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The Dual Effects of Alcohols on the Kinetic Properties of Guinea Pig Liver Cytosolic β-Glucosidase*

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This report demonstrates the effect of primary alcohols on the kinetic properties of guinea pig liver cytosolic β-glucosidase. Lineweaver-Burk analyses of the kinetic data revealed a biphasic response; at low concentrations the alcohols increased the V_{max} 5–7-fold while at higher concentrations they caused a purely competitive type of inhibition. For example, with *n*-butyl alcohol, increasing the alcohol's concentration in the assay medium from 0 to 0.14 M (0–1% (v/v)) resulted in a progressive increase in V_{max} to a value 7-fold above the basal level without affecting the K_m. However, between 0.14 and 0.54 M (1 and 4% (v/v)) *n*-butyl alcohol, the K_m for 4-methylumbelliferyl-β-D-glucopyranoside increased significantly from 0.14 to 0.93 mM. In contrast to *n*-butyl alcohol or isobutyl alcohol, which are potent activators, structurally related compounds like *sec*-butyl alcohol, *tert*-butyl alcohol, butylurea, and butanesulfonic acid did not stimulate the activity of the cytosolic β-glucosidase. In the concentration range where activation was observed, conventional secondary replots of 1/Δ slope *versus* 1/[alcohol] yielded perfect straight lines, demonstrating that binding of a single molecule of alcohol to the β-glucosidase was responsible for the initial phase of activation. Furthermore, the glycohydrolase displayed a propensity to bind the longer chain alcohols, as reflected by the K_A (binding constant) values of 555, 146, 34.1, and 7.47 mM for ethanol, *n*-propyl alcohol, *n*-butyl alcohol, 1-pentanol, respectively.

This phenomenon of nonessential activation by alcohols has led us to speculate on the presence of a physiologic activator for the β-glucosidase in mammalian tissues which contain this enzyme.

An outstanding feature of the prominent cytosolic β-glycosidase of mammalian liver is its broad specificity (Daniels *et al.*, 1981). This broad specificity applies to both the glycone and aglycone moieties of the enzyme's substrates, for the glycosidase catalyzes the hydrolysis of a variety of nonphysiological glycosides of *p*-nitrophenol and 4-methylumbelliferone including β-D-glucosides, β-D-galactosides, α-L-arabinosides, β-D-fucosides, and β-D-xylosides. Furthermore, this striking aspect of its catalytic flexibility is underscored by the recent finding that several plant aryl glycosides with widely differing aglycone moieties could serve as potential physiologic substrates (LaMarco and Glew, 1986). The latter observation has led to the suggestion that this relatively nonspecific glycosidase could play a role in the detoxification of xenobiotic glycosides which might find their way into the diet of man or animals. Since the highest specific activity is usually obtained with aryl β-D-glucoside substrates, this enzyme is referred to by many investigators as a β-glucosidase.

The broad specificity β-glucosidase has been purified to homogeneity from a variety of sources including human liver (Daniels *et al.*, 1981) and guinea pig liver (LaMarco and Glew, 1986). The guinea pig liver β-glucosidase is fully active as a 60,000-dalton, single chain protein. Since it has maximum activity in the pH 5.7–6.5 range and in order to distinguish it from lysosomal glucocerebroside:β-glucosidase, which has a more acidic pH optimum, it is sometimes referred to as a "neutral" β-glucosidase.

Although its physiologic function remains to be established, we are interested in the structure of this soluble cytosolic β-glucosidase, particularly the hydrophobic domains located in its active site and elsewhere, because of several observations: first, its nominal (2–3-fold) activation by gangliosides such as G_{D1A}[1]; second, the mixed-type inhibition caused by micromolar concentrations of physiologically relevant amphiphilic compounds like glucosylsphingosine and galactosylsphingosine (LaMarco and Glew, 1985); and finally, its high affinity for hydrophobic affinity columns (*e.g.* phenyl- and octyl-Sepharose) (LaMarco and Glew, 1986).

