MECHANISM OF ACTION OF 5'-NUCLEOTIDASE*

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The enzyme 5'-nucleotidase was originally discovered in the venom of snakes (1) and later shown to be present in mammalian tissues (2-5) and bull seminal plasma (6). It has recently been extensively purified and characterized by Heppel and Hilmoe (7). The enzyme was shown to hydrolyze 5'-nucleotides rapidly, ribose-5-phosphate slowly, and other phosphate esters extremely slowly, if at all. Because of its importance in nucleotide metabolism and its interesting specificity pattern, a study of the mechanism of action of the enzyme was initiated.

In order to determine whether carbon-oxygen or phosphorus-oxygen bond rupture occurred during the hydrolytic action, the hydrolysis of adenosine-5-phosphate was followed in H$_2$O$^{18}$ (see Equation 1). After the cleavage point was determined, exchange experiments of adenosine-C$^{14}$, Adenosine-5'-phosphate in H$_2$O$^{18}$:

\[
\text{Ad(OH)} + \text{HO}^{18}\text{PO}_3^{-} \rightarrow \text{Ad}^{18}\text{O} + \text{H}^{18}\text{PO}_3^{-}
\]

KH$_2$P$^{32}$O$_4$, and H$_2$O$^{18}$ with AMP$^1$ and of H$_2$O$^{18}$ with KH$_2$PO$_4$, were performed to test the type and life time of the enzyme-substrate intermediates.

EXPERIMENTAL

Hydrolysis of Adenosine-5-phosphate in H$_2$O$^{18}$. The enzyme solution (528 units of a purified preparation kindly supplied by Dr. Heppel and Dr. Hilmoe) was added to 50 ml. of H$_2$O$^{18}$ (about 1.2 atom per cent excess) containing sufficient solid materials to make the final solution 0.04 M in adenylic acid, 0.08 M in glycine, and 0.14 M in magnesium chloride. (Radioactive KH$_2$PO$_4$ was added as an analytical control for the H$_2$O$^{18}$-AMP exchange experiment described below.) The solution, after adjustment to

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$^1$ The following abbreviations were used: AMP for adenosine-5-phosphate, Ad for adenosine, E for enzyme.
pH 8.5 with solid potassium hydroxide, was allowed to stand for 2 hours at 37°. Samples were then removed for determining the O\textsuperscript{18} content of the medium and the per cent hydrolysis, which was usually about 50 per cent (see below). The inorganic phosphate was precipitated as the barium salt, dissolved in 0.2 N HBr, and reprecipitated three times to remove impurities. The barium phosphate was then converted to KH\textsubscript{2}PO\textsubscript{4} with Dowex 50 ion exchange resin (or by sulfate precipitation), isolated, reprecipitated, and pyrolyzed in a manner similar to that described by M. Cohn (8). The O\textsuperscript{18} content of the inorganic phosphate was calculated from the O\textsuperscript{18} content of the CO\textsubscript{2} measured in the mass spectrometer by using a formula similar to the one developed by Dostrovsky and Klein (9).

**Exchange Experiment between H\textsubscript{2}O\textsuperscript{18} and Adenylic Acid**—After removal of the barium phosphate in the hydrolysis experiment described above, the remaining solution was lyophilized. The dry residue was dissolved in ordinary distilled water, and unlabeled phosphate was added and removed as the barium salt. Samples were removed to determine the O\textsuperscript{18} content of the medium and the amount of inorganic phosphate. The adenylic acid present in the supernatant liquid was then hydrolyzed with alkaline phosphatase or 5'-nucleotidase. The phosphate produced in this second hydrolysis was precipitated as the barium salt, purified, and analyzed as described above. Counts of the P\textsuperscript{32} activity were made to correct for any phosphate produced in the initial hydrolysis in H\textsubscript{2}O\textsuperscript{18} which had "leaked through" into the unhydrolyzed ester determination.

