ENZYMATIC PHOSPHORYLATION OF ADENOSINE AND 2,6-DIAMINOPURINE RIBOSIDE

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Knowledge concerning the nature of the biologic synthesis of purine or pyrimidine mononucleotides is relatively limited. A significant contribution was the demonstration by Ostern and coworkers (3) of the phosphorylation of adenosine to adenosine-5'-phosphate (A-5'-P) and adenosine-triphosphate (ATP) by means of crude yeast extracts. The energy source for this reaction was supplied by the fermentation of hexose diphosphate or phosphoglyceric acid. These extracts did not act on guanosine or ribose and failed to form yeast adenylic acid. Recently, Sable (4) observed the disappearance of ATP in the presence of crude yeast fractions and adenosine. He suggested the direct phosphorylation of adenosine by ATP and the name adenosine phosphokinase for this enzyme.

In order to provide some definitive information on the mechanism of conversion of adenosine to adenosine-5'-phosphate, we have purified this activity from yeast autolysates. An enzyme preparation has been obtained which catalyzes the reaction

\[ \text{Adenosine} + \text{ATP} \rightarrow \text{adenosine-5'-phosphate} + \text{adenosinediphosphate (ADP)} \]

In conjunction with phosphopyruvate, pyruvate phosphokinase from muscle, and myokinase, this enzyme preparation catalyzes the synthesis of ATP from adenosine according to Equation 4 (sum of Equations 1, 2, and 3).

\[ 3 \text{phosphopyruvate} + 3\text{ADP} \xrightarrow{\text{pyruvate phosphokinase}} 3 \text{pyruvate} + 3\text{ATP} \]

\[ \text{ATP} + \text{A-5'-P} \xrightarrow{\text{myokinase}} 2\text{ADP} \]

(4) \[ \text{Adenosine} + 3 \text{phosphopyruvate} \rightarrow \text{ATP} + 3 \text{pyruvate} \]

The same enzyme preparation under similar conditions also brings about the phosphorylation of 2,6-diaminopurine riboside (2-aminoadenosine) to 2-aminoATP, as in Equation 5.

* These findings were reported by one of us (1). In the same month, similar findings were reported by Caputto (2), who demonstrated the phosphorylation of adenosine by ATP to yield adenosine-5'-phosphate with enzymes derived from liver and kidney as well as from yeast.
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(5) 2-Aminoadenosine + 3 phosphopyruvate → 2-aminoATP + 3 pyruvate

Of seventeen nucleosides tested, the enzyme reacts only with adenosine and 2-aminoadenosine and the transfer of phosphate is limited to the 5' carbon of the nucleoside.

Methods

Materials—ATP and ADP were purified from commercial products (Sigma) by ion exchange chromatography (5). Adenosine (recrystallized twice from water), guanosine, uridine, cytidine, yeast adenylc acid, and d-ribose were products of the Nutritional Biochemicals Corporation. Desoxyadenosine was prepared from desoxyadenylc acid and inosine from inosinic acid by quantitative removal of the phosphate with lyophilized snake venom (Crotalus adamanteus), which contains a 5'-nucleotidase (6). 2,6-Diaminopurine riboside and other synthetic nucleosides were generously provided by Dr. J. Davoll and Dr. G. B. Brown. Reduced di-phosphopyridine nucleotide (DPNH) (purity 0.68) was prepared by Ohlmeyer's method (7). Triphosphopyridine nucleotide (TPN) (purity 0.86) was obtained by ion exchange chromatography of a crude liver fraction. Phosphopyruvic acid was prepared by Ohlmeyer's modification of Kiesling's method and kindly supplied to us by Dr. F. Ohlmeyer.

Pyruvate phosphokinase, the enzyme catalyzing the transfer of phosphate from phosphopyruvate to ADP to form pyruvate and ATP, was purified from rabbit muscle by a modification of the procedure described by Kubowitz and Ott for human muscle (8).

