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pH-DEPENDENT INHIBITORY EFFECTS OF TRIS AND LITHIUM ION ON INTESTINAL BRUSH-BORDER SUCRASE

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Tris and two of its hydroxylated amine analogs were examined in a metal-free, universal n-butylamine buffer, for their interaction with intestinal brush border sucrase. Our recent three-proton- families model (Vasseur, van Melle, Frangne and Alvarado (1988) *Biochem. J.*, 251, 667–675) has provided the sucrase pK values necessary to interpret the present work.

At pH 5.2, 2-amino-2-methyl-1-propanol (PM) causes activation whereas Tris has a concentration-dependent biphasic effect, first causing activation, then fully competitive inhibition. The amine species causing activation is the protonated, cationic form. The difference between the two amines is related to the fact that Tris has a much lower pK_a value than PM (respectively, 8.2 and 9.8). Even at pH 5.2, Tris (but not PM) exists as a significant proportion of the free base which, by inhibiting the enzyme fully competitively, overshadows the activating effect of the cationic, protonated amine.

Above pH 6.8, both Tris and PM act as fully competitive inhibitors. These inhibitions increase monotonically between pH 6.5 and 8.0 but, above pH 8, inhibition by 2.5 mM Tris tends to diminish whereas inhibition by 40 mM PM increases abruptly to be essentially complete at pH 9.3 and above. As pH increases from 7.6 to 9.0, the apparent affinity of the free amine bases decreases whereas that of the cationic, protonated amines, increases. In this way, the protonated amines replace their corresponding free bases as the most potent inhibitors at high pH.

The pH-dependent inhibition by 300 mM Li^+ is essentially complete at pH 8, independent of the presence or absence of either 2.5 mM Tris or 40 mM PM. Even at pH 7.6, an excess (300 mM) of Li^+ causes significant increases in the apparent K_i value of each Tris, PD (2-amino-2-methyl-1-3-propanediol) and PM, suggesting the possibility of a relation between the effects of Li^+ and those of the hydroxylated amines which in fact are mutually exclusive inhibitors.

The inhibitory results are interpreted in terms of a mechanistic model in which the free bases bind at two distinct sites in the enzyme's active center. Binding at the glucosyl sub-site occurs through the amine's free hydroxyl groups. This positioning facilitates the interaction between the lone electron pair of the deprotonated amino group with a proton donor in the enzyme's active center, characterized by a pK_0 around 8.1. When this same group deprotonates, then the protonated amines acting as proton donors replace the free bases as the species giving fully competitive inhibition of sucrase.

KEY WORDS: Sucrase, tris, lithium ion, pH.

INTRODUCTION

Tris is a known fully competitive inhibitor of glycosidases from various sources.[1-9] In some instances, however, additional effects of Tris have been reported, e.g., noncompetitive inhibition of insect trehalase at pH 9.2.[7]

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Abbreviations: Tris, tris (hydroxymethyl) aminomethane; PD, 2-amino-2-methyl-1-3-propanediol; PM, 2-amino-2-methyl-1-propanol.

With a pK_a of about 8.24 at 37°C,[5] Tris is at neutral pH a mixture of a protonated, cationic form (Tris-H^+) and a deprotonated, neutral form (Tris base). Using hog intestinal maltase (α-glucosidase) and oligo- 1,6-glucosidase (iso-maltase), Larner and Gillespie were the first to show that Tris inhibition is competitive with the substrate and is stronger at alkaline pH values.[1] Their conclusion that Tris base is the active species seems to have been confirmed by work with both yeast α-glucosidase[2] and intestinal sucrase.[3,5] However, this conclusion is by no means accepted generally. On the basis of a detailed study of Tris inhibition of barley α-glucosidase as a function of pH, Jorgensen and Jorgensen[4] concluded that no decision could be made as to the ionic form of the inhibiting substance. Because their experiments indicated that if Tris base were the active species its inhibitory constant would be very low, they rejected the idea that this form is the active one because this "great affinity between enzyme and Tris base . . . perhaps makes the Tris inhibition unlikely". Other workers who used as experimental material either insect trehalase,[7] rat intestinal trehalase,[8] or the glucosidasic activity of mammalian glycogen debranching enzyme[9] have added new uncertainty to the problem by suggesting that the active species is Tris-H^+.

