-enzyme intermediate proceeds through alcoholysis

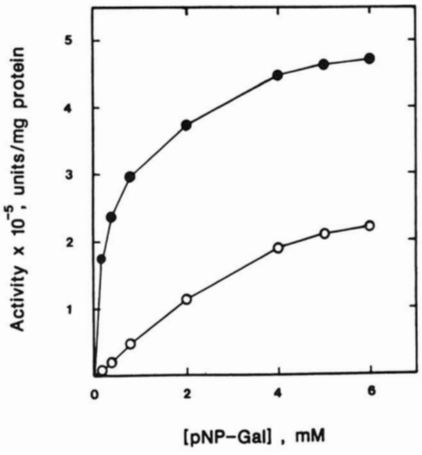

FIG. 5. **The transglycosylation activity of cytosolic β-glucosidase using pNPGal as the substrate.** The activity was measured using reversed-phase HPLC procedure (see "Experimental Procedures") that simultaneously provided estimates for the amounts of the aglycone, pNP (●), and the transglycosylation product, pNPGal(6→1)β-Gal (○), produced in the β-glucosidase assay.

TABLE II

Summary of Michaelis-Menten constants for pNP and pNPGalGal formation from pNPGal

K_m and V_{max} values were determined by nonlinear regression analysis using the Enzfitter program (Elsevier-Biosoft). The numbers in parentheses represent the standard error for these kinetic constants.

Product	K_m	$V_{max} \times 10^{-3}$
	mM	*units/mg*
pNP	0.46 (0.06)	600 (60)
pNPGalGal	6.45 (1.39)	360 (34)

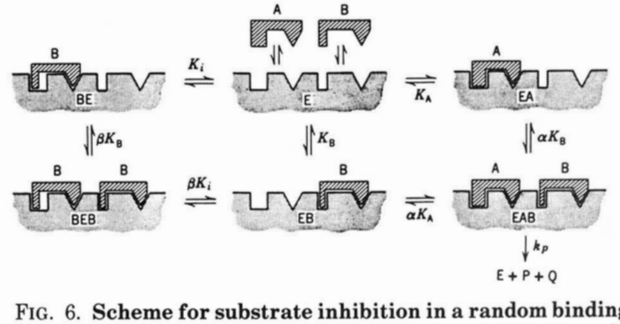

FIG. 6. **Scheme for substrate inhibition in a random binding rapid equilibrium system as proposed by Segel (22).** The substrate B binds to the A site as well as to the normal B site. The binding of B to site B and site A are designated K_B and K_I, respectively. When this model is applied to our data, A refers to 4-MUGlc and B refers to a linear alkanol such as *n*-butyl alcohol (see text for more details).

rather than hydrolysis validates this assumption. Fig. 7 depicts a typical replot of slope *versus* 1/[*n*-propyl alcohol]; the slope, K_m/V_{max}, is calculated from Lineweaver-Burk analyses of 1/v (where v represents the velocity of formation of propyl-β-D-glucoside) *versus* 1/[4-MUGlc] at various concentrations of *n*-propyl alcohol. As described by Segel (22), the position of the asymptote (Fig. 7, *dashed line*) is adjusted until its slope is half that of the line (Fig. 7, *dotted line*) which connects the minimum of the original plot with the 1/slope intercept of the asymptote. The *x* intercept is equal to $-1/K_B\{1 + 1/\gamma\}$, where $\gamma = K_I/K_B$. In our previous study (8), we had calculated the K_B values by fitting the data to the scheme for nonessential activation described by Segel (22) (Fig. 6) for the interaction of ethanol, *n*-propyl alcohol, *n*-butyl alcohol, or *n*-pentanol with a hydrophobic binding site (designated as *site B* in Fig. 1, *Scheme II*) on the cytosolic β-glucosidase and determined them to be 555, 146, 34.1, and 7.47 mM, respectively (24). Using the *x* intercept value of -7.8 mM^{-1} (Fig. 7) and a K_B value of 146 mM (data from Ref. 8), a γ value of 6 was obtained (Table III). Hence, the K_I value for the binding of *n*-propyl alcohol to the subsite of the catalytic center that interacts with the 4-MU moiety of the substrate 4-MUGlc is $6 \times K_B$, or 971 mM. Similar analyses with ethanol, *n*-butyl alcohol, and *n*-pentanol yielded γ values of 6.8, 5.6, and 5.8, respectively (Table III). Based on these γ values the inhibition constants (K_I) for ethanol, *n*-butyl alcohol, and *n*-pentanol, with the cytosolic β-glucosidase were calculated to be 3763, 189, and 43.7 mM, respectively. It is evident that there is a progressive decrease in the K_I value with increasing carbon number of the alkyl moiety of the alcohol (Fig. 8). This result corroborates previously articulated conclusions regarding the hydrophobic character of this site on the β-glucosidase (25).