(a) Assay—The incubation mixture (3.0 cc.) for enzyme assay contained 0.1 cc. of phosphopyruvate (0.005 M), 0.1 cc. of MgCl₂ (0.1 M), 0.1 cc. of DPNH₂ (0.002 M), 0.2 cc. of ADP (0.0015 M), 0.2 cc. of phosphate buffer (0.5 M, pH 7.0), and 0.02 cc. of lactic dehydrogenase (final fraction diluted 1:40). A unit of enzyme activity was defined as the amount producing 1 μM of pyruvate (determined as the oxidation of 1 μM of DPNH₂ at 340 mp) per minute at 21–23°, corrected when necessary for a blank value owing to the contamination of the lactic dehydrogenase preparation by this activity.

(b) Purification—Ground rabbit muscle (631 gm.) was stirred at 2° for 30 minutes with 3 volumes of 0.1 M KH₂PO₄ and then centrifuged. The turbid extract (1730 cc., 30 units per cc., 3.3 units per mg. of protein) was fractionated with acetone (8). The acetone fraction (Acetone I, 71 cc., 800 units per cc., 11.5 units per mg. of protein) was stable for months when stored at -15°. (Fractional precipitation with nucleic acid at this point, as in the method of Kubowitz and Ott, was not successful.) An

1 Kindly furnished by Dr. C. E. Carter and Dr. W. E. Cohn.
2 Kornberg, A., and Horecker, B. L., unpublished.
inactive precipitate which appeared on thawing the acetone fraction was removed and a second acetone precipitation was carried out. 60 cc. were diluted with water to 240 cc. Acetone (112 cc.) was added slowly while the temperature was maintained at about $-2^\circ$, and the resulting precipitate, collected by centrifugation, was dissolved in water with the aid of a few drops of 0.1 N NaOH (Acetone II, 60 cc., 440 units per cc., 26.2 units per mg. of protein). This fraction was diluted with an equal volume of cold water and treated with 180 cc. of calcium phosphate gel (1.42 gm., dry weight, aged for several months) (9). After 10 minutes at room temperature the gel was removed by centrifugation and the supernatant adjusted to pH 4.6 with acetate buffer (1 M, pH 4.0). Ammonium sulfate solution (saturated at $2^\circ$, 333 cc.) was added and the precipitate which formed was removed by centrifugation. 67 cc. of saturated ammonium sulfate were added and the resulting precipitate, collected by centrifugation, was dissolved in water with the aid of a few drops of 0.5 M phosphate buffer, pH 7.0 (ammonium sulfate fraction, 30 cc., 424 units, 57.3 units per mg. of protein). This fraction, free of myokinase, approximates in purity the crystalline preparation of Kubowitz and Ott and retains its activity for several months on storage at $-15^\circ$.

Adenosine deaminase was obtained by dissolving 100 mg. of Armour intestinal phosphatase (stated to contain 15 Schmidt-Thannhauser units (10) of phosphatase activity per mg.) in 10 cc. of ammonium acetate buffer (0.02 M, pH 8.0) and dialyzing this solution against 0.04 M sodium acetate for 3 hours at $2^\circ$. This solution can be stored at $-15^\circ$. When tested in the presence of 0.05 M phosphate buffer, pH 7.4, the deamination of adenosine is rapid, while phosphatase activity (determined with adenosine-5'-phosphate as substrate under similar conditions of incubation) is not detectable. The use of phosphate buffer thus avoids the phosphatase interference, and, in this instance, the need for further purification steps (11).

Lactic dehydrogenase was prepared by ammonium sulfate fractionation of rabbit muscle extract.³

(a) Assay—The incubation mixture (3.0 cc.) for enzyme assay contained 0.1 cc. of pyruvate (0.01 M), 0.1 cc. of DPH$_2$ (0.002 M), 1.0 cc. of phosphate buffer (0.1 M, pH 7.4), and enzyme previously diluted with cold 0.01 M NaCl. A unit of enzyme activity was defined as the amount required to oxidize 1 $\mu$M of DPH$_2$ (determined at 340 m$\mu$) per minute at 21-23$^\circ$.

