Enzymes with Two Optimum pH Values

André Kimura Okamoto, Márcio André Miranda, José Mauro Granjeiro, Hiroshi Aoyama

Abstract— **Different chemical compounds were tested as buffers for the bovine milk acid phosphatase catalyzed reactions to elucidate the presence of an additional optimum pH value. Imidazole, histidine, alanine, glycine, malate, oxalate, succinate, citrate and acetate were studied as buffers, using p-nitrophenylphosphate or inorganic pyrophosphate as substrates. An optimum pH range between 4.5 and 6 was obtained for all the buffers studied. However, with glycine, and alanine, an additional pH optimum around 3 was observed. The specificity constant value (Vmax/Km), at pH 3, for glycine, was about twice the value obtained at pH 5 with acetate. The additional activity at pH 3 was also observed for other acid phosphatases. Our results suggest the existence of an inverse ratio between the negative character of the buffer and the reaction velocity.**

Index Terms— **acid phosphatase, bovine milk, buffers, pH.**

I. INTRODUCTION

pH is a highly useful parameter in chemical, biological and medical research as much as in laboratory or industry applications. For instance, wondering know more about dental diseases, it is of great importance to study the relationship of 24-h intraoral pH and temperature with circadian rhythm [1] . pH can affect the mechanism of infertility by decreasing sperm movement and capacitation [2]. The pH control of tumor acidity improves the efficacy of immunotherapy [3]. Determination of pH is of great importance in industrial research areas. High ethanol concentrations and low pH values are essential for the growth of strains in the wine production [4]. Structural and mechanical properties of latex particles can be supervied by controlling the pH [5]. In some cases highly sensitivity pH measurements are required. For these purposes pH sensors have always been developed [6,7].

The goal of this work is to present a little more about pH applications in chemical and biological research.

The best assay conditions for reactions catalyzed by enzymes have been described by several authors [8,9]. In this context, pH is a parameter that greatly affects the properties of the enzymes due to the sensitivity of their amino acid functional groups essential for a more adequate substrate binding and catalysis [10-12]. Usually, the enzyme activity must be determined at the pH optimum value, which is given by the maximum obtained from bell-shaped curve in the graph enzyme activity *versus* pH. This behavior depends on

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the state of protonation of amino acids groups involved in the catalysis and on the three-dimensional structure of the enzyme. At the pH optimum value, the active site of the enzyme is adequately prepared for the binding of the substrate; at a pH value far from the optimum, however, not only the enzyme structure could be altered but even also of the substrate, making it difficult the catalysis.

Another important study in the pH-related enzyme reactions is the pH of the enzyme stability. In this case, the experimental design is to pre-incubate the enzyme at different pH values, verifying the remained activity, using the optimum pH of the reaction.In general, the pH optimum of activity and of stability are very correlated values, as observed by Talley and Alexov [13].

Buffers, compounds that resist the addition of acids and alkalis, are used to adjust the desired pH value in an enzymatic reaction. Developing the equation of Henderson-Hasselbach the chosen pH value is desired to be within the range of pK $\pm$ 1. For instance, to prepare a phosphate buffer at pH 6, the $pK_2 = 7.2$ should be considered, with $[(HO)_2PO_2]^-$ and $[(HO)PO_3]^{2-}$, as the weak acid and the salt forms, respectively.

In general, the enzymes have good activities at physiological pH values, around 7.0. However, there are several exceptions, e.g., pepsin with an optimum pH value of 2.0 and trypsin, at pH 8.0.

Despite the same chemical reaction used in the catalysis for one enzyme, the optimum pH value can be different depending on several factors, including origin of the enzyme [14], presence of materials [15], enzyme immobilization [16], and enzyme mutation [17].

Phosphatases, enzymes that catalyze the hydrolysis of orthophosphate monoesters, can be classified as acid, alkaline and protein phosphatases [18,19]. Acid phosphatases (E.C. 3.1.3.2) have been found largely distributed in vertebrates [20,21], plants [22,23] and microorganisms [24,25]. Some tissues contain more than one type of acid phosphatase differing in molecular mass, localization in the cells, substrate specificity and sensitivity to inhibitors [26,27]. Acid phosphatases are poorly specific enzymes, recognizing a broad kind of orthophosphate monoesters as substrates [22, 23, 28]. Mammal acid phosphatases can be classified according to their relative molecular mass (Mr) as high Mr (above 100 kDa), intermediate Mr (20 to 100 kDa), and low Mr (lower than 20 kDa), and are respectively inhibited by tartrate, fluoride and *p*-chloromercuribenzoate [20].