In addition, in a recent comparative study (Gopalan *et al.*, 1989) of the inhibitory effects of a homologous series of *n*-alkyl-β-D-glucosides, which act at the active sites of the human liver cytosolic β-glucosidase and placental lysosomal glucocerebrosidase, we observed that the inhibition constants (K_i values) for the former were at least 100-fold lower than those of glucocerebrosidase, which is an intrinsic membrane protein. This latter observation indicates that the active site of the broad specificity β-glucosidase may be as apolar or perhaps even more so than that of glucocerebrosidase which catalyzes the hydrolysis of the highly hydrophobic glucocerebroside.

In an effort to learn more about these hydrophobic binding sites on the cytosolic β-glucosidase, we investigated the effects of alcohols on the kinetic properties of the enzyme. In this report we show that the enzyme possesses two distinct binding sites for alcohols: a high affinity activator site and a low affinity site which is probably a part of the hydrophobic domain in the active site.

EXPERIMENTAL PROCEDURES

Materials

Cytosolic β-glucosidase was purified from the liver of the English short-hair (albino) guinea pig as described elsewhere (LaMarco and

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[1] K. L. LaMarco and R. H. Glew, unpublished observation.

Glew, 1986); the final preparation exhibited a specific activity of 2.4–2.6 × 10^6 units/mg protein in the standard β-glucosidase assay described below. 4-Methylumbelliferyl-β-D-glucoside (MUGlc) was purchased from Sigma.

n-Butyl alcohol, isobutyl alcohol, ethanol, *n*-propyl alcohol, isopropyl alcohol, sodium citrate, ammonium hydroxide, sodium phosphate, sodium chloride, 2-mercaptoethanol, sodium azide, and ethylene glycol were purchased from Fisher. Sodium butyrate, butylurea, butanesulfonic acid (sodium salt), butyramide, 1-pentanol, and 1,4-butanediol were purchased from Aldrich. *sec*-Butyl alcohol was purchased from Kodak. *tert*-Butyl alcohol was obtained from J. T. Baker Chemical Co. All solvents were distilled immediately before use.

Methods

The Standard β-Glucosidase Assay—The standard β-glucosidase assay medium contained 5 mM MUGlc,[2] 0.2 M sodium citrate, pH 6.0, and enzyme protein (0.01–0.05 μg) in a final volume of 0.1 ml. The reaction was initiated by the addition of enzyme and the incubation was performed at 37 °C for 10–30 min. Reactions were terminated by the addition of 2.9 ml of 0.3 M ammonia-glycine buffer, pH 10.5. Fluorescence was determined as described elsewhere (Peters *et al.*, 1975). One unit of activity corresponds to the release of 1 μmol of 4-methylumbelliferone/min.

Protein Determination—Protein concentration was determined using the Coomassie dye-binding method of Bradford (1976) with bovine serum albumin serving as the standard.

Evaluation of Kinetic Data—Lineweaver-Burk analyses were performed in the presence of fixed alcohol concentrations using eight different MUGlc concentrations in the range 0.5–5.0 mM. The initial reaction rates and their corresponding substrate concentrations were subjected to a computer analysis which fits the data to a rectangular hyperbola using the mathematical procedure described by Cleland (1979) and one which places greater weight on values obtained at the higher substrate concentrations. The computer program calculates K_m, V_{max}, and K_m/V_{max} values and the standard errors for these data. The means of the standard errors for K_m and V_{max} values in all our kinetic analyses averaged 10 and 4%, respectively.

Binding constants (K_A values) for the alcohol-β-glucosidase interaction were calculated from secondary replots of 1/Δ slope or 1/Δ intercept *versus* 1/[alcohol] (Segel, 1975). The Δ slope values were obtained from individual Lineweaver-Burk plots. The constants α and β refer to the -fold change in K_m and V_{max}, respectively, obtained in the presence of a nonessential activator. In the 1/Δ slope *versus* 1/[alcohol] replot, the *x* and *y* intercepts correspond to $-\beta/\alpha K_a$ and $\beta V_{max}/K_s(\beta - \alpha)$, respectively. By assigning values for α and β it is possible to estimate accurately the K_A values.

