On the Nature of the Inhibitor of Urinary Alkaline Phosphatase

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The inhibition of urinary alkaline phosphatase has been shown to be caused principally by the inorganic phosphates which are naturally excreted in urine. An inorganic phosphate concentration of 15 mg./100 ml. resulted in approximately 50% inhibition, and that of 120 mg./100 ml. in an inhibition of about 90%. Evidence is presented which shows that the inhibition is of the competitive type.

The determination of alkaline phosphatase activity in human urine has recently been shown to serve as a valuable index of the presence of renal parenchymal disease (1, 2). Accurate assay of this enzyme in urine depends on the removal of a naturally occurring dialyzable inhibitor (1). In the present report the nature of this inhibitor and the mechanism of its action are elucidated.

Materials and Methods

Preparation of the Urine

Urine specimens collected at random from normal individuals were immediately centrifuged at 2000 rpm for 5 min., and the supernatant was then brought to a boil, thus destroying all alkaline phosphatase activity without inactivating the inhibitor (2). The boiled urine was then divided into 2 aliquots, one of which was used undialyzed and the other used after being dialyzed against running tap water for 2 hr. for removal of inorganic phosphate.

Unboiled urine was processed by collecting overnight specimens from normal individuals, centrifuging, and dialyzing it according to the procedure of Amador et al. (1).

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The alkaline phosphatase used in this report was Type III bacterial (E. coli) obtained from the Sigma Chemical Company, St. Louis, Mo.
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Inorganic Phosphate Determinations

All specimens were assayed for inorganic phosphate by the methods of Fiske and SubbaRow (3).

Assay of Alkaline Phosphatase Activity

The alkaline phosphatase activity was measured by a modification of the procedure described by Amador et al. (1) when boiled urine was used. Into a cuvet were added 1 ml. of 1.5 M TRIS (Tris[hydroxymethyl]aminomethane) buffer (pH 10.3), 1 ml. of 9 mM p-nitrophenyl phosphate, and 1 ml. of the specimen. This mixture was incubated for 30 min. in a water bath at 37°C, then 10 ml. of 0.02 N sodium hydroxide was added and the absorbance read in a spectrophotometer at 410 m.μ. The activity was expressed as μM substrate hydrolyzed per mg. of enzyme per 30 min. When unboiled urine was used, the alkaline phosphatase activity was assayed by the spectrophotometric procedure given by Amador et al. (1).

Experimental Procedures and Results

Inasmuch as inorganic phosphates have been shown to inhibit the assay of purified alkaline phosphatase in aqueous solutions (4) and inorganic phosphate is normally excreted in urine (70–835 mg./day) (5), a similar inhibition was postulated for the urinary enzyme. This hypothesis was tested by the following experimental procedures.

Inhibitory Effect of Increasing Concentration of Inorganic Phosphate on Alkaline Phosphatase Activity

Four boiled urine specimens were collected and prepared in the manner described above. The inorganic phosphate level in each of these specimens was determined, and corresponding concentrations of sodium phosphate in aqueous solutions were prepared. Both the aqueous solutions and urines were then serially diluted with water, resulting in phosphate levels of 1.9–130 mg./100 ml., and to equal aliquots of these diluted solutions was added 0.6 μg. of commercially obtained, purified E. coli alkaline phosphatase.

All specimens were assayed for inorganic phosphate and alkaline phosphatase activity by the procedures described above. Figure 1, typical of all 4 studies, is a composite representation of 2 such experiments. As can be seen, the inhibition of alkaline phosphatase activity by added sodium phosphate in the aqueous solutions was comparable to that noted in urine. An inorganic phosphate concentration of 15 mg./100 ml. resulted in 50% inhibition, and that of 120 mg./100 ml. in about 90% inhibition of the original alkaline phosphatase activity. In another series of experiments,
boiled urine, having been first dialyzed for the removal of inorganic phosphate, was used. In these studies, 0.6 µg. of alkaline phosphatase and graded amounts of inorganic phosphate were subsequently added to the dialyzed urine, and inhibition of the enzyme comparable to that in the undialyzed specimens was again demonstrated (Fig. 1). That the inhibition of the alkaline phosphatase activity was not due to the high concentration of sodium was shown by 150 mg./100 ml. of sodium chloride having no inhibitory effect.