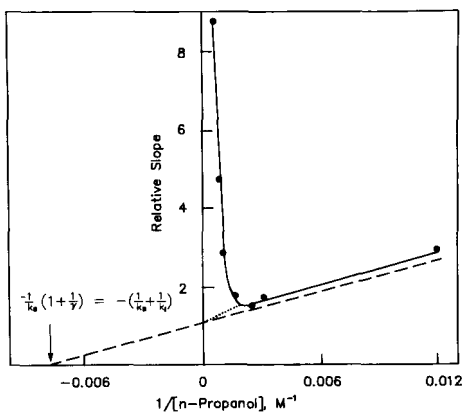

FIG. 7. **Calculation of the affinity constant, K_I, for the interaction of *n*-propyl alcohol with the active site of the cytosolic β-glucosidase.** Replot of slope (K_m/V_{max}) values obtained from Lineweaver-Burk analyses performed with 4-MUGlc as the substrate in the presence of varying concentrations of *n*-propyl alcohol *versus* the reciprocal concentration of *n*-propyl alcohol. These values were obtained form the data contained in an earlier report (8). The position of the asymptote (*i.e.* the *dashed line*) is adjusted until its slope is half that of a line (*i.e.* the *dotted line*) connecting the minimum point of the original plot with the *y* axis.

TABLE III

Summary of affinity constants of primary alcohols for two subsites designated A and B in the active site of the guinea pig liver cytosolic β-glucosidase

Alcohol	K_B[a]	K_I[b]	γ[c]
	mM	*mM*	
Ethanol	555	3763	6.8
n-Propyl alcohol	146	964	6.6
n-Butyl alcohol	34.1	189	5.6
n-Pentanol	7.47	43.6	5.8

[a] These values were calculated as described and reported elsewhere (8).

[b] These values were calculated based on the kind of kinetic analysis presented in Fig. 7.

[c] $\gamma = K_I/K_B$ (refer to Fig. 6).

Effect of n-Butyl Alcohol on the Substrate Specificity of the Cytosolic β-Glucosidase—We next investigated if the increase in the overall rate of 4-MUGlc or pNPGlc disappearance in the presence of *n*-butyl alcohol could also be achieved with the three other glycosides (*i.e.* 4-MUGal, 4-MUXyl, and 4-MUAra) that are also substrates of the cytosolic β-glucosidase. We exploited the phenomenon of transglycosylation to inquire into two questions: (i) is the rate-determining step in the β-glucosidase reaction independent of the glycone moiety for different glycoside substrates which possess the same aglycone, and (ii) is the relationship between the rates of glycosylation (k_2) and deglycosylation (k_3) for the four different glycosides invariant? To this end we utilized the β-D-glucose, β-D-galactose, β-D-xylose, and α-L-arabinose derivatives of 4-MU. We discovered that *n*-butyl alcohol increased the rate of aglycone (4-MU) release from 4-MUGlc, 4-MUGal, 4-MUXyl, and 4-MUAra 8-, 4-, 15-, and 30-fold, respectively. Furthermore, we observed that low concentrations of *n*-butyl alcohol (0.05–0.2 M) increased the rate of aglycone release with 4-MUGal, 4-MUXyl, and 4-MUAra, while higher concentrations (0.2–0.6 M) of *n*-butyl alcohol were inhibitory (Fig. 9). We documented the occurrence of such a biphasic response in our previous report (8) in which we analyzed the effect of *n*-butyl alcohol on the enzyme-catalyzed breakdown of 4-MUGlc.