RESULTS

The Effects of Various Alcohols and Related Substances on β-Glucosidase Activity—It has been suggested (Schrier and Scheraga, 1962) that when one investigates the apolar interactions between a protein and an organic solvent the ambiguity in interpreting the biological response induced by changes in the dielectric constant and pH of the medium can be minimized by analyzing the effects at relatively low concentrations of individual members of a homologous series of organic compounds on the protein's structure or function. Hence, we undertook to examine the effect of a family of straight-chain alcohols on the kinetic properties of cytosolic β-glucosidase.

The standard β-glucosidase assay was used to investigate the effects of various concentrations of ethanol, *n*-propyl alcohol, *n*-butyl alcohol, and 1-pentanol on the specific activity of guinea pig liver β-glucosidase. From the results of this experiment which are presented in Fig. 1, one can draw at least three conclusions: 1) The alcohols brought about a biphasic activity *versus* concentration curve; at lower concentrations they stimulated β-glucosidase activity (5–7-fold) while at higher concentrations they began to inhibit activity. 2) The concentration of each alcohol at which maximum stimulation was achieved was lowest for 1-pentanol, the most apolar member of the series. 3) The order of their effectiveness as β-glucosidase activators was 1-pentanol > *n*-butyl alcohol > *n*-propyl alcohol > ethanol. The biphasic activity *versus* concentration curve observed with the alcohols has been observed in several other systems; it is noteworthy, however, that the concentrations of alcohols, especially 1-pentanol and *n*-butyl alcohol, used in our studies were much lower than those used by others (Tan and Lovrien, 1972 and references therein).

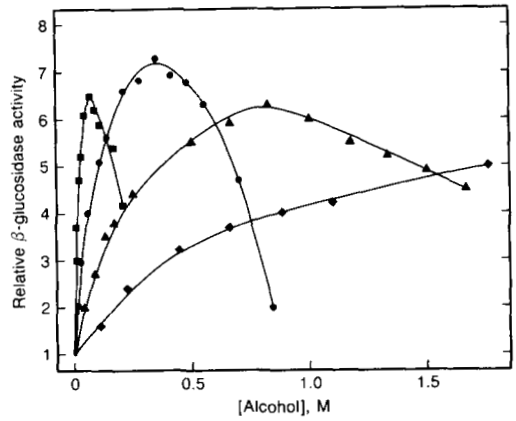

FIG. 1. **Effect of primary alcohols on β-glucosidase activity.** Activity was determined using the standard β-glucosidase assay. Relative activity of β-glucosidase is plotted *versus* the concentration of ethanol (♦), *n*-propyl alcohol (▲), *n*-butyl alcohol (●), or 1-pentanol (■).

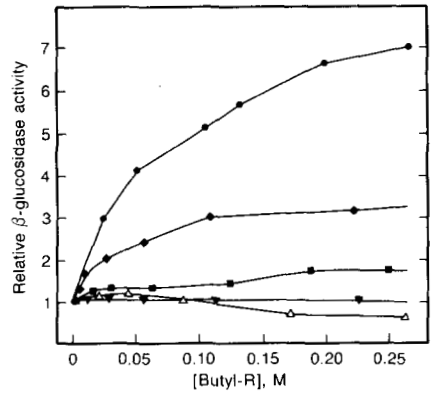

FIG. 2. **Effect of *n*-butyl alcohol and related four-carbon compounds on β-glucosidase activity.** The relative activity of β-glucosidase is plotted *versus* the concentration of *n*-butyl alcohol (●), butyric acid (▼), butanediol (♦), butanesulfonic acid (■), or butylurea (△).

In order to gain insight regarding the specificity of the effects of these alcohols, we compared the ability of several other four-carbon compounds against *n*-butyl alcohol for their ability to stimulate the β-glucosidase. As shown in Fig. 2, whereas *n*-butyl alcohol activated the enzyme 7-fold, compounds in which the hydroxyl group of *n*-butyl alcohol was replaced by a carboxyl (butyric acid), sulfonyl (butanesulfonic acid), urea (butylurea), or amino (butylamine) group were ineffective as activators of the β-glucosidase. Thus, the hydroxyl group of *n*-butyl alcohol appears to be critical for the β-glucosidase-stimulating effects of this alcohol. 1,4-Butanediol was only 30% as effective as *n*-butyl alcohol in this regard, suggesting that the methyl group of *n*-butyl alcohol enhances binding of *n*-butyl alcohol to the enzyme.

