MECHANISM OF INHIBITION OF PHOSPHATASE ACTIVITY BY GLYCINE

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Glycine and other \( \alpha \)-amino acids in very low concentrations have been shown to increase the apparent activity of several enzymes: urease, the various amylases, pancreatic lipase, tyrosinase, yeast peptidase, and the phosphatases (1–3). Occasional observations in the literature indicate, however, that higher concentrations of \( \alpha \)-amino acids exert an inhibiting effect on enzyme action (1, 4–7). Thus Kato's study of the effect of glycine on urease activity contains data showing that glycine inhibits this enzyme at concentrations greater than about 0.04 \( \text{M} \) (4). Bodansky observed that tissue phosphatase activity was retarded by glycine and other \( \alpha \)-amino acids in concentrations greater than about 0.01 to 0.001 \( \text{M} \), depending upon the particular \( \alpha \)-amino acid employed (1).

In view of these instances of enzyme inhibition by \( \alpha \)-amino acids, it was considered that the nature of the effect warranted more complete investigation. The present paper is concerned with the mechanism of inhibition of bone and intestinal phosphatase preparations by glycine.

EXPERIMENTAL

The methods for preparing the phosphatase extracts and for measuring their activity have been previously described (1, 8). Dialyzed phosphatase extracts of rat bone, rat intestine, human intestine, and cat bone were employed. The concentration of the substrate used, sodium \( \beta \)-glycerophosphate, depended upon the particular experiment. The concentration of the buffer, sodium diethyl barbiturate, was 0.5 gm. per 100 cc. of hydrolysis mixture. All reaction velocity determinations were carried out at optimal pH (9.0 to 9.2), attained by incorporating the necessary amount of acid or alkali in the buffered mixture. Since it had been previously shown that concentrations of about 0.01 to 0.001 \( \text{M} \) magnesium ion and 0.006 \( \text{M} \) glycine are necessary for optimal activity of phosphatase and for direct proportionality between such activity and enzyme concentration (1), the inhibitory effects of glycine were determined in mixtures containing these concentrations of magnesium ion and glycine. Thus, any concentration of glycine noted in the text represents that present in addition to 0.006 \( \text{M} \) glycine. The sources of the compounds were as follows: glycine (Eastman
GLYCINE INHIBITION OF PHOSPHATASE

Kodak, monomethylglycine (Hoffmann-La Roche), glycine ethyl ester (Hoffmann-La Roche). Dimethylglycine was prepared according to the method of Michaelis and Schubert (9).

The reaction velocity was determined as previously described (8), usually in duplicate hydrolyses. The amount of phosphorus liberated as inorganic phosphate per cc. of hydrolysis mixture was determined at three time intervals, spaced as equally apart as possible, during the zero order portion of the reaction. All hydrolyses were conducted at 25° in a water thermostat regulated to within 0.01–0.05°.

### Results

**Inhibition of Bone Phosphatase**—Table I shows the effect of 0.0625 M glycine on the velocities of action of rat bone phosphatase, Preparation RBM-d, as the concentration of substrate, sodium β-glycerophosphate, was varied. The reciprocals of the reaction velocities in the absence of glycine were plotted against the reciprocals of the substrate concentrations, in accordance with the Lineweaver-Burk transposition of the Michaelis-Menten expression (10, 11),

\[
\frac{1}{V_0} = \frac{K_s}{V_{\text{max.}}} \cdot \frac{1}{S} + \frac{1}{V_{\text{max.}}}
\]  

(1)
where \( V_0 \) is the reaction velocity in the absence of inhibitor at concentration \( S \) of the substrate, \( V_{\text{max}} \) is the reaction velocity in the absence of inhibitor at maximal (theoretically infinite) concentration of substrate, and \( K_s \) is the dissociation constant of the assumed, intermediate enzyme-substrate complex. A straight line was drawn through the experimental points and the following values were obtained from the plot: \( K_s = 0.0019 \) mole per liter; \( V_{\text{max}} = 2.00 \) \( \gamma \) of P liberated per cc. per minute.

A similar plot was made of the reaction velocities in the presence of 0.0625 m glycine in accordance with the following Lineweaver-Burk expression.