We had documented previously (8) that when 4-MUGlc served as the substrate, the increase in the rates of aglycone release occurring at relatively low *n*-butyl alcohol concentrations could be accounted for entirely by an increase in V_{max}. The inhibition observed at the higher alcohol concentrations was competitive in nature, as evidenced by the increased K_m for cytosolic β-glucosidase and 4-MUGlc. Lineweaver-Burk analyses performed with 4-MUGal and increasing *n*-butyl alcohol concentrations revealed a kinetic pattern similar to that obtained with 4-MUGlc (data not shown); however, when 4-MUXyl and 4-MUAra served as substrates, both V_{max} and K_m increased simultaneously as the *n*-butyl alcohol concentration was increased (data not shown). Due to differences in the effects of *n*-butyl alcohol on the kinetic constants for the various glycoside substrates used, it is possible that the specificity constant of the enzyme could be altered by *n*-butyl

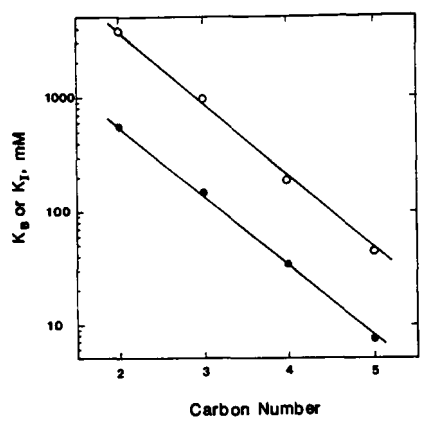

FIG. 8. **A plot of the affinity constants of a series of normal alcohols *versus* the carbon number of the alcohol.** The K_B (●) and K_I (○) values represent the affinity constants (mM) for the interaction of alkanols with sites B and A, respectively, on the β-glucosidase (refer to Fig. 6).

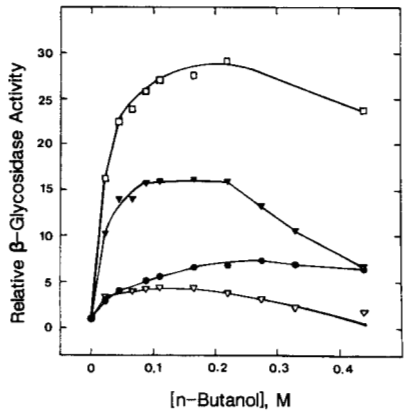

FIG. 9. **Effect of *n*-butyl alcohol on β-glycosidase activity.** The enzyme activity was determined using the β-glucosidase assay. The relative activity of cytosolic β-glucosidase toward 4-MUGlc (●), 4-MUGal (△), 4-MUXyl (▼), and 4-MUAra (□) is plotted *versus* the concentration of *n*-butyl alcohol.

TABLE IV

Summary of kinetic constants for the four 4-MU glycoside substrates in the absence and presence of 0.11 M n-butyl alcohol

Substrate	(−) *n*-Butyl alcohol		(+) 0.11 M *n*-Butyl alcohol	
	K_m	$V_{max} \times 10^{-3}$	K_m	$V_{max} \times 10^{-3}$
	mM	*units/mg*	*mM*	*units/mg*
4-MUGlc	0.15 (0.03)[a]	550 (30)	0.28 (0.04)	4800 (200)
4-MUGal	0.48 (0.08)	503 (33)	1.01 (0.16)	1910 (125)
4-MUXyl	0.08 (0.008)	50 (1.5)	0.98 (0.11)	450 (23)
4-MUAra	0.04 (0.005)	110 (3.5)	0.43 (0.03)	1730 (34)

[a] The numbers in parentheses represent the standard error for the respective kinetic constants.