The existing disparities in the interpretation of the mechanism of action of Tris are indeed puzzling. Moreover, the apparently universal role of Tris, a rather remote sugar analog, as a strong fully competitive inhibitor of saccharidases is also intriguing and suggests that further study of the mechanism(s) of action involved might be rewarding. We have therefore undertaken a reinvestigation of Tris and two of its hydroxylated analogs on intestinal sucrase (sucrose-α-D-glucohydrolase, EC 3.2.1.48), an ectoenzyme characteristic of the brush border membrane of the enterocyte.[9] As our theoretical basis we used a previous study from our laboratory, showing that the pH-dependent activity of sucrase fits a three-proton-families model[10–12] where, depending on the cation concentration, the enzyme is specifically activated and/or inhibited by the alkali metal ions[10–16] and inhibited by certain organic cations.[17]

In order to understand fully the effect(s) of Tris on sucrase, we felt it necessary to start by distinguishing between: (i) a possible competition of either the neutral or the cationic form of Tris, a polyol, with the glucosyl-binding site of the enzyme, and (ii) the equally possible competition of the Tris-H^+ cation with the alkali metal ion-binding site(s). A distinction between these different possibilities is not readily apparent. For instance, as previously shown for harmaline, an organic cation competing with Na^+ for the metal-binding activator site may exhibit fully competitive inhibition kinetics for the sucrose-binding site.[17]

In the present article we show that, although at pH 5.2 the protonated amine cations appear to activate sucrase by interacting with the (activating) alkali-metal ion binding sites, such activations can be easily overshadowed by the powerful effects the free bases have as true, fully competitive inhibitors of the enzyme in the pH range 5.7 to 8.

The possible molecular basis of the interaction of both the free bases and the amine cations with the enzyme's active center is discussed in the light of current views on the mechanism of action of disaccharidases. Preliminary accounts of this work have been given.[18,19]

MATERIALS AND METHODS

Reagents and Buffers

All chemicals used were of analytical-reagent grade. The n-butylamine, metal-ion-free universal buffer covering the pH range 5.2 to 9.6 was prepared as previously described.[11,14] The alkali-metal ions were added as the chlorides.[11,12]

Enzyme preparation

Brush-border membranes from California rabbit small intestine were isolated as described by Kessler.[20] The sucrase-isomaltase complex was purified to electrophoretic homogeneity by a four-step procedure developed by Vasseur.[21] The purified enzyme was stocked at 4°C in a 10 mM phosphate buffer contaning 100 mM NaCl and 0.02% NaN_3. Immediately before use, the stock enzyme was exhaustively dialysed with the n-butylamine buffer at the appropriate pH. No changes in enzyme stability under the conditions of our assay were observed in the pH range studied.[10,11]

Determination of Sucrase Activity and Expression of Results

Sucrase was assayed by measuring its D-glucose product after stopping the reaction with 0.5 M Tris. We used a glucose oxidase/peroxidase reagent containing 4-amino-antipyrine instead of *o*-dianisidine as the chromogen.[21]

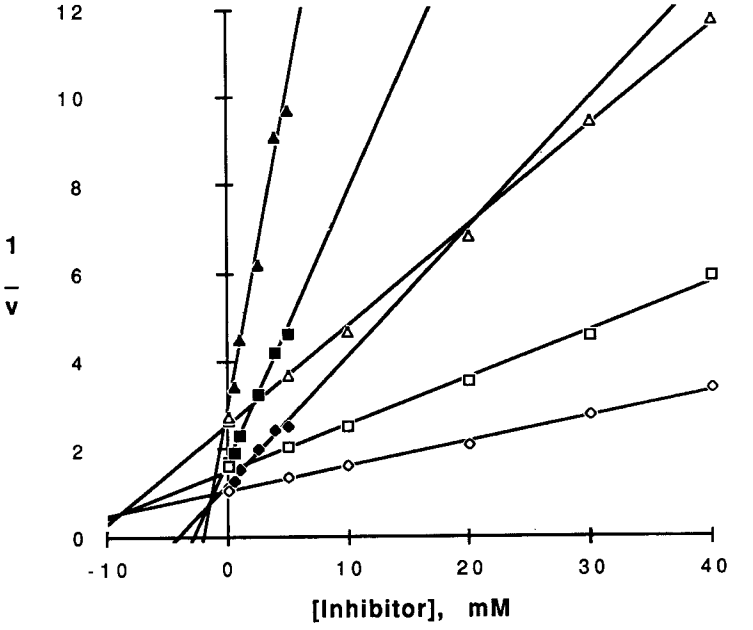

FIGURE 1 Sucrase inhibition by Tris and PM at pH 9. The effect of different concentrations of either Tris (black symbols) or PM (empty symbols) was determined at 10 (▲, △), 20 (■, □) and 40 (◆, ◇) mM sucrose concentrations. The buffer composition and other details are given in the text.