We next compared the four isomers of butanol to determine if there might be structural requirements for the interaction

[2] The abbreviation used is: MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside.

between the four-carbon alcohol and the cytosolic β-glucosidase. As shown in Fig. 3, other than *n*-butyl alcohol, only isobutyl alcohol approached *n*-butyl alcohol in terms of its ability to enhance β-glucosidase activity; the *sec* and *tert* isomers of butanol were essentially inactive in this regard.

Taken together, these data suggest that a relatively specific interaction occurs between these primary alcohols and the enzyme. Furthermore, they show that the length of the carbon chain, the shape of the molecule, and the presence of the hydroxyl group seem to be critical determinants of this interaction.

Lineweaver-Burk Analysis of the Effects of Primary Alcohols on Cytosolic β-Glucosidase—The interactions of the four normal alcohols ranging in carbon number from C_2 (ethanol) to C_5 (1-pentanol) with the β-glucosidase were analyzed kinetically. A typical Lineweaver-Burk analysis of the effects of *n*-butyl alcohol on the kinetic properties of the enzyme is shown in Fig. 4. It is apparent from the results shown in *Panel A* that at relatively low concentrations (0–0.14 M) the effect of *n*-butyl alcohol is exclusively to increase V_{max} without altering the K_m for MUGlc. Interestingly, as the concentration of *n*-butyl alcohol in the assay medium was increased from 0.14 to 0.54 M (Fig. 4, *Panel B*), a very different pattern was observed; in this case, the slopes increased with increasing alcohol concentration and the Lineweaver-Burk plots intersected on the ordinate, indicating purely competitive inhibition. Similar kinetic patterns were obtained when the effects of ethanol, *n*-propyl alcohol, and 1-pentanol on β-glucosidase activity were examined by Lineweaver-Burk analysis.

All of the kinetic data described above were expressed in terms of relative V_{max}/K_m ratios and plotted *versus* alcohol concentration; from the resultant patterns obtained, the biphasic nature of the alcohol effects on the kinetic properties of the enzyme are readily apparent (Fig. 5). For each of the four alcohols, at the lower concentrations, the replots exhibited a positive slope, indicating that at these concentrations the alcohols act as activators. Approximately the same normalized V_{max}/K_m ratio (5.0–7.0) is reached for all of the alcohols. Higher alcohol concentrations result in a negative

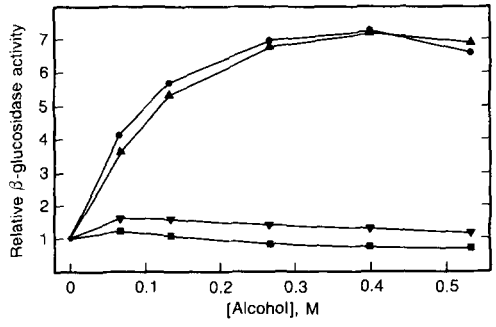

FIG. 3. **Effect of the four isomers of butanol on β-glucosidase activity.** The relative activity of β-glucosidase is plotted *versus* the concentration of *n*-butyl alcohol (●), isobutyl alcohol (▲), *sec*-butyl alcohol (■), or *tert*-butyl alcohol (▼).