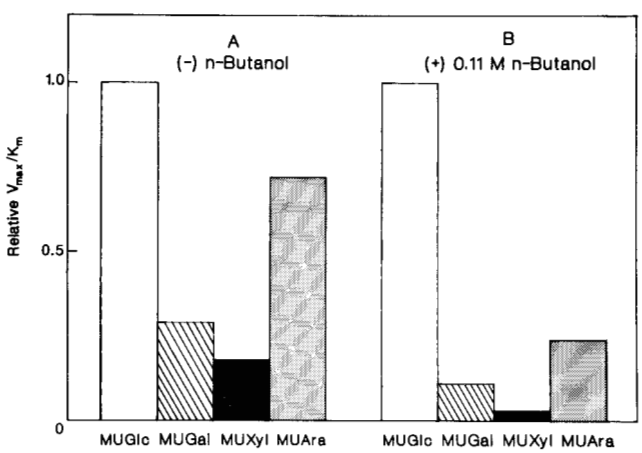

FIG. 10. **Effect of *n*-butyl alcohol on the specificity constant (V_{max}/K_m) of the cytosolic β-glucosidase.** The relative V_{max}/K_m values were calculated from the respective Lineweaver-Burk analyses for each substrate in the absence (*panel A*) and presence (*panel B*) of 0.11 M *n*-butyl alcohol. The absolute values for these kinetic constants are summarized in Table III.

alcohol. In fact, the specificity constant (V_{max}/K_m) data (Table IV) indicate that 0.11 M *n*-butyl alcohol in the assay medium narrows the broad specificity exhibited by the cytosolic β-glucosidase, causing it to be more specific for β-D-glucosides. Fig. 10 depicts the relative V_{max}/K_m values for the various 4-MU glycoside substrates in the absence and presence of 0.11 M *n*-butyl alcohol.

DISCUSSION

The main purpose of this study was to investigate the mechanism of catalysis and delineate the rate-limiting step for the β-glucosidase-catalyzed hydrolysis of aryl β-D-glucosides. To this end we took advantage of the well-established capability of many glycosidases to catalyze transglycosylation reactions in the presence of alcohols. This study establishes that guinea pig liver cytosolic β-glucosidase generates a common glucosyl-enzyme intermediate from a variety of aryl β-D-glucoside substrates. Furthermore, we document that the glucosyl-enzyme intermediate thus generated can react with different acceptors to form distinct products at rates that are dependent on the structure, nucleophilicity, and concentration of the acceptor. Specifically, we report here that water and linear alkanols (*e.g. n*-butyl alcohol) can react with the glucosyl-enzyme intermediate to form D-glucose and alkyl-β-D-glucoside (*e.g.* butyl-β-D-glucoside), respectively. Moreover, at high concentrations a substrate molecule (*e.g.* pNPGal) itself can also serve as an acceptor and attack the galactosyl-enzyme intermediate to form a disaccharide molecule (pNPGalGal). These observations have yielded insights regarding the mechanism of action and the structure of the catalytic center of the cytosolic β-glucosidase.