Protein was determined according to Lowry *et al.*[22] Velocities are given as units per milligram of protein (1 unit = 1μmole glucose formed per minute under standard conditions). The kinetic parameters ($k_{i(\text{Amine})}$, K_m and V_{max}) were calculated by appling both linear[23,24] and nonlinear[10-12] regression analysis. Both approaches gave essentially identical results, but the linear tranformations are used in the Figures because of their recognized visual impact.

RESULTS AND DISCUSSION

Above pH 6.8, the Lithium Ion interferes with the Fully Competitive effect of Tris and its Analogs on Sucrase

Sucrase inhibition by Tris and its hydroxylated analogs is fully competitive in the pH range from 6.8 to 9.0. As an example, the inhibitory effects of Tris and PM at pH 9.0 are illustrated in Figure 1 by using the classical Dixon[23] transformation. The results yielded as apparent K_i values: $K_{i(\text{Tris})} = 1.43 \pm 0.12$ mM and $K_{i(\text{PM})} = 8.81 \pm 0.10$ mM. However, by correcting for their respective pK_a values (8.24 for Tris[5] and 9.80 for PM), the inhibition constant for each free base was found to be same: 1.22 and 1.23 mM for Tris and PM respectively.

To establish whether or not the amines and Li^+ behave as mutually exclusive inhibitors, the inhibition by variable concentrations of either Tris or its analogs was

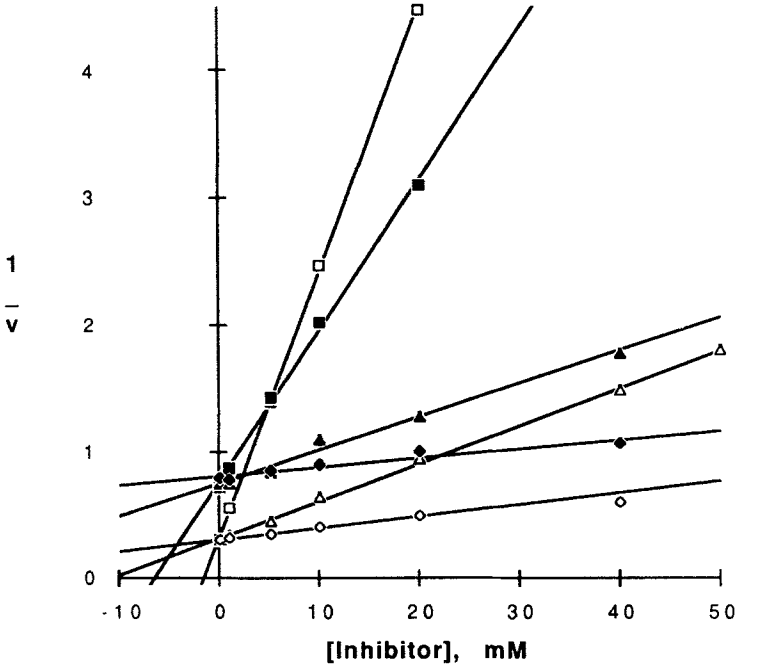

FIGURE 2 The effect of excess lithium ion on the fully competitive inhibition of sucrase by Tris, PD and PM at pH 7.6. Effects of Tris (■, □), PD (▲, △) and PM (◆, ◇) in the absence (empty symbols) or presence (black symbols) of 300 mM Li^+. The data were obtained at a single (20 mM) sucrose concentration. The buffer composition and other details are given in the text.

TABLE I

Kinetic parameters deduced from results (Figure 2) involving mixtures of two different inhibitors. The velocity equations used correspond to system B6 of Segel,[25] where we assume Tris and its analogs act as type-Ia inhibitors and Li^+ as a type-IIIb inhibitor. The apparent inhibition constants, $K_{i(app)}$ were calculated for 20 mM sucrose concentrations by applying $K_{m(sucrose)}$ values previously determined.[11,12] Further details are given in the text.

