also for the simple models described by eq 3.

Registry No. Mg, 7439-95-4; PP_i, 9024-82-2; EC 3.6.1.1, 19262-94-3.

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Reversible Inhibitors of β-Glucosidase†

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ABSTRACT: A variety of reversible inhibitors of sweet almond β-glucosidase were examined. These included simple sugars and sugar derivatives, amines and phenols. With respect to the sugar inhibitors and, indeed, the various glycoside substrates, the enzyme has what can be considered a "relaxed specificity". No single substituent on glucose, for example, is essential for binding. Replacement of a hydroxyl group with an anionic substituent reduces the affinity while substitution with a cationic (amine) substituent enhances the affinity. Amines, in general, are good inhibitors, binding more tightly than the corresponding alcohols: $pK_i^{RNH_3^+} = 0.645pK_i^{ROH} + 1.77$ ($n = 9$, $r = 0.97$). The affinity of a series of 10 primary amines was found to be strongly influenced by substituent hydrophobicity: $pK_i = 0.52\pi + 1.32$ ($r = 0.95$). The major binding determinant of the glycoside substrates is the aglycon moiety. Thus, the K_i values of phenols are similar in magnitude to the K_s values of the corresponding aryl β-glucoside. The pH dependence for the inhibition by various phenols indicates that it is the un-ionized phenol which binds to the enzyme when an enzymic group of $pK_a = 6.8$ (±0.1) is protonated. The affinity of the phenol inhibitor is dependent on its basicity with a Brønsted coefficient for binding of $\beta = -0.26$ ($n = 14$, $r = 0.98$). The pH dependence of the binding of two particularly potent β-glucosidase inhibitors was also examlned. 1-Deoxynojirimycin (1;5-dideoxy-1,5-imino-D-glucitol) has a pH-corrected $K_i = 6.5$ μM, and D-glucono-1,5-lactam has a pH-corrected $K_i = 29$ μM. Although these are two of the most effective reversible inhibitors of the enzyme, they are not transition-state analogues. The evidence for this is that their affinities for β-glucosidase do not change with changing pH in a way that completely resembles the influence of pH on k_{cat}/K_m for normal substrates as might be expected for an ideal transition-state analogue inhibitor.

Although almond β-glucosidase (EC 3.2.1.21) was one of the first enzymes studied (Wohler & Liebig, 1837; Fischer, 1898), relatively little is known about its structure or catalytic mechanism (Legler, 1975). One striking feature of the enzyme, however, is its remarkably broad specificity. This broad specificity refers not only to the aglycon portion of the substrate but also to the glycon moiety as well. Thus, in addition to

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catalyzing the hydrolysis of a wide variety of β-D-glucopyranosides, sweet almond β-glucosidase also catalyzes the hydrolysis of β-D-glucofuranosides (Yoshida, 1966), β-D-galactosides (Heyworth & Walker, 1962; Walker & Axelrod, 1978), β-D-xylosides (Kiss et al., 1981), β-D-2-deoxyglucosides (Legler, 1975), and α-L-arabinosides (Conchie et al., 1968). This broad specificity facilitates the use of structure–reactivity studies to explore both the active site topology and the mechanism of action of the enzyme. This study, which involves an examination of the interactions of a variety of reversible inhibitors with β-glucosidase, was undertaken in order to learn more about the nature of interactions that can potentially occur between the enzyme and the substrate during the course of the catalytic reaction. Reversible inhibitors are useful for probing the binding properties of enzymes and may also help in elucidating mechanisms of catalysis [see, for example, Wolfenden (1978)]. Therefore, among the inhibitors examined were some generally thought to bear a structural resemblance to putative chemically activated intermediates in the enzymic reaction.

Materials and Methods

Most of the sugar derivatives were obtained from Sigma Chemical Co. The monomethylated glucose derivatives (2-, 3-, 4- and 6-*O*-methylglucopyranose) were obtained from Supelco, Inc. The amines and phenols were also commercially available products. The 1-deoxynojirimycin and D-glucono-1,5-lactam were prepared as described by Inouye et al. (1968). The 1-methyl-1-deoxyglucose was prepared from 2,3,4,6-tetrabenzylglucose by treatment with methylenetriphenylphosphorane and reaction with mercuric trifluoroacetate in tetrahydrofuran (THF) followed by quenching with sodium borohydride. The 1-*n*-octyl-1-deoxyglucose was prepared by treatment of 2,3,4,6-tetrabenzylgluconolactone (Fletcher et al., 1967) with octynyllithium followed by reduction with triethylsilane and BF_3·Et_2O and then catalytic hydrogenolysis over $Pd(OH)_2$ in acetic acid as described by Lancelin et al. (1983). The 1-(9-fluorenyl)-1-deoxyglucose (mp 86–88 °C) was prepared similarly from lithiofluorene and 2,3,4,6-tetrabenzylgluconolactone. The *N*-benzyl-2-amino-2-deoxyglucose derivatives were prepared by reductive amination of 2-amino-2-deoxyglucose as described by Wacker (1967).

The β-glucosidase, from sweet almonds, was obtained from Sigma Chemical Co. (type I, specific activity usually ~24 units/mg). The enzyme yielded a single band on gel filtration (Fractogel HW 55) corresponding to a molecular weight of ~135 000 (cf. Grover & Cushley, 1977) and a single band on both native and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Enzyme concentration was determined by absorbance at 277 nm with $E^{1\%} = 7.25$ (Grover & Cushley, 1977).

β-Glucosidase was generally assayed by following the hydrolysis of *p*-nitrophenyl β-D-glucoside spectrophotometrically on a Beckman Model 3600 spectrophotometer. Assays were carried out at 27 °C. The reactions were initiated by addition of enzyme to a solution of substrate in buffer which had been thermally equilibrated. The buffer system usually consisted of 0.01 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.02 M sodium acetate (NaOAc), and 0.1 mM (ethylenedinitrilo)tetraacetic acid (EDTA). The buffer system used for some studies carried out at pH 6.2 consisted of 0.05 M 4-morpholineethanesulfonate (MES) and 0.1 M NaCl. The enzyme activity was essentially identical in this buffer and the PIPES/NaOAc buffer at pH 6.2.

The kinetic parameters for the enzymic reaction under initial velocity (zero-order) conditions (K_M, V_{max}) were determined graphically by the method of Eisenthal & Cornish-Bowden (1974). The dissociation constant of the enzyme–inhibitor complex, K_{is}, was calculated from the effect of the inhibitor on the ratio of the two steady-state parameters:

$$(K/V)_+ = (K/V)_0(1 + [I]/K_{is}) \quad (1)$$

$(K/V)_+$ is the ratio of K_M to V_{max} in the presence of the inhibitor. The parameter K/V could also be determined conveniently under first-order conditions ($[S] \ll K_M$). The first-order rate constants, V/K, were evaluated graphically from a plot of log $(A_\infty - A_t)$ vs. time. These plots were linear for at least three half-lives. The absorbance was monitored at a wavelength which gave a convenient absorbance change (e.g., 400 nm at pH 6.2). When pH effects were studied, the reaction was usually monitored at the *p*-nitrophenol/*p*-nitrophenoxide isosbestic point (347.5 nm). At this wavelength, $\epsilon = 3500$ M^{-1} cm^{-1}.

In order to test for transglucosylation (i.e., transfer of glucose from *p*-nitrophenol to an acceptor other than water), the ratio of released products, [glucose]/[*p*-nitrophenol], was determined. This ratio is unity in the absence of transglucosylation. The *p*-nitrophenol concentration was determined directly by absorbance at 400 nm where $\epsilon = 18\,300$ M^{-1} cm^{-1} for the phenolate ion. The glucose concentration was determined by the hexokinase/glucose-6-phosphate dehydrogenase coupled assay. Aliquots from the β-glucosidase reaction were diluted at various times into Tris buffer (pH 8) containing the glucose assay system. The absorbance (340 nm) of the NADH produced, which is proportional to the glucose concentration, was then measured. The assay conditions are essentially those described by Schachter (1975). This procedure was also used to monitor the rate of the enzyme-catalyzed hydrolysis of phenyl β-glucoside and methyl β-glucoside.

The hydrophobicity substituent constants (π) were determined from, or calculated by, the method described in Hansch & Leo (1979).