We demonstrated in a previous study (8) that low concentrations of primary alcohols exerted a stimulatory effect on the activity of the cytosolic β-glucosidase. Our investigation was directed primarily toward elucidating the mechanism for the increase in V_{max} of the β-glucosidase-catalyzed reaction elicited by a nonessential activator molecule such as *n*-butyl alcohol. It is generally accepted that alcohols are more effective nucleophiles than water in reactions involving nucleophilic substitutions. Thus, in the solvolysis of the glycosyl-enzyme intermediate generated by any glycosidase, the rate of alcoholysis (k_4, Fig. 1, *Scheme I*) will be greater than the rate of hydrolysis (k_3, Fig. 1, *Scheme I*). If the rate-limiting step in the cytosolic β-glucosidase-catalyzed cleavage of aryl-β-D-glucosides is the deglucosylation of the glucosyl-enzyme intermediate (k_3), then the rate of glucosylation k_2 must be greater than k_3. If the rate of glucosylation is rapid, the glucosyl-enzyme intermediate will be replenished as rapidly as it reacts with a nucleophile. Hence, an increased rate of solvolysis of the glucosyl-enzyme intermediate in the presence of alcohols manifests as an increase in the k_{cat} value. It follows, then, that for a substrate whose rate of breakdown is determined by the rate of glucosylation (*i.e.* $k_2 < k_3$), increasing the rate of solvolysis of the intermediate would have no effect on the overall rate of substrate disappearance.

The theoretical expectations outlined above are in complete agreement with the results obtained in our study of the effect of *n*-butyl alcohol on the β-glucosidase-catalyzed breakdown of pNPGlc and phenyl-β-D-glucoside (Table I). The 8-fold increase in the rate of pNP (aglycone) release elicited by *n*-butyl alcohol is consistent with k_3 being the rate-determining step in the enzymatic hydrolysis of pNPGlc in the absence of alcohols. A considerable increase in overall rate is possible because k_2 is greater than k_3. The lower steady-state k_{cat} value for phenyl-β-D-glucoside compared to pNPGlc indicates that k_3 is not rate determining for the cytosolic β-glucosidase-catalyzed hydrolysis of phenyl-β-D-glucoside (*i.e.* $k_2 < k_3$). Therefore, only a small increase in the overall rate of substrate disappearance would be expected in the presence of normal alcohols. In fact, only a 2-fold increase in phenol (aglycone) release was observed. On the basis of these results we attribute the observed enhancement in overall rate of breakdown of aryl-β-D-glucosides by the cytosolic β-glucosidase by alcohols to the phenomenon of transglucosylation.

The above-mentioned results are consistent with the findings of other investigators who have studied analogous systems (1, 2, 5). For example, in the case of pig liver serine

esterase Greenzaid and Jencks (5) documented that the overall rate of phenylacetate disappearance was increased 5.5-fold by 0.5 M methanol with little change in the hydrolysis rate; their results also indicated that the less reactive *p*-nitrophenyl acetate (pNP-acetate) displayed a much smaller rate acceleration in the presence of methanol. The reaction pathway specified in Fig. 1, *Scheme I*, is completely applicable to the mechanism of serine esterase if the glucosyl-enzyme intermediate is replaced by an acyl-enzyme intermediate. Greenzaid and Jencks (5) concluded that the rate of acylation (k_2) is greater than the rate of deacylation (k_3) for the esterase-catalyzed breakdown of phenylacetate while $k_2 < k_3$ for the catalysis of pNP-acetate.

Consider *Schemes I* and *II* of Fig. 1 for competing hydrolysis and alcoholysis with water and *n*-butyl alcohol as acceptors, where *X*, *Enz.Glc-X*, and *Enz-Glc*, represent the aglycone moiety in the substrate, the Michaelis complex, and the putative common glucosyl-enzyme intermediate, respectively. There are two routes the cytosolic β-glucosidase-catalyzed reaction could follow: the hydrolysis and alcoholysis reactions that occur with β-glucosidase could either derive from nucleophilic attack on an glucosyl-enzyme intermediate (Fig. 1, *Scheme I*) or the Michaelis complex (Fig. 1, *Scheme II*). If the cytosolic β-glucosidase catalyzes the hydrolysis of various aryl β-D-glucosides through a common Enz-Glc intermediate (Fig. 1, *Scheme I*), it follows then that in the presence of water and a fixed concentration of *n*-butyl alcohol, the butyl-β-D-glucoside/β-D-glucose ratio should be independent of differences in the chemical and physical properties of the leaving group. Alternatively, if these nucleophiles directly attack the Michaelis complex (Fig. 1, *Scheme II*), then the nature of the aglycone would be expected to alter the alcoholysis/hydrolysis product ratio obtained with different substrates. That this approach is both precedented and conventional is borne out by the fact that it has enabled others to identify a common intermediate in the reactions catalyzed by chymotrypsin (2, 24), almond β-glucosidase (11), and *E. coli* β-galactosidase (3, 4). We have demonstrated that at least four substrates possessing very different leaving groups (with pK_a values varying from 7.1 to 10.0) yielded an invariant product ratio (24:1, alkyl-β-D-glucoside:D-glucose) for the parallel processes of alcoholysis and hydrolysis (Table I). This result points to the generation of a common glucosyl-enzyme intermediate in the mammalian cytosolic β-glucosidase-catalyzed breakdown of various aryl-β-D-glucosides.

