THE ENZYMATIC CLEAVAGE OF ADENYLC ACID TO
ADENINE AND RIBOSE 5-PHOSPHATE

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This report is concerned with the purification and properties of an ade-
nine-forming enzyme from Azotobacter vinelandii which shall be called
5'-AMP ribosidase. Evidence has been obtained that the reaction cata-
lyzed by this enzyme is

\[
\text{Adenosine 5'-monophosphate} \rightarrow (\text{ATP}) \rightarrow \text{adenine + ribose 5-phosphate}
\]

with ATP acting catalytically. Of a large number of compounds tested,
only AMP was cleaved. The requirement for ATP could also be met by
adenosine tetraphosphate, inorganic pyrophosphate, and inorganic tri-
polyphosphate, although not by metaphosphate or other nucleoside poly-
phosphates.

Methods

The various nucleosides and nucleotides used in this study were com-
mmercial preparations. This was also true for inorganic pyrophosphate,
triphosphosphate, and adenosine tetraphosphate.

ATP was a crystalline preparation; UTP was further purified by ion
exchange chromatography. 1-Pyrophosphoryl ribose 5-phosphate was
isolated by the procedure of Kornberg, Lieberman, and Simms (1) to whom
we are indebted for a generous sample of this material. Ribose 5-pyro-
phosphate and ribose 5-triphosphate were synthesized as described else-

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1 The enzyme was encountered in the course of a study of polynucleotide phos-
phorylase of A. vinelandii, being carried out by one of us (L. A. H.) in collaboration
with Dr. S. Ochoa and Miss P. J. Ortiz. We are grateful to Dr. Ochoa for helpful
discussions and for supplying various enzyme fractions and compounds used in
preliminary exploration.

2 The following abbreviations are used: 5'-monophosphates of adenosine, inosine,
guanosine, uridine, and cytidine, AMP, IMP, GMP, UMP, and CMP; adenosine
5'-diphosphate, ADP; 5'-triphosphates of adenosine, inosine, guanosine, uridine, and
cytidine, ATP, ITP, GTP, UTP, and CTP; reduced diphosphopyridine nucleotide,
DPNH; triphosphopyridine nucleotide, TPN; tris(hydroxymethyl)aminomethane,
Tris; ethylenediaminetetraacetic acid, EDTA.
where (2). Phosphoenolpyruvate was generously provided by Mr. W. E. Pricer, Jr., of this Institute.

Hexokinase was a commercial preparation from the Sigma Chemical Company, St. Louis. Glucose 6-phosphate dehydrogenase was isolated from yeast (3). Crystalline lactic dehydrogenase (Worthington Biochemical Corporation, Freehold, New Jersey) also served as a source of pyruvic kinase.

Spinach phosphoribulokinase was prepared as previously described (4). Myokinase (adenylate kinase) was purified from muscle according to the procedure of Colowick and Kalckar (5). Myokinase activity was determined with ATP and AMP as starting substrates, by measuring the formation of ADP with pyruvic kinase and lactic dehydrogenase, as described by Kornberg and Pricer (6). ATP was estimated by the reduction of TPN after the action of hexokinase and glucose 6-phosphate dehydrogenase. AMP was measured by the combined action of myokinase, pyruvic kinase, and lactic dehydrogenase (6), in the presence of catalytic amounts of ATP. When myokinase is added last, the presence of ADP does not interfere in this determination. The method is sensitive for AMP, since 2 moles of DPNH are oxidized per mole of AMP present.

Phosphate was determined by the method of Fiske and Subbarow (7) or by the Lowry and Lopez (8) procedure. Pentose phosphate was measured by the method of Mejbaum (9) with a 20 minute heating period. Reducing sugar was determined by the procedure of Park and Johnson (10) with ribose 5-phosphate as the standard. It was found that high phosphate concentrations interfered with the method. Protein was determined as described by Sutherland et al. (11).

The following solvent systems were used for paper chromatography:

Solvent 1, isopropanol-water (70:30, v/v), with NH₃ in the vapor phase (12); Solvent 2, saturated (NH₄)₂SO₄-isopropanol-1 M sodium acetate (80:2:18, v/v/v) (12); Solvent 3, 750 ml. of 95 per cent ethanol-300 ml. of 1 M ammonium acetate (13); Solvent 4, 100 ml. of isobutyric acid-60 ml. of 1 M NH₄OH-1 ml. of 0.1 M EDTA (14).

C¹⁴ samples were counted at infinite thinness with a gas flow counter. P³² counting was done with conventional Geiger-Müller counters.