(b) Purification—Rabbit muscle extract was fractionated with ammonium sulfate between saturations of 0.52 and 0.72 according to Cori et al.

³ This method was suggested by Dr. C. F. Cori and developed in his laboratory by one of us (A. K.).
NUCLEOSIDE PHOSPHORYLATION (12) with or without prior crystallization of aldolase (13). The precipitate may be stored as a paste at 3° for many months. 57.5 gm. of the 0.52 to 0.72 fraction, derived from 500 gm. of muscle, were dissolved in cold water and the small amount of insoluble material was removed by filtration (92 cc., 725 units per cc., 12 units per mg. of protein). Three successive ammonium sulfate fractionations were then carried out, the lactic dehydrogenase appearing each time in the least soluble fraction. 39 cc. of ammonium sulfate (saturated at 2° and kept cold) were added slowly at 0-2°. After exposure to room temperature for 15 minutes, the temperature of the solution rose to 12° and the resulting precipitate was collected by centrifugation and dissolved in water (16.5 cc., 3430 units per cc., 54 units per mg. of protein). To 15 cc. of this fraction, 5.0 cc. of saturated ammonium sulfate were added dropwise and the slightly turbid solution was exposed to room temperature for 30 minutes (during which time the temperature of the solution rose to 18°). The resulting precipitate was centrifuged and dissolved in water (15 cc., 2950 units per cc., 114 units per mg. of protein). This last fractionation was then repeated, yielding a final fraction (7.5 cc., 4180 units per cc., 220 units per mg. of protein). The over-all yield was approximately 50 per cent and the purity, based on the turnover number cited by Kubowitz and Ott (14) for the crystalline enzyme from rat muscle, was estimated to be about 80 per cent. The final rabbit muscle fraction is stable for months at 3° or -15°, but is unstable in high dilutions. Lactic dehydrogenase, once recrystallized, was prepared in this laboratory from beef heart according to Straub's procedure (15). This preparation contained only 136 units per mg. of protein and the yield, referred to fresh weight of muscle, was only one-tenth as great as that obtained from rabbit muscle by our procedure.

**Hexokinase (Fraction 3)** was prepared according to Berger et al. (16), myokinase (ammonium sulfate fraction) according to Colowick and Kalckar (17), and **Zwischenferment** as previously described (18).

**Determinations**—ATP was determined spectrophotometrically as previously described (19). In the absence of myokinase, the successive action of hexokinase and **Zwischenferment** on glucose and glucose-6-phosphate, respectively, resulted in the reduction of 1 mole of TPN per mole of ATP. In the presence of myokinase, ADP produced by the action of hexokinase is activated (17, 20) and 2 moles of TPN are reduced per mole of ATP. The term "kinase-labile phosphate" refers to the phosphate transferred to glucose (as determined by TPN reduction) by ATP and ADP when both hexokinase and myokinase are present.

**ADP** was determined in this system by carrying out the reaction with and without the use of myokinase. ADP was estimated with greater accuracy by the use of pyruvate phosphokinase freed of myokinase and phosphatases. ADP is the specific acceptor and, for every mole present,
1 mole of pyruvate appears, the latter being measured spectrophotometrically by the oxidation of DPNH₂ in the presence of lactic dehydrogenase. With the same system, phosphopyruvate was determined, an excess of ADP being present. Estimations of iodine-labile phosphate (21) as a measure of phosphopyruvate were in close agreement with enzymatic assay values.