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} \left( K_s + \frac{K_s I}{K_I} \right) \frac{1}{S} + \frac{1}{V_{\text{max}}}.
\]  

\( V \) is the reaction velocity in the presence of concentration of inhibitor, \( I \), at substrate concentration; \( S \cdot V_{\text{max}} \) is the reaction velocity at maximal substrate concentration in the presence of inhibitor. \( K_I \) is the dissociation constant of the assumed intermediate enzyme-inhibitor complex and \( K'_s = K_s + \frac{K_s I}{K_I} \) is the dissociation constant of the intermediate enzyme-substrate complex in the presence of inhibitor. A straight line drawn through the experimental values gave the following: \( V_{\text{max}} = 0.83 \) \( \gamma \) of P liberated per cc. per minute; \( K'_s = 0.0019 \) mole per liter. The third and fifth columns of Table I show the values for the reaction velocities calculated by using these values for \( V_{\text{max}} \) in the presence and absence of glycine and for \( K_s \) and \( K'_s \). Except for the reaction velocities at 0.00319 and 0.00635 m substrate in the absence of glycine, these calculated values are in very good agreement with the experimentally determined reaction velocities. The findings that \( V_{\text{max}} \) in the presence of glycine was less than \( V_{\text{max}} \) without inhibitor and that \( K'_s \) had, within experimental error, the same value as \( K_s \) show that the inhibition of rat bone phosphatase by glycine was wholly non-competitive.

In order to obtain the value for the dissociation constant of the assumed bone phosphatase-glycine complex, the velocities of action were determined at varying glycine concentrations and constant substrate concentration, 0.0127 m sodium \( \beta \)-glycerophosphate (Table II). The values for \( K'_I \), the dissociation constant of an enzyme-inhibitor complex formed non-competitively, were calculated in accordance with the following transposition of the Michaelis-Menten expression (10),

\[
K'_I = \frac{I}{V} \frac{V_0}{V - 1}
\]  

\( V_0 \) is the velocity without inhibitor and \( V \) the velocity at the concentration, \( I \), of the inhibitor. It may be seen from Table II that the values of \( K'_I \)
were the same, within experimental error, when the concentration of gly-
cine was varied from 0.00625 to 0.250 M. The average value of \( K' r \) is
0.039 (s.d. = 0.0045). Since Equation 3 does not contain any terms in-
volving substrate concentration, the value for \( K' r \) is the same at all sub-
strate concentrations.

**Inhibition of Intestinal Phosphatase**—Table III, Column 2, shows the
velocities of action of the rat intestinal phosphatase, Preparation RIK-d,
as the concentration of substrate was varied. The reciprocals of these

<table>
<thead>
<tr>
<th>Concentration of glycine (M)</th>
<th>( \gamma )</th>
<th>( V_2 )</th>
<th>( K' r ) (mole per l.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.81</td>
<td>1.19</td>
<td>0.033</td>
</tr>
<tr>
<td>0.00625</td>
<td>1.52</td>
<td>1.28</td>
<td>0.045</td>
</tr>
<tr>
<td>0.0125</td>
<td>1.42</td>
<td>1.95</td>
<td>0.033</td>
</tr>
<tr>
<td>0.0313</td>
<td>0.93</td>
<td>2.52</td>
<td>0.041</td>
</tr>
<tr>
<td>0.0625</td>
<td>0.72</td>
<td>6.97</td>
<td>0.039</td>
</tr>
<tr>
<td>0.125</td>
<td>0.43</td>
<td>6.97</td>
<td>0.042</td>
</tr>
<tr>
<td>0.250</td>
<td>0.26</td>
<td>6.97</td>
<td>0.042</td>
</tr>
</tbody>
</table>

velocities in the absence of glycine were plotted against the reciprocals of
the corresponding concentrations of substrate and a straight line drawn
through the experimental points in accordance with Equation 1. The
following values were obtained: \( K_s = 0.0029 \) mole per liter, \( V_{\text{max}} = 3.12 \gamma \)
of P liberated per cc. per minute. Column 3 shows that the reaction veloc-
ities calculated on the basis of these values are in good agreement with the
experimentally determined velocities. Column 4 shows the reaction veloc-
ities in the presence of 0.0625 M glycine. The reciprocals of these velocities
were plotted against the reciprocals of the substrate concentrations and a
straight line was drawn through the resulting points in accordance with
Equation 2. According to this plot, \( V_{\text{max}} \) in the presence of glycine was
1.42 \( \gamma \) of P liberated per cc. per minute, and \( K' s = K_s + (K_s I/K_I) \) was
0.0062 mole per liter. That in this case also the experimental points fell
very close to the straight line drawn through them is attested by the excel-
lence of the agreement between the calculated (Column 5) and the experi-
mentally determined velocities (Column 4).