**Effect of Endogenous and Exogenous Phosphate on Urinary Alkaline Phosphatase**

Three unboiled urine specimens, collected and processed as described above were each divided into 2 aliquots, one of which was dialyzed against running tap water. The phosphate and alkaline phosphatase concentrations were determined in each specimen both before and after dialysis. As shown in Table 1, dialysis removed the urinary phosphate and resulted in enzyme levels which were substantially higher than those demonstrated in the undialyzed urine. The degree of alkaline phosphatase inhibition correlated well with the quantity of inorganic phosphate present in the initial urine specimen. When amounts of phosphate com-

![Fig. 1. Inhibitory effect of increasing concentration of inorganic phosphate on alkaline phosphatase activity. Alkaline phosphatase activity was assayed, as described in text, in 3 different solutions containing increasing concentrations of inorganic phosphate and 0.6 µg. of added alkaline phosphatase. Percent inhibition, calculated from relative activities of the enzyme in solutions containing phosphate as compared with that without phosphate, was plotted against inorganic phosphate concentrations.](image-url)
parable to those present in the undialyzed urine were added to the dialyzed specimens, the inhibition of the enzyme observed in the undialyzed urine was reproduced (Table 1).

Table 1. Effect of Endogenous and Exogenous Phosphate on Urinary Alkaline Phosphatase Activity

<table>
<thead>
<tr>
<th>Urine spec. No.</th>
<th>Dialyzed urine</th>
<th>Undialyzed urine</th>
<th>Dialyzed urine + added phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic phosphate (mg./100 ml.)</td>
<td>Alkaline phosphatase (U.)</td>
<td>Inorganic phosphate (mg./100 ml.)</td>
<td>Alkaline phosphatase (U.)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12.3</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>13.8</td>
<td>62.5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>7.4</td>
<td>96.5</td>
</tr>
</tbody>
</table>

*One unit is equal to a change in absorbance of 0.001 per ml. of urine per min.
†Per cent inhibition refers to units of alkaline phosphatase in undialyzed urine and in dialyzed urine with added phosphate, as compared with the enzyme activity in the dialyzed specimens, containing no phosphate.

Inhibition of Serum Alkaline Phosphatase

Using the Bessey-Lowry-Brock (BLB) procedure (6), serum alkaline phosphatase activity was also found to vary with the phosphate concentration. Since, however, the inorganic phosphate concentration of normal serum is only 3–4.7 mg./100 ml., inhibition of the serum enzyme is correspondingly slight. In a specimen initially containing 4.1 mg./100 ml. phosphate and 4.2 BLB units of alkaline phosphatase, dialysis of the serum with the removal of essentially all phosphate, resulted in a 13.8% increase in enzyme activity.

![Fig. 2. Lineweaver-Burk plot of inhibition of alkaline phosphatase activity by inorganic phosphate. All tubes contained 0.6 μg. alkaline phosphatase, given concentrations of p-nitrophenyl phosphate, and 0.5 M TRIS buffer (pH 10.3). Reaction mixtures were incubated for 30 min. at 37°. Velocity of reactions was measured as μM of substrate hydrolyzed per mg. of enzyme per 30 min. Reciprocal of velocities is plotted against reciprocal of substrate concentrations.](image-url)
Lineweaver and Burk Plot of the Inhibition of Alkaline Phosphatase Activity by Inorganic Phosphate

Variation of the substrate (p-nitrophenyl phosphate) concentration and the plot of its reciprocal versus the reciprocal of the alkaline phosphatase activity, the method of Lineweaver and Burk (7), is shown in Fig. 2. These experimental data are consistent with competitive type of inhibition. In this type of inhibition, which is reversible, the phosphate inhibitor of alkaline phosphatase activity and the substrate are both believed to attach to the same site on the enzyme (8).

Discussion

The results presented in this study demonstrate that inorganic phosphate, naturally excreted in urine, is an inhibitor of urinary alkaline phosphatase. In addition, evidence is presented that the inhibition is of a reversible and competitive type. It is probable that other naturally occurring substances in urine and serum will also be found to participate in inhibition of the enzyme. Indeed, citrate has recently been shown to inhibit alkaline phosphatase in serum (9). This investigation, however, indicates that phosphate is the major, if not the only, significant inhibitor of the enzyme in urine.

These findings are of clinical interest for two reasons: (1) the elimination of phosphate from urine by dialysis, as suggested elsewhere (1), is essential for accurate measurement of the urinary enzyme in human renal disorders; (2) the presence of a wide variation in serum inorganic phosphorus in certain disease states (such as uremia or renal tubular disease) may result in inaccurate measurement of alkaline phosphatase activity actually present in serum.

References