Adenosine was determined spectrophotometrically by the reduction in density at 265 μm resulting from the action of adenosine deaminase (22). In 3.0 cc. of phosphate buffer (0.05 M, pH 7.4) at room temperature, 20 γ of the deaminase preparation completed the deamination of 0.15 μM of adenosine in 8 minutes. 2-Aminoadenosine is also acted on by adenosine deaminase preparations to yield guanosine. The ratio of the guanosine and 2-aminoadenosine extinction coefficients was observed to be 1.45 at 255 μm, 1.00 at 274 μm, and 0.47 at 290 μm. The deaminase action on 2-aminoadenosine was weaker than on adenosine and therefore about 10 times as much enzyme was used in estimations of 2-aminoadenosine. Orthophosphate was determined by the method of Fiske and Subbarow (23); acid-labile phosphate was the phosphate liberated after 10 minutes hydrolysis in 1 N HCl at 100°, and total phosphate was that determined after ashing with a sulfuric acid-nitric acid mixture. Ion exchange chromatography was carried out on Dowex-1 (200 to 400 mesh, chloride form) columns according to Cohn (24). Protein was determined nephelometrically (25) at 340 μm and by a modified phenol method (26).

Assay of Adenosine Phosphokinase—The incubation mixture contained 0.1 cc. of succinate buffer (0.33 M, pH 6.0), 0.05 cc. of adenosine (0.017 M), 0.01 cc. of MgCl₂ (0.1 M), 0.01 cc. of MnCl₂ (0.02 M), 0.02 cc. of glutathione (0.16 M), 0.01 cc. of ATP (0.002 M), 0.025 cc. of phosphopyruvate (0.06 M), 0.02 cc. of myokinase, 0.05 cc. of pyruvate phosphokinase (Acetone I), and water and adenosine phosphokinase to a volume of 0.5 cc. (Glutathione was included to activate myokinase (17).) After 20 minutes at 21-23° the incubation mixture was placed in a boiling water bath for 3 minutes, centrifuged, and the supernatant analyzed for kinase-labile phosphate. A unit of enzyme activity was defined as the amount producing 1 μM of kinase-labile phosphate during the test period. When ADP and ATP were to be determined separately, the incubation mixture was treated with 0.5 cc. of 6 per cent perchloric acid and 0.5 cc. of bovine serum albumin (10 mg. per cc.) and centrifuged; the neutralized supernatant was used for assay.

Results

Purification of Enzyme—Autolysates of dried bakers’ yeast and of several dried beer and ale yeasts were all found to be active. 25 gm. of the dried

4 Personal communication from Dr. G. B. Brown.
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lager beer yeast,\textsuperscript{5} which yielded the most active autolysate, were sus-
pended in 75 cc. of 0.1 M sodium bicarbonate and incubated for 6 hours at 34°C. The mixture was centrifuged and yielded approximately 40 cc. of clear yellow autolysate (51.2 units per cc., 1.1 units per mg. of protein). All subsequent operations were performed at 2°C, unless otherwise specified. To 40 cc. of autolysate were added 40 cc. of water, 20 cc. of 1 N acetic acid, and then 8 cc. of nucleic acid solution (Merck, 50 mg. per cc., pH 5.0). After 5 minutes, 40 cc. of 2 N acetic acid were added and the precipitate was removed by centrifugation. The pH of the clear supernatant, which was approximately 4.4, was raised to 6.3 by the addition of about 12 cc. of 2 N NaOH. Salmine sulfate (Lilly, 100 mg. in 4 cc.) was added; 5 minutes later the precipitate was removed by centrifugation, yielding 120 cc. of supernatant (12.6 units per cc., 3.8 units per mg. of protein). The pH was adjusted to 5.1 with 7.0 cc. of 2 N acetic acid, the solution cooled to $-0.5^\circ$, and ethanol added with mechanical stirring. The temperature was maintained just above the freezing point during the early ethanol addition and at $-5^\circ$ thereafter. The precipitates were centrifuged at $-5^\circ$. 45 cc. of 50 per cent ethanol and then 7.0 cc. of zinc chloride (0.5 M) were added (27). After 5 minutes the precipitate was removed by cen-
trifugation. To the supernatant were added 10 cc. of 50 per cent ethanol, and after 5 minutes the precipitate was again removed by centrifugation. The addition of 60 cc. of ethanol to the supernatant produced a precipitate which was collected by centrifugation and dissolved in citrate buffer (0.05 M, pH 6.2) to a volume of 50 cc. (14.5 units per cc., 13.1 units per mg. of protein). Lyophilization yielded 930 mg. of a white powder which when stored in a vacuum desiccator over CaCl$_2$ at 2°C was stable for 8 months or more. (In the liquid state at 2°C about one-third of the activity was lost overnight.) The dry preparation was used throughout these studies.