	Slope	1/Intercept (y axis)	Intercept (x axis)	K_{iapp} (mM)	$K_{iapp(Base)}$ (mM)	$K_{iapp(Acid)}$ (mM)
Tris	0.2076	2.94	1.64	0.73	0.135	0.591
Tris + Li	0.1385	1.34	5.38	1.25	0.233	1.017
PD	0.0294	3.11	10.96	4.86	0.232	4.626
PD + Li	0.0273	1.30	28.25	6.57	0.313	6.256
PM	0.0077	3.12	41.45	18.38	0.115	18.26
PM + Li	0.0083	1.27	95.40	22.18	0.139	22.04

further studied at pH 7.6, both in the absence and in the presence of 300 mM Li^+ (Figure 2). By applying to these results Segel's procedure for multiple inhibition analysis,[25] we ascertained that they fit best system B-6, where Tris and its analogs are fully competitive inhibitors (type-Ia of Dixon and Webb[26]) and Li^+ a linear mixed-type (type-IIIb) inhibitor binding to a different site.

The apparent (overall) inhibition constants for the amines, $K_{i(app)}$, were calculated from the intercepts on the [I] axis, assuming a 20 mM substrate concentration and $K_{m(sucrose)}$ values previously determined,[11] namely, $K_{m(Li=O)} = 15.93$ mM and $K_{m(Li=300mM)} = 6.06$ mM at pH 7.6. The results indicate that the $K_{i(app)}$ of each amine increases by approximately the same factor (between 1.2 and 1.7) when an excess of 300 mM Li^+ is present (Table I). The free bases are the strongest inhibitors, as indicated by the $K_{iapp(acid)}/K_{iapp(base)}$ ratios which were always greater than unity: respectively, 4.4, 20 and 159 for Tris, PD and PM, independent of the presence or absence of Li^+.

Further inspection of Figure 2 reveals that the Dixon plots for each PD and PM in the presence and absence of Li^+ are parallell (the respective slopes are essentially identical: Table I), this fact permitting by itself the conclusion that both amines behave as mutually exclusive inhibitors with respect to Li^+. The same conclusion can also be reached for Tris, even though the corresponding Dixon plots in the absence or presence of Li^+ are not parallel. To permit reaching the contrary conclusion, the slope in the presence of Li^+ should have increased[25] whereas the experimentally observed fact is that it decreased (Table I).

To ascertain why Li^+ interferes with amine inhibition, we considered next the possibility of a direct effect of Li^+ on the enzyme itself, causing a decrease on the overall affinity of sucrase for the amines. To address this question, we can analyze as follows the effects of Li^+ on amine inhibition in terms of our recently-proposed three-proton-families sucrase model.[11] This model explains the alkali-meta-lion- and the H^+-dependent activation and/or inhibition of rabbit brush border sucrase in terms of substrate- and effector-induced pK shifts.[12] Independent of the absence or the presence of 20 mM substrate, 300 mM Li^+ causes inhibition both by displacing upwards the macroscopic value of pK_1 and, downwards, the microscopic pK_0 value (see Figure 3 in Ref. 12). Because, independent of the presence or the absence of Li^+, the two prototropic groups pertaining to pK_1 will be deprotonated at pH 7.6,[11] it can be

assumed that the effects induced by Li^+ on $K_{i(amines)}$ at pH 7.6 are not related to any effect of Li^+ at this level. To the contrary, Li^+ causes pK_0 to shift either from 8.1 to 6.9 in the absence of substrate or from 8.2 to 6.7 in the presence of 20 mM sucrose, facilitating enzyme deprotonation from the fully active form, EH, to the inactive one, E.

pH-dependent Effects of Tris and Li^+ on Sucrase

The pioneer studies of Larner and Gillespie,[1] confirmed by Semenza and von Balthazar,[6] have indicated that Tris inhibits intestinal glycosidases (maltase, isomaltase, sucrase) more strongly at basic than at acid pH values, suggesting that the active inhibitor species is the Tris base. Similar conclusions apply to other glycosidases, notably, yeast α-glucosidase.[2] However, all of this work was performed at rather narrow ranges of pH, never above pH 8.