Table I: Relative Reactivities of *p*-Nitrophenyl Glycosides with β-Glucosidase (pH 5.6,[a] 27 °C)

glycon	K_m (mM)	$(V/K)_{rel}$[b]
D-fucose	0.93	2.6
D-glucose	2.5	1.0[c]
D-galactose	15.7	5.6×10^{-2}
D-xylose	3.1	1.9×10^{-2}
D-mannose		$\leq 3 \times 10^{-4}$

[a] The K_m values are essentially identical (±20%) at pH 6.2 and 5.6. [b] The V/K value for each glycoside relative to that of glucoside [i.e., $(V/K)_{rel}$] is essentially independent of pH from 4 to 7. [c] On the basis of a molecular weight of 135 000 for the enzyme (Grover & Cushley, 1977), the value of $(V/K)/[E]$ $(=k_{cat}/K_M)$ for *p*-nitrophenyl β-D-glucoside is 3.2×10^6 M^{-1} min^{-1} at pH 5.6.

Results

Interactions of β-Glucosidase with Sugars and Sugar Derivatives. β-Glucosidase from sweet almonds ("emulsin") has a broad substrate specificity, catalyzing the hydrolysis of a wide range of glycosides (Nath & Rydon, 1954). Table I summarizes the kinetic properties of some of the *p*-nitrophenyl glycoside substrates.

Table II summarizes the inhibition constants of a variety of sugars. These sugars were all found to be linear competitive inhibitors with respect to *p*-nitrophenyl β-D-glucoside as substrate. No significant amount of transglycosylation (transfer of glucose from *p*-nitrophenol to the sugar inhibitor) was found either at pH 6.2 or at pH 5.6 (the pH optimum of the enzyme), under conditions used to determine the K_i values, with any of the sugars for which this was checked (i.e., D-glucose, D-

Table II: Binding of Some Sugars to β-Glucosidase at pH 6.2

	K_i[a] (mM)		K_i[a] (mM)
aldohexoses		aldopentoses	
D-idose	95	D-arabinose	40
D-glucose	160	L-xylose	120
L-glucose	160	D-lyxose	300
D-galactose	280	L-ribose	300
L-galactose	300	D-ribose	310
D-talose	300	L-arabinose	475
D-altrose	360	L-lyxose	480
D-allose	450	D-xylose	495
L-mannose	550		
D-gulose	1200	deoxyaldohexoses	
D-mannose	1460	1-deoxy-D-glucose[b]	60
		6-deoxy-D-galactose[c]	70
ketohexoses		6-deoxy-L-mannose[d]	110
D-fructose	135	1-deoxy-D-galactose	140
D-sorbose	145	2,6-dideoxy-D-allose[e]	170
D-tagatose	300	2-deoxy-D-glucose	180
		6-deoxy-L-galactose[c]	190
		6-deoxy-D-glucose[f]	290
		2-deoxy-D-galactose	790

[a] The relative errors for each of the K_i values were generally <10%. [b] 1,5-Anhydroglucitol. [c] Fucose. [d] Rhamnose. [e] Digitoxose. [f] Quinovose.

galactose, D-mannose, D-fructose, D-arabinose, D-xylose, or D-fucose).

In order to evaluate the role of the glycon hydroxyl groups in the interaction of the sugar with the enzyme, we examined a variety of glucose derivatives. The K_i values, listed in Table III, were generally determined under first-order conditions (i.e., $[S] \ll K_m$, $K_i = K_{is}$). Many of these derivatives were also tested under zero-order conditions at various substrate concentrations, and these were all found to be linear competitive inhibitors with K_i values within 20% of those obtained under first-order conditions. These inhibitors include 1-deoxy-1-methylglucose, glucal, 2-deoxy-2-aminoglucose, 1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol), and δ-gluconolactam. The pH dependence for the binding of these last two inhibitors to the enzyme is illustrated in Figure 1. The catalytic activity of the enzyme (V/K) yields a bell-shaped pH profile showing the dependence on two ionizing groups with pK_a's of 4.4 and 6.7 (Dale & Byers, 1982; Dale, 1983). The binding of gluconolactam shows a dependence only on the group with the higher pK_a (=6.8, in this case). The pH profile for binding of gluconolactam to the enzyme is given by eq 2.

$$1/K_i = 34.5\ \text{mM}^{-1}/(1 + 10^{\text{pH}-6.8}) \quad (2)$$

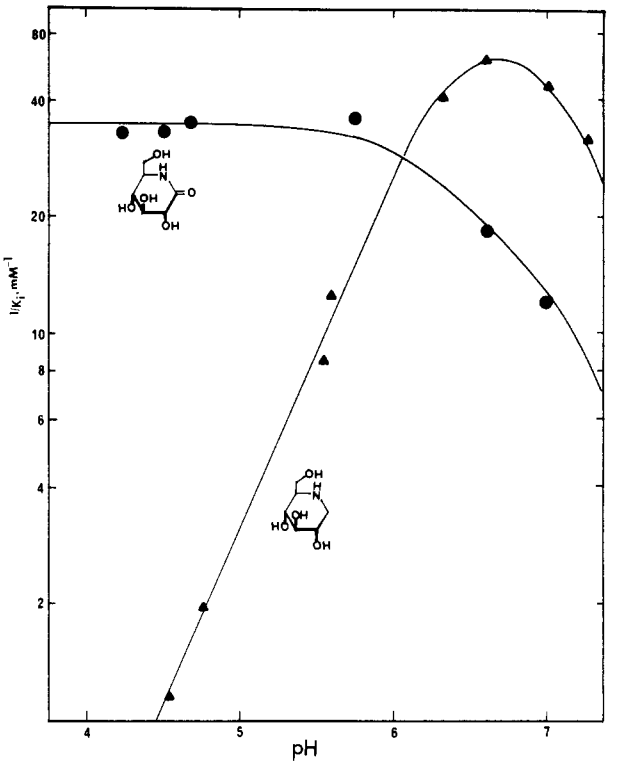

FIGURE 1: pH dependence for the binding of D-glucono-1,5-lactam (●) and 1-deoxynojirimycin (▲) to β-glucosidase (27 °C, μ = 0.02 M). The solid lines are the theoretical curves for the dependence on an ionizing group with a pK_a of 6.8 (in the case of the lactam) or on a pK_a of 6.8 and a pK_a of 6.7 (=pK_a of 1-deoxynojirimycin).

Thus, as the pH is lowered below 6, the K_i value levels off at 29 (±5) μM.

The pH profile for 1-deoxynojirimycin binding to the enzyme is bell shaped, and this reflects the ionization of the piperidine nitrogen group in the inhibitor. The pK_a of 1-deoxynojirimycin was determined by potentiometric titration to be 6.7 (±0.1) under the reaction conditions (27 °C, μ = 0.02 M). The pH profile for binding of this inhibitor is given by eq 3. This implies that it is the unprotonated inhibitor

$$1/K_i = 154.5\ \text{mM}^{-1}/(1 + 10^{\text{pH}-6.8} + 10^{6.7-\text{pH}}) \quad (3)$$

(pK_a = 6.7) that binds to the protonated enzyme (pK_a = 6.8). The data can be interpreted equally well, however, as reflecting the binding of the protonated inhibitor to the unprotonated

Table III: Inhibition of Glucosidase by D-Glucose Derivatives (27 °C, pH 6.2)

