ALKALINE PHOSPHATASE ACTIVITY AND pH OPTIMA

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High pH values have been found necessary for optimum activity of alkaline phosphatase in vitro. The pH values in the living cell structures, on the other hand, are near neutrality: cytoplasm 6.9, nucleus 7.6 (1-3). This definite difference between the pH required for optimum activity and the pH of the living cell indicates that alkaline phosphatase is not optimally active in vivo. However, the pH required for optimum activity in vivo may not be the same as that in vitro.

The pH optimum for alkaline phosphatase activity has been shown to vary with the substrate concentration (4, 5), the type of substrate (6), and the buffer employed (7, 8). A pH optimum less than 8.0, however, had never been reported for alkaline phosphatase.

The possibility of lowering the pH optimum to physiological values by lowering the substrate concentration was investigated. As the concentrations of the substrates were lowered, the pH optima for alkaline phosphatase activity were found to be nearer neutral pH values.

EXPERIMENTAL

Normal male rats of the Wistar strain, each weighing 175 to 200 gm., were used. The small intestine was removed immediately after decapitation, freed of pancreatic and mesenteric remnants, opened longitudinally to expose the mucosa, washed in cold tap water, and macerated separately. After addition of acetone and further maceration, the muscularis was removed, the mucosa centrifuged, and the supernatant acetone discarded. The mucosa was washed four more times with 40 ml. of acetone and air-dried at room temperature. A fine white powder was obtained which averaged 650 mg. per intestine.

A suspension of 1.2 gm. of the powder in 50 ml. of distilled water was refrigerated at 4° for 30 minutes and centrifuged. The supernatant extract, filtered and diluted to 500 ml. with distilled water, contained large quantities of inorganic and hydrolyzable organic phosphate contaminants (Table I) which could not be completely removed by dialysis and which interfered in enzyme determinations. Solid MgSO₄ and sodium veronal were therefore added to the intestinal mucosa filtrate to final concentrations of 0.01 and 1/30 M, respectively, and the pH was adjusted to 7.8 with 1.2 N HCl. The solution was incubated at 37° for 2 hours, placed
in viscose tubing, and dialyzed for 60 hours at 4° against frequently changed distilled water to which a small amount of toluene was added. No hydrolyzable organic phosphates were then present.

56 buffered substrates were prepared by adding 50 ml. portions of each of seven sodium β-glycerophosphate (C₃H₇O₅PO₃Na₂·5H₂O) solutions to 50 ml. portions of eight buffers, M/15 sodium veronal, adjusted with HCl to values ranging from pH 6.5 to 9.5. Each of the acid volumes required for adjusting the pH was augmented with water to give a total volume equal to that of the acid required for the lowest pH.

Adenylic acid and ribonucleic acid were neutralized with NaOH before the addition of the buffer.

1 ml. of MgSO₄ (0.1 M) and 7 ml. of the buffered substrate were introduced into test-tubes and placed in a constant temperature bath at 37° for 10 minutes. 2 ml. of the enzyme solution were then added and the tubes sealed with Parafilm. Duplicate tubes for pH determinations were prepared from the same solutions and with the same pipettes for every phosphatase determination. Enzyme activity determinations for each substrate were made within 1 day with the same sample of enzyme.

If the concentration of the hydrolyzed substrate was greater than 1 mg. per ml. and if the solution contained 1 γ or more of inorganic phosphorus per ml., 2 ml. of 50 per cent trichloroacetic acid were added to the enzyme-buffer-magnesium-substrate mixture and to the blanks to inactivate the enzyme. The inorganic phosphorus content in a 5 ml. aliquot portion of the mixture was determined by the method of King (10).

If the concentration of the hydrolyzed substrate was less than 1 mg. per ml. and if the solution contained less than 2 γ of inorganic phosphorus per ml., 10 ml. of 0.02 M sodium molybdate in 1.2 N sulfuric acid were added to inactivate the enzyme. To the entire sulfuric acid-molybdate-enzyme-substrate mixture, 0.1 ml. of 0.2 N stannous chloride in concentrated HCl was added according to Norberg’s modification (11) of the technique of Denigès, by means of a Phipps-Bird dispenser. Because the

### Table 1

**Extract of Intestinal Mucosa**

<table>
<thead>
<tr>
<th></th>
<th>Phosphatase activity*</th>
<th>Organic P content γ per ml.</th>
<th>Inorganic P content γ per ml.</th>
<th>Nitrogen content γ per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation and dialysis .......</td>
<td>18.4</td>
<td>17.0</td>
<td>32.5</td>
<td>99.0</td>
</tr>
<tr>
<td>After &quot; &quot; &quot; &quot; .........</td>
<td>12.3</td>
<td>0.48</td>
<td>0.8</td>
<td>7.79</td>
</tr>
</tbody>
</table>

* Expressed as micrograms of phosphorus per ml. liberated at pH 9.1 and 37° in 1 hour from sodium β-glycerophosphate (10 mg. per ml.).
† Determined according to the method of Ma and Zuazaga (9).
SnCl₂ deteriorated rapidly, it was added as quickly as possible and the pipette was rinsed each time with fresh reagent.