Consequently, using a metal-free universal buffer with n-butylamine as the base, we have undertaken a more detailed analysis of Tris, PM and/or Li^+ inhibition covering the range from pH 5.0 to 9.6. The results are presented as percentage activations and/or inhibitions in Figure 3. Confirming previous work,[11,14] 300 mM Li^+ activates sucrase between pH 5.4 and pH 7.0 but behaves as an inhibitor at either side of these pH values. In the basic pH range, Li^+ leads to 90% enzyme inhibition at pH 8 or above, independent of the presence of either 2.5 mM Tris or 40 mM PM. It should be noted that, at pH 8, in the presence of 20 mM sucrose and 300 mM Li^+, 95% of the

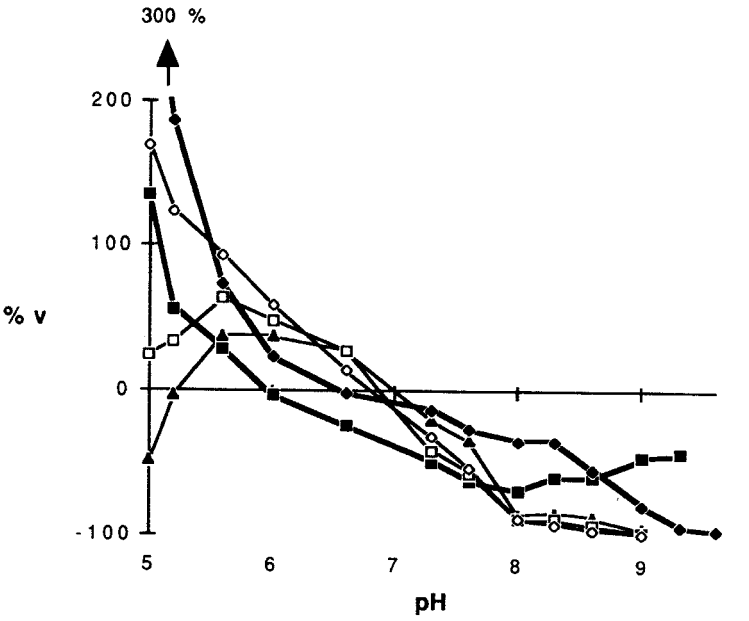

FIGURE 3 pH-dependent effects of Tris, PM and/or Li^+ on sucrase. Sucrase activity was measured in the indicated pH range at a constant (20 mM) sucrose concentration, either in the absence (v_0) or in the presence (v_i) of the following inhibitors: 300 mM Li (▲); 2.5 mM Tris either alone (■) or with 300 mM Li^+ (□); 40 mM PM either alone (◆) or with 300 mM Li^+ (◇). The results are expressed as percentage velocities according to the formula: $\%\, v = [100*(v_i - v_0)/v_0]$.

enzyme exists in the fully deprotonated form whereas, under the same conditions but in the absence of Li^+, the enzyme consists of a mixture of 67% EHS and 33% ES.[11]

Below pH 6, in the absence of Li^+, 2.5 mM Tris activates whereas, above pH 6, a clear inhibition sets in. This inhibition increases with pH to reach its maximum (60% inhibition) at pH 8. Above pH 8 the inhibition decreases to reach a 40% level at pH 9.3.

With 40 mM PM, the results are roughly parallel to those just described for Tris, namely, activation below and inhibition above pH 6.6. In contrast with Tris, however, above pH 8 PM inhibition increases abruptly to become essentially complete at or above pH 9.3. By applying the equation for fully competitive inhibition, simple arithmetic permits the conclusion that the different behaviour of either Tris or PM above pH 8.6 (Tris inhibition decreases and PM inhibition increases), results simply from a difference in the $[I]/K_i$ ratio which is greater for PM than it is for Tris: respectively, 4.54 and 1.75 at pH 9.0.

Nonetheless, independent of the ionization state of the amines, the results in Figure 3 indicate that a key change occurs at or above pH 8. In the following we shall consider the possibility of a direct interaction between the amines and pK_0, the enzyme's basic ionization constant previously shown to be strongly affected by the presence of lithium ions.[11,12]

Below pH 8.0, inhibition by both amines becomes apparent as soon as a small proportion of the total inhibitor present changes to the deprotonated form. We find, for instance, 3% inhibition with 0.014 mM Tris base at pH 6.0 and 2% inhibition with 0.024 mM PM base at pH 6.6. At higher amine concentrations, the inhibition increases with pH, roughly in parallel with the curve describing formation of either free