Uniformly C¹⁴-labeled AMP was obtained from the Schwarz Laboratories, Inc., Mount Vernon, New York. Uniformly C¹⁴-labeled ATP was prepared from randomly labeled AMP. 9 μmoles of C¹⁴-AMP were incubated in a volume of 2.2 ml. with 30 μmoles of phosphoenolpyruvate, 80 μmoles of Tris buffer, pH 7.8, 10 μmoles of MgCl₂, 2.5 units of myokinase, 2 μmoles of ATP, and 0.05 ml. of pyruvic kinase. After 60 minutes at 38°, the reaction was stopped by the addition of 0.1 ml. of 8 N HClO₄, and the nucleotides were adsorbed on charcoal (0.2 ml. of 30 per cent suspension). The charcoal was washed two times with 3 ml. of H₂O, and the nucleotides
were eluted with ethanolic NH$_3$ (ethanol-water-concentrated NH$_3$, 1:1:0.08). The solution (3 ml.) was concentrated to a small volume and chromatographed with Solvent 4, and the area corresponding to ATP was quantitatively eluted with water. Measurement of the absorption at 260 mµ indicated that 4.0 µmoles were obtained.

**Assay of AMP-Cleaving Enzyme**—Two independent methods were used for the determination of AMP cleavage. The first procedure was based on the liberation of the free base and the second on the appearance of reducing sugar. For free base formation, the following assay mixture was used: 0.02 ml. of 0.1 M MgCl$_2$, 0.03 ml. of 0.4 M Tris buffer, pH 7.95, 0.04 ml. of 0.02 M ATP, 0.04 ml. of 0.02 M AMP, 0.03 to 1.3 units of enzyme, and water to give a volume of 0.2 ml. Incubation was for 15 minutes at 37.5° in 12 ml. conical centrifuge tubes. After incubation, 0.03 ml. of 25 per cent barium acetate solution was introduced, followed by 0.92 ml. of absolute ethanol. The tubes were briefly shaken, kept in ice for 20 minutes, and centrifuged at 2° for 10 minutes at about 2000 r.p.m. The optical density was determined at 250 mµ after the addition of 1.17 ml. of 0.15 N HCl to a 0.7 ml. aliquot. It was found necessary to measure both adenine and hypoxanthine together because adenase was present at all stages of purification. At 250 mµ both adenine and hypoxanthine have identical molar extinction coefficients of 1.03 × 10$^4$ (15).

For reducing sugar, the following assay mixture was used: 0.02 ml. of 0.02 M AMP, 0.02 ml. of 0.02 M ATP, 0.01 ml. of 0.1 M MgCl$_2$, 0.025 ml. of 0.4 M Tris buffer, pH 7.95, enzyme, and water to give a volume of 0.1 ml. After 10 minutes at 37.5°, an aliquot was removed, and the reducing power was measured by using ribose 5-phosphate as a standard. A unit of enzyme activity was defined as equal to the amount required for the formation of 1 µmole of reducing sugar or adenine per hour at 38° under the above conditions.

In both assays, controls lacking enzyme were run. However, the early enzyme fractions ("Crude extract" to "Ammonium sulfate I") contained considerable ultraviolet spectrum-absorbing as well as -reducing material, and additional controls containing enzyme, but lacking substrate, were included when these fractions were assayed.

With the above conditions, the reaction rate was found to be linear with time (Table I) and directly proportional to the enzyme concentration (Fig. 1). This was true with either assay and helped to establish that reducing sugar and purine base were formed simultaneously.

**Results**

**Purification of 5'-AMP Ribosidase**—The enzyme was purified from extracts of A. vinelandii strain O. The organism was grown and harvested as described by Grunberg-Manago, Ortiz, and Ochoa (16). The cells were
washed with cold H₂O and centrifuged at 13,000 × g, and the pellet was stored at -15°C. Cells frozen up to 2 months showed no loss of ability to cleave AMP. A summary of the purification procedure is given in Table II. The details follow.

### TABLE I

**Formation of Reducing Sugar and Free Base As Function of Time**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Reducing sugar μmole per 0.2 ml.</th>
<th>Free base μmole per 0.2 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>10</td>
<td>0.13</td>
<td>0.15</td>
</tr>
<tr>
<td>15</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>20</td>
<td>0.29</td>
<td>0.27</td>
</tr>
<tr>
<td>30</td>
<td>0.33</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The conditions were as follows: 0.8 μmole of ATP, 0.8 μmole of AMP, 12 μmoles of Tris buffer, pH 7.95, 10 μmoles of MgCl₂, 5.9 γ of protein (Ammonium sulfate IV, 1600 units per ml., specific activity = 140), and water to give a volume of 0.2 ml.; temperature 37.5°C.