The optimum pH of the reactions catalyzed by acid phosphatases is around 5 and sodium acetate is generally used as buffer. However, it has been reported that some acid phosphatases could hydrolyze phosphomonoesters at an optimum pH of 2.5-3.0 and not at pH 5.0. In this context, *Escherichia coli* acid phosphatase hydrolyzed the synthetic substrate *p*-nitrophenylphosphate (*p*-NPP) with an optimum

pH of 2.5, using glycine as buffer, and an optimum pH of 3.0, using phtalate as buffer [29]. Recombinant acid phosphatases of *Aspergillus niger* [30] and *Escherichia coli* [31] hydrolyzed *p*-NPP and sodium phytate at pH 2.5.

We have previously observed in our laboratory significant activities at pH 3.0 for acid phosphatase purified from bovine kidney, using glycine as buffer. In this work we described the existence of this additional optimum pH around 3.0, using glycine as buffer, besides pH 5.0, with acetate buffer, for bovine milk acid phosphatase. In order to determine wether the enzyme activity at pH 3.0 could be due to an activating effect of glycine or due to the chemical nature of the buffer, we performed a more detailed pH study, with *p*-NPP and inorganic pyrophosphate as substrates, and different chemical compounds as buffers. The implications on kinetic parameters of the reactions were also discussed.

II. MATERIAL AND METHODS

A. Enzymes

The bovine milk intermediate molecular weight (42.000) acid phosphatase was purified in our laboratory to homogeneity, about 22,700-fold with a specific activity of 19.2 Units/mg of protein. All other acid phosphatases used in this work were also purified in our laboratory from shark liver (intermediate molecular weight), bovine lung (low molecular weight) and guinea pig (low and high molecular weight enzymes).

All the chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

B. Enzyme assay

The enzyme activity, using *p*-NPP as substrate, was performed as previously described [32]. The reaction mixture consisted of 20 mM of different compounds as buffer, 5 mM *p*-NPP and 0.35 μg/ml of enzyme in a volume of 1 ml. After 10 min incubation at 37°C, the reaction was stopped by the addition of 1 ml of 1 M NaOH. Acid phosphatase activity was measured at 405 nm by monitoring the release of *p*-nitrophenol (molar extinction coefficient of 18,300 $M^{-1}cm^{-1}$).

When inorganic pyrophosphate (PPi) was used as substrate, the enzyme activity was assayed by measuring the amount of phosphate released [33]. The assay conditions were the same as described for *p*-NPP, excepting for inorganic phosphate determination as product. The reactions were terminated by the addition of 1 mL 3% (w/v) ammonium molybdate (in 200 mM acetate buffer, pH 4.0), followed by addition of 0.1 ml of 1% ascorbic acid (in 200 mM acetate buffer, pH 4.0). The color was developed for 30 min and the absorbance was read at 700 nm. The amount of inorganic phosphate produced was calculated using a molar extinction coefficient of 4000 M^{-1} cm^{-1} at 700 nm.

One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per minute.

III. RESULTS AND DISCUSSION

pH is an environmental parameter that affects the properties of all proteins and enzymes. Van Etten [34] and Van Etten and McTigue [35] have observed, for human prostatic acid phosphatase, that the parameters Km and Vmax must be determined at various pH values, taking into account the ionization of the substrates. These authors observed that the "typical" bell-shaped pH-rate profile with a maximum at pH 5 was mainly the result of increased Km values associated with substrate ionization. Behzadi *et al*. [36] reported an inverse relationship between the storage pH of alkaline phosphatase and its pH-optimum. For storage pH values larger than 8.3, they observed "bell-shaped" pH-profiles with a pH optimum that decreased with increasing storage pH. Leone *et al*. [37] have shown that pH might affect the modulation of rat osseous plate alkaline phosphatase by metal ions. Modification in the growth medium pH induced variation in the phosphorylation status of several proteins in *Leishmania pifanoi*, as a consequence of activation of both tyrosine kinases and phosphatases [38].

While working on the purification and characterization of acid phosphatases which pH optimum is around 5, we observed, in some cases, an additional pH optimum of 2.5-3.0 with glycine as buffer. In order to contribute to a better understanding of this activity at pH 3.0, we tested different compounds as buffers, in the reactions catalyzed by bovine milk acid phosphatase, using as substrates *p*-nitrophenyl phosphate and inorganic pyrophosphate.