**Exchange Experiment between H\textsubscript{2}O\textsuperscript{18} and Inorganic Phosphate**—In a typical exchange experiment the enzyme (442 units) was added to 49 ml. of H\textsubscript{2}O\textsuperscript{18} containing 0.02 M KH\textsubscript{2}PO\textsubscript{4} and 0.008 M tris(hydroxymethyl)aminomethane buffer (pH 8.5). After 29 hours at 37°, more enzyme (442 units) was added, and an aliquot was removed to determine the O\textsuperscript{18} content of the medium. The reaction was stopped after 52 hours by adding barium chloride and removing the phosphate precipitate by centrifugation. The inorganic phosphate was separated and analyzed as described above.

In some of the experiments the enzyme was partially inactivated by heating at 60° or 70° for varying lengths of time. The amount of inactivation was measured by assay under standard conditions after the heat treatment.

**Exchange Experiment between Adenosine-C\textsuperscript{14} and Adenylic Acid**—Commercial C\textsuperscript{14}-labeled adenosine was purified by chromatographing and rechromatographing with additions of inactive adenylic acid. The purified adenosine-C\textsuperscript{14}, sufficient to give final concentration 0.008 M, was added to a solution containing 9.8 units of enzyme per ml., 0.039 M AMP, 0.08 M glycine, and 0.14 M MgCl\textsubscript{2} at pH 8.5. The solution (0.5 ml. total) was incubated for 2 hours at 37° until 48 per cent of the adenylic acid had been hydrolyzed and was then added to the Dowex 1 column. The fractions
were eluted by a modification of the procedures of Cohn and Carter (10). The principal changes were the introduction of larger wash volumes and of inactive carrier adenosine to eliminate contamination of the adenylic acid fraction by adenosine-C^14.

Exchange Experiment between \( \text{KH}_2\text{P}^{32}\text{O}_4 \) and Adenylic Acid—To a solution containing 0.04 M adenylic acid, 0.08 M glycine, 0.16 M MgCl₂, and 8.6 units of enzyme per ml. at pH 8.6 was added 0.08 mc. of \( \text{P}^{32} \) activity as \( \text{KH}_2\text{PO}_4 \). After 2 hours incubation at 37°, the adenylic acid was hydrolyzed to the extent of 42.5 per cent. The inorganic phosphate formed was then eliminated as the barium salt. Inactive inorganic phosphate was added to the supernatant liquid and again precipitated as the barium salt. This procedure was repeated until adenylic acid of constant specific activity was obtained.

Table I

Hydrolysis of Adenosine-5-phosphate in \( \text{H}_2\text{O}^{18} \) Catalyzed by 5'-Nucleotidase

The conditions were 0.04 M adenylic acid, 0.08 M glycine, 0.14 M MgCl₂, 10.6 units of enzyme per ml.; total volume = 50 ml., pH 8.5, 2 hours at 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>( \text{O}^{18} ) atom per cent excess medium</th>
<th>( \text{O}^{18} ) atom per cent excess of phosphate produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calculated for P—O splitting</td>
</tr>
<tr>
<td>1</td>
<td>1.13</td>
<td>0.28</td>
</tr>
<tr>
<td>2</td>
<td>1.24</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Results

The results of the hydrolysis and exchange experiments are presented in Tables I to V.

In Table I, the \( \text{O}^{18} \) content of the inorganic phosphate is seen to agree with the theoretical value calculated for cleavage of the P—O bond during hydrolysis (see Equation 1, a). The theoretical value is one-fourth that of the medium because of the symmetrical nature of the phosphate ion and, the presence of the 3 unlabeled oxygen atoms. The slight discrepancy between theoretical and observed values is within experimental error and does not indicate an alternate pathway of hydrolysis.