	K_i (mM)		K_i (mM)
α-glucose	127 (±25)	C-3 derivatives	
β-glucose	189 (±36)	3-deoxy-3-aminoglucose	8 (±1)
C-1 derivatives		3-methoxyglucose	930 (±150)
N-methylglucamine	1.0 (±0.1)	C-4 derivatives	
1-deoxy-1-(9-fluorenyl)glucose	1.6 (±0.3)	4-methoxyglucose	320 (±150)
1-deoxy-1-*n*-octylglucose	1.8 (±0.3)	C-5 derivatives	
1-deoxy-1-methylglucose	7.3 (±0.4)	5-deoxy-5-thioglucose	245 (±10)
gluconic acid	20 (±1)	C-6 derivatives	
phenyl α-glucoside	80 (±5)	6-deoxy-6-aminoglucose	40 (±3)
methyl α-glucoside	~400	6-methoxyglucose	165 (±12)
p-nitrophenyl α-glucoside	>500	glucuronic acid	>900
cellobiose	630 (±40)	glucose 6-phosphate	>900
sucrose	1000 (±100)	lactones, lactams, etc.	
glucal	20 (±1)	1-deoxynojirimycin	0.029 (±0.002)
C-2 derivatives		δ-gluconolactam	0.037 (±0.006)
2-deoxy-2-[(*p*-chlorobenzyl)amino]glucose	3.0 (±0.6)	δ-gluconolactone	0.20 (±0.02)
2-deoxy-2-[(*p*-methoxybenzyl)amino]glucose	5.9 (±0.9)	L-ascorbic acid	2.0 (±0.1)
2-deoxy-2-aminoglucose	6.9 (±0.4)	cytosine	20 (±4)
2-deoxy-(3-methyl-3-nitrosoureido)glucose (streptozotacin)	60 (±1)	piperidine	22 (±2)
2-methoxyglucose	120 (±6)	α-gluconoheptanoic acid γ-lactone	55 (±3)
2-deoxy-2-acetamidoglucose	>900	δ-valerolactam	65 (±8)

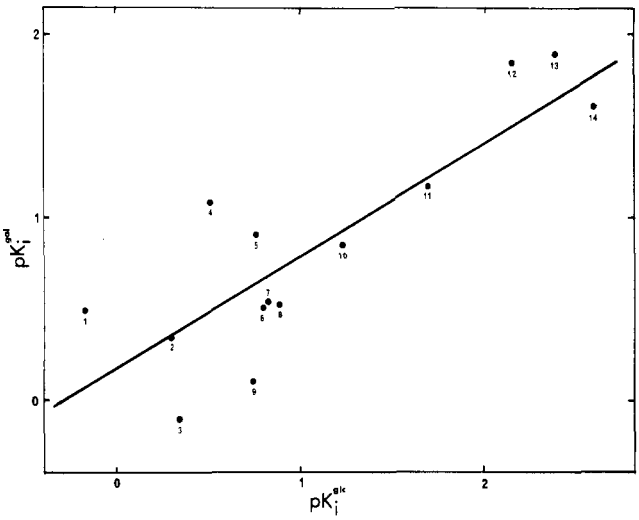

FIGURE 2: Trend showing the effect of stereochemistry at C-4 on the binding of sugars and derivatives to β-glucosidase (27 °C, pH 6.2, 0.01 M MES, 0.1 M NaCl). $-\log K_i$ for the glucose derivative is represented on the abscissa. The points represent the following modifications on each glucose/galactose pair: (1) inversion of configuration at C-2, (2) replacing C-5 hydroxymethylene with H, (3) inversion of configuration at C-3, (4) 6-deoxy, (5) methyl β-glycoside, (6) none, (7) enantiomer (i.e., L sugar), (8) ketose, (9) 2-deoxy, (10) 1-deoxy, (11) glycal, (12) 2-deoxy-2-amino, (13) *p*-nitrothiophenyl β-glycoside, and (14) *p*-nitrophenyl β-glycoside. The correlation equation for the line (least-squares fit) is $pK_i^{gal} = 0.62pK_i^{glc} + 0.19$ with $r = 0.83$.

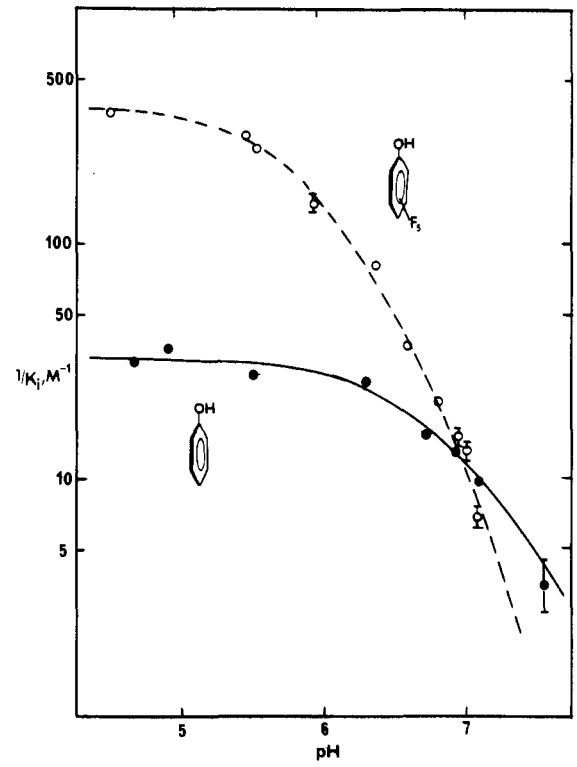

FIGURE 3: pH dependence of pentafluorophenol and phenol binding to β-glucosidase (27 °C). K_i values ($=K_{is}$) were obtained under pseudo-first-order conditions (S = *p*-nitrophenyl glucoside; [S] = 0.14 mM). The solid line is the theoretical fit for phenol binding to the enzyme form with a protonated group of $pK_a = 6.8$. The dashed line is the theoretical fit for the un-ionized form of pentafluorophenol ($pK_a = 5.35$) binding to the protonated enzyme. See eq 4 in the text.

enzyme. Because of the near identity of the two pK_a's, a distinction between these two interpretations is not readily accessible experimentally (see Discussion). At pH 6.75 the interaction between the enzyme and 1-deoxynojirimycin is maximal, and the K_i value is 18 (±1) μM. A comparison of eq 2 and 3 shows the pH-independent association constant for 1-deoxynojirimycin is about 4.5 times larger than that for gluconolactam.

The C-1 alkyl glucoside derivatives in Table III show a trend that is paralleled by the substrates for the enzyme. 1-Deoxy-1-*n*-octylglucose binds more tightly to the enzyme than does 1-deoxy-1-methylglucose. *n*-Octyl β-glucoside is a good substrate for the enzyme with $K_M = 3.5$ (±0.3) mM and a V/K value approximately 3% of that for *p*-nitrophenyl glucoside (at pH 6.2). Methyl β-glucoside has a $K_M = 175$ (±15) mM and a V/K value approximately 0.04% of that for *p*-nitrophenyl glucoside. The K_M for *n*-amyl β-glucoside is 6.5 (±0.5) mM. The larger alkyl substituents attached to the C-1 position result in a tighter binding of the inhibitors and a more favorable interaction between the enzyme and substrate (as reflected in the K_M and V/K values).

The effect of inversion of stereochemistry at C-4 on the affinity of a variety of glucose derivatives is summarized in Figure 2. The data suggest that the effect of inversion at C-4 is not constant but depends on the affinity of the derivative. For example, with the parent pair of derivatives, D-glucose has a K_i of 160 mM (at pH 6.2) and binds 75% more tightly than does D-galactose. The *p*-nitrothiophenyl β-glycosides (which are poor substrates for the enzyme) bind more tightly than the free sugars. The K_i for *p*-nitrothiophenyl glucoside is 4.5 mM and the K_i for the galactoside is 14 mM, indicating that the glucose derivative binds about 3 times more tightly to the enzyme than its C-4 epimer. Inversion of stereochemistry at C-2 in glucose (i.e., mannose) results in a diminished affinity for the enzyme ($K_i = 1.46$ M). The galactose derivative with stereochemical inversion at C-2 (i.e., talose) actually binds about 5 times *more tightly* than its C-4 epimer (mannose).

Interaction of β-Glucosidase with Phenols. Aryl glycosides are particularly good substrates for β-glucosidase from sweet almond [see, e.g., Nath & Rydon (1954)]. The hydrolysis products of aryl glycosides are linear competitive inhibitors, with the phenol being the better inhibitor, for example, the K_i values, at pH 5.6, for the hydrolysis products of phenyl glucoside [$K_m = 36$ (±4) mM] are 36 (±3) mM for phenol and 217 (±4) mM for glucose.