A. Acetate, citrate, glycine and alanine as buffers

Figure 1 shows that, independently of the substrate, the pH optima for acetate and citrate used as buffers were around 5. Acetate and *p*-NPP are, respectively, the buffer and the substrate most commonly used in the reactions catalyzed by acid phosphatases. Besides acetate, citrate can also be used as buffer at optimum pH values around 5.0-5.5 [31]. These compounds were used by Andrews and Pallavicini [39] that obtained a single pH optimum peak for bovine milk acid phosphatase.

Glycine and alanine promoted high enzyme activities around pH 3 with *p*-NPP as substrate, however, no significative activity was observed with PPi (Fig. 1). These results with *p*-NPP and PPi correlate well with the findings of Taga and Van Etten [40] where human liver acid phosphatase could preferentially act on some aryl rather than aliphatic phosphomonoesters. The amino acid glycine can be used as buffer in the pH values around its pK of 2.3 (carboxilic acid group) and 9.6 (amino group). This buffer was used to maintain the optimum pH of 2.5 in the reactions catalyzed by several acid phosphatases [29-31]. However, for these enzymes only a single pH optimum peak has been obtained, in contrast to our results with the bovine milk acid phosphatase displaying two optimum pH values: 5.0 (acetate buffer) and 3.0 (glycine buffer) (Fig. 1). Optimum pH values of 2.5 and 5.5 were detected for a phytase expressed in *Aspergillus niger*, however no pertinent discussion was performed [31].

B. Histidine and glutamine as buffers

The *p*-NPP-directed acid phosphatase reactions using histidine and glutamine as buffers showed a maximum value of activity at pH 5.0-6.0; no activity was obtained at pH 3.0 (Fig. 2). With PPi as substrate and glutamine as buffer, lower

values of activity were obtained around pH 3, with no activity at pH 5.0; under the same conditions with histidine as buffer, the bovine milk enzyme showed a broader range of pH optimum, including the values of 3 and 5. Imidazole, a compound that has the same heterocyclic ring as histidine and frequently used as buffers, showed the same pattern of pH optimum as obtained for histidine (not shown). Our data suggest that depending on the amino acids used as buffers, there is only one optimum pH value around 5.0.

C. *Succinate, malate and oxalate as buffers*
Using succinate, malate and oxalate as buffers and *p*-NPP as substrate, optimum pH values were obtained only in the range 5.0-6.0 (Fig. 3). With PPi as substrate, a shift of optimum pH to more acidic values were obtained, around 3.0-4.0; at pH 5.0 the activity was reduced. As observed for the amino acids histidine and glutamine, no activities were detected at pH 3 using the dicarboxilic acids succinic, malic and oxalic as buffers, and *p*-NPP as substrate.For this class of compounds, depending on the substrate used, PPi for instance, only one peak could be observed at acidic pH values (below pH 4).

D. *Activity at pH 3 with glycine buffer*
The enzyme activity at pH 3 with glycine buffer could be ascribed to some factors including activation and increase in the affinity of the enzyme by the substrate in the presence of this amino acid. However, no acid phosphatase activation was observed by increasing glycine concentration (not shown). On the other hand, the dipeptide of glycine, glycyl-glycine, activated two isoforms of avian pectoral muscle acid phosphatase [41].

E. *Effect of buffers on the specificity constants*
Using *p*-NPP as substrate, specificity constant (Vmx/Km) values were determined at different buffers in order to dertenine the affinity of the enzyme by the substrate (Table 1). Considering the pH range of 5.0-6.0, acetate was the best buffer, promoting the highest value of specificity constant; under the same conditions, citrate buffer was the less effective. Interestingly, with glycine as buffer at pH 3.0, the specificity constant value was about 2-fold higher than the obtained with acetate buffer at pH 5.0. Our results suggest that the significative activity with glycine buffer at pH 3.0 can be explained by the increase in the specificity of bovine milk acid phosphatase by the substrate *p*-NPP, when compared with the activity at pH 5.0, with acetate as buffer. . Considering the fact that the Vmax values did not significantly alter for the buffers tested, the enzyme-substrate affinity, through the Km values, acquired great importance, which explains the sharp difference between the specificity constant values obtained for glycine and citrate. Revaluating the data described by Van Etten and McTigue [35], for human prostatic acid phosphatase, we calculated the specificity constants for *p*-NPP, which value at pH 3 was 1.37-fold higher than that determined at pH 5.0. The Km value (0.27 mM) obtained for *p*-NPP at pH 5.0 (Table 1) was 3-fold lower than that described by Andrews and Pallavicini [39] for bovine milk acid phosphatase, at pH 4.8 (0.81 mM). The Km and Vmax values are highly dependent on the

ionization of the substrates. In this context, *p*-NPP, which is not a "physiological substrate", is an unusual acidic substrate because of the electron-withdrawing effect of the *p*-nitro group [34].

