

Effects of Tricine, Glycine and Tris Buffers on Alkaline Phosphatase Activity

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The effect of Tris, Glycine and Tricine on alkaline phosphatase activity was tested at a pH of 8.6, 8.85 and 9.1, using pNPP as substrate. Alkaline phosphatase activity was measured by monitoring the increase in NP absorbance at 420nm using a spectrophotometer. Alkaline phosphatase in Tris buffer at pH 9.1 was found to have the highest activity. Michaelis-Menten enzyme kinetic analysis revealed that the presence of Tris produced the highest V_{max} and K_m values for alkaline phosphatase at each pH tested, while the presence of Glycine produced the lowest. Also, increasing the pH was found to increase both the V_{max} and K_m .

Alkaline phosphatase is an important enzyme used in many scientific and clinical assays. Researchers are actively attempting to improve the assay system by methods such as locating heat stable versions in thermophilic bacteria (3,6). Greater understanding of its mechanism of action is therefore important both scientifically and commercially.

Alkaline phosphatase activity is dependent on a complex interaction of numerous factors, including: pH, concentration and purity of the enzyme, ionic environment, temperature and buffering solution. Previous studies suggest that at pH 9, conversion of p-nitrophenol phosphate (pNPP) to nitrophenol (NP) and phosphate by alkaline phosphatase is higher in a Tris-buffered solution than in a Glycine-buffered solution (4).

We hypothesized that this difference in alkaline phosphatase activity is a result of Glycine "screening" the substrate. Although Tris and Glycine both have an amine group as their buffering side chains (Fig. 1), at pH 9, the amine group of Tris is predominantly in the NH_2 form while the amine group of Glycine is predominantly in the NH_3^+ form. The positively charged amine group of Glycine could thus interact with the negatively charged phosphate group of pNPP, forming an ionic bond and preventing efficient recognition of pNPP by alkaline phosphatase. On the other hand, the neutral amine group of Tris would not form an ionic bond with pNPP, thus allowing for efficient recognition. The additional use of Tricine buffer in this study may help elucidate the buffers' mechanism of interaction with the enzyme or substrate as it contains the functional groups of both Tris and Glycine conjugated together (Fig. 1).

The rate of an enzyme has two major elements, both of which can be determined experimentally: the enzyme's affinity for its substrate (K_m) and the enzyme's theoretical maximal cleavage rate (V_{max}). This paper investigates the effect of Tris, Glycine, and Tricine buffers on alkaline phosphatase activity at pH 8.6, 8.85, and 9.1.

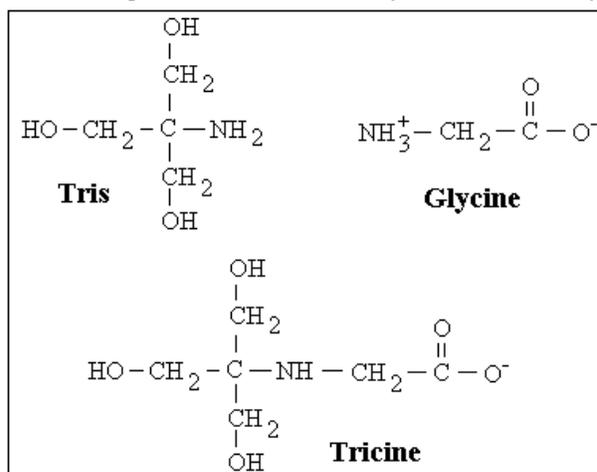
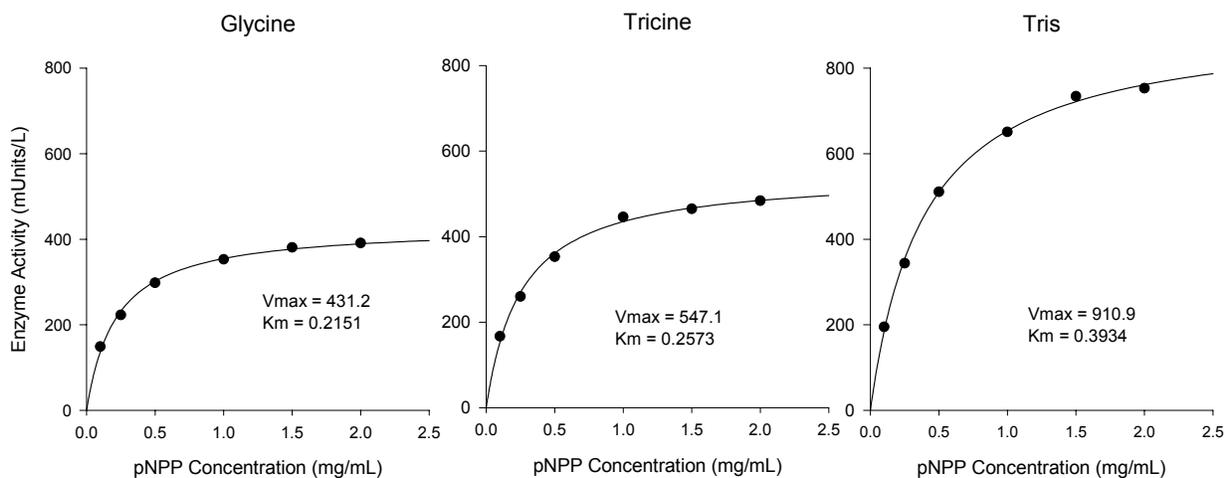