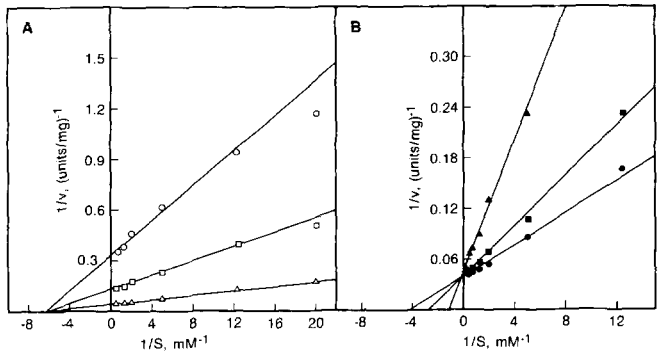

FIG. 4. **Lineweaver-Burk analysis of the dual effects of β-glucosidase activity by *n*-butyl alcohol.** Activity was measured using the standard β-glucosidase assay over at least a 100-fold range of MUGlc concentrations in the absence (○) or presence of 0.05 M (□) and 0.14 M (△) *n*-butyl alcohol (*Panel A*) and in the presence of 0.20 M (●) 0.35 M (■), or 0.54 M (▲) *n*-butyl alcohol (*Panel B*).

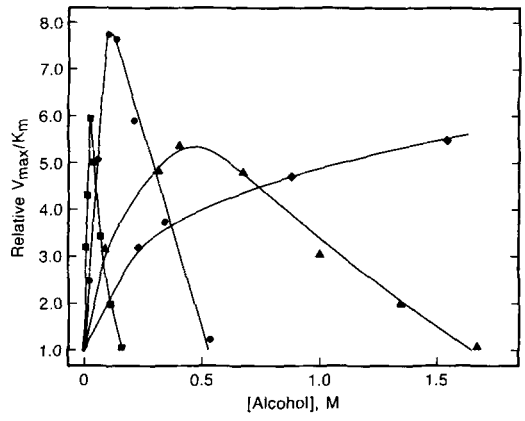

FIG. 5. **The effect of primary alcohols on the relative V_{max}/K_m ratio of β-glucosidase.** The V_{max}/K_m values were calculated from the respective Lineweaver-Burk analyses for each alcohol. The relative slope value is plotted against the concentration of ethanol (♦), *n*-propyl alcohol (▲), *n*-butyl alcohol (●), or 1-pentanol (■).

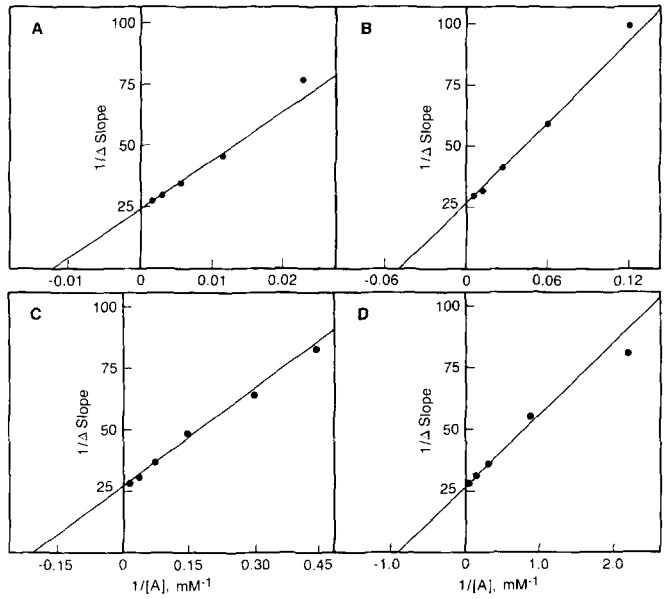

FIG. 6. **Demonstration of a single molecule of alcohol binding to the activator site on the β-glucosidase.** The figure shows secondary replots of 1/Δ slope *versus* 1/[*A*] for ethanol (*Panel A*), *n*-propyl alcohol (*Panel B*), *n*-butyl alcohol (*Panel C*), and 1-pentanol (*Panel D*) as nonessential activators ($\alpha = 1$, $\beta = 7$).

TABLE I

Summary of affinity constants and the free energies of binding of primary alcohols to β-glucosidase

Alcohol	K_A	ΔG^0 [K_A]
	mM	*cal/mol*
Ethanol	555 (74)[a]	362
n-Propyl alcohol	146 (20)	1190
n-Butyl alcohol	34.1 (3.4)	2082
1-Pentanol	7.47 (1.0)	3020

[a] The number in parentheses indicates the standard error.