* This includes both adenine and hypoxanthine.

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**Fig. 1.** Proportionality between rate of formation of free base from AMP and amount of enzyme added. The assay conditions are described in the text. The enzyme solution was Ammonium sulfate IV diluted 100-fold. The concentrated solution contained 13 mg. of protein per ml. and had a specific activity of 206 units per mg.

*Alumina Extract*—28 gm. of cells were ground in a mortar with 56 gm. of Alumina A-301 (325 mesh, Aluminum Company of America) for 10 minutes, and the paste was extracted with 112 ml. of water. The turbid supernatant solution obtained by centrifugation for 15 minutes at 13,000 × g was decanted, and the sediment was reextracted with 56 ml. of water. The supernatant solutions were combined (alumina extract, 160 ml.). The
above operations were performed at room temperature. All subsequent steps were carried out at 2\(^\circ\) except where indicated.

**Protamine Treatment**—160 ml. of alumina extract were diluted with 160 ml. of water, and 12.8 ml. of a 2 per cent solution of protamine sulfate (Nutritional Biochemicals Corporation, Cleveland, Ohio) in 0.2 M sodium acetate buffer, pH 5.0, were added. The solution was rapidly stirred during the addition of protamine, kept standing for 10 minutes, and centrifuged, yielding 315 ml. of supernatant solution ("Protamine fraction").

**Ammonium Sulfate I, Heated**—The Protamine fraction was treated with 150 gm. of solid ammonium sulfate. After 15 minutes, the solution was centrifuged, and the precipitate was dissolved in 89 ml. of water. The en-

<table>
<thead>
<tr>
<th>Table II</th>
<th>Purification of 5'-AMP Ribosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>Units per ml.*</td>
</tr>
<tr>
<td>Crude extract</td>
<td>20.7</td>
</tr>
<tr>
<td>Protamine fraction</td>
<td>34.2</td>
</tr>
<tr>
<td>Ammonium sulfate I, heated</td>
<td>85</td>
</tr>
<tr>
<td>&quot; &quot; II</td>
<td>360</td>
</tr>
<tr>
<td>&quot; &quot; III</td>
<td>970</td>
</tr>
<tr>
<td>&quot; &quot; IV, heated</td>
<td>4400</td>
</tr>
</tbody>
</table>

* The assay conditions were those described for the reducing sugar procedure under "Methods."

† The crude extract had a relatively low activity in this preparation. Such extracts usually contained a total of 10,000 units with a specific activity of 5.

tire solution (98 ml.) was heated to 55\(^\circ\) in about 2.5 minutes by immersion in a water bath at 55\(^\circ\) and held at this temperature for 5 more minutes; the solution was then rapidly cooled to 3\(^\circ\). The voluminous precipitate was removed by centrifugation and washed with about 15 ml. of water. The supernatant and wash solutions were combined ("Ammonium sulfate I, heated," 110 ml.).

**Ammonium Sulfate II**—In all subsequent fractionations ammonium sulfate solution saturated at room temperature and adjusted with concentrated NH\(_3\) to pH 7.4 was used. The salt concentration of the Ammonium sulfate I, heated fraction was measured with the Barnstead purity meter and found to be about 0.10 saturated. This solution (110 ml.) was treated with 27.5 ml. of water and 27.5 ml. of 0.2 M Tris buffer, pH 7.7. To the mixture (165 ml.) were added 55 ml. of ammonium sulfate solution. After centrifugation, the supernatant solution (216 ml.) was treated with 33.2 ml. of saturated ammonium sulfate solution. Both precipitates were dis-
carded. The supernatant solution (244 ml.) was treated with 73.2 ml. of saturated ammonium sulfate, and the heavy precipitate obtained was dissolved in water ("Ammonium sulfate II," 20.5 ml.). This solution was found to be 0.05 saturated with ammonium sulfate.

**Ammonium Sulfate III**—The above fraction (20.5 ml.) was heated for 1 hour at 60°. The resulting suspension was cooled, diluted with 50 ml. of 0.04 M Tris buffer, pH 7.7, and treated with 30 ml. of ammonium sulfate solution. The heavy precipitate was removed by centrifugation. The supernatant solution (96 ml.) was treated with 16 ml. of ammonium sulfate solution, and the precipitate again was discarded. To the second supernatant solution (111 ml.) were added 79 ml. of ammonium sulfate solution. The flocculent precipitate was collected and dissolved in water ("Ammonium sulfate III," 7.1 ml.).