Figure 1. Structures of Tris, Glycine, and Tricine in the form found predominantly between pH 8.6 and 9.1.

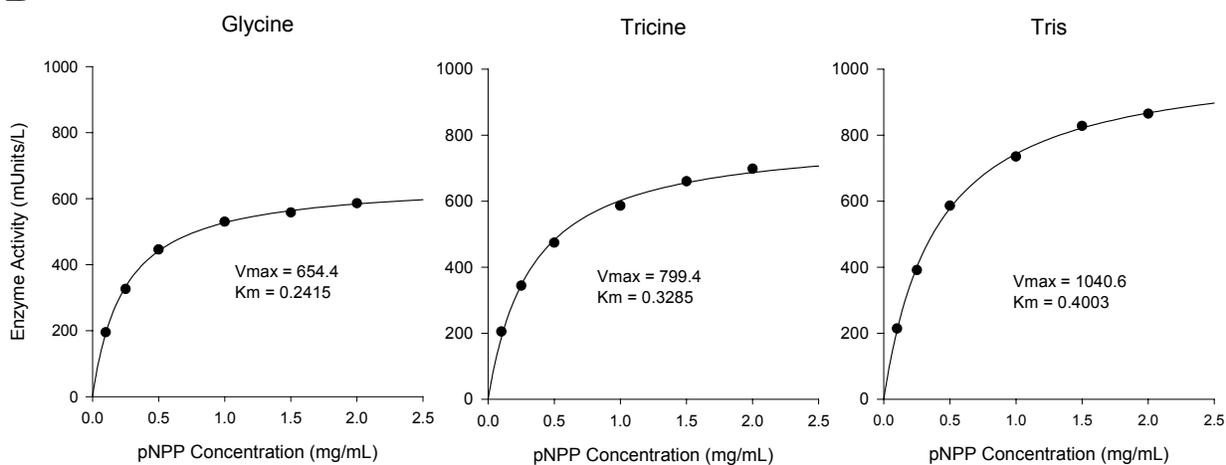
MATERIALS AND METHODS

Enzymatic Assays. The enzymatic assay used was conducted as previously described (4) with a number of modifications. Each 3.1 mL reaction contained 100 μ L alkaline phosphatase (2 mg/mL, Sigma) for a final concentration of 0.68 μ M, 500 μ L distilled water, 1.5 mL buffer, and 1.0 mL pNPP (Sigma) substrate at various concentrations (2.0, 1.5, 1.0, 0.5, 0.25, 0.10 and 0 mg/mL for a final concentration of 2.6, 1.9, 1.3, 0.6, 0.3, 0.1 and 0 mM respectively). 10 mM Glycine (BDH Chemicals), 50 mM Tricine (Sigma), and 500 mM Tris (Sigma) were used as buffers, each at pH 8.6, 8.85, and 9.1 and containing 2 mM $MgCl_2$ (Fisher). The concentrations selected for Glycine and Tris are those used in the previous study (4), while the concentration for Tricine was selected as a median value from a wide variety of studies as no precedent was available for this assay. Kinetic measurements of alkaline phosphatase cleavage were calculated from the enzyme's initial velocity as a function of the six different substrate concentrations with the help of Sigma Plot 2000 software, assuming Michaelis-Menten kinetics.