When barium fructose-6-phosphate in high concentration was used as the substrate, the precipitate (probably BaSO₄) was removed by filtration after the addition of the sulfuric acid.

When ribonucleic acid in high concentration was used as the substrate, precipitation occurred upon the addition of trichloroacetic acid (unused ribonucleic acid) and ammonium molybdate (probably products from ribonucleic acid resulting from activity of contaminating enzymes). 2 ml. of 2 per cent uranyl acetate in 50 per cent trichloroacetic acid solution were therefore added to the hydrolyzed substrate and to the blanks. The precipitate was removed by filtration. 1 ml. of the filtrate was diluted to 10 ml. with distilled water and then 10 ml. of H₂SO₄-Na₂MoO₄ reagent and 0.1 ml. of SnCl₂ were introduced.

Substrate and enzyme blanks were made by the method appropriate to the concentration of the substrate. Reagent blanks and standard phosphorus samples were included with every series. Color was permitted to develop for 30 minutes and was measured with the Klett-Summerson photoelectric colorimeter equipped with a red filter No. 66.

**Results**

At pH 9.1 at different initial glycerophosphate concentrations (10 mg. per ml. to 3 γ per ml.), various amounts of phosphate were liberated by alkaline phosphatase activity in 1 hour at 37°. Much smaller amounts, however, were found at concentrations below 5 mg. per ml. (Fig. 1). At this pH, at a concentration of 10 mg. per ml., the rate of hydrolysis during
the 1st hour was the same as the initial rate, \(4.33 \times 10^{-3}\) \(\gamma\) of phosphorus per second per ml. The value of the Michaelis constant, \(K_m\), calculated on the assumption that the velocity of the reaction at the end of 1 hour is proportional to the initial velocity, was found to be 0.0022.

Optimum activity was found to occur at pH 9.13 when the enzyme was incubated with 10 mg. per ml. of glycerophosphate at different pH values for 1 hour (Fig. 2). At lower glycerophosphate concentrations, however, the optimum activity was less and occurred at a lower pH value. At a substrate concentration of 5000 \(\gamma\) per ml. the optimum pH was 9.08, but at a substrate concentration of 5 \(\gamma\) per ml. the optimum pH was 7.50 and at 3 \(\gamma\) per ml. it was 7.35. The relationship between the optimum pH and the substrate concentration expressed logarithmically is shown in Fig. 3. Extrapolation to a substrate concentration of 1 \(\gamma\) per ml. indicates 7.1 to be its optimum pH.

At any pH within the range of 7 to 8, there was only a slight difference in the amount of phosphate liberated at glycerophosphate concentrations of 10, 5, and 1 mg. per ml., but at these concentrations above pH 8.0, there were definite differences in the amounts liberated. Over the entire pH range, the amount of phosphate liberated differed with different enzyme preparations, but the pH optimum for substrate concentrations from 10 to 1 mg. per ml. did not differ.

The rate of hydrolysis was found to be almost constant during 1 hour at a concentration of 5 \(\gamma\) per ml. and at pH 7.5. At values other than the pH optimum 7.5, the amount of phosphate released during periods of less
than 1 hour were too minute to measure. The rate of hydrolysis of the substrate concentrations of 5 γ per ml. at its optimum pH, 7.52, was $1.3 \times 10^{-4} \gamma$ of phosphorus per second per ml., in contrast to the $43.3 \times 10^{-4}$

![Graph showing the relationship between pH for optimum alkaline phosphatase activity and concentration of sodium β-glycerophosphate.](image)

**Fig. 3.** Relationship between pH for optimum alkaline phosphatase activity and concentration of sodium β-glycerophosphate.

![Graph showing the percentage of phosphate released at optimum pH against substrate concentration.](image)

**Fig. 4.** Sodium β-glycerophosphate hydrolyzed by alkaline phosphatase at optimum pH. Incubation period, 1 hour. γ of phosphorus per second per ml. for the substrate concentration of 10,000 γ per ml. determined at its optimum pH.

The percentage of the phosphate liberated in 1 hour at different substrate concentrations was shown to be greater when the concentration was smaller (see Fig. 4). 1 γ per ml. of glycerophosphate would therefore be completely hydrolyzed in 1 hour, if the initial rate were maintained, and less than 1 γ per ml. would be completely hydrolyzed in less time, as can be seen by the extrapolation of the semilogarithmic curve in Fig. 4. Since
progressively smaller amounts of substrate were available to the enzyme at low substrate concentrations (e.g. 5 γ per ml.), a reduction in the rate of hydrolysis was found; at high substrate concentrations (e.g. 10 mg. per ml.), however, since only a small proportion of the substrate was hydrolyzed and only a small change in pH optimum occurred, the reduction in rate was not as pronounced.