The finding that \( V_{\text{max}} \) with glycine was lower than \( V_{\text{max}} \) without glycine
indicated the non-competitive nature of the inhibition by glycine. On the
other hand, the finding that $K' > K$ showed that there was also a competitive component in this inhibition. That enzyme inhibitors need not be either wholly competitive or wholly non-competitive has, of course, been previously noted in a number of instances (12).

The relative magnitude of the competitive and non-competitive components of the inhibition of rat intestinal phosphatase by glycine was determined by the following calculations and experiments. At maximal, i.e. theoretically infinite, substrate concentration, whatever inhibition is present is due entirely to the non-competitive component (11, 12). As may be calculated from Table III, the ratio $V : V_0$ at this concentration was 0.45. The reaction velocities at the other substrate concentrations in the absence of glycine were multiplied by this factor to give the velocities that would result if the inhibition by glycine were solely non-competitive (Table III, Column 6). These velocities were then compared with those actually obtained in the presence of 0.0625 M glycine. The inhibitions were then calculated, and the non-competitive component expressed as a fraction of the total inhibition. For example, at 0.00127 M substrate, the total inhibition amounted to 0.96 $- 0.24$ or 0.72 $\gamma$ of P per cc. per minute; the calculated inhibition that would result if it were solely non-competitive would be 0.96 $- 0.43$ or 0.53 $\gamma$ of P per cc. per minute. Hence, the non-competitive component was 74 per cent in this instance. As may be seen

\[ \begin{array}{cccccc}
\text{Concentration of Na $\beta$-glycerophosphate} & \text{Phosphorus liberated as phosphate per cc. per min.} & \text{Ratio of non-competitive to total inhibition*} \\
\hline
\text{(1)} & \text{No inhibition} & 0.0625 \text{ M glycine} & \text{If inhibition were solely non-competitive*} & \text{(6)} & \text{(7)} \\
\hline
\gamma & \gamma & \gamma & \gamma & \gamma & \gamma \text{ per cent} \\
M & 0.96 & 0.94 & 0.24 & 0.24 & 0.43 & 74 \\
0.000254 & 1.39 & 1.45 & 0.41 & 0.41 & 0.63 & 78 \\
0.00635 & 2.09 & 2.13 & 0.71 & 0.72 & 0.94 & 83 \\
0.0127 & 2.47 & 2.55 & 0.95 & 0.95 & 1.13 & 88 \\
0.0254 & 2.97 & 2.81 & 1.17 & 1.13 & 1.34 & 91 \\
0.0508 & 3.59 & 2.96 & 1.17 & 1.13 & 1.34 & 91 \\
\infty & 3.12\dagger & 3.12 \dagger & 1.42\dagger & 1.42 \dagger & 1.42 \dagger & 100 \\
\end{array} \]

* See the text for the method of calculating these values.
\dagger Extrapolated.

The relative magnitude of the competitive and non-competitive components of the inhibition of rat intestinal phosphatase by glycine was determined by the following calculations and experiments. At maximal, i.e. theoretically infinite, substrate concentration, whatever inhibition is present is due entirely to the non-competitive component (11, 12). As may be calculated from Table III, the ratio $V : V_0$ at this concentration was 0.45. The reaction velocities at the other substrate concentrations in the absence of glycine were multiplied by this factor to give the velocities that would result if the inhibition by glycine were solely non-competitive (Table III, Column 6). These velocities were then compared with those actually obtained in the presence of 0.0625 M glycine. The inhibitions were then calculated, and the non-competitive component expressed as a fraction of the total inhibition. For example, at 0.00127 M substrate, the total inhibition amounted to 0.96 $- 0.24$ or 0.72 $\gamma$ of P per cc. per minute; the calculated inhibition that would result if it were solely non-competitive would be 0.96 $- 0.43$ or 0.53 $\gamma$ of P per cc. per minute. Hence, the non-competitive component was 74 per cent in this instance. As may be seen

\[ \begin{array}{cccccc}
\text{Concentration of Na $\beta$-glycerophosphate} & \text{Phosphorus liberated as phosphate per cc. per min.} & \text{Ratio of non-competitive to total inhibition*} \\
\hline
\text{(1)} & \text{No inhibition} & 0.0625 \text{ M glycine} & \text{If inhibition were solely non-competitive*} & \text{(6)} & \text{(7)} \\
\hline
\gamma & \gamma & \gamma & \gamma & \gamma & \gamma \text{ per cent} \\
M & 0.96 & 0.94 & 0.24 & 0.24 & 0.43 & 74 \\
0.000254 & 1.39 & 1.45 & 0.41 & 0.41 & 0.63 & 78 \\
0.00635 & 2.09 & 2.13 & 0.71 & 0.72 & 0.94 & 83 \\
0.0127 & 2.47 & 2.55 & 0.95 & 0.95 & 1.13 & 88 \\
0.0254 & 2.97 & 2.81 & 1.17 & 1.13 & 1.34 & 91 \\
0.0508 & 3.59 & 2.96 & 1.17 & 1.13 & 1.34 & 91 \\
\infty & 3.12\dagger & 3.12 \dagger & 1.42\dagger & 1.42 \dagger & 1.42 \dagger & 100 \\
\end{array} \]