**Ammonium Sulfate IV, Heated**—The protein concentration was determined (11), and the solution was diluted with 0.1 M sodium acetate, pH 7.7, to a protein concentration of 4 mg. per ml. To this solution (14.2 ml.) 12.9 ml. of acetone (−10°) were added rapidly, and the mixture was centrifuged for 3 minutes at 13,000 × g. Acetone (4.2 ml.) was added to the supernatant solution (25.2 ml.) in the same way. Both fractions were dissolved in water (2.5 ml.) and immediately assayed.³

The first acetone fraction (2.6 ml.) was treated with 6.5 ml. of 0.04 M Tris buffer, pH 7.7, and 6.13 ml. of ammonium sulfate solution. The precipitate was removed by centrifugation, and 10.3 ml. of ammonium sulfate solution were added to the supernatant solution (14.5 ml.). The precipitate was dissolved in water (1.0 ml.). This fraction was then heated for 1 hour at 60°, after which the small precipitate was removed by centrifugation at 13,000 × g for 5 minutes ("Ammonium sulfate IV, heated," 0.95 ml.).

The above procedure results in a purification of approximately 50-fold (Table II).

**Properties of Enzyme Preparation**—The final preparation is stable when stored at −10°. After 2 months under such conditions, no loss of activity has been noted. While the enzyme is stable to prolonged heating at 60° at pH 6.5, it is completely destroyed in 10 minutes at this temperature at pH 5.1. At 70° the enzyme is rapidly destroyed even at pH 6.5.

The final preparation is free of myokinase activity, but is still contaminated with inorganic pyrophosphatase and adenase. The latter enzyme is very active in extracts of *A. vinelandii*; a procedure for its purification will be published separately.

³ This procedure is not reproducible, and the enzyme has been found to appear in either acetone fraction. The yield has varied from 40 to 100 per cent of the activity initially present in Ammonium sulfate III. The acetone fraction is unstable, and the enzyme cannot be stored at this stage.
**Effect of pH and Metal Requirement**—The reaction is most rapid at about pH 7.8. The rate drops sharply above this pH and below pH 7.0. As shown in Fig. 2, the enzyme is completely inactive in the absence of Mg++. The dissociation constant, $K_m$, for Mg++ calculated from the Lineweaver and Burk plot (17) was about $1.1 \times 10^{-4}$ M. Other divalent metals were

![Graph](image)

**Fig. 2.** The determination of $K_m$ for Mg++. The conditions used were as follows: 0.6 μmole of AMP, 0.1 μmole of ATP, 10 μmoles of buffer, 0.5 μl. of Ammonium sulfate III, and Mg++ in a total volume of 0.1 ml.

**TABLE III**

<table>
<thead>
<tr>
<th>Metal addition</th>
<th>Activity (μmoles base per ml. enzyme per hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>650</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>595</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>580</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>450</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0</td>
</tr>
</tbody>
</table>

The conditions used were as follows: 0.4 μmole of AMP and ATP, 1 μmole of metal salts, and 12 μmoles of Tris buffer, pH 7.8, in a total volume of 0.2 ml. Temperature 37.5°. The enzyme was Ammonium sulfate IV (specific activity = 140).

It was observed that Mn++ markedly inhibits adenase, and this ion was useful for preventing the deamination of adenine to hypoxanthine, since the removal of adenase from 5'-AMP ribosidase was not complete.
Nature of Substrate—With crude extracts of *A. vinelandii* there was formation of reducing sugar and free base in the presence of ADP. However, after removal of myokinase by prolonged heating, it became evident that ADP was not a substrate for 5'-AMP ribosidase. In order to obtain reducing sugar, or free base, both AMP and ATP were necessary, and no other combination of adenine nucleotides would serve. With increasing concentration of ATP, the rate of free base formation increased (Fig. 3).

![Graph](image)

**Fig. 3**. Effect of ATP concentration on the rate of base formation from AMP. The incubation mixture contained 0.8 μmole of AMP, ATP as indicated, 1 μmole of MgCl₂, 12 μmoles of Tris buffer, pH 7.95, 4.8 γ of enzyme (Ammonium sulfate III), and water up to 0.2 ml. Assay for total free base was carried out as described under "Methods." In another experiment the concentration of MgCl₂ was reduced to 5 × 10⁻⁴ M, and the same *Kₘ* value for ATP was obtained. Ordinate values are per ml. of enzyme.

**Fig. 4**. Effect of AMP concentration on the rate of base formation. The incubation mixture contained 0.2 μmole of ATP and varying amounts of AMP as indicated. The enzyme added was 1 μl. of Ammonium sulfate III. All other conditions were as in Fig. 3.