The pH profile for phenol binding (Figure 3) is similar to that for gluconolactam binding. Thus, it appears that the phenol binds to the enzyme when a group on the enzyme with a $pK_a = 6.8$ is protonated. Since the pK_a of phenol is outside the pH range examined ($pK_a = 10$), it cannot be concluded with certainty that it is the un-ionized phenol which binds to the enzyme. It is important to determine the ionization state of the phenol that binds to the enzyme since structure–reactivity studies (Nath & Rydon, 1954; Kopfler & Byers, 1984) and the large ^{18}O kinetic isotope effect (Rosenberg & Kirsch, 1981) suggest that the immediate product of the enzyme-catalyzed hydrolysis of aryl glucosides is the phenoxide. We, therefore, examined the pH profile for the binding of a more acidic phenol, pentafluorophenol ($pK_a = 5.35$). This is also shown in Figure 3. The data fit the equation:

$$1/K_i = \frac{(1/K_i)^{lim}}{1 + K_e/[H^+] + K_a/[H^+] + K_eK_a/[H^+]^2} \quad (4)$$

where K_e is the ionization constant for the group on the enzyme ($K_e = 10^{-6.8}$) and K_a is the acid dissociation constant for the pentafluorophenol. This equation is based on the assumption that it is the un-ionized phenol that binds to the protonated form of the enzyme. Thus, if it is indeed the phenoxide that is the immediate product of the reaction, the inhibition by the

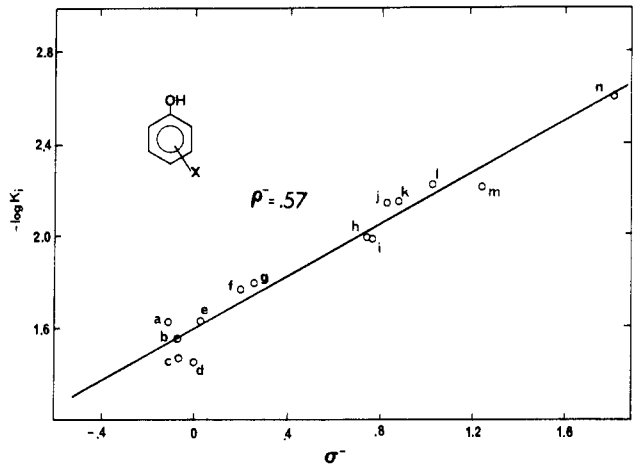

FIGURE 4: Hammett plot for binding of substituted phenols to β-glucosidase (27 °C, pH 5.6, μ = 0.02 M). The various substituted phenols are (a) 4-OCH_3, (b) 4-CO_2^-, (c) 4-$CH_2CO_2^-$, (d) H, (e) 4-OH, (f) 4-$NHC(O)CH_3$, (g) 3,5-$(OH)_2$, (h) 4-CF_3, (i) 4-CO_2CH_3, (j) 4-$C(O)CH_3$, (k) 4-CN, (l) 4-CHO, (m) 4-NO_2, and (n) F_5. The K_i values (M) are the pH-corrected K_{is} values for the un-ionized phenol.

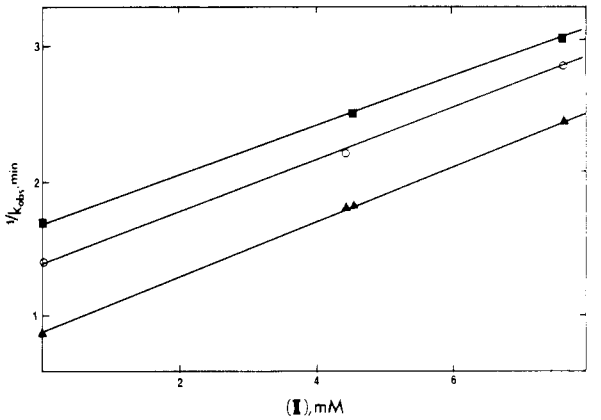

FIGURE 5: Yonetani–Theorell plot showing the inhibition of β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl glucoside (0.14 mM) by pentafluorophenol (I) at various fixed levels of 1-deoxyglucose (1,5-anhydroglucitol). The 1-deoxyglucose concentrations were 0 (▲), 41 (○), and 61 mM (■). The reactions were carried out under pseudo-first-order conditions at pH 5.65 (0.01 M PIPES, 0.02 M NaOAc, and 0.1 mM EDTA) at 27 °C with an enzyme concentration of 4×10^{-7} M.

phenol (which, the data suggest, must bind at least 50-times more tightly than the phenoxide) is more correctly considered as dead-end, rather than product, inhibition.

Figure 4 illustrates the dependence of the affinity of the enzyme for a series of phenols on the electron-withdrawing effect of the substituent. The correlation equation for the line is

$$-\log K_i \text{ (M)} = 0.57\sigma^- + 1.6 \qquad n = 14,\ r = 0.978 \qquad (5)$$

This correlation is not significantly improved by including a hydrophobicity term (π). Indeed, the single σ^- parameter accounts for over 95% ($r^2 = 0.956$) of the variance in the data. There was no correlation found ($r < 0.7$) when either the hydrophobicity substituent constant (π) or the steric substituent constant (E_s) was used in place of the electronic substituent constant. Thus, the data show that the major factor which influences the substituent sensitivity of the binding of phenols is electronic in origin. This is equivalent to saying that the more acidic the phenol the more tightly it binds to the enzyme. The measured ρ value of 0.57 is equivalent to a Brønsted coefficient of 0.26 [=0.57/(−2.23), where the denominator is the ρ^- value for the ionization constants of phenols (Biggs & Robinson, 1961)].

In order to determine the possible mutual influence of binding of the two products of hydrolysis of a typical substrate, the rate of hydrolysis of *p*-nitrophenyl glucoside was measured as a function of pentafluorophenol concentration at different levels of glucose (100 and 250 mM). A plot of $1/k$ (k = the pseudo-first-order hydrolysis rate constant) vs. [I] (I = pentafluorophenol) yielded a pair of lines that were parallel to the one obtained in the absence of any added glucose. This indicates that the binding of glucose and pentafluorophenol is mutually exclusive (Yonetani & Theorell, 1964). This might reflect an unfavorable steric interaction between the phenolic hydroxyl group and the C-1 hydroxyl group on glucose (with either the α- or β-anomer). To test this possibility, the experiment was repeated but with 1-deoxyglucose (1,5-anhydro-D-glucitol) replacing glucose. The results are shown in Figure 5. Again, the lines appear to be parallel, suggesting that 1-deoxyglucose and pentafluorophenol cannot be bound simultaneously to the enzyme. This mutually exclusive binding of 1-deoxyglucose and pentafluorophenol is in contrast to the results obtained with 1-deoxyglucose and various amine inhibitors. When a Yonetani–Theorell plot is constructed for the inhibition by benzylamine or methylamine at different fixed levels of 1-deoxyglucose, the lines appear to intersect near the abscissa, suggesting that the presence of 1-deoxyglucose does not appreciably affect the binding of the amine inhibitor.

Inhibition by Amines. Since the early observations of Larner & Gillespie (1956) that 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) is an inhibitor of several glycosidases, Tris and a variety of other amines have been shown to be competitive inhibitors of many glycosidases (Halvorson & Ellias, 1958; Jørgensen & Jørgensen, 1966; Shinitzky et al., 1966). Lai & Axelrod (1973) have taken advantage of these observations to develop potent and specific inhibitors of several glycosidases, viz., 1-aminoglycosides. For example, D-glucosylamine is an effective competitive inhibitor of β-glucosidase, binding 4 orders of magnitude more tightly to the enzyme than does D-glucose (Walker & Axelrod, 1978). Legler (1978) has shown, with a series of N-substituted β-glucosylamines, that sweet almond β-glucosidase (isozyme B) is more strongly inhibited by basic (protonatable) glucosyl derivatives than by the corresponding neutral analogues. Quaternary ammonium glucosyl derivatives (e.g., β-glucosylpyridinium ion), however, are only weakly inhibitory (Legler, 1978), particularly in comparison to their corresponding neutral C-glucoside analogues (Loeffler et al., 1974). In order to further explore the factors responsible for the enhanced binding of nonquaternary amines, relative to neutral compounds, we decided to look at the interaction of a series of amines with β-glucosidase.