In Table II are presented the analyses of the unhydrolyzed adenylic acid in the hydrolytic experiments run to about 50 per cent hydrolysis. The observed values must be corrected for the atom per cent excess of the medium (0.014) in which the second (analytical) hydrolysis was performed. Although the \( \text{H}_2\text{O}^{18} \) had been removed by lyophilization, enough water of crystallization remained to give a slight but appreciable added \( \text{O}^{18} \) content.
MECHANISM OF 5'-NUCLEOTIDASE ACTION

### Table II

*Exchange of Oxygen between Adenosine-5-phosphate and H$_2$O$^{18}$ Catalyzed by 5'-Nucleotidase*

The conditions were 0.04 M adenylie acid, 0.08 M glycine, 0.14 M MgCl$_2$, 10.6 units of enzyme per ml.; total volume = 50 ml., pH 8.5, 2 hours at 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Initial hydrolysis in H$_2$O$^{18}$</th>
<th>Atom per cent of AMP hydrolyzed</th>
<th>Atom per cent excess of medium during analytical hydrolysis</th>
<th>Observed atom per cent excess of phosphate from AMP</th>
<th>Corrected atom per cent excess in unhydrolyzed AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.6</td>
<td>1.13</td>
<td>0.014</td>
<td>0.005</td>
<td>+0.001</td>
</tr>
<tr>
<td>2</td>
<td>42.5</td>
<td>1.24</td>
<td>0.014</td>
<td>0.001</td>
<td>-0.003</td>
</tr>
</tbody>
</table>

### Table III

*Exchange of Oxygen between H$_2$O$^{18}$ and KH$_2$PO$_4$ Catalyzed by 5'-Nucleotidase*

The conditions were 0.02 M KH$_2$PO$_4$, 0.008 M tris(hydroxymethyl)aminomethane, pH 8.5; total volume = 52 ml., 52 hours at 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Total amount of enzyme protein*</th>
<th>Per cent inactivation of enzyme</th>
<th>Atom per cent excess of medium</th>
<th>Atom per cent excess of inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>884</td>
<td>0</td>
<td>1.33</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>884</td>
<td>85</td>
<td>1.19</td>
<td>0.014</td>
</tr>
<tr>
<td>3</td>
<td>1460</td>
<td>10</td>
<td>1.16</td>
<td>0.029</td>
</tr>
<tr>
<td>4</td>
<td>1460</td>
<td>98</td>
<td>1.17</td>
<td>0.016</td>
</tr>
<tr>
<td>5</td>
<td>730</td>
<td>0</td>
<td>1.23</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td>730</td>
<td>0</td>
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<td>0.009</td>
</tr>
<tr>
<td>7</td>
<td>730</td>
<td>0</td>
<td>1.21</td>
<td>0.005</td>
</tr>
<tr>
<td>8</td>
<td>730</td>
<td>99</td>
<td>1.21</td>
<td>0.005</td>
</tr>
<tr>
<td>9</td>
<td>365</td>
<td>0</td>
<td>1.36</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*Total units present in initial enzyme solution before thermal inactivation.

Thus, in Experiment 2 the actual solution added contained 133 active units of enzyme and 751 units of enzyme which had been thermally inactivated.

### Table IV

*Exchange between Adenosine-C$^{14}$ and Adenylic Acid Catalyzed by 5'-Nucleotidase*

The conditions were 0.039 M adenylie acid, 0.008 M adenosine-C$^{14}$, 0.08 M glycine, 0.14 M MgCl$_2$, pH 8.5; total volume = 0.5 ml., 2 hours at 37°.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Per cent hydrolysis</th>
<th>Per cent C$^{14}$ activity in adenylic acid fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme added</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>9.8 units enzyme per ml. added</td>
<td>48</td>
<td>0.05</td>
</tr>
</tbody>
</table>
to the water. The final corrected figure, therefore, is the observed atom per cent excess minus one-fourth that of the medium in the analytical hydrolysis. These values in the last column are clearly zero within experimental error, and hence show that no appreciable exchange of the type seen in Equation 2 is catalyzed by the enzyme.