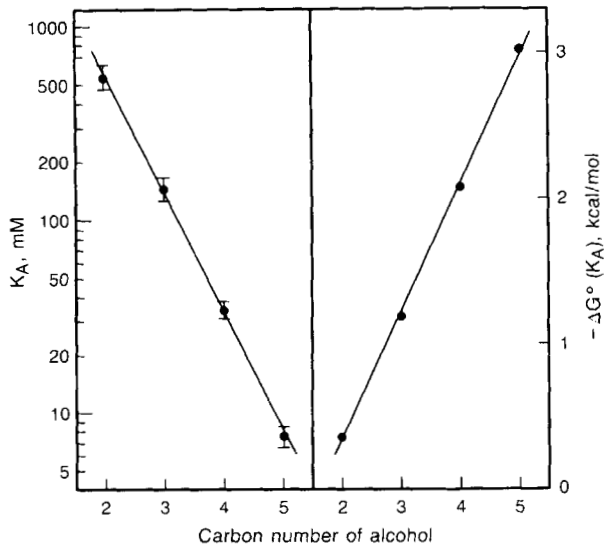

FIG. 7. **A plot of the affinity constants (K_A values) and the free energies of binding ($\Delta G°(K_A)$) *versus* the carbon number of alcohols.** The affinity constants (K_A values) were determined from secondary replots of $1/\Delta$ slope *versus* $1/[A]$ for each alcohol and plotted *versus* their carbon number (*Panel A*). The free energies of binding of alcohols to β-glucosidase were determined using the Gibbs equation, $\Delta G° = -RT \ln K_{eq}$ ($K_{eq} = 1/K_A$). *Panel B* depicts the linear increase in the free energy of binding with increasing chain length of the alcohols ($r = 0.999$); the slope $\Delta\Delta G° (K_A)/CH_2 = 887$ cal/mol represents the increase in free energy of binding gained by increasing the length of the alkyl chain by one carbon.

slope, indicating that the alcohols act as inhibitors. From the replots shown in Fig. 5, it is apparent that the most stimulatory and inhibitory alcohol was 1-pentanol, followed in order by *n*-butyl alcohol, *n*-propyl alcohol, and ethanol.

The activation phase was characterized more extensively in a separate experiment by performing Lineweaver-Burk analyses using at least five different concentrations of each alcohol. This enabled us to obtain secondary replots of $1/\Delta$ slope *versus* 1/[alcohol], from which we could determine the binding constants (K_A values) for each alcohol. As shown in Fig. 6, the replots were perfectly linear for all the alcohols. The K_A values and the respective free energies of binding ($\Delta G° (K_A)$) are summarized in Table I and depicted graphically in Fig. 7.

DISCUSSION

The present study demonstrates that amphipathic primary alcohols exert either a stimulatory or an inhibitory effect on the broad specificity cytosolic β-glucosidase of mammalian liver, depending on the alcohol concentration of the assay medium. At low concentrations the alcohols stimulate β-glucosidase activity, while at higher concentrations they inhibit its activity (Fig. 1). The enhancement of activity obtained with the lower concentrations of the *n*-alkyl alcohol series is entirely the result of a V_{max} effect, while the decrease in activity observed at the higher concentrations is completely accounted for by competitive inhibition (Fig. 4).

The results of the comparative study of the effects of the four members of the butanol family on the activity of the broad specificity β-glucosidase provide evidence against a general solvent effect on the enzyme's conformation or on the assay medium (Fig. 3); instead, they favor a structurally specific interaction between the perturbant molecule (*i.e.* the alcohol) and the enzyme. Furthermore, the structural specificity of the enzyme-alcohol interaction is borne out by the observation that compounds like butyric acid, butanesulfonic acid, butylamine, and butylurea did not stimulate the activity of cytosolic β-glucosidase (Fig. 2).

A model which would explain the data and account for the interactions of the various amphipathic alcohols with the β-glucosidase consists of two elements: first, an anchoring component, which we attribute to the interaction of the hydrophobic domain of the alcohol with a hydrophobic site on the enzyme, and second, the proper orientation of the hydrophilic domain of the alcohol such that the hydroxyl group can interact with some polar group on the enzyme.