Figure 6 illustrates a comparison of the binding energies for a series of alcohols and corresponding primary amines. The binding energies were calculated from the K_{is} values obtained under pseudo-first-order conditions (i.e., [S] $\ll K_m$). Each of the amines was found to be a competitive inhibitor. The correlation equation for the data in Figure 6 can be rearranged and expressed in terms of dissociation constants ($pK_i = -\log K_i$, K_i in M):

$$pK_i^{ROH} = 1.55pK_i^{RNH_3^+} - 2.75 \qquad (6)$$

This reveals several features: (a) Primary amines (with $K_i > 10^{-5}$M) bind more tightly to the enzyme than do the corresponding alcohols. (b) The affinity of alcohols for the enzyme is somewhat more sensitive to substituent effects than is the affinity of amines. (c) The enhanced binding of the amine

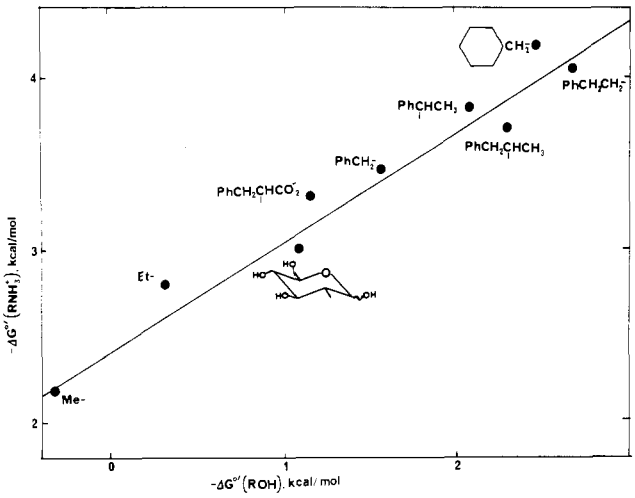

FIGURE 6: Relationship between the binding energy of various alcohols and the corresponding amines (27 °C, pH 6.2; 0.01 M MES and 0.1 M NaCl). The correlation equation for the line is $-\Delta G^{\circ\prime}(RNH_3^+) = -0.645\Delta G^{\circ\prime}(ROH) - 2.41$, with $n = 9$ and $r = 0.97$.

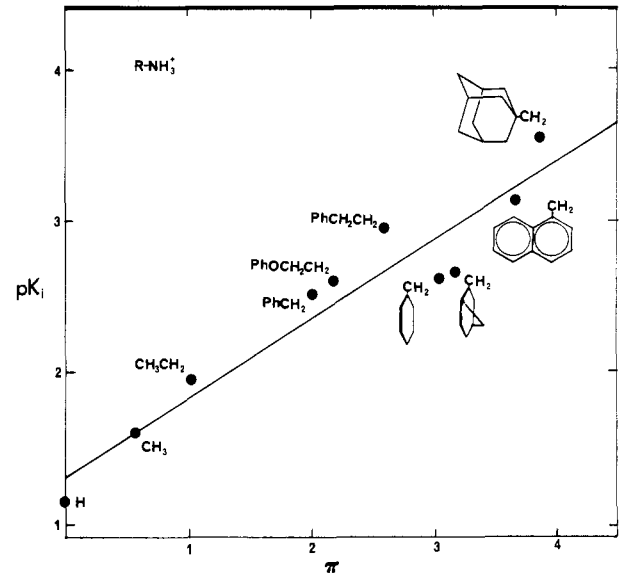

FIGURE 7: Dependence of the binding of primary amines on hydrophobicity (27 °C, pH 6.2; 0.01 M MES and 0.1 M NaCl). The correlation equation for the line is $pK_i = 0.52\pi + 1.32$, with $n = 10$ and $r = 0.95$.

over the alcohol [i.e., $K_i(ROH)/K_i(RNH_3^+)$] becomes greater as the affinity is reduced. Another interesting point available from eq 6 is that it enables us to calculate a dissociation ("inhibition") constant for water. Water can be considered as an alcohol with R = H. The corresponding amine is NH_4^+, and its K_i was found to be 0.073 (±0.005) M at pH 6.2. Substituting this value into eq 6 yields a value of $K_i = 9.7$ M for water.

To further examine the substituent effects on the binding of amines to the enzyme, a series of 10 structurally homogeneous amines (i.e., compounds containing a primary amino group attached to a primary carbon) were tested as inhibitors. The results, illustrated in Figure 7, show that for this series of amines, the substituent property that exerts a major effect on the sensitivity of the binding constant is the hydrophobicity. Furthermore, the enzyme is quite accommodating with respect to the steric bulk of the substituent. Thus, neither 1-naphthylmethylamine nor 1-adamantylmethylamine shows a deviation from the correlation line in Figure 7.

The data presented so far do not allow a distinction to be made between the possibilities that inhibition is due to the protonated form of the amine or to the unprotonated species (which, necessarily, will be present at relatively low concentrations in the pH range examined). In order to distinguish between these two possibilities, we looked at the rate of onset of inhibition in the stopped-flow spectrophotometer. The amine inhibitor which we examined was (−)-ephedrine [(1*R*,2*S*)-1-phenyl-2-(methylamino)propanol] because it has a reasonably high affinity for the enzyme (Kern & Byers, 1980). The K_i for (−)-ephedrine is 0.2 mM (pH 5.7, 25 °C), assuming it is the protonated amine that is the inhibitory species. Since the pK_a of ephedrine is 9.6, the K_i would be $\sim 2 \times 10^{-8}$ M if the inhibition observed at pH 5.7 was entirely due to the unprotonated amine. In a typical experiment the contents of one syringe containing the enzyme is mixed with the contents of the second syringe containing substrate (*p*-nitrophenyl glucoside) plus the inhibitor. The final concentrations in the reaction chamber were 4 mM substrate, 1 mM inhibitor, and ~15 units/mL β-glucosidase at 25 °C, pH 5.7. Under these conditions the steady-state velocity of *p*-nitrophenol release was approximately 35% of the value in the absence of any inhibitor. Furthermore, there was no detectable lag in the attainment of this steady-state velocity (i.e., the steady-state rate was reached within the 3 ms dead time of the instrument). Under the reaction conditions, the concentration of the protonated amine was 1 mM, and the concentration of the free amine was about 10^{-7} M. Assuming a maximum, diffusion-controlled, rate constant for amine binding of 10^8 M^{-1} s^{-1} [in fact, the rate of substrate binding may be about 2 orders of magnitude below this limit (Kopfler & Byers, 1984)], the pseudo-first-order rate constant for binding of the *free amine* would be $\leq(10^8\ M^{-1}\ s^{-1})(10^{-7}\ M) = 10\ s^{-1}$. This corresponds to a half-life of ≥69 ms for the onset of inhibition. Since the onset of inhibition was complete within 3 ms, this would require a large and improbable second-order rate constant for the binding of the inhibitor ($k \sim 10^{10}$ M^{-1} s^{-1}) if the neutral amine was the inhibitory species. It thus appears that, for (−)-ephedrine at least, it is the cationic amine which is the inhibitory species. This is probably also true for all of the noncyclic amines as well. Some additional evidence for this can be seen in the inhibition by the quaternary amine, *N*,*N*,*N*-trimethylbenzylamine. The K_i value for this inhibitor at pH 6.2 is 29 (±4) mM which is only 10 times larger than the K_i for benzylamine (assuming it is the protonated species that binds to the enzyme).

The K_i value for a member of the series Ph–$(CH_2)_n$–NH_3^+ is fairly insensitive to the number of interspersed methylene groups. Thus, at pH 6.2 the following K_i values were obtained: benzylamine, 2.8 mM; phenylethylamine, 1.1 mM; phenylpropylamine, 0.7 mM; phenylbutylamine, 1.5 mM. The K_i measured for aniline at pH 5.6 was found to be 44.5 (±0.7) mM. On the basis of a pK_a of 4.58, this corresponds to K_i = 3.5 mM if it is assumed that inhibition is due entirely to the protonated species. The effect of N-methylation on the K_i value of amines can be seen in the following data obtained at pH 6.2: ammonia, 73 (±5) mM; methylamine, 25 (±4) mM; dimethylamine, 7 (±1) mM; trimethylamine, 4.1 (±0.8) mM. The tetramethyl- or tetraethylammonium ions do not appear to inhibit the enzyme ($K_i > 10$ M).