* See the text for the method of calculating these values.
\dagger Extrapolated.
from Table III, this component constituted from 74 to 91 per cent of the total inhibition at various substrate concentrations, being greater at the higher substrate concentrations.

Table IV shows the inhibition of intestinal phosphatase activity by various concentrations of glycine at a substrate concentration of 0.0127 M. In the third column, these velocities are calculated as fractions of the velocities without glycine (V : V₀). Application of Equation 3, the expression for non-competitive inhibition, to the reaction velocities at maximal substrate concentration in the presence and absence of 0.0625 M glycine yielded a value of 0.051 mole per liter for Kᵢ, the dissociation constant. By substituting this value and the velocity, 2.47 V₀ of P per cc. per minute, at 0.0127 M substrate concentration in the absence of glycine (V₀) in Equation 3, it was possible to obtain, within the experimental errors involved, the reaction velocities that would exist at the various glycine concentrations if the inhibition were solely non-competitive. The ratios of non-competitive to total inhibition were then calculated (last column, Table IV). For example, at 0.250 M glycine, the total inhibition was 2.47 - 0.36 or 2.11 V₀ of P per cc. per minute; the inhibition that would exist if it were solely non-competitive would be 2.47 - 0.42 or 2.05 V₀ of P per cc. per minute. Hence the non-competitive component in this instance was 98 per cent of the total. It may be seen that, at a concentration of 0.0127 M substrate, the non-competitive inhibition constituted

TABLE IV

<table>
<thead>
<tr>
<th>Concentration of glycine (M)</th>
<th>Phosphorus liberated as phosphate per cc. per min.</th>
<th>If inhibition were solely non-competitive*</th>
<th>Ratio of non-competitive to total inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed [\gamma] per cent</td>
<td>Fraction of uninhibited velocity [\gamma] per cent</td>
<td>Fraction of uninhibited velocity [\gamma] per cent</td>
</tr>
<tr>
<td>0.0</td>
<td>2.47 100</td>
<td>2.47 100</td>
<td>80</td>
</tr>
<tr>
<td>0.0125</td>
<td>1.84 75</td>
<td>1.98 80</td>
<td>97</td>
</tr>
<tr>
<td>0.0313</td>
<td>1.53 62</td>
<td>1.54 63</td>
<td>90</td>
</tr>
<tr>
<td>0.0625</td>
<td>0.95 39</td>
<td>1.11 45</td>
<td>90</td>
</tr>
<tr>
<td>0.125</td>
<td>0.64 26</td>
<td>0.75 29</td>
<td>90</td>
</tr>
<tr>
<td>0.188</td>
<td>0.50 20</td>
<td>0.52 22</td>
<td>98</td>
</tr>
<tr>
<td>0.250</td>
<td>0.36 15</td>
<td>0.42 17</td>
<td>98</td>
</tr>
</tbody>
</table>

* See the text for the method of calculating these values.
### Table V

**Inhibition of Phosphatase Activity by Glycine and Glycine-Substituted Compounds**

Concentration of phosphatase, 12.5 per cent by volume in hydrolysis mixture. Concentration of sodium β-glycerophosphate, 0.0127 M.

<table>
<thead>
<tr>
<th>Phosphatase preparation</th>
<th>Concentration of inhibitor</th>
<th>Phosphorus liberated as phosphate per cc. per min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glycine</td>
</tr>
<tr>
<td>CaBA-d, cat bone</td>
<td>0.0</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>0.0125</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0375</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>0.0625</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.425</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>MID-d, human intestinal</td>
<td>0.0</td>
<td>1.13</td>
</tr>
<tr>
<td>phosphatase</td>
<td>0.00625</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>0.0125</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>0.0250</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>0.0625</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.156</td>
<td>0.24</td>
</tr>
</tbody>
</table>

### Table VI

**Concentrations of Glycine and Substituted Glycine Compounds Required to Produce 60 Per Cent Inhibition of Phosphatase Activity**