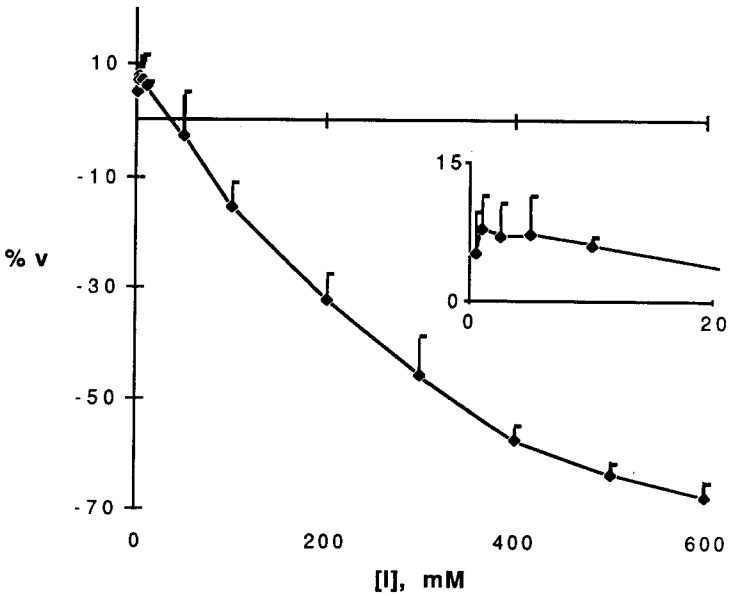

FIGURE 4 Biphasic effect of Tris on sucrase at pH 5.2. The concentration-dependent effect of Tris on sucrase was measured under conditions similar to those in Figure 3. The activating effect of Tris at low concentrations (up to 20 mM) is shown enlarged in the insert.

base. Taken as a whole, these results demonstrate that, for each amine, the free base is the inhibitory species although, at about pH 8 or higher, the enzyme tends to deprotonate and, in this case, it is the amine cations that behave as fully competitive inhibitors of sucrase (further details below).

The Dual Effects of Tris at Acid pH Values

Recently, Chen *et al.*[8] reported that Tris inhibition on rat intestinal trehalase was either much diminished or absent at or below pH 5. Accordingly, we have performed at pH 5.2 a detailed analysis of the Tris and PM effects on sucrase. The results indicate that PM activates sucrase whereas Tris has a more complex, concentration-dependent biphasic effect, namely, activation at low concentrations, followed by inhibition at 100 mM or higher concentrations (Figure 4; because of the difference in scale, the PM results are not illustrated). It should be noted however that, at pH 5.2 and 100 mM or higher total Tris concentration, the free base will be present at 0.1 mM or higher concentrations. If, as has been seen, Tris base is a strong fully competitive inhibitor, the activating effect of the amine cation can be expected to be totally overshadowed under these conditions. To test this hypothesis, a more detailed study of these inhibitions was undertaken. The results (not illustrated) confirm that, at pH 5.2, Tris is indeed a fully competitive inhibitor of sucrase, with an apparent $K_{i(\text{total Tris})} = 110.9 \pm 1.6$ mM and $K_{i(\text{Tris base})} = 0.1$ mM.

The simple activation kinetics exhibited by PM can be explained as follows. In contrast with Tris, due to the difference in pK_a, practically 100% of the PM will be in the activatory, cationic form in the concentration range studied (up to 600 mM). To reach a free base concentration as low as 0.1 mM, a total 4 M PM concentration would have been required. We therefore conclude that, at pH 5.2, each Tris-H^+ and PM-H^+ resemble the alkali-metal ions and activate sucrase, probably by inducing pK_1 to decrease. Whether or not, similar to the alkali-metal ions,[12] the amine cations at high concentrations do inhibit the enzyme by inducing pK_1 again to increase, cannot be tested experimentally because any such effects, if they exist, will be overshadowed by the strong, fully competitive inhibitor effects of the free bases.

Role of the Deprotonated Amino Group of Tris Base

The results just described confirm and extend the notion that, as with other glycosidases, Tris base is the species acting as a fully competitive inhibitor of rabbit brush border sucrase in the pH range separating pK_1 from pK_0 (that is, in the 5.8 to 8.0 pH range[11,12]).

The data in Table I show that, at pH 7.6, the apparent affinity of Tris base (defined as $1/K_{i(\text{Tris base})}$) is about 120 times higher than that of the natural substrate, sucrose ($K_m = 15.9$ mM[12]). This advantage of Tris base drops by about 5-fold but remains high (26-fold) when 300 mM Li^+ is present (here, $K_{m(\text{sucrose})} = 6.1$ mM[12]). It seems unlikely that the presence of three hydroxyl groups in Tris suffices to explain its exceedingly high affinity for the sucrose-binding site. In fact, by taking into consideration differences in pK_a values, Larner and Gillespie[1] have already indicated that, for the hydroxylated amines to attain maximal inhibitory capacity, presence of a single hydroxyl next to the amino group was sufficient. Our data confirm that, at either pH 7.6 (Table I) or 9.0 (Figure 1) the apparent K_i of the free base is the same for either PM or Tris. However, the hydroxyl groups cannot be eliminated altogether since

tert-butylamine, which is structurally very close to Tris, is essentially inert as an inhibitor of sucrase[14] and other disaccharidases.[1]