1. *F. Activity at pH 3 for other acid phosphatases*
Other acid phosphatases, purified in our laboratory were tested in relation to the additional activity at pH 3.0 (Fig. 4). In this context, acid phosphatases purified from shark liver (intermediate molecular weight), bovine lung (low molecular weight), and guinea pig (low and high molecular weights) have shown considerable activities at both pH values, around 3 (glycine buffer) and 5 (acetate buffer). These results suggest that the optimum pH of 3.0 with glycine buffer was independent of the molecular weight of mammal acid phosphatases. On the other hand, plant acid phosphatases did not show this activity at pH 3.0 with glycine as buffer [22,23].

G. *Mechanisms of action for the activities at pH 3.0 and 5.0*
The enzyme activity at pH 3.0 was restricted to only some compounds such as glycine and alanine, but not for acetate, dicarboxilic acids, citrate, histidine and lysine. This optimum pH was also observed for different substrates (with *p*-nitrophenyl phosphate as more efficient substrate than inorganic pyrophosphate) and for different acid phosphatases (bovine milk and lung, shark liver and guinea pig). When compared with enzyme activity at pH 5.0, the higher specificity constant value with glycine at pH 3.0 suggests that the presence of this amino acid promoted a more favorable condition for the interaction between enzyme and the substrate *p*-NPP. However, the difference in enzyme affinity at pH 3 and 5 was not sufficient to change the effect of some compounds, including known acid phosphatases inhibitors (not shown). In relation to the enzyme-substrate interactions, Kostrewa *et al*. [42] showed through crystal structure of *Aspergillus niger* acid phosphatase that the singly highly acidic pH optimum of 2.5 could be explained by the charge distribution at the substrate specificity site. These authors also observed that a less constraints on the substrate's charge distribution allowed for a wide variety of phosphomonoesters as substrates.

In face of the highest enzyme activity in the presence of acetate as buffer and taking into account the pH of the reaction mixture and the pK of the chemical compounds used as buffer, we can suggest the existence of an inverse relationship between the negative character of the buffer and the rate of the enzymatic reaction.

IV. CONCLUSION

Independently of the buffers used, an optimum pH range between 4.5 and 6 was obtained for bovine milk acid phosphatase. However, with glycine and alanine as buffers, an additional pH optimum around 3 was observed.
At pH 3 the efficience of catalysis was higher than at pH 5, since the specificity constant (Vmax/Km) value at pH 3, with glycine, was about twice the value obtained at pH 5, with acetate.

No significant differences were observed at pH 3 and 5 in the presence of known acid phosphatase inhibitors, suggesting that the action of these compounds was pH-independent. The additional optimum pH value of 3 observed for other acid phosphatases could be due to the similarity of active sites. This fact correlates well with a possible existence of an inverse relation between the negative character of the buffer and the reaction velocity.

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Table 1: Kinetic parameters for bovine milk acid phosphatase[a].

Buffer	pH			
Glycine	3.0	0.10±0.01	9.04±0.58	90.4
Histidine	5.5	0.64±0.06	16.38±0.49	25.59
Citrate	6.0	2.25±0.36	15.07±0.90	6.69
Succinate	6.0	0.48±0.05	15.30±0.54	31.87
Acetate	5.0	0.27±0.02	13.32±0.31	49.33

[a] Reaction conditions: *p*-NPP as substrate, 20 mM of different compounds as buffer at pH values where the activities were maxima.