The value of ½*Vₘₐₓ* for ATP was about 1 × 10⁻⁴ M, with complete saturation at approximately 3 × 10⁻⁴ M ATP. The corresponding curve for AMP is presented in Fig. 4. Here the value of ½*Vₘₐₓ* is about 6 × 10⁻⁴ M.

Conclusive evidence has been obtained that the reducing sugar and purine base arise by cleavage of AMP. As shown in Fig. 5, 0.91 μmole of AMP in the presence of 4 μmoles of ATP yielded 0.93 μmole of base (Curve A), and doubling the AMP concentration resulted in exactly twice as much free base formation. As shown in Table IV, the role of ATP appears to be catalytic. In the presence of 8 μmoles of AMP and 2 μmoles of ATP, 7 μmoles of base were formed. Further evidence that AMP was actually the only source of base and reducing sugar was obtained by using C¹⁴-la-
beled nucleotides. Only in the presence of C^{14}-AMP is there a formation of labeled base and labeled ribose 5-phosphate (Table V). When C^{14}-ATP was used with unlabeled AMP, no C^{14} appeared in the free base or ribose 5-phosphate.

![Graph](image)

**Fig. 5.** Formation of free base as a function of time and AMP concentration. Each ml. of incubation mixture contained 10 μmoles of MgCl₂, 40 μmoles of Tris buffer, pH 7.95, enzyme, and nucleotides. Curve A, 0.91 μ mole of AMP and 4 μmoles of ATP per ml. were present; Curve B, 1.82 μmoles of AMP and 4 μmoles of ATP per ml. were added. Incubation was at 37.5°. Aliquots were removed at the times indicated, and determinations for free base (adenine plus hypoxanthine) were performed. The arrows indicate the concentrations of free base equivalent to the AMP added.

**TABLE IV**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Base formed, μmoles per ml.</th>
<th>7 min.</th>
<th>20 min.</th>
<th>100 min.</th>
<th>180 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, 8.0 + AMP, 2.0</td>
<td></td>
<td>0.4</td>
<td>1.4</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>&quot; 2.0 + &quot; 8.0</td>
<td></td>
<td>0.6</td>
<td>1.8</td>
<td>5.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

The additions were as follows: nucleotide as above, 0.001 M Mg^{2+}, 0.06 M Tris buffer, pH 7.9, and 2 μl. of enzyme (Ammonium sulfate III, 516 units per ml., specific activity = 100) in a total volume of 0.2 ml.

**Specificity of Reactants**—The following compounds did not replace AMP: adenosine, inosine, adenosine 3-phosphate, deoxyadenosine, deoxyuridine, deoxycytidylate, thymidylate, deoxyguanosine, deoxyadenylate, GMP, IMP, CMP, UMP, and AMP polymer (16). The dinucleotides 5′-phosphoadenosine 3′-adenosine 5′-phosphate and 3′-phosphouridine 5′-adenosine 3′-phosphate were also inactive. The following compounds did not replace ATP: GTP, ITP, CTP, UTP, 1-pyrophosphoryl ribose 5-phosphate, ribose triphosphate. In addition, various combinations such as GTP and GMP, UTP and UMP, and CTP and CMP did not yield either reducing sugar or free base.
It was found that inorganic pyrophosphate, tripolyphosphate, and adenosine tetraphosphate could partially or completely replace ATP. In the case of adenosine tetraphosphate, the maximal rate was the same as that with ATP, and the concentration required to give $\frac{1}{2}V_{\text{max}}$ was approximately $5 \times 10^{-4}$ M, compared with $1 \times 10^{-4}$ M for ATP. These results would be obtained if adenosine tetraphosphate were contaminated with 

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Total counts, c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base</td>
</tr>
<tr>
<td>C$^{14}$-AMP + ATP + enzyme (no enzyme)</td>
<td>10,000</td>
</tr>
<tr>
<td>C$^{14}$-ATP + AMP + enzyme (no enzyme)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The following additions were made in the case of C$^{14}$-AMP experiment: 0.4 μmole of C$^{14}$-AMP (uniformly labeled), 0.4 μmole of ATP, 1 μmole of Mg$^{2+}$, 10 μmoles of Tris buffer, pH 7.8, and 1.5 units of enzyme (Ammonium sulfate IV, heated, 11.8 g of protein) in a total volume of 0.145 ml. The reaction was terminated after 60 minutes at 38° by the addition of 0.05 ml. of 1 N HClO$_4$. The adenine compounds were adsorbed on charcoal (0.05 ml. of a 30 per cent suspension). The supernatant solution from the mixture incubated with enzyme contained 0.31 μmole of orcinol-reactive material. This was counted as such and is designated above as the “Ribose 5-phosphate” fraction. Charcoal pellet was washed three times with 3 ml. aliquots of H$_2$O. The charcoal was then treated with 0.5 ml. of ethanolic NH$_3$. Aliquots were chromatographed on Whatman No. 3 MM paper for 18 hours (descending) in Solvents 3 and 4 (see under “Methods”). In the neutral solvent adenine and hypoxanthine do not separate. This area was eluted and is listed as “Base” above. The ultraviolet spectrum-absorbing material corresponding to AMP and ATP was eluted quantitatively from the isobutyric acid solvent and counted. In the case of the C$^{14}$-ATP experiment with enzyme, 0.25 μmole of orcinol-reactive material was found in the supernatant solution after charcoal treatment. All other conditions were the same as those used in the C$^{14}$-AMP experiment.