Legler and Bieberich (26) reported that the cytosolic β-glucosidase from calf liver released only β-D-glucose during hydrolysis of pNPGlc. They estimated the amount of pNP released from pNPGlc by the enzyme using a spectrophotometric method. When the amount of D-glucose was estimated polarimetrically they found good agreement with the amount predicted on the basis of aglycone release only when their calculations were based on the specific rotation of the β-anomer rather than the α-anomer. We have provided evidence in this study to support that the cytosolic β-glucosidase preserves the anomeric configuration of the original β-D-glucoside substrate molecule in the final product of alcoholysis, *i.e.* alkyl β-D-glucoside (Fig. 3). This observation is consistent with a reaction course which follows a double-displacement mechanism involving two sequential S_N2 substitutions and one which necessitates a sequential liberation of products (aglycone release followed by the discharge of the glycone) in the cytosolic β-glucosidase-catalyzed breakdown of aryl β-D-glucosides. The product of the first nucleophilic substitution must be a common glucosyl-enzyme intermediate.

Since the β-glucosidase can use linear alkanols as acceptors in the transglucosylation reaction, is it possible that a primary hydroxyl group in the sugar moiety of a substrate molecule (*e.g.* pNPGlc) or any other glycoside molecule could also serve as an acceptor? Indeed, we have documented herein that at high concentrations (in the range 2.5–10.0 mM) appropriate β-glucosidase substrates such as pNPGlc and pNPGal do function as nucleophilic acceptors and react with the glucosyl-enzyme intermediate to form pNP-disaccharide glycosides (Fig. 5). Since we have unequivocally identified the disaccharide products as possessing a β(1→6) linkage between their constituent sugar units, it is reasonable to conclude that the C(6) primary hydroxyl group in these substrates serves as the nucleophilic acceptor. It is interesting that the disaccharide formed contains exclusively the β(1→6) linkage between the two sugar units. It has been demonstrated with *E. coli* β-galactosidase that when lactose is cleaved to D-glucose and D-galactose, the D-glucose molecule could use its C(6) primary hydroxyl group to attack the galactosyl moiety to generate allolactose (galactose β1→6 glucose) (27). The basis for this intermolecular transgalactosylation was attributed to the increased nucleophilicity of the C(6) primary hydroxyl group in D-glucose.

We observed differences in the amount of disaccharide glycoside generated when pNPGlc or pNPGal served as the acceptor; the amount of pNPGalGal and pNPGlcGlc (pNP-gentiobioside) were 60 and 15%, respectively, of the amount of pNP released from their respective substrates (Fig. 4). The accumulation of disaccharide glycosides in the assay medium is dependent on both their rate of formation and degradation. The rates of formation of both pNP-β-Gal(6→1)β-Gal and pNP-β-Glc(6→1)β-Glc are likely to be very similar owing to the fact that the two substrates, pNPGal and pNPGlc, possess the same nucleophilic acceptor moiety *i.e.* the C(6) primary hydroxyl group. Therefore, a plausible explanation for these discrepant yields must focus on differences in the rate of their disappearance. Indeed, data[2] confirm that the cytosolic β-glucosidase is capable of hydrolyzing pNPGlc-(6→1)β-Glc but not pNPGal(6→1)β-Gal. This observation explains the higher amount of disaccharide accumulated in the transglycosylation reaction when pNPGal served as the acceptor instead of pNPGlc.