\[
H_2O^{18} + AdOPO_4^{--} \rightleftharpoons AdOPO \rightleftharpoons AdOPO^{15} + H_2O
\]  

(2)

In Table III are some of the experiments designed to test the possibility of exchange of oxygen between water and inorganic phosphate (Equation 3). In Experiments 1 to 4 the experimental error was larger than in the subsequent experiments. It is seen, however, that in the presence of varying amounts of enzyme, activated or inactivated, the \("O^{18}\) content\) of the phosphate was essentially constant and indicated no exchange. Since this persistent positive blank appeared to be caused by organic impurities, additional purification steps were introduced, and Experiments 5 to 9 confirm the lack of exchange even when the experimental error is one-fifth of the previous value.

In Table IV the experiments on exchange of adenosine-C\(^{14}\) and adenylic acid are shown, and it is found that the exchange is less than 0.05 per cent when the hydrolysis has proceeded to about 50 per cent. To obtain the low values illustrated, it was necessary (a) to purify the commercially obtained adenosine-C\(^{14}\) extensively to remove adenine and adenylic acid impurities and (b) to purify the adenylic acid fraction from the column carefully to prevent contamination by small leaks from the adenosine fraction. Previous experiments with less purification had given agreement within experimental error between the enzyme experiment and the control without enzyme but at a higher contamination level (\(\sim 0.2\) per cent). The results with the improved purification show a slight difference between the control and the enzyme run which is of the order of the experimental error.

\[
Ad-O-PO_4^{--} + H_2O \rightleftharpoons Ad-OH + HOPO_4^{--}
\]  

(4)

A rough calculation of the amount of labeled adenylic acid produced by a reversal of the over-all reaction of Equation 4 gives a figure of 0.016 per cent which is in agreement with the observed difference within experimental error.

The exchange of inorganic phosphate with AMP is shown in Table V.
One possible route for such an exchange is by a reversal of the over-all reaction (Equation 4). A rough calculation indicates that 0.009 per cent of the originally added activity would be added to the AMP by this pathway. The observed result (0.015 per cent) is within experimental error of this value and indicates no alternate avenue for exchange of any importance.

**DISCUSSION**

The O\textsuperscript{18} experiments clearly establish that the phosphorus-oxygen bond is broken during the hydrolytic action catalyzed by the very specific 5'-nucleotidase. Experiments with O\textsuperscript{18} have already established that the non-specific acid and alkaline phosphatases (11, 12) and the moderately specific acetyl phosphatase (13) react through phosphorus-oxygen cleavage. Thus,

**TABLE V**

| Exchange of Phosphate between KH\textsubscript{2}P\textsuperscript{32}O\textsubscript{4} and Adenosine-5-phosphate Catalyzed by 5'-Nucleotidase |
| --- | --- | --- |
| Purification step | AMP | Calculated per cent of total P\textsuperscript{32} activity in AMP fraction |
| 1st purification | 57.1 | 1.47 |
| 2nd " | 1.45 | 0.037 |
| 3rd " | 0.62 | 0.013 |
| Final AMP fraction | 0.57 | 0.015 |

all the phosphatases examined so far for cleavage point have been found to have P—O splitting, regardless of the specificity pattern of the particular enzyme.

In previous publications evidence has been presented to show that the enzymatic substitution reactions proceed by displacement mechanisms (14, 15). In the case of 5'-nucleotidase, the O\textsuperscript{18} data show that the displacement occurs on the phosphorus atom but do not establish whether the primary attack is made by water, *i.e.* a single displacement mechanism, or enzyme, *i.e.* a double displacement mechanism. (The frontside displacement mechanism can be excluded for phosphate cleavage because of the evidence that the non-enzymatic reactions proceed by bimolecular nucleophilic substitution mechanisms (16).) Morton (17) has shown that the enzyme does not have transferase properties with glycerol as acceptor. Since glycerol is more similar to water than adenosine, it can be concluded that adenosine would be even less likely to substitute for water as an ac-
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ceptor. Likewise, according to the specificity for 5'-nucleotides, water cannot replace adenosine at the donor site. Thus exchange of water with \( \text{KH}_2\text{PO}_4 \) or of adenosine with AMP would have to occur via a phosphoryl intermediate, and an observed exchange in either case would establish the mechanism in Equations 5 and 6. Neither was observed.