To explain the progression from activation to inhibition as the concentration of the alkyl alcohol in the assay medium is increased, it is helpful to conceive that the cytosolic β-glucosidase possesses two distinct apolar alkyl binding domains, with greatly differing affinities for nonpolar effectors. We designate the high affinity site as the activator site and envision the low affinity site to be an integral part of the enzyme's active site. Using this model, we envision that at low concentrations, alcohols such as *n*-butyl alcohol bind to the high affinity site and convert the β-glucosidase from one conformation (State I) to another (State II) (Fig. 8); the latter form is catalytically more efficient than the alcohol-free form of the enzyme, as is evident from the 5–7-fold increase in the V_{max}. We propose that in this alternate conformation the essential functional groups at the active center which affect the actual catalytic process are transformed to assume a more favorable orientation. The transition from State I to State II entails removing or decreasing certain kinetic or thermodynamic restrictions inherently imposed on State I. An identical situation has been reported by Dimroth *et al.* (1970) for the biotin carboxylase component of the acetyl-CoA carboxylase of *Escherichia coli*. Their Lineweaver-Burk analysis revealed that 10% (v/v) ethanol increased the V_{max} 6–7-fold without causing a detectable change in the K_m values for ATP-Mg, HCO_3^-, or *d*-biotin.

We propose that in the present study the inhibition caused by the higher concentrations of alcohols, which is largely competitive in nature, is the result of a second alcohol molecule binding to the alkyl binding subsite at the active center of the β-glucosidase when it is in State II. These kinetic data support a model which envisions that the broad specificity β-glucosidase contains a second hydrophobic binding site, one that is distinct from the aglycone binding subsite at the enzyme's catalytic center. Furthermore, this second hydrophobic site on the enzyme has a higher affinity for *n*-butyl alcohol, than does the active site. Hence, at low concentrations the alcohols bind to this higher affinity (stimulatory) activator site whereas at higher concentrations they also bind to the lower affinity (inhibitory) site that corresponds to the active site, thus accounting for the biphasic kinetic pattern we observed. Of course, direct binding studies are needed to confirm the postulate of two alcohol molecules binding to the cytosolic β-glucosidase.

Since the β-glucosidase-catalyzed reaction proceeds rapidly in the absence of alcohol, the alcohol-induced increase in the reaction velocity causes us to classify this phenomenon as a case of nonessential activation (Segel, 1975). In the alcohol-induced β-glucosidase transition from State I to State II there

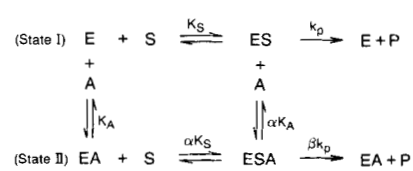

FIG. 8. **Scheme for nonessential activation proposed by Segel (1975).**

is no change in K_m, but a 5–7-fold increase in V_{max}. From our extensive Lineweaver-Burk analyses of the β-glucosidase reaction at different concentrations of each alcohol throughout the activation phase, we plotted the reciprocal of the change in slope (1/Δ slope) *versus* the reciprocal of the activator (alcohol) concentration. These perfectly linear replots (Fig. 6) obtained for all four alcohols provide convincing kinetic evidence for the binding of one molecule of alcohol to the activator site. Since the activator caused only an increase in V_{max}, replots of 1/Δ intercept *versus* 1/[alcohol] are also linear (data not shown).

In the extensive kinetic analyses of 1/Δ slope *versus* 1/[alcohol] (Fig. 6), the *Y* intercept $\beta V_{max}/K_s(\beta - \alpha)$ is the same for all alcohols. This supports the hypothesis that the alcohols are interacting with the same activator site on the glycohydrolase and that when they bind this site they all induce the same transition from State I to State II.