DISCUSSION

Binding of Sugars. Two features readily apparent from an inspection of Tables II and III are (1) glucose does not bind particularly tightly to the enzyme and (2) β-glucosidase is not very selective for the sugar residue. From these data it does not appear as if any single substituent on glucose is absolutely

essential for binding. Nevertheless, some general trends can be discerned. For the aldohexoses, the sugars with the higher affinity for the enzyme have the hydroxyl groups at C-2 and C-3 diequatorial in their most stable conformation. The stereochemical configuration at C-2 and C-3 appears to be the most crucial for optimizing the interaction between the sugar and the enzyme. Thus, the C-2 epimer of glucose (i.e., mannose) binds about 9 times less tightly than glucose, and the C-3 epimer (i.e., allose) binds about 3 times less tightly than glucose. Inversion of configuration at C-1 or C-4 has little effect on the affinity. Both the ketohexoses and the aldopentoses exist predominantly in the pyranose form (Capon, 1969). The K_i values obtained with these compounds show that the removal of the C-6 hydroxymethyl substituent (in pentoses) or the transfer of this group from C-5 to C-1 (i.e., the pyranose form of the ketohexose) has little effect on the affinity. It is interesting to note that removal of the C-2 hydroxyl group in glucose slightly reduces the affinity, but removal of the C-1 hydroxyl group enhances the affinity. This suggests a favorable interaction between the C-2 hydroxyl group and the enzyme (e.g., hydrogen bonding) and an unfavorable interaction between the C-1 hydroxyl group (either α- or β-anomer) and the enzyme (e.g., steric compression). Replacing either the C-2 or the C-3 hydroxyl group with an ammonium group enhances the binding of glucose by more than 20-fold, consistent with a stronger hydrogen bonding (or an electrostatic) interaction between the enzyme and the glucosamine. Methylation of the C-3 hydroxyl group abolishes binding, suggesting that in glucose the C-3 hydroxyl group is probably involved as a hydrogen bond donor with the enzyme. Similar modifications of the C-6 substituent do not result in as great an alteration of the affinity. Thus, 6-deoxy-6-aminoglucose binds about 4 times more tightly to the enzyme than does glucose while 6-methoxyglucose binds about as well as glucose. Removal of the hydroxyl group at C-6 (i.e., quinovose) or removal of the hydroxymethyl group (i.e., xylose) reduces the affinity by less than a factor of 3. The lack of binding of glucuronic acid or of glucose 6-phosphate suggests the presence of a negatively charged group on the enzyme near the C-6 hydroxyl binding site.

The sugar binding data suggest potential interactions between the glucose portion of the substrate and the enzyme. Unfortunately, it is difficult to get a reliable quantitative estimate of the energetics of these interactions without knowledge of the conformation of the bound sugar. Furthermore, the sugars can potentially bind in a variety of orientations. For example the two enantiomers of glucose have identical affinities for the enzyme (Table II). Some other data that suggest a multiplicity of binding orientations are presented in Table III. As previously mentioned, replacing the C-2 hydroxyl substituent in glucose with an ammonium group enhances the binding. Acetylation of the amino group abolishes binding. However, if this polar acetyl group is replaced by a nonpolar benzyl group the binding is enhanced. This may be due to the benzyl group occupying the "aromatic binding site" on the enzyme. The *p*-chlorobenzylamino derivative binds about twice as tightly as the *p*-methoxybenzylamino derivative, and this is the same binding ratio observed with the *p*-chlorophenyl and *p*-methoxyphenyl β-glucosides (Natch & Rydon, 1954). Another difficulty in estimating the energetics of the specific interactions of the sugar substituents with the enzyme is that these values depend on the total binding energy of the molecule. This is illustrated in Figure 2 which shows the relative binding constants for 14 pairs of sugar derivatives differing from each other only in the configuration at C-4. The clear, but imperfect, trend in the data suggests that, for derivatives which have a dissociation constant of less than $\sim$320 mM, the glucose derivative binds more tightly than the galactose derivative. The more tightly the compound binds, the greater the discrimination between the glucose and galactose derivatives. In this regard it is interesting to note that with the *p*-nitrophenyl substrates the glucoside binds about 6 times more tightly than the galactoside (based on K_m values which, in this case, are equal to the K_s values; M. P. Dale et al., unpublished results). In the transition state, however, the glucoside binds 18 times more tightly than the galactoside (based on the k_{cat}/K_m ratio). This suggests a strengthening of the interactions between the sugar portion of the substrate and the enzyme as the reactants progress from the ground state to the transition state.

Binding of Phenols. The observation that aryl glycosides bind more tightly to the enzyme than do the corresponding free sugars suggests that the major binding determinant of the substrate is the aglycon. For example, at pH 5.6 and 27 °C the K_m ($=K_s$) for *p*-nitrophenyl glucoside (=2.5 mM) is comparable to the K_{is} for *p*-nitrophenol (=6.1 mM) but is much less than the K_{is} for glucose (=217 mM at this pH). Similarly, the K_{is} for phenol (=36 mM) is equal to the K_m for phenyl glucoside. The pH dependence of K_i (Figure 3) indicates that it is the protonated (i.e., neutral) species of the phenol which binds to the enzyme. This is consistent with the results obtained with thiophenols. For example, pentafluorothiophenol is not an inhibitor ($K > 170$ mM). This thiophenol is very acidic ($pK_a = 2.7$) and is completely ionized at the pH values used in the assay. The pH profile of all the phenols tested shows that binding depends on a group on the enzyme with a pK_a of 6.8 ($\pm$0.1). This group must be protonated in order for the phenol to bind. The pH profile of V/K (or k_{cat}/K_m) also shows a dependence on an acidic group with $pK_a = 6.7$ (Dale & Byers, 1982).

The dependence of the K_i value on electronic substituent effects is illustrated in Figure 4. The ρ value for the binding equilibrium is +0.57 (corresponding to a Brønsted β value of −0.26). This can be compared to the ρ value of +0.66 for the binding of meta- and para-substituted phenyl glucosides to the enzyme (Hansch et al., 1965). The observation that the phenol binds at least 50 times more tightly to the enzyme than does the phenoxide suggests the possibility of electrostatic repulsion (i.e., the presence of an anionic group on the enzyme near the phenol binding site). The observation of a substantial sensitivity of phenol binding to electronic effects ($\rho = 0.57$) suggests the possibility of hydrogen bonding. The ρ value for phenol ionization is 2.23 (Biggs & Robinson, 1961). Thus, the ρ value for phenol binding suggests that the extent of proton transfer in this process is about one-fourth (=0.57/2.23). This value is typical of those obtained from correlations of hydrogen bonding equilibrium constants between meta-substituted and para-substituted phenols and a variety of acceptors (Hine, 1975).

It is noteworthy that the Yonetani–Theorell plot (Figure 5) indicates mutually exclusive binding of the phenol and glucose analogue, 1,5-anhydroglucitol (1-deoxyglucose). This is most easily rationalized in terms of a double-displacement mechanism where the reaction products are not normally present simultaneously on the enzyme. (The competitive nature of the inhibition by the phenol would then be a reflection of this species functioning as a dead-end inhibitor.) Other explanations for this mutually exclusive binding of the hydrolytic products seem less likely. For example, phenol binding and glucose binding would be mutually exclusive if

the major mode of glucose binding was not in the sugar subsite of the substrate binding site but in the aryl leaving group subsite. However, it is improbable that glucose has a higher affinity for the aromatic, or hydrophobic, binding site than for the sugar site. This is indicated by the observation that disaccharides such as cellobiose have *lower*, rather than enhanced, affinities for the enzyme than does glucose. Similarly, phenol binding and glucose binding would be mutually exclusive if the phenol bound better at the sugar subsite than at the aryl leaving group subsite. This also seems improbable. As mentioned earlier, the K_i for a phenol is comparable to the K_s for the corresponding glucoside. Unless the K_s reflects predominant nonproductive binding of the substrate, the phenol K_i is a direct (inverse) measure of the intrinsic affinity of the aglycon for the leaving group subsite.