When other phosphoesters were used as substrates at various concentrations, different pH values for optimum activity were also found (Table II). In each case the activity at the low substrate concentrations was much less than that at the high substrate concentrations and there was no sharply defined optimum. The activity also differed among substrates:

<p>| Table II |
|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>pH for Optimum Alkaline Phosphatase Activity on Different Concentrations of Substrates</th>
<th>Concentration (m × 10^-4)</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl disodium phosphate</td>
<td>15.14</td>
<td>9.55</td>
</tr>
<tr>
<td></td>
<td>0.01514</td>
<td>7.75</td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>21.09</td>
<td>9.35</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>8.30</td>
</tr>
<tr>
<td></td>
<td>0.02109</td>
<td>7.65</td>
</tr>
<tr>
<td>Fructose-1-6-phosphate</td>
<td>13.51</td>
<td>9.50</td>
</tr>
<tr>
<td></td>
<td>0.01351</td>
<td>7.70</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>11.22</td>
<td>9.05</td>
</tr>
<tr>
<td></td>
<td>0.01122</td>
<td>7.75</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>2.711</td>
<td>8.75</td>
</tr>
<tr>
<td></td>
<td>0.002711</td>
<td>7.60</td>
</tr>
</tbody>
</table>

on fructose-1-6-phosphate it was less than on other substrates; on phenyl phosphate, on the other hand, it was so great that the enzyme concentration had to be reduced. It was found that the pH optima for the various substrates were relatively close to one another at the same molarity over the entire range of concentrations.

When the pH was maintained at the optimum for each particular concentration of substrate, the amounts of phosphate liberated in 1 hour from glycerophosphate were different from those liberated when a constant pH was maintained for all substrate concentrations. The Michaelis constant, $K_m$, calculated from the phosphate liberated when each concentration of substrate was at the optimum pH, was 0.0030.

**DISCUSSION**

Enzyme activity has heretofore been studied with individual substrates in vitro, but there are many substrates available in vivo. When the sub-
strate is added in vitro in such amounts as to saturate the enzyme, erroneous conclusions may be drawn regarding the activity of the enzyme in the cell or in body fluids, for it does not seem likely that these substrate concentrations exist in vivo. Moreover, alkaline phosphatase activity had been studied primarily in the range pH 8 to 10, but the pH limits of the living cell structures are much lower than this. Also, the cell apparently contains many activators and inhibitors whose combined effect should be considered. The enzyme activity is no doubt influenced by the dynamics in the cell where substrates are continuously being supplied and hydrolyzed and end-products are continuously being removed from the cell or utilized in the cell.

The nucleic acids are probably the most important substrates for alkaline phosphatase in the nucleus. Since alkaline phosphatase activity against desoxyribonucleic acid (DNA) appears to be limited to terminal phosphate groups (12), the greater the molecular weight of the molecule, the less substrate is available for enzymic hydrolysis. Calculated from the average amount of DNA, 1.02 X 10⁻⁸ mg. (13, 14), in a normal rat liver nucleus, with a volume of 700 cu.μ and the assumed molecular weight of 500,000, this nucleus would contain 6.5 X 10⁻¹⁸ γ of DNA phosphorus, which would be available to the enzyme. At this concentration of phosphorus, the pH optimum for alkaline phosphatase activity would be approximately 7.6. The actual molecular weight may be as much as 5 times the 500,000 value and at this lower molarity the pH for optimum activity is 7.25. On the other hand, DNA cannot be considered as the sole substrate for alkaline phosphatase, and the pH value for optimum activity is probably somewhat higher.

Since it has been shown that the pH optimum can be reduced from a non-physiological to a physiological pH, it is logical to assume that there is optimum alkaline phosphatase activity in vivo. It may be that other enzymes whose pH optima change with variation in substrate concentration, e.g. urease (15, 16), also have as their pH optima in vivo the same pH as that of the cell.

SUMMARY

The optimum pH for alkaline phosphatase activity has been shown to be dependent upon the initial substrate concentration. At the optimum pH the rate of hydrolysis was found to be lower when the concentration of substrate was lower, but the small amount of phosphate released represented a greater proportion of available phosphate. A direct relation has been found between the pH optima and the substrate concentrations expressed logarithmically. At a concentration of 10 mg. per ml. of glycerophosphate, the optimum pH was 9.13, but at a concentration of 3 γ per ml., the optimum pH was 7.35.
BIBLIOGRAPHY

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