The results of our kinetic analyses on the formation of alkyl-β-D-glucoside in the presence of varying concentrations of alcohols conforms precisely to the model proposed by Segel (22) for a random bisubstrate rapid equilibrium system in which one of the substrates binds to its usual site B but also competes for the binding site A of the second substrate (Fig. 6). Using this approach we have obtained evidence that there are two hydrophobic sites in the catalytic center of the cytosolic β-glucosidase, and that one of these sites (B) binds to the alcohol with a higher affinity than does the other site (A). We designated the affinity constants for interaction of an alcohol molecule with the A and B site as K_I and K_B. The interaction of an alcohol molecule with the A site causes competitive inhibition and is therefore designated as a K_I value. The K_I and K_B values for the enzyme-alcohol interaction exhibit a 4.0–4.5-fold decrease with each additional carbon atom in the alkyl chain of the alcohol molecule (Fig. 8). The apparent higher K_I values displayed by the alcohols, relative to their K_B values, could be accounted for by the higher affinity of the substrate for its usual binding site A.

The data regarding the rate of formation of pNP and pNPGalGal with increasing pNPGal concentration provide additional evidence for the presence of two distinct hydropho-

[2] V. Gopalan, A. Pastuszyn, W. R. Galey, and R. H. Glew, manuscript submitted.

bic sites in the active site of the cytosolic β-glucosidase. Two different hyperbolic curves were observed when the rates of formation of pNP and pNPGalGal were plotted *versus* the pNPGal concentration (Fig. 5). Based on the rate of pNP formation a K_m value of 0.46 mM for the interaction of pNPGal and the cytosolic β-glucosidase was obtained. The K_m for disaccharide product formation was determined to be 6.5 mM. These data indicate that pNPGal binds to its usual substrate site A at the lower concentrations while at higher concentrations it binds to the site B where it serves as a nucleophilic acceptor (Fig. 11).

The relative affinity of pNPGal for the second site on the β-glucosidase is less than that of an alcohol molecule such as *n*-butyl alcohol. We hypothesize that in the case of *n*-butyl alcohol, due to its higher affinity for site B, it acts as an acceptor at low concentrations while at high concentrations it causes competitive inhibition by binding to the less hydrophobic site A. In contrast, a hydrophilic substrate molecule such as pNPGal binds with a higher affinity to site A while at higher concentrations it occupies site B and participates in the transglycosylation reaction (Fig. 11).

In this report we have also examined the effect of *n*-butyl alcohol on the substrate specificity of the cytosolic β-glucosidase. The contention that alcoholysis of the glucosyl-enzyme intermediate could permit an increase in overall rate of substrate disappearance only when $k_2 > k_3$ is validated by the results of our study on the effect of *n*-butyl alcohol on the enzyme-catalyzed breakdown of pNPGlc and phenyl-β-D-glucoside. In fact, the requirement that k_2 must be greater than k_3 for the stimulatory effect of alcohols to be observed allows one to utilize the magnitude of the stimulatory effect of alcohols to determine the rate-limiting step in the enzyme-catalyzed breakdown of aryl-β-D-glycosides. From our data it is evident that the extent to which *n*-butyl alcohol can stimulate the rate of substrate disappearance is dependent on the nature of the glycone moiety conjugated to the aglycone; specifically, 8-, 4-, 15-, and 30-fold increases in the steady-state k_{cat} value for the catalysis of 4-MUGlc, 4-MUGal, 4-MUXyl, and 4-MUAra, respectively, were observed when 0.17 M *n*-butyl alcohol was included in the β-glycosidase assay medium. From this observation one can draw at least two inferences. First, $k_2 > k_3$ for all of the glycoside substrates. It follows, then, that deglycosylation of the glycosyl-enzyme intermediate is the rate-determining step in the cleavage of 4-MUGlc, 4-MUGal, 4-MUXyl, and 4-MUAra by the cytosolic β-glucosidase. Second, the differences we observed in the fold increase in the steady-state k_{cat} values for hydrolysis of the various glycoside substrates permitted us to predict the relative k_2/k_3 ratios for the four substrates; the k_2/k_3 ratio should decrease in the following order: 4-MUAra > 4-MUXyl > 4-MUGlc > 4-MUGal. Based on these data we conclude that the differences in K_m values for the substrates 4-MUGlc, 4-MUGal, 4-MUAra, and 4-MUXyl are related to variations in the kinetic rate constants for the hydrolysis of the various glycosides by the cytosolic β-glucosidase and not due to subtle changes in the structural complementarity of the enzyme-substrate interaction.