Binding of Amines. Two conspicuous properties of amines are that they (or at least those with $K_i > 10^{-5}$ M) bind more tightly to the enzyme than do the corresponding alcohols (Figure 6) and that their binding is strongly influenced by the hydrophobicity of their substituents (Figure 7). The higher affinity of the amine than of the corresponding alcohol suggests the presence of an anionic site on the enzyme. This is consistent with the conclusion, discussed earlier, based on the pH dependence of phenol binding. It is also consistent with the demonstration by Legler & Hasnain (1970) of the presence of an ionized carboxylate group at the active site of sweet almond β-glucosidase. In addition to the role of Coulombic interactions, the importance of dispersion energies in the interaction of amines with the enzyme is evident from the results obtained from the series of primary, secondary, and tertiary amines. The relative affinities ($1/K_i$) of trimethyl-, dimethyl-, and methylamine and ammonia are 18:10:3:1. The contribution of the methyl group is also indicated in the N-methylated amphetamine and ephedrine series where there is a decrease in binding energy as the methyl groups are removed from the nitrogen atom (Kern & Byers, 1980). In this case, as in the methylamine series, the average contribution to the binding energy per methyl group is −0.63 (±0.06) kcal/mol. This is in reasonable agreement with the calculated value of −0.58 kcal/mol for the dispersion energy for interaction of a methyl group with protein (Webb, 1963).

It is interesting to note that while the simple aliphatic quaternary amines, tetramethylammonium and tetraethylammonium, do not bind to the enzyme, the corresponding trialkylbenzylammonium ions do bind to the enzyme. Thus, the replacement of either a hydrogen atom (in tetramethylammonium) or a methyl group (in tetraethylammonium) with a phenyl group supplies sufficient interaction with the enzyme to overcome the unfavorable interactions of the simple aliphatic quaternary amines with the enzyme. This is consistent with the conclusion that there is an aryl binding site on the enzyme. In the phenylalkylamine series, $Ph-(CH_2)_n-NH_3^+$, for $1 \leq n \leq 4$, the K_{is} values are similar (within a factor of 4). This suggests that while there is a hydrophobic binding site and at least one anionic site on the enzyme, a precise fit on the enzyme is not critical for the inhibitor. This probably reflects a fairly large hydrophobic binding region on the enzyme. This is apparent from the data in Figure 7 which show effective inhibition of the enzyme by amines with bulky substituents. A large hydrophobic region on the enzyme, which probably corresponds to the aglycon binding site, is also indicated by the effective inhibition by the C-glucosides fluorenyl- and octyl-1-deoxy-D-glucose (Table III) and by the observation that *n*-amyl, *n*-octyl, and 4-methylumbelliferyl β-D-glucosdes are efficiently hydrolyzed by the enzyme.

Gluconolactam and 1-Deoxynojirimycin as Potential Reactive Intermediate Analogue Inhibitors. Inhibition of glycosidases by aldono-1,5-lactones of appropriate configuration has been known for some time (Conchie et al., 1967a,b; Legler, 1968). The high affinity of the lactone for the enzyme was attributed to an electronic and structural resemblance to the putative oxocarbonium ion intermediate in the hydrolytic reaction (Leaback, 1968; Reese et al., 1971). This was based on the assumption that D-glucono-1,5-lactone can assume a distorted half-chair conformation in solution as it does in the crystal (Hackert & Jacobson, 1971). This assumption was recently validated by Walaszek et al. (1982), who showed, by ^1H and ^{13}C NMR, that the conformational equilibria for gluconolactone in solution strongly favors the $^4H_3(D)$, *gg*, or half-chair conformation.

Inhibitors that are analogues of reactive intermediates (many commonly called transition-state analogues) can be useful in elucidating enzymatic reaction mechanisms (Wolfenden, 1978; Byers, 1978). One property of these compounds is that they are generally quite potent inhibitors. However, there are other important criteria for a reactive intermediate analogue, or a transition-state analogue, inhibitor. One of these is that the binding is influenced by factors (e.g., pH, temperature, etc.) in the same way that V/K (or k_{cat}/K_m) is influenced by these factors (Wolfenden, 1977). For example, the pH dependence of V/K for the β-glucosidase reaction is bell shaped, which shows a dependence on two ionizable groups with apparent pK_a's of 4.4 and 6.7 (Dale, 1983). Thus, the affinity of a transition-state analogue inhibitor for the enzyme should be optimal where the activity is optimal (pH 5.6) and would show the same pH dependence as does V/K. In order to apply this test to β-glucosidase, we used D-glucono-1,5-lactam which is a structural, but more stable, analogue of gluconolactone. The K_i for the lactam is similar to that of the lactone. [This is in contrast to the results of Legler & Witassek (1974), who found that, with isozyme A of sweet almond β-glucosidase, the K_i for gluconolactam was about 40 times larger than the K_i for gluconolactone (pH 5.0, 35 °C).] Gluconolactam is a very good inhibitor of the enzyme, binding about 4000 times more tightly to the enzyme than does glucose. The K_i value for the lactam, like the enzymic activity, shows a dependence on an ionizable group with $pK = 6.8$ but, unlike the catalytic activity, does not depend on any other ionizable group (Figure 1). This suggests that the lactam is not a transition-state analogue inhibitor of β-glucosidase. Evidence that D-glucono-1,5-lactone, like the lactam, is not a transition-state analogue can be obtained from the results of Legler & Witassek (1974). They found that, at pH 5.0, the lactone binds 60% more tightly to β-glucosidase at 25 °C than at 35 °C. This is just the opposite of the influence of temperature on V/K. We find (M. P. Dale et al., unpublished results) that as temperature is increased from 25 to 35 °C, the k_{cat} of *p*-nitrophenyl glucoside is increased 78% (while the K_m remains unchanged). Therefore, the affinity of the enzyme for a perfect transition-state analogue should increase, not decrease, as the temperature is raised over this range.

1-Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol), like the lactam, is a fairly potent inhibitor of β-glucosidase (Lalegeire et al., 1982). At pH 6.1, for example, the K_i value of 1-deoxynojirimycin (34 μM) is equal to the K_i value of D-glucono-1,5-lactam. It was originally thought that the potent inhibition by 1-deoxynojirimycin was due to the similarity of the protonated inhibitor ($pK_a = 6.7$) and the putative oxocarbonium ion intermediate. Our studies with reversible inhibitors (phenols and amines) as well as Legler's studies with

irreversible inhibitors (Legler & Hasnain, 1970; Legler, 1973) indicate the presence of an anionic group (a carboxylate ion) at the active site of the enzyme. It is, thus, surprising that the pH profile for inhibition by 1-deoxynojirimycin (Figure 1) suggests that the unprotonated inhibitor is binding to the enzyme. This is similar to the results of Saul et al. (1984), who found that inhibition of almond β-glucosidase by castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was due to the unprotonated (pK_a = 6.1) form of the indolizidine alkaloid. There is an ambiguity in the interpretation of the results with 1-deoxynojirimycin, however. The pH dependence of the enzymic activity indicates the following ionizations (Dale, 1983; Legler & Hasnain, 1970; Conchie et al., 1967a,b):

$$EH_2 \underset{H^+}{\overset{pK_1}{\rightleftharpoons}} EH^- \underset{H^+}{\overset{pK_2}{\rightleftharpoons}} E^{2-}$$

where EH is the active form of the enzyme. For 1-deoxynojirimycin, the pK_a of 6.7 can be represented by

$$IH^+ \rightleftharpoons I + H^+$$

The observed pH profile (Figure 1) can be the result either of I binding to EH (the active form of the enzyme) or of IH^+ binding to E (an inactive form of the enzyme). The significant point, however, is that if I is the form of the inhibitor which binds to the enzyme, it must bind equally well to EH and to EH_2 since, as with the lactam, its binding is not influenced by the ionization corresponding to pK_1 (=4.4). Thus, if I is the species that binds to the enzyme, it is not a transition-state analogue inhibitor since it binds equally well to an inactive and an active form of the enzyme. Similarly, if IH^+ is the species that binds to the enzyme, then it too cannot be a transition-state, or reactive intermediate, analogue inhibitor since it does not bind to the active form of the enzyme. Thus, 1-deoxynojirimycin, while a potent inhibitor of the enzyme, cannot be considered a transition-state analogue inhibitor.