These concentrations have been estimated from the experimental values recorded in Table V and in accordance with the method described in the text.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bone phosphatase, Preparation CaBA-d</th>
<th>Intestinal phosphatase, Preparation MID-d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.030</td>
<td>0.043</td>
</tr>
<tr>
<td>&quot; ethyl ester</td>
<td>0.059</td>
<td>0.082</td>
</tr>
<tr>
<td>Monomethylglycine</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>1.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

80 to 98 per cent of the total inhibition, being higher at the higher concentrations of glycine.
Inhibition of Phosphatase Activity by Substituted Glycine Compounds—

In Table V are shown the inhibitions of cat bone phosphatase, Preparation CaBA-d, and human intestinal phosphatase, Preparation MID-d, by glycine, glycine ethyl ester, monomethylglycine, and dimethylglycine. The reciprocals of the reaction velocities were plotted against the concentrations of these various inhibitors; from the straight lines drawn through the experimental points, the concentrations at which 50 per cent inhibition occurred were estimated. Table VI shows that about twice as high a concentration of glycine ethyl ester as of glycine was necessary to produce the same degree of inhibition. The introduction of methyl groups into the amino group led to a much more marked decrease in the inhibition. Thus, about 10 times as high a concentration of monomethylglycine and about 50 times as high a concentration of dimethylglycine as of glycine were necessary to produce 50 per cent inhibition of cat bone and human intestinal phosphatase activity.

DISCUSSION

Michaelis and Menten (10) assumed that enzyme inhibition was due to the formation of an inactive but dissociable enzyme-inhibitor complex. A considerable number of studies have substantiated this assumption (10–14). Michaelis and Menten (10) also pointed out that the inhibitor may combine with the enzyme either so as to decrease its active mass without displacing it from its combination with the substrate (non-competitive inhibition) or so as to compete with the substrate (competitive inhibition). In the present study it has been shown that the inhibition of rat bone phosphatase by glycine is wholly non-competitive and in good agreement with the mathematical formulations for such inhibition. The dissociation constant of the rat bone phosphatase-glycine complex was found to be 0.039 (S.D. = 0.0045) mole per liter.

In contrast, the inhibition of rat intestinal phosphatase by glycine is mixed, being both competitive and non-competitive, but largely the latter. At a concentration of 0.0625 M glycine, the non-competitive component ranged from 74 per cent of the total inhibition at 0.00127 M substrate to 91 per cent at 0.0254 M substrate. This relative increase in the magnitude of the non-competitive component is to be expected, since competitive inhibition decreases with increasing substrate concentration.

The formation of the phosphatase-glycine complex appears to involve both the carboxyl and the amino groups of glycine. Esterification of glycine reduced the inhibition considerably, and substitution of two methyl groups for the hydrogen atoms in the amino radical practically nullified the inhibition. The extension of the studies reported in the present work to bone and intestinal phosphatases of other species, to other tissue phos-
phatases, and to α-amino acids other than glycine naturally suggests itself. It would also be of interest to elaborate the present observations on inhibition so as to determine whether blocking the carboxyl and amino groups may alter not only the degree, but also the type of inhibition.

**SUMMARY**

1. The Michaelis-Menten dissociation constant of the intermediate enzyme-substrate complex has been found to be 0.0019 mole per liter for the action of rat bone phosphatase on sodium β-glycerophosphate, and 0.0029 mole per liter for that of rat intestinal phosphatase on this substrate.

2. Glycine in concentrations higher than the 0.006 M necessary for optimal activity inhibits the action of phosphatase. The glycine inhibition of rat bone phosphatase is entirely non-competitive in type; the dissociation constant of the assumed rat bone phosphatase-glycine complex is 0.039 mole per liter. The glycine inhibition of rat intestinal phosphatase is mixed, being both competitive and non-competitive. Under the conditions of variation in the concentrations of substrate and glycine here investigated, the non-competitive component of the inhibition ranged from 74 to 98 per cent of the total inhibition.

3. The inhibition of phosphatase action by glycine depends to a very considerable degree upon the intactness of the carboxyl and amino groups of glycine. Esterification of the carboxyl group reduced the inhibition to about one-half its value. Introduction of methyl groups into the amino radical decreased the inhibition much more markedly.

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1. Bodansky, O., *J. Biol. Chem.*, **114**, 273 (1936); **115**, 101 (1936). (These articles also contain references to α-amino acid activation of enzymes, other than phosphatase.)
MECHANISM OF INHIBITION OF PHOSPHATASE ACTIVITY BY GLYCINE
Oscar Bodansky


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