[b] Km and Vmax values were calculated from Lineweaver-Burk double-reciprocal plots, and represent the means (±standard error) of triplicate determinations.
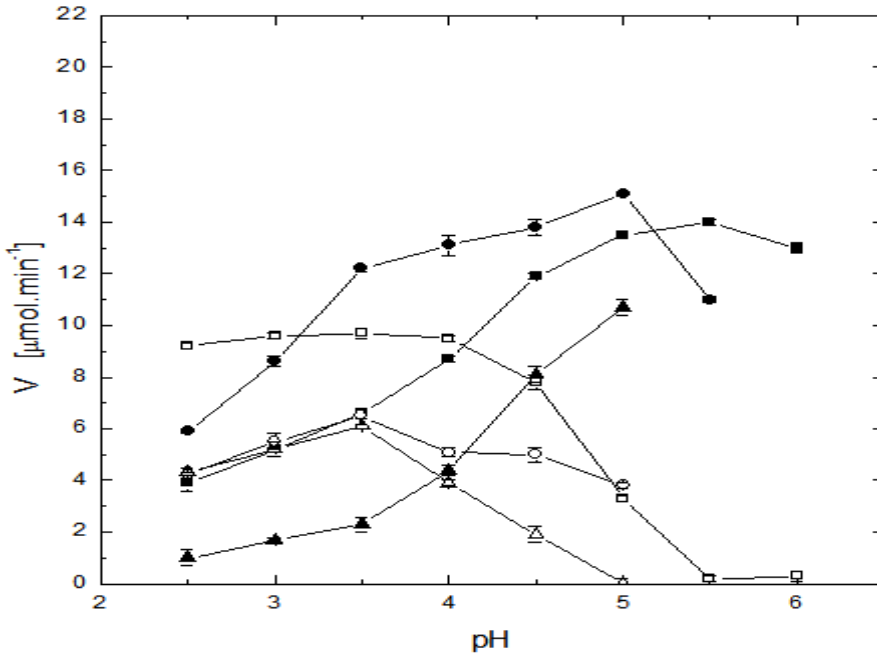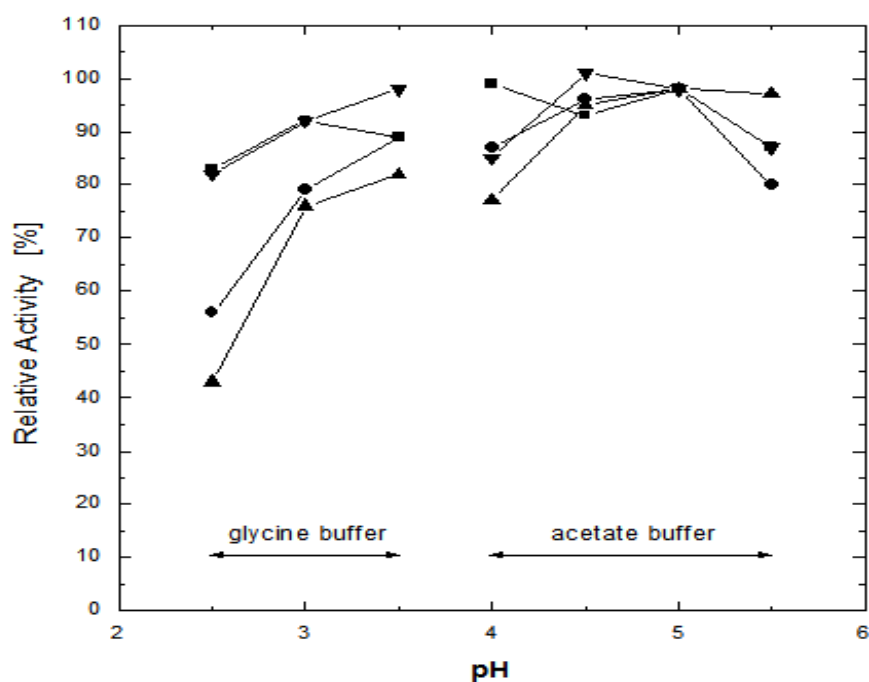
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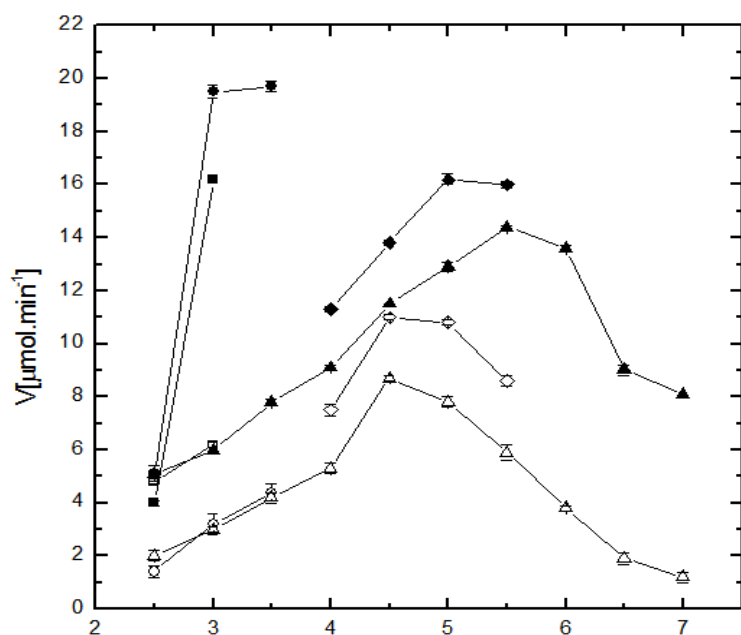
Fig. 1: pH dependence for the bovine milk acid phosphatase with acetate, citrate, glycine and alanine as buffers. The assay conditions were the same as described in Methods in the presence of 20 mM acetate (◊), citrate (Δ), glycine (□), or alanine (o) as buffer and 5 mM *p*-NPP (closed symbols) or PPi (open symbols) as substrate. Each point represents the mean (± standard error) of triplicate determinations.
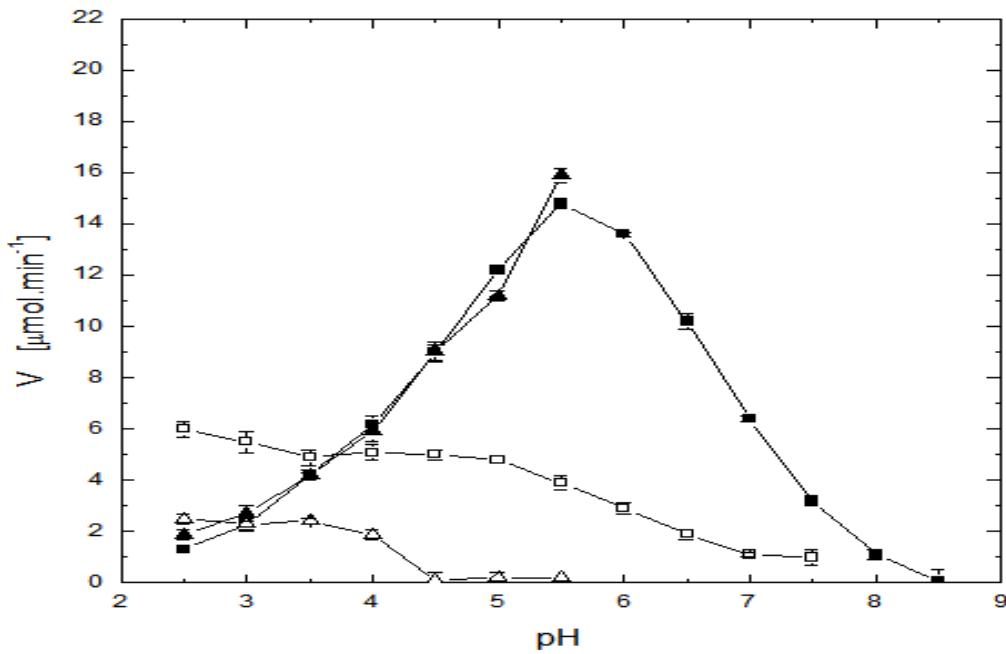
Fig. 2: pH dependence for the bovine milk acid phosphatase with histidine and glutamine as buffers. The assay conditions were the same as described in Methods in the presence of 20 mM hisitidine (□) or glutamine (Δ) as buffer and 5 mM *p*-NPP (closed symbols) or PPi (open symbols) as substrate. Each point represents the mean (± standard error) of triplicate determinations.

Fig. 3: pH dependence for the bovine milk acid phosphatase with malate, oxaloacetate and succinate as buffers. The assay conditions were the same as described in Methods in the presence of 20 mM malate (□), oxalate (Δ) or succinate (o) as buffer and 5 mM *p*-NPP (closed symbols) or PPi (open symbols) as substrate. Each point represents the mean (± standard error) of triplicate determinations

Fig. 4: pH dependence for different acid phosphatase with acetate and glycine as buffers.The assay conditions were the same as described in Methods with *p*-NPP as substrate in the presence of 20 mM glycine pH 3.0 or acetate pH 5.0 and the following acid phosphatases: shark liver intermediate molecular weight (■), bovine lung low molecular weight (●), guinea pig low molecular weight (▲) and guinea pig high molecular weight (▼). Each point represents the mean (± standard error) of triplicate determinations