A



B



C

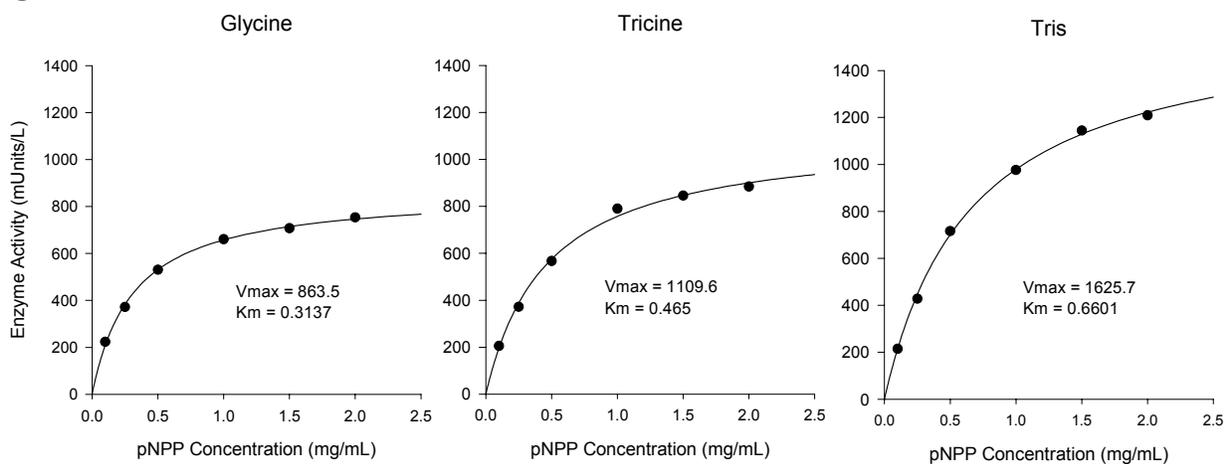


Figure 2. Michaelis-Menten Plots of Initial Velocity (V_o) Against pNPP Substrate Concentrations for Tricine, Glycine, and Tris Buffers. A) pH 8.6, B) pH 8.85, C) pH 9.1.

RESULTS

As expected from previous data (1,4,7), the activity of alkaline phosphatase was found to be greater in Tris buffer than in Glycine buffer. The enzyme activity in the third buffer, Tricine, was intermediate between Tris and Glycine. The results obtained were consistent at all three of the pH values tested. At all pH values tested, the data obtained fit the Michaelis-Menten curve very well (Fig. 2). The V_{max} of alkaline phosphatase was highest in Tris buffer, followed by Tricine buffer, and lowest in Glycine buffer (Figure 3). Surprisingly, the K_m of alkaline phosphatase was highest in Tris buffer, followed by Tricine buffer, and lowest in Glycine buffer (Fig. 4).

Changing the pH value appears to affect our results with the three different buffers in the same way. Both the V_{max} and K_m values for all three buffers increased with increasing pH.

DISCUSSION

Originally we hypothesized that the enzyme would have a similar V_{max} value in Tris and Glycine buffers, but the K_m in Tris buffer would be lower, accounting for the better activity. However, this is not the case. Observed V_{max} values do not remain constant but are higher in Tris than in Glycine. Furthermore, the K_m values are lower in Glycine than in Tris, which contradicts our prediction that the positive charge on the Glycine would interact with the negative charge on the pNPP and decrease the affinity of the enzyme for the substrate. Values for Tricine, the conjugated form of Tris and Glycine, are intermediate between Glycine and Tris (Fig. 3, Fig. 4). Therefore, the model proposed initially is incorrect and a new model will need to be developed in order to explain the observation that while affinity between substrate and enzyme is higher in Glycine than in Tris, the cleavage rate of pNPP by alkaline phosphatase is higher in Tris than in Glycine.

At the pH range used, only half or less of the amino groups on Glycine (pKa 9.6) molecules would be deprotonated (the other half would retain a positive charge), whereas most of the amino groups on the Tris (pKa 8.3) and Tricine (pKa 8.15) molecules would be deprotonated (and neutrally charged). If it was indeed this charge that interfered with the reaction, one would expect that the V_{max} and K_m data for Tris and Tricine to be almost identical, given their similar pKa and chemical structures (the amino group attached to Tris). However, Tricine consistently has a V_{max} and K_m lying in between those of Tris and Glycine. These results suggest there is something in the Glycine structure (shared by Tricine and Glycine) that increases K_m , perhaps due to the presence of the negatively charged carboxyl group while there is something in the Tris structure (shared by Tricine and Tris buffers) that increases V_{max} .