The optical density, at 280 m$\mu$ in a light path of 1 cm., of a purified enzyme solution containing 1 mg. of protein per cc., was 6.4. The ratio of the density at 280 m$\mu$ to that at 260 m$\mu$ was 0.53, a value corresponding to that of nucleic acid. Efforts to remove this nucleic acid, as by the use of metal salts, protamine, and ion exchange resins, were unsuccessful.

Myokinase activity was identified in the final adenosine phosphokinase preparation, both by the production of ADP (from adenosine-5'-phosphate and ATP) and by the conversion of ADP to ATP (Fig. 1). The production of ADP was determined by phosphorylation with phosphopyruvate to yield ATP and pyruvate (the latter estimated spectrophotometrically by the oxidation of DPNH$_2$ in the presence of lactic dehydrogenase), a phosphate transfer which is specific for ADP (Curve 1). Since little ATPase activity was present in the purified enzyme preparation, this re-

\textsuperscript{5} Schwarz Laboratories, Inc.
suit can be attributed to myokinase-like action; an experiment in which adenosine-5'-phosphate was omitted initially (Curve 2) served as an additional control. The conversion of ADP to ATP was demonstrated by the phosphorylation of glucose by hexokinase, the glucose-6-phosphate formed being assayed spectrophotometrically by the reduction of TPN in the presence of Zwischenferment (Curve 3).

Like the muscle enzyme, yeast myokinase is relatively resistant to heating in acid solution. After treatment of muscle myokinase with 0.1 N HCl at 100° for 10 minutes (17), 66 per cent of the activity remained, while the yeast enzyme under identical conditions retained 50 per cent of its activity.

Phosphorylation of Adenosine by ATP—This reaction was demonstrated by the removal of adenosine and ATP and by the appearance of adenosine-5'-phosphate and ADP (Table I). While the presence of myokinase prevented a simple stoichiometric balance, the essential nature of the reaction was apparent. In Experiments I and II, the disappearance of adenosine is matched by the removal of phosphate transferable by hexokinase-myokinase action (kinase-labile phosphate) or labile to acid hydrolysis. In Experiment III the reaction mixture was analyzed chromatographically.

![Graph of Myokinase in yeast fractions](http://www.jbc.org/)
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on Dowex-1 by separation of discrete adenosine, adenosine-5'-phosphate, ADP, and ATP fractions. It may be noted that the values obtained were

Table I

Direct Phosphorylation of Adenosine by ATP

The values are expressed in micromoles.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Adenosine</th>
<th>A-5'-P</th>
<th>ADP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Controls</td>
<td>0.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ</td>
<td>-0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II Controls</td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ</td>
<td>-0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III 0 min.</td>
<td>12.5</td>
<td>0.0</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>30 &quot; *</td>
<td>6.9</td>
<td>7.1</td>
<td>5.4</td>
<td>2.5</td>
</tr>
<tr>
<td>30 &quot; †</td>
<td>-5.7</td>
<td>+7.1</td>
<td>+5.2</td>
<td>-7.7</td>
</tr>
</tbody>
</table>

* Assays of perchloric acid supernatant.
† Ion exchange analysis.
‡ The 30 minute value used in this calculation is an average of the values obtained by ion exchange analysis and assays of the perchloric acid supernatant.