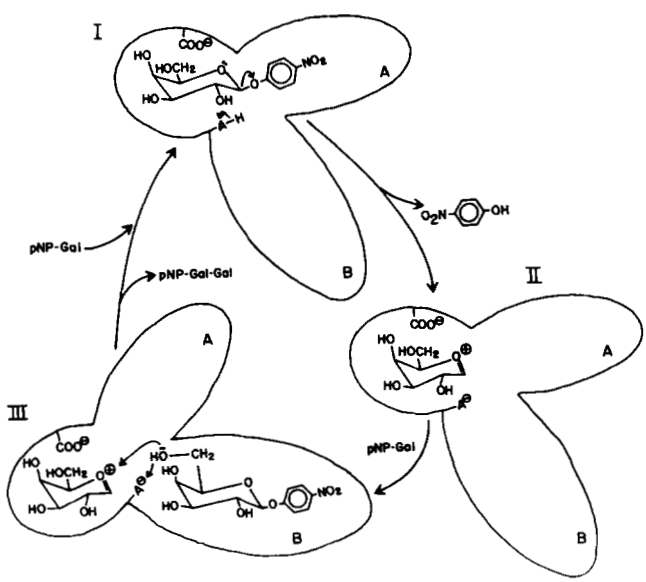

FIG. 11. **Model depicting the synthesis of pNP-β-Gal(6→1)β-Gal when pNP-β-Gal serves not only as a substrate but also as an acceptor in the cytosolic β-glucosidase-catalyzed transglycosylation reaction.**

The literature contains a number of reports which have noted the organic solvent-dependent enhancement of substrate specificity in enzyme-catalyzed reactions. For example, Schuster (28) demonstrated that 20% methanol inhibited beef heart mitochondrial ATPase-catalyzed hydrolysis of inosine triphosphate by 50% whereas the rate of hydrolysis of ATP was increased 3-fold compared to that of the aqueous control.

Does the cytosolic β-glucosidase-catalyzed transglucosylation reaction characterized in this study using the linear alkanols have a parallel reaction *in vivo*? A recent study by Li and co-workers (23) has demonstrated that a ceramide glycanase isolated from the leech *Macrobdella decora* transfers oligosaccharides *en bloc* from various glycosphingolipids to suitable acceptors including alkanols such as *n*-octanol. The authors suggested that the oligosaccharide-transferring reaction could be used in the synthesis of neoglycoconjugates and in studies aimed at elucidating the biological functions of glycan chains in glycosphingolipids. In a related vein, it is possible that the transglycosylation reaction catalyzed by the cytosolic β-glucosidase toward appropriate glycoside acceptors could generate novel mammalian glycoconjugates hitherto undiscovered. For example, it is possible that amphipathic galactosylsphingosine might serve as an acceptor in the β-glucosidase-catalyzed transglucosylation reaction to form sphingosine-conjugated disaccharides.

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