Tris is a nucleophile and has a lone pair of electrons on its sole nitrogen atom. With two hydrogen and only one carbon attached to this nitrogen (see Fig. 1), Tris may act as an effective nucleophile for the phosphate, which (after cleavage of pNPP) remains bound to the active site of alkaline phosphatase and must be removed in order for the enzyme to bind another molecule of substrate. In this scheme, one Tris molecule would covalently interact with enzyme-bound phosphate through nucleophilic attack on the phosphate-enzyme bond (5). This transphosphorylation from the substrate to Tris would accelerate phosphate's cleavage from the enzyme and clear the enzyme's active site

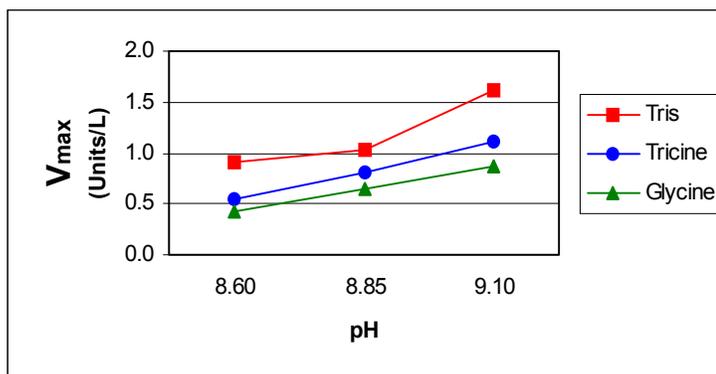


Figure 3. V_{max} values for the nine buffer/pH combinations tested.

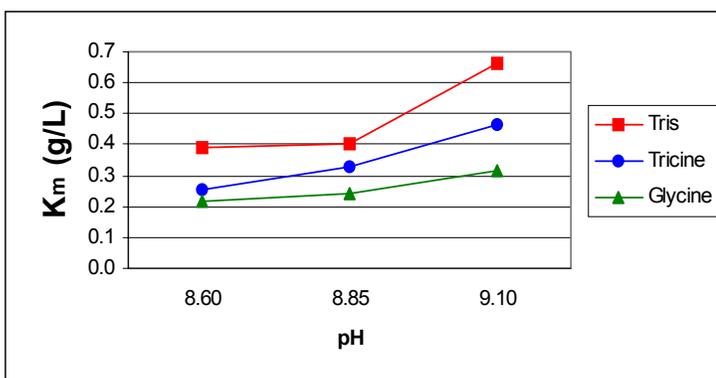


Figure 4. K_m values for the nine buffer/pH combinations tested.

so another substrate molecule could bind. This mechanism also suggests that after cleavage Tris would covalently bind the liberated phosphate, which presumably due to the cleavage between its phosphorus and oxygen, is now lacking an oxygen atom, is electrophilic and thus attracted to Tris's lone pair (5). This would remove some of the phosphate from the mixture and perhaps push the reaction even further in favour of cleaving more pNPP and result in a larger V_{max} .

Tricine also has a lone pair of electrons on its sole nitrogen and, like Tris, can be considered a nucleophile. However, alkaline phosphatase's activity was not as high in Tricine as in Tris. This may simply be because Tricine's nitrogen is bound to two carbon atoms and only one hydrogen. The extra carbon (and the atoms bound to it) in place of the hydrogen may not only decrease Tricine's nucleophilic strength by attracting the lone pair of electrons to itself more, but also sterically hinder Tricine's ability to act as effective of a nucleophile as Tris and thus be much poorer at removing the phosphate group from alkaline phosphatase's active site. Note too that Glycine has no nitrogen group and no lone pair of electrons. It cannot act as an acceptor nucleophile and thus could not remove phosphate from alkaline phosphatase and would not increase alkaline phosphatase's activity.