Experiments I and II—The experimental incubation mixture contained 0.05 cc. of adenosine (0.017 M), 0.08 cc. (Experiment I) or 0.04 cc. (Experiment II) of ATP (0.011 M), 0.01 cc. of MgCl₂ (0.1 M), 0.01 cc. of MnCl₂ (0.02 M), 0.10 cc. of succinate buffer (0.33 M, pH 6.0), 0.20 cc. of adenosine phosphokinase (10 units per cc.), and water to a final volume of 0.50 cc. The incubation mixture in one control lacked adenosine and in the other it lacked ATP. After incubation for 20 minutes at 23°, 1.5 cc. of water were added and the mixture was immersed in a boiling water bath for 3 minutes.

Experiment III—The incubation mixture contained 0.75 cc. of adenosine (0.017 M), 0.40 cc. of ATP (0.025 M), 0.10 cc. of MgCl₂ (0.1 M), 0.20 cc. of MnCl₂ (0.01 M), 1.0 cc. of succinate buffer (0.33 M, pH 6.0), 2.0 cc. of adenosine phosphokinase (10 units per cc.), and water to a final volume of 5.0 cc. After 30 minutes incubation at 23°, an aliquot of 0.5 cc. was added to 0.5 cc. of 6 per cent cold perchloric acid. The neutralized perchloric acid supernatants were used for enzymatic and phosphate assays. The remainder of the mixture was immersed in a boiling water bath for 1.5 minutes. The heated sample was chromatographed on a Dowex-1 chloride column (5 cm. X 1 sq. cm.) (5). Adenosine and A-5'-P were eluted in succession with 0.008 N HCl (0 to 128 and 339 to 507 cc., respectively). ADP was eluted with 0.02 M NaCl in 0.01 N HCl (0 to 146 cc.) and ATP with 0.2 M NaCl in 0.01 N HCl (0 to 78 cc.).

in fair agreement with enzymatic assays of an aliquot of the reaction mixture. 22.5 μM of adenosine + ATP were added initially and 21.5 μM of adenosine + adenosine nucleotides were recovered at the end of the
reaction. It is noteworthy that no substance corresponding to adenylic acids a or b (presumably adenosine-2'-phosphate and adenosine-3'-phosphate, respectively) appeared in the chromatogram.

Starting with ADP, the phosphorylation of adenosine was 25, 34, and 39 per cent as rapid as with ATP in three experiments. The persistence of myokinase action in the purified enzyme preparation makes it difficult to determine whether ADP is a substrate for adenosine phosphokinase. Phosphorylation of Adenosine by Transfer from Phosphopyruvate—Phosphorylation of adenosine with phosphopyruvate as donor confirmed and amplified the results with ATP as phosphorylating agent. With crude yeast extracts and fractions rich in ATPase, the use of phosphopyruvate provided a feasible way of following adenosine phosphorylation, since it obviated the need for ATP (except in trace amounts as a primer) (Equations 1 to 4). With the purified enzyme preparation, no reaction took place unless additions of pyruvate phosphokinase and a priming amount of adenine nucleotide were made; additional myokinase did not influence the rate of reaction. In Experiments I and II (Table II), the disappearance of phosphopyruvate was matched by the appearance of pyruvate, and no orthophosphate was released. Adenosine was consumed and kinase-labile phosphate produced, but the data are inadequate from the standpoint of reaction balance. Experiment III, which includes chromatographic separation of the products as well as their enzymatic assay, provides a simple balance between the phosphopyruvate and adenosine utilized and the ATP formed. The consumption of 3 moles of phosphopyruvate in the conversion of adenosine to ATP is shown more clearly in an experiment in which an excess of phosphopyruvate was provided (Fig. 2).