The 1/Δ slope *versus* 1/[alcohol] replots for each alcohol yields the K_A value for the respective enzyme-alcohol interaction. From *Panel A* of Fig. 7, it is evident that for each additional carbon atom, the K_A value for the enzyme alcohol interaction decreases by a factor of 4.0–4.5. This 4-fold decrease in K_A values translates into an additional 887 cal/mol ($\Delta\Delta G^\circ(K_A)/CH_2$) in the free energy of binding ($\Delta G^\circ(K_A)$) to the activator site on the cytosolic glycohydrolase (Fig. 7, *Panel B*).

The binding of alcohols to a hydrophobic site on the cytosolic β-glucosidase is a thermodynamically favorable process, due to entropic stabilization resulting from a decrease in what Schrier and Scheraga (1962) refer to as the organized "ice-like" structure of water molecules surrounding the nonpolar moiety of the alcohol and also, of those which had been in contact with the exposed hydrophobic side chains of this alcohol binding site. It is well established that the tendency of amphipathic substances to partition from the aqueous bulk phase into the apolar interior of a protein increases with the hydrophobicity of their nonpolar moiety (Tanford, 1980). Hence, the greater thermodynamic potential of the longer chain alcohols, manifested as a larger entropic contribution to their free energy of binding to the activator site, may account for their higher affinity for this site relative to the lower alcohols (Table I). Therefore, it is not unusual to observe an inverse relationship between the chain length of the alcohols and the breakpoint in the biphasic V_{max}/K_m *versus* alcohol concentration plot (Fig. 5).

The theory of hydrophobic bonding proposed by Nemethy and Scheraga (1962) predicts that upon increasing the hydrocarbon length of the organic material added to the assay medium there should be an increase in the magnitude of any observed effect on the biological properties of the protein. For instance, the lowering of the transition temperature of ribonuclease for a given concentration of alcohol was found to increase as the chain length of the straight chain alcohol was increased (Schrier *et al.*, 1965). Alternatively, for a homologous series of apolar compounds, it may require lower concentrations of a higher analog and higher concentrations of a lower member to evoke the same quantitative response.

What might be the role of the high affinity alcohol binding site on the β-glucosidase? The term "ambiquitous enzyme" is applied to those enzymes whose subcellular distribution (*e.g.* cytosolic *versus* membrane-bound) is dictated by the metabolic status of the cell (Wilson, 1980). It has been suggested (Wilson, 1980) that the reversible binding of "soluble" enzymes to particular subcellular membranes may achieve the dual objectives of activation and regulation of catalytic activity. The observation that short-chain primary alcohols can activate the cytosolic β-glucosidase *in vitro* 5–7-fold raises an interesting question: Do mammalian cells which produce this enzyme contain cofactors or regulatory substances which resemble these alcohols in their ability to influence the catalytic capacity, substrate specificity, and, in addition, affect the subcellular distribution of the cytosolic β-glucosidase? It is conceivable that such natural substances could alter the overall kinetics of a subcellular membrane-bound system of enzymes by juxtaposing the β-glucosidase with another sequential enzyme of a metabolic pathway.

Our speculation regarding the existence of a physiological activator for the cytosolic β-glucosidase is supported by precedents in the literature (Dimroth *et al.*, 1970; Sanwal *et al.*, 1966). In one such instance, the activation of phosphoenolpyruvate carboxylase by ethanol replaced the requirement for acetyl-CoA as the allosteric activator, thereby demonstrating that ethanol could elicit the same favorable conformational change in the enzyme as the *in vivo* allosteric effector (Dimroth *et al.*, 1970). Hence, it seems reasonable to postulate that the alcohols mimic some *in vivo* effector of the cytosolic β-glucosidase.

It may be that the ability of the cytosolic β-glucosidase to hydrolyze its true physiologic substrate *in vitro* can be demonstrated only when the assay medium is supplemented with this natural activator. This idea should stimulate investigators to conduct a meticulous search for the putative activator substance in cells which contain the broad specificity β-glucosidase. We recommend that, until this hypothetical activator is identified, investigators seeking to identify natural substrates of the β-glucosidase include an optimal concentration of some nonphysiologic activator like *n*-butyl alcohol in their assay media.

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