There are numerous sources of alkaline phosphatases; the one used in this study was isolated from calf intestine, the most common source. Activity of alkaline phosphatase isolated from calf intestine has previously been shown to be higher in Tris buffer than Glycine buffer (2). The results presented in this study agree with these previous results. Interestingly, alkaline phosphatase isolated from one thermophilic bacteria, *Bacillus stearothermophilus*, worked equally well in Tris and Glycine buffers (3), while alkaline phosphatase isolated from a second thermophile, *Thermus aquaticus*, had much higher activity in Tris buffer than in Glycine buffer (7). Yeh and Trela propose that the higher activity of *T. aquaticus* alkaline phosphatase in Tris is due to a putative transphosphorylation activity between Tris and substrate (7). It is possible then that the reason that the alkaline phosphatase of *B. stearothermophilus* gave similar results in both Tris and Glycine is because it is simply not capable of such a putative transphosphorylation activity in Tris. This inability may stem from its shape differing from that of the *T. aquaticus* alkaline phosphatase in such a way that it causes steric hindrance of any such activity in Tris. In any event, the buffers seem to have different effects, depending on the source of the enzyme, and a pure buffer-substrate mechanism can be ruled out.

Although the pH range over which we conducted the experiment is small, it is the only range where Tris and Glycine buffer are compatible and therefore comparable. Above pH 9.1 the buffering ability of Tris decreases rapidly, while below pH 8.6 the buffering capability of Glycine declines. These measurements can be complicated by the fact that pH can also affect nitrophenol colour (4). Figure 3 shows that over this small pH range, the activity of alkaline phosphatase does increase even though the colour of nitrophenol decreases (A. Flynn, personal communication). As A. Flynn reports that the molar extinction coefficient of nitrophenol only decreases around 10% over this pH range, the decrease in nitrophenol colour cannot account for the near two fold increase in enzyme activity from pH 8.60 to pH 9.10. The enzyme's activity, therefore, must actually increase as the pH increases in this small range. Regardless of these small changes, increasing activity of the enzyme is seen as the pH increases for all three buffers, so the increase in pH must directly affect the enzyme and/or substrate. This result is confirmed by information provided by the manufacturer (Boehringer Mannheim) stating that both V_{max} and K_m of the enzyme has been observed to increase as the pH increases.

The data collected here is insufficient to suggest a new model in place of the model that was tested and found to be incorrect. Either Tris or Glycine or both are interacting with the substrate and/or enzyme to yield the complex results found here. Future studies could involve varying the concentrations of the buffer to see whether or not changes in Tris molarity have a greater affect than changes in Glycine concentration on enzyme/substrate affinity and enzyme cleavage of substrate. For example, if alkaline phosphatase activity increases as Tris molarity increases but not as Glycine molarity increases, then that would provide stronger evidence for the Tris transphosphorylation model. The increased amount of Tris per volume would presumably lead to an increased rate of phosphate removal from the enzyme and thus an increased rate of enzyme activity while an increased amount of Glycine per volume would have no effect on enzyme activity since it is not capable of transphosphorylation.

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Appendix A

The following equation for Enzyme Activity was obtained from the *Microbiology 421 Manual of Experimental Microbiology* (4):

$$\text{Enzyme Activity (Units/L)} = (\Delta A / \Delta t) \times (10^3 / 20,000) \times (N_v / E_v)$$

Where:

- 1 unit = 1 μ mole of nitrophenol product formed per minute.
 ΔA = change in absorbance at a wavelength of 420 nm.
 Δt = time in minutes for the observed change in absorbance.
20,000 = the molar extinction coefficient of p-nitrophenol in Spectronic 20 tube (the absorbance caused by one molar solution of p-nitrophenol). It is derived by making a standard curve which relates the concentration of nitrophenol in molar units to the concentration in absorbance units and then using this standard curve to make predict the absorbance of a one molar solution.
 10^3 = a correction to change the extinction coefficient from liter per mole to milliliters per micromole.
 N_v = total assay volume (L) at the time of the absorbance reading.
 E_v = volume of enzyme (L) in assay.

For Glycine at pH 8.6 and pNPP = 1.0 mg/mL, $\Delta A = 0.0038$ and $\Delta t = 1$ sec.:

$$\begin{aligned} \text{Thus, Alkaline Phosphatase Activity} &= (.0038 / 1 \text{ sec}) \times (60 \text{ sec} / 1 \text{ min}) \times (10^3 / 20,000) \times \\ &\quad (3.1 \text{ mL} / 0.1 \text{ mL}) \\ &= 2.9 \text{ mUnits/L} \end{aligned}$$

By performing an inverse transformation of the Michaelis-Menten equation to generate the Lineweaver-Burk equation, the V_{\max} and K_m for each pH-buffer combination can be determined.

$$1/V_o = (K_m / V_{\max}) \times \{ (1 / \text{substrate concentration}) + (1 / V_{\max}) \}$$

So $1/V_{\max}$ = y-intercept and $-1/K_m$ = x-intercept of the graph.