ATP to the extent of 20 per cent. However, this degree of contamination is unlikely, and it is probable that adenosine tetraphosphate is active in the reaction. With inorganic pyrophosphate and tripolyphosphate, the reaction was carried out in the presence of 0.025 M sodium fluoride in order to inhibit pyrophosphatase. This level of fluoride was found to have no effect on 5'-AMP ribosidase when tested with ATP. The activity with inorganic pyrophosphate and tripolyphosphate depended largely on their ratio to Mg$^{2+}$. With 5 μmoles per ml. of both Mg$^{2+}$ and inorganic pyrophosphate, a rate of AMP cleavage of 170 units per ml. of enzyme was
obtained. With ratios of Mg$^{++}$ to pyrophosphate of 2:1 and 1:2, the rate
was only one-third as fast. With 0.01 M Mg$^{++}$ and 0.01 M inorganic pyro-
phosphate, the rate was 310 units per ml., which is to be compared with
890 units per ml. obtained with ATP. Variations in the concentration
of MgCl$_2$ had similar but quantitatively smaller effects with tripolyphos-
phate. The $K_m$ for tripolyphosphate was $1 \times 10^{-3}$ M, and the optimal
rate was 50 per cent of that obtained with ATP.

**Stoichiometry of Reaction**—Starting with AMP and ATP, there was a
disappearance of AMP with the appearance of equal amounts of reducing
sugar and free base. In addition, ATP was quantitatively recovered,
indicating that this compound was not utilized during the course of the
reaction (Table VI). Chromatography of reaction mixtures (Dowex 1,
Cl$^-$) after incubation with 5'-AMP ribosidase confirmed this finding.
Quantitative elution of the ATP area as described by Cohn and Carter (18)
yielded a compound with a base-pentose-labile P-organic P ratio of approxi-
mately 1:1:2:3. Ion exchange chromatography gave no evidence for any
intermediate reaction product derived from ATP and AMP. In other
experiments, the reaction was halted after various time intervals, and the
reaction mixture was examined by paper chromatography in Solvents 1,
2, 3, and 4. The only changes detected by ultraviolet examination were
AMP disappearance matched by the appearance of adenine and hypoxan-
thine. No ultraviolet spectrum-absorbing material of unique $R_F$, which
might represent an intermediate of the reaction, was found.

Similar data for inorganic pyrophosphate and tripolyphosphate are also
shown in Table VI. In addition, there was no change in the 10 minute
labile P, suggesting that no utilization of these compounds occurred during
the reaction.

**Identification of Products**—The evidence for ribose 5-phosphate as one
of the products of AMP cleavage is summarized in Table VII. The amount
of reducing sugar formed with ribose 5-phosphate as the standard agreed
well with that obtained by using the phosphoribulokinase assay. In the
latter case, ribulose 5-phosphate, as well as ribose 5-phosphate, is active
due to the presence of phosphoribosimerase in the kinase preparation.
That the product is not ribulose 5-phosphate is indicated by the results in
the orcinol test, since the ketopentose esters yield only 57 per cent of the
color of the aldopentose esters (19). In addition, the product did not give
the cysteine-carbazole test (20) which is given by ketopentoses (21). In
view of the agreement between the methods used for determining the
pentose phosphate, the reducing sugar is most likely ribose 5-phosphate.