Why do Tris and its hydroxylated analogs, which appear to resemble the sugars merely by having one OH group, exhibit such great affinity for sugar-binding sites of glycosidases? As an example, erythritol is thought to inhibit barley α-glucosidase because it mimics the configuration of D-glucose at carbons C-3 to C-6.[27] But Tris base (K_i = 0.013 mM at pH 6.85) clearly outperforms erythritol (K_i = 86 mM) as an inhibitor of barley α-glucosidase (see Table 2 in Ref. 5). It seems striking that Tris base would exhibit K_i values 100 times smaller than the K_m of the natural substrates of two phylogenetically distant enzymes, barley α-glucosidase and rabbit intestinal sucrase. The logical suggestion is that similar principles must be involved both in the mechanism of action of these enzymes and in their inhibition by Tris and its hydroxylated analogs.

The exceedingly high affinity of the Tris-, PD- and PM- free bases for glycosidases must depend on their having some chemical characteristic that is absent in the sugars: the existence of a deprotonated amino group next to a free hydroxyl could very well be this characteristic. This idea agrees with earlier suggestions that the inhibitory effect of Tris analogs on glycosidases is due to the combined influence of the amino group and the polyhydroxyl constellation.[2] Lai and Axelrod[28] were probably the first to predict that "a compound possessing, at once, the essential configuration of a specific glycone as well as an amino group" should exhibit strong affinity for glycosidases. Their demonstration that glycosylamines meet these specifications has been extended by others to include a long list of amino sugars, all of them acting in the deprotonated form.[29,30]

Taken as a whole, available information indicates sugar analogs to exhibit the following order of apparent affinity for sucrase and all other glycosidases so far studied: 5-deoxy-5-aminosugars > acarbose > 1-amino-glycosides = Tris analogs > neutral sugars. The fact that the hydroxylated Tris analogs appear in this list at the same level as the 1-aminoglycosides is indeed striking. It is clear that Tris analogs cannot adopt the half-chair conformation considered to be what the glycosylamines have in common with the transition state of the substrate, the glycosylamonium ion. If inhibitors resembling the transition state bind much better than direct analogs of the substrates,[31] it seems reasonable to suggest that Tris and its analogs behave as transition state analogs of glycosidases.

Consequently, in the following we will treat Tris inhibition in terms of currently-held ideas on the mechanim of action of sucrase,[32,33] which is similar to that of other glycosidases.[34,35] In each case, an active-center prototropic group pertaining to pK_0 is thought to make an electrophylic attack on the glycosidic oxygen to give a glycosyl-carbonium ion. This is followed by formation of an oxocarbonium ion and aglycone release. The intermediate carbonium ions may be further stabilized by a neighboring carboxylate ion, more precisely, one of the two groups postulated to be involved in pK_1.[11] Consistent with this model, Tris base would inhibit by binding first through its OH-group(s) to the glucosyl subsite, thereby anchoring the lone electron pair of the deprotonated amino group in a position suitable for binding to the proton donor of pK_0 (Figure 5). Destabilization of this particular proton would cause the apparent affinity of the enzyme for the free base of Tris and its analogs to decrease. Firstly, even at pH 7.6, Li^+ would act by destabilizing the proton of pK_0, thus explaining the increase caused by this ion on the $K_{i(base)}$ value of the amines. Secondly, for both Tris and PM, the $K_{i(base)}$ values would increase from around 0.14 mM to pH 7.6 to 1.22 mM