\[
\text{AdOPo}_4^- \text{EH} \xrightleftharpoons[k_{-1}]{k_1} \text{EPO}_3^- + \text{AdOH} \tag{5}
\]

\[
\text{EPO}_3^- + \text{H}_2\text{O} \xrightleftharpoons[k_{-2}]{k_2} \text{EH} + \text{HOPO}_4^- \tag{6}
\]

The results exclude certain alternatives: a stable phosphorylated enzyme intermediate, a rapid reversible first step (Equation 5) followed by a slow second step (Equation 6), or a slow first step followed by a rapid reversible second step. With the currently available data, however, a transient enzyme-phosphate intermediate is still possible.

A single displacement mechanism is also consistent with the data. In this case a direct attack of water on the phosphorus of the adenylic acid would occur in the hydrolysis, and any exchanges of \( \text{H}_2\text{O}^{18} \) with \( \text{KH}_2\text{PO}_4 \) or of adenosine-C\(^{14}\) with AMP would require that water could occupy the adenosine site or adenosine occupy the water site. In view of the specificity pattern, neither would be possible (for a discussion of the specificity requirements for exchange, see Koshland (14)), and hence no exchange would be expected as is observed. The lack of exchange of oxygen between \( \text{H}_2\text{O}^{18} \) and the unhydrolyzed AMP, a criterion which has previously been used for hydrolytic reactions (18), indicates that no free addition intermediate of the type \( \text{HO}^{18} \cdot \text{P} \cdot \text{OR} \) is formed in the reaction. The single displacement, if it is ultimately shown to be the actual mechanism, probably proceeds as in Equation 7 with simultaneous breaking and forming of the bonds indicated by the dotted lines.

\[
\text{H}_2\text{O} + \text{OPO}_3\text{Ad} \rightarrow \text{H}_2\text{O} \cdots \text{P} \cdots \text{OAd} \rightarrow \text{HOPO}_3^- + \text{AdOH} \tag{7}
\]

The lack of exchange between \( \text{KH}_2\text{P}^{32}\text{O}_4 \) and adenylic acid is consistent with the previous conclusions. Since a long lived \( \text{EPO}_3 \) intermediate was excluded, an exchange reaction of the type of Equation 8 would not be expected to compete successfully with the other modes of decomposition

\[
\text{EPO}_3 + \text{KH}_2\text{P}^{32}\text{O}_4 \rightarrow \text{EP}^{32}\text{O}_4 + \text{KH}_2\text{PO}_4 \tag{8}
\]
of the intermediate. Similarly, exchange by a single displacement mechanism, i.e. by direct attack of phosphate on AMP, would be excluded by the cleavage point evidence and by the specificity pattern of the enzyme. Thus, it can be concluded that the 5'-nucleotidase catalyzes a displacement on phosphorus by either water or enzyme with formation of a transient intermediate.

SUMMARY

1. The point of bond rupture as a result of 5'-nucleotidase action has been studied during the hydrolysis of adenosine-5-phosphate in H2O18. The presence of a stoichiometric amount of O18 in the inorganic phosphate produced is consistent with a displacement mechanism involving an attack on the phosphorus atom.

2. No appreciable exchange was observed on incubating H2O18 with either KPiPO4 or adenylic acid. The very small exchanges observed on incubating adenosine-C14 with adenylic acid or KH2P2O4 with adenylic acid agree with the calculations for the amount incorporated by reversal of the over-all reaction and indicate no appreciable exchange by a partial reaction. The exchange experiments establish that the enzyme intermediate must be short lived with respect to decomposition to products.

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BIBLIOGRAPHY

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