The other product is adenine. This was established by paper chro-
matography, as well as by the action of the enzyme adenase which con-
taminates the enzyme preparation. Chromatographic identification was
TABLE VI
Stoichiometry of Splitting of 5'-AMP by 5'-AMP Ribosidase

<table>
<thead>
<tr>
<th>Compound analyzed</th>
<th>With sodium pyrophosphate</th>
<th>With sodium tripolyphosphate</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles per ml.</td>
<td>μmoles per ml.</td>
<td>μmoles per ml.</td>
<td>μmoles per ml.</td>
</tr>
<tr>
<td>ΔAMP</td>
<td>-2.50</td>
<td>-3.55</td>
<td>-1.75</td>
<td>-3.40</td>
</tr>
<tr>
<td>Δ adenine</td>
<td>+2.4</td>
<td>+3.2</td>
<td>+2.16</td>
<td>+3.30</td>
</tr>
<tr>
<td>Δ reducing sugar</td>
<td>+2.75</td>
<td>+3.75</td>
<td>+2.07</td>
<td>+3.78</td>
</tr>
<tr>
<td>ΔATP</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Δ inorganic phosphate</td>
<td>+0.3</td>
<td>+0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Δ acid-stable phosphate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Δ acid-labile</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The reaction mixture (total volume = 1.5 ml.) contained 90 μmoles of Tris buffer, pH 7.8, 60 μmoles of MgCl₂, 37.5 μmoles of NaF, 6 μmoles of 5'-AMP, 6.0 μmoles of either sodium pyrophosphate or sodium tripolyphosphate, adjusted to pH 7.8, and 6.4 γ of enzyme (Ammonium sulfate IV, heated). The incubation was for 3.5 hours at 37.5°. In the reaction with ATP, 2.2 μmoles of ATP, 4.4 μmoles of AMP, 100 μmoles of Tris buffer, 5 μmoles of MgCl₂, and 5 μl. of Ammonium sulfate IV, heated, in a total volume of 1 ml. were incubated for 20 and 55 minutes in Experiments 1 and 2, respectively. All procedures used are summarized under “Methods.” The results are given as change (Δ) in concentration of the various substances measured.

TABLE VII
Identification of Ribose 5-Phosphate

<table>
<thead>
<tr>
<th>Method</th>
<th>μmoles per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugar formed</td>
<td>5.02</td>
</tr>
<tr>
<td>Orcinol-reactive material</td>
<td>5.54</td>
</tr>
<tr>
<td>Phosphoribokinase activity</td>
<td>5.14</td>
</tr>
</tbody>
</table>

20 μmoles of AMP, 20 μmoles of ATP, 20 μmoles of Tris buffer, pH 7.8, 2 μmoles of Mg²⁺, and 23 γ of Ammonium sulfate IV, heated (30 units), in a total volume of 0.27 ml. were incubated for 90 minutes at 38°. The reaction was terminated with 0.015 ml. of 1 N HClO₄ and 0.1 ml. of 30 per cent charcoal; after 1 hour at 0°, the charcoal was collected by centrifugation and washed with H₂O. The supernatant solutions were combined and neutralized with KOH; after 15 minutes at 0°, KClO₄ was removed by centrifugation. A total of 14 μmoles of orcinol-reactive material was present. The incubation with phosphoribokinase was carried out as previously described (4), and the reaction was followed by ADP formation.
obtained by using Solvents 1 and 2 as described under "Methods." Further, the compound had the electrophoretic mobility (12) and absorption spectrum characteristic of adenine.

**Inhibitors of Reaction**—As shown in Table VIII, orthophosphate markedly inhibited the cleavage of AMP when ATP was used as the cofactor. This inhibition appears to be due to a competition between ATP and orthophosphate for the enzyme. $7.5 \times 10^{-3}$ M phosphate gave 100 per cent inhibition when the APP concentration was $0.5 \times 10^{-3}$ M; with increasing concentrations of ATP, less inhibition was obtained. All attempts to demonstrate the uptake of phosphate were negative. No phosphate disappeared as measured with the Lowry and Lopez phosphate procedure (8).

**TABLE VIII**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Rate of purine base formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>$\mu \times 10^8$</td>
<td>$\mu \times 10^8$</td>
</tr>
<tr>
<td>0.5</td>
<td>4.5</td>
</tr>
<tr>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

To the above nucleotides 12 $\mu$moles of Tris buffer, pH 7.95, 2 $\mu$moles of Mg$^{++}$, 2 $\mu$l of Ammonium sulfate III (15 $\gamma$ of protein), orthophosphate, and water in a total volume of 0.2 ml. were added. The incubation time was 16 minutes at 37.5°. Controls lacking phosphate were run at each level of nucleotide indicated. The final phosphate concentration when added was $7.5 \times 10^{-2}$ M.

or by the use of P$^{32}$. Arsenate was also found to inhibit, but was only approximately 50 per cent as effective as phosphate under identical conditions.

IMP was also found to inhibit the cleavage of AMP. With $2 \times 10^{-8}$ M IMP and $5 \times 10^{-3}$ M ATP and $2 \times 10^{-3}$ M AMP, inhibition was 40 per cent. This inhibition was overcome by increasing the concentration of either AMP or ATP. UMP was without effect under the conditions found to be inhibitory with IMP.