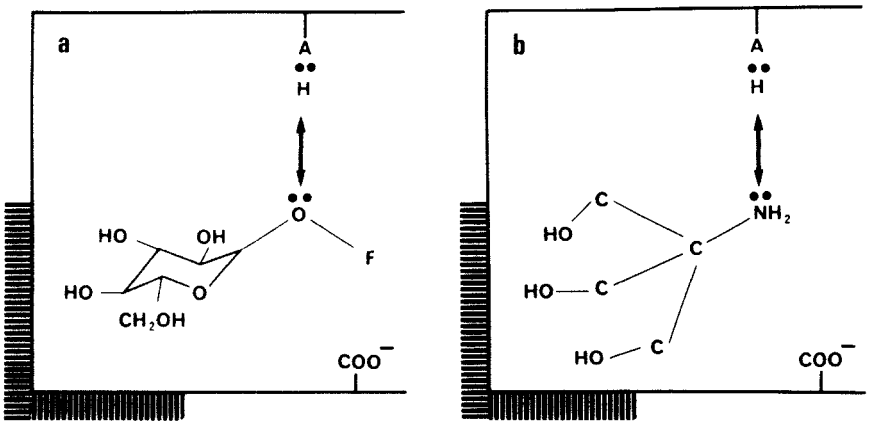

FIGURE 5. Proposed mechanism for the interaction of Tris base with the active center of sucrase in comparison with that of the natural substrate, sucrose. Shaded areas represent the glycosyl subsite of the enzyme. F is the fructosyl residue of sucrose. -A:H represents the prototropic group involved in pK_0. $-COO^-$ is one of the two groups corresponding to pK_1, believed to participate in catalysis in its deprotonated form. The vertical double-headed arrows indicate the hydrogen bond that forms between -A:H and either the glucosidic oxygen of sucrose (first step in the catalytic reaction) or the lone electron pair of Tris base. No attempt has been made to draw the model to scale.

at pH 9.0, when more than 86% of the enzyme exists in the fully deprotonated form, E.

Two mutually exclusive interactions can be postulated to exist here. When the pK_0 group is protonated (EH enzyme form), the free amine base will be the competitive inhibitor acting as an electron donor. To the contrary, the deprotonated pK_0 group characterizing E will be the electron donor and the amine cation the electron acceptor.

The interaction of the amine-H^+ cations with sucrase is unusual as far as the inhibitor is postulated to bind to an inactive form of the enzyme, the fully deprotonated one. However, the calculations mentioned above indicate that, at pH 9, 14% of the enzyme exists in the active form EH. Binding of the amine cations to E would displace the EH $\rightleftarrows$ E equlibrium to the right, resulting in fully competitive inhibition.

At present, no definitive proof exists as to the identity of the group involved in pK_0. If a carboxylic group with an unusually high pK_a were involved,[32,33] then its interaction with Tris base would generate a neutral enzyme inhibitor (E–I) complex. In contrast, if pK_0 involved a lysine ε-amino group,[36] a positively charged E–I complex would result. In the latter case, the E–I complex could be further stabilized by electrostatic interaction with the deprotonated groups of pK_1. More work will be needed, however, to establish which of these two alternatives is correct. Nevertheless, the proposal seems warranted that the key step in Tris inhibition is the formation of a hydrogen bond between the free amine and the prototropic group of pK_0. It should be noted that Legler[29] and Hanozet *et al.*[30] have proposed a somewhat similar mechanism for the competitive effect of Tris and other amines on glycosidases, namely, that a prototropic group at (or nearby) the enzyme's active center transfers its proton to the amine base, resulting in formation of the charged form of the inhibitor which is the active species. In contrast, our mechanism proposes no charge transfer but simple formation of a hydrogen bond between the amine and the pK_0 group. This mechanism has the advantage of explaining quite simply how indistinctly the free base and the

protonated amine can behave as fully competitive inhibitors; which depends on whether the pK_0 group is protonated or not.

With regard to the mechanism proposed by us, the following calculations are worth mentioning here. Firstly, as concerns Tris and PM, it has been seen that their respective, apparent $K_{i(base)}$ values are identical, and both increase (affinity decreases) by the same factor, about 10-fold, as the pH increases from 7.6 to 9. Secondly, in sharp contrast, the apparent affinity of the protonated amines increases under the same conditions. In effect, for Tris and PM at pH 7.6 the $K_{i(cation)}$ parameters equal 0.59 and 18.26 mM but, as pH increases to 9, they drop by about the same factor to yield $K_{i(cation)}$ values of 0.2 and 7.6, respectively. Once more in contrast with the free bases (where, we have seen, one single OH is sufficient to achieve maximal inhibitory potency), for the protonated amines Tris-H^+ is 31- and 36-fold stronger than PM-H^+, respectively at pH 7.6 and 9.

In conclusion, the double attachment of Tris base to the sucrase active center, further stabilized or not by electrostatic interaction with the deprotonated groups in pK_1, appears to lock the enzyme into a rather stable conformation, resembling a transition state analog. However, more work is needed to determine what is peculiar to their structure that allows Tris and its analogs to behave in this manner.

Acknowledgements

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