Another good inhibitor of β-glucosidase is nojirimycin (5-amino-5-deoxyglucopyranose). This compound binds about 50 times more tightly to almond β-glucosidase than does its 1-deoxy derivative (Legler & Julich, 1984). However, nojirimycin, like 1-deoxynojirimycin and gluconolactam, does not appear to be a transition-state analogue inhibitor. The evidence for this comes from the recent results of Legler & Julich (1984). They prepared the 2-epimer of nojirimycin, 5-amino-5-deoxymannopyranose, and found that at pH 5 it binds only 20 times less tightly to the enzyme than does nojirimycin. This is similar to the 9-fold lower affinity of mannose than of glucose for the enzyme (Table II). This is much smaller, however, than the glucosidase/mannosidase activity ratio of β-glucosidase (Table I). The k_{cat}/K_m value for hydrolysis of *p*-nitrophenyl β-D-glucoside is at least 3500-fold larger than the corresponding value for hydrolysis of *p*-nitrophenyl β-D-mannoside. Thus, inverting the stereochemistry at C-2 in the glucoside substrate reduces the catalytic activity (and, hence, transition-state stability) by more than 3 orders of magnitude. Inverting the stereochemistry at C-2 in the glycon product, however, reduces the stability of the binary complex by only an order of magnitude. Thus, the effect of inversion of stereochemistry at C-2 in nojirimycin on the affinity for the enzyme more closely parallels that for the product than for the transition state. By this reasoning then, nojirimycin appears to be more a product analogue than a transition-state analogue inhibitor.

Nojirimycin, 1-deoxynojirimycin, δ-gluconolactam, and δ-gluconolactone are all fairly potent inhibitors of β-glucosidase, yet none of them appear to be a transition-state analogue. How then can we account for their unusual affinity for the enzyme? The reason a transition-state analogue is such a potent enzyme inhibitor is that it can take advantage of favorable interactions which occur between the enzyme and the substrate in the transition state. An inhibitor that can take advantage of some of these interactions should then have an enhanced affinity for the enzyme. The pH profile for 1-deoxynojirimycin binding indicates the requirement of at least one proton in the binary E·I complex (e.g., I binding to EH or IH binding to E). This can result in either a stronger hydrogen bond than might be present or an ionic interaction that would not be present, in the complex with the corresponding oxygen analogue, 1-deoxyglucose. A similar situation might also apply to the case of nojirimycin where replacement of the –O– (in glucose) with a –NH– (or a NH_2^+) results in an enhanced affinity of somewhere between 80-fold (at pH 6; Grover & Cushley, 1977) and 6.7 × 10^4-fold (at pH 5; Legler & Julich, 1984). The enhanced binding of gluconolactone and of gluconolactam over that of glucose must also be the result of additional interactions between the enzyme and the inhibitor not present in the enzyme–glucose complex. To some extent, a reduced unfavorable steric interaction with the lactone and the lactam may be responsible for the enhanced interactions, but this cannot be too significant since the binding of these carbonyl-containing inhibitors is substantially better than the binding of the various deoxyglucose derivatives (Table II). Whatever these favorable interactions are, however, they cannot be perfect mimics of the interactions between the enzyme and the substrate in the transition state. To what extent these interactions resemble those between the enzyme and the reactive intermediates will be seen only after a more detailed elucidation of the mechanism of action of sweet almond β-glucosidase.

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Registry No. Phenol, 108-95-2; 4-methoxyphenol, 150-76-5; 4-carboxyphenol, 99-96-7; 4-carboxymethylphenol, 156-38-7; 4-hydroxyphenol, 123-31-9; 4-acetamidophenol, 103-90-2; 3,5-dihydroxyphenol, 108-73-6; 4-trifluoromethylphenol, 402-45-9; 4-hydroxybenzoic acid methyl ester, 99-76-3; 4-acetylphenol, 99-93-4; 4-cyanophenol, 767-00-0; 4-formylphenol, 123-08-0; 4-nitrophenol, 100-02-7; pentafluorophenol, 771-61-9; ammonia, 14798-03-9; benzeneethanamine, 64-04-0; 2-phenoxyethanamine, 1758-46-9; benzenemethanamine, 100-46-9; ethanamine, 75-04-7; methanamine, 74-89-5; cyclohexanemethanamine, 3218-02-8; bicyclo[2.2.1]heptane-2-methanamine, 14370-50-4; 1-naphthalenemethanamine, 118-31-0; 1-adamantylmethanamine, 17768-41-1; methanol, 67-56-1; ethanol, 64-17-5; α-hydroxybenzenepropanoic acid, 156-05-8; benzenemethanol, 100-51-6; α-methylbenzenemethanol, 98-85-1; α-methylbenzeneethanol, 698-87-3; cyclohexanemethanol, 100-49-2; benzeneethanol, 60-12-8; α-aminobenzenepropanoic acid, 63-91-2; α-methylbenzenemethanamine, 98-84-0; α-methylbenzeneethanamine, 60-15-1; D-idose, 5978-95-0; D-glucose, 50-99-7; L-glucose, 921-60-8; D-galactose, 59-23-4; L-galactose, 15572-79-9; D-talose, 2595-98-4; D-altrose, 1990-29-0; D-allose, 2595-97-3; L-mannose, 10030-80-5; D-gulose, 4205-23-6; D-mannose, 3458-28-4; D-fructose, 57-48-7; D-sorbose, 3615-56-3; D-tagatose, 87-81-0; D-arabinose, 10323-20-3; L-xylose, 609-06-3; D-lyxose, 1114-34-7; L-ribose, 24259-59-4; D-ribose, 50-69-1; L-arabinose, 5328-37-0; L-lyxose, 1949-78-6; D-xylose, 58-86-6; 1-deoxy-D-glucose, 154-58-5; 6-deoxy-D-galactose, 3615-37-0; 6-deoxy-L-mannose, 3615-41-6; 1-deoxy-D-galactose, 3971-48-0; 2,6-dideoxy-D-allose, 527-52-6; 2-deoxy-D-glucose, 154-17-6; 6-deoxy-L-galactose, 2438-80-4; 6-deoxy-D-glucose, 7658-08-4; 2-deoxy-D-galactose, 1949-89-9; α-glucose, 492-62-6; β-glucose, 492-61-5; *N*-methylglucamine, 6284-40-8; 1-deoxy-1-(9-fluorenyl)glucose, 96326-81-7; 1-deoxy-1-*n*-octylglucose, 96392-39-1; 1-deoxy-1-methylglucose, 96392-40-4; gluconic acid, 526-95-4; phenyl α-glucoside, 4630-62-0; methyl α-glucoside, 97-30-3; *p*-nitrophenyl α-glucoside, 3767-28-0; cellobiose, 528-50-7; sucrose, 57-50-1; glucal,

13265-84-4; 2-deoxy-2-[(*p*-chlorobenzyl)amino]glucose, 96326-82-8; 2-deoxy-2-[(*p*-methoxybenzyl)amino]glucose, 96326-83-9; 2-deoxy-2-aminoglucose, 3416-24-8; 2-deoxy-2-(3-methyl-3-nitrosoureido)-glucose, 18883-66-4; 2-methoxyglucose, 4132-40-5; 2-deoxy-2-acetamidoglucose, 7512-17-6; 3-deoxy-3-aminoglucose, 576-44-3; 3-methoxyglucose, 146-72-5; 4-methoxyglucose, 4132-38-1; 5-deoxy-5-thioglucose, 20408-97-3; 6-deoxy-6-aminoglucose, 576-47-6; 6-methoxyglucose, 2461-70-3; glucuronic acid, 6556-12-3; glucose 6-phosphate, 56-73-5; 1-deoxynojirimycin, 19130-96-2; δ-gluconolactam, 14904-83-7; δ-gluconolactone, 90-80-2; L-ascorbic acid, 50-81-7; cytosine, 71-30-7; piperidine, 110-89-4; α-glucoheptanoic acid γ-lactone, 89-67-8; δ-valerolactam, 675-20-7; *p*-nitrophenyl-β-D-fucose, 1226-39-7; *p*-nitrophenyl-β-D-glucose, 2492-87-7; *p*-nitrophenyl-β-D-galactose, 55529-86-7; *p*-nitrophenyl-β-D-xylose, 2001-96-9; *p*-nitrophenyl-β-D-mannose, 35599-02-1; β-glucosidase, 9001-22-3.

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