**DISCUSSION**

The mechanism whereby AMP is hydrolyzed to adenine and ribose 5-phosphate is clearly not due to any previously described enzymatic reaction. The system described by Kornberg, Lieberman, and Simms (1), in which AMP is phosphorolytically cleaved to form adenine and 1-pyrophosphoryl ribose 5-phosphate, was excluded for several reasons. No evidence for
the formation of AMP from adenine and 1-pyrophosphoryl ribose 5-phosphate was found after prolonged incubation. Furthermore, 1-pyrophosphoryl ribose 5-phosphate, which is non-reducing, was not cleaved by the 5'-AMP ribosidase preparation to yield a reducing sugar and therefore could not have been formed as an intermediate in the reaction.

Another possibility to consider is hydrolysis of AMP to form adenosine, phosphorolysis of adenosine to give ribose 1-phosphate, and conversion of ribose 1-phosphate to ribose 5-phosphate. This was also excluded, for no phosphorolysis of adenosine occurred, nor could any adenosine be detected chromatographically during the course of the reaction. Further evidence against this mechanism is the finding that C\textsuperscript{14}-ribose 5-phosphate was not incorporated into a material adsorbable on charcoal (adenosine).

The conversion of AMP to adenine and ribose 5-phosphate appears to be irreversible, for all attempts to demonstrate the formation of AMP from adenine and ribose 5-phosphate were unsuccessful. With C\textsuperscript{14}-labeled ribose 5-phosphate or adenine, no radioactivity was incorporated into AMP or ATP during the course of the reaction. In addition, as shown in Table V, incubation with randomly labeled AMP and unlabeled ATP did not result in the incorporation of radioactivity into the ATP. The reverse experiment with labeled ATP and unlabeled AMP demonstrated that C\textsuperscript{14}-ATP did not yield any radioactivity in the ribose 5-phosphate and free base formed, thus eliminating the interconversion of ATP and AMP. In view of the activity with pyrophosphate, experiments with P\textsubscript{32}-labeled inorganic pyrophosphate\textsuperscript{4} were also carried out. However, no counts were incorporated into any of the nucleotides or into ribose 5-phosphate.

At present the nature of the ATP requirement is not known. This requirement cannot be met by preliminary incubation of the enzyme preparation with ATP, followed by removal of the ATP with hexokinase and glucose. The possibility that ATP, as well as adenosine tetraphosphate, pyrophosphate, and tripolyphosphate, exerts its effect by forming a complex with some potent inhibitor present in the enzyme preparation does not appear reasonable in view of the inactivity of other nucleoside triphosphates and EDTA.

It is interesting to note that Muntz (22) has described a deaminase for AMP which also requires ATP for activity. It has also been found that in this reaction there is no utilization of ATP (23, 24). Ishikawa and Komita (25, 26) have obtained a ribose ester and free base by the action of fractions from calf spleen and other sources on guanosine 2'- or 3'-phosphate. However, as Schlenk (27) has pointed out, their data can be explained adequately by dephosphorylation of the nucleotide, followed by

\textsuperscript{4} P\textsubscript{32}-labeled pyrophosphate was synthesized by a procedure kindly supplied by Dr. Paul Berg.
phosphorolysis of the resulting nucleoside. The reaction described in the present investigation thus represents the first clear-cut instance of mononucleotide hydrolysis at the glycosidic linkage.

SUMMARY

1. An enzyme preparation has been purified 50-fold from Azotobacter vinelandii. This preparation catalyzes the cleavage of adenosine 5'-monophosphate (AMP) to adenine and ribose 5-phosphate. The enzyme shows no activity with AMP unless catalytic quantities of adenosine 5'-triphosphate (ATP) are added. The activating effect of ATP is also shown by adenosine tetraphosphate, inorganic pyrophosphate, and inorganic tripolyphosphate. Metaphosphate and other nucleoside polyphosphates are inactive. ATP and adenosine 5'-diphosphate (ADP) are not cleaved by the enzyme even in the presence of an excess of AMP. Isotope experiments have shown that ATP and inorganic pyrophosphate do not participate in the reaction, and the nature of the activating effect is not clear. Under the conditions studied, the reaction is irreversible.

2. Of a large number of compounds tested, only AMP is cleaved.

BIBLIOGRAPHY

THE ENZYMATIC CLEAVAGE OF ADENYLIC ACID TO ADENINE AND RIBOSE 5-PHOSPHATE
Jerard Hurwitz, Leon A. Heppel and B. L. Horecker


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