Phosphorylation of 2-Aminoadenosine—The pyruvate phosphokinase system was also effective in the phosphorylation of 2-aminoadenosine to 2-aminoATP. In the presence of this system and adenosine phosphokinase, 2-aminoadenosine was completely removed and somewhat more than 3 moles of phosphopyruvate were consumed per mole of nucleoside (Fig. 2). Ultraviolet spectra (240 to 340 μm) of aliquots removed at 30 minute intervals during the course of the experiment showed no change. With prolonged incubations and the use of larger amounts of enzyme necessary for completion of the phosphorylation of 2-aminoadenosine, the formation of some inorganic orthophosphate was observed, presumably a result of ATPase action. This probably accounts for the fact that more than 3 equivalents of phosphopyruvate were consumed. The direct phosphorylation of 2-aminoadenosine by ATP was demonstrated under conditions exactly comparable to those described for the direct phosphorylation of adenosine (Experiment II, Table I). The transfer of kinase-labile phosphate was, respectively, 0.28, 0.28, and 0.36 μm after 1, 2, and 3 hours of incubation.
### Table II

**Phosphorylation of Adenosine by Transfer from Phosphopyruvate**

The values are expressed in micromoles.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Phosphopyruvate</th>
<th>Pyruvate</th>
<th>Adenosine</th>
<th>A-5'-P</th>
<th>ADP</th>
<th>ATP</th>
<th>Kinase-labile phosphate</th>
<th>Orthophosphate</th>
</tr>
</thead>
</table>
| I
| Controls       | 1.29            | 0.32     | 0.80      |        |     |     | 0.12                   | 2.71           |
| Experimental   | 0.06            | 1.77     | 0.00      |        |     |     | 0.81                   | 2.71           |
| Δ              | -1.23           | +1.45    | -0.80     |        |     |     | +0.69                  | 0.00           |
| II
| Controls       | 1.28            | 0.33     | 0.94      |        |     |     | 0.11                   | 2.52           |
| Experimental   | 0.17            | 1.42     | 0.32      |        |     |     | 0.80                   | 2.44           |
| Δ              | -1.11           | +1.09    | -0.62     |        |     |     | +0.69                  | -0.08          |
| III
| Control*       | 32.2            |          |           | 12.5   | 0.0 | 0.0 | 0.3                    | 39.1           |
| Experimental*  |                |          |           |        |     |     | 0.0                    | 12.1           |
| ""            | 0.5             |          |           | 0.0    | 0.0 | 0.4 | 12.6                   | 39.8           |
| Δ†             | -31.7           | -12.5    | +0.2      | +12.1  |     |     | +0.7                   |                |

* Assays of perchloric acid supernatant.
† Ion exchange analysis.
‡ The experimental value used in this calculation is an average of the values obtained by ion exchange analysis and assays of the perchloric acid supernatant.

**Experiments I and II**—The experimental incubation mixture contained 0.05 cc. of adenosine (0.016 M), 0.10 cc. of phosphopyruvate (0.014 M), 0.01 cc. of ATP (0.002 M), 0.01 cc. of MgCl₂ (0.1 M), 0.01 cc. of MnCl₂ (0.02 M), 0.10 cc. of succinate buffer (0.33 M, pH 6.0), 0.02 cc. of glutathione (0.15 M), 0.10 cc. of adenosine phosphokinase (10 units per cc.), 0.02 cc. of myokinase, 0.02 cc. of pyruvate phosphokinase (Acetone I), and water to a final volume of 0.50 cc. The incubation mixture in one control lacked adenosine and in the other it lacked phosphopyruvate. After incubation for 20 minutes at 23°, 1.5 cc. of water were added and the mixture was immersed in a boiling water bath for 3 minutes.

**Experiment III**—The experimental incubation mixture contained 0.75 cc. of adenosine (0.016 M), 0.60 cc. of phosphopyruvate (0.06 M), 0.30 cc. of ATP (0.001 M), 0.15 cc. of MgCl₂ (0.1 M), 0.30 cc. of MnCl₂ (0.01 M), 1.5 cc. of succinate buffer (0.33 M, pH 6.0), 0.30 cc. of glutathione (0.15 M), 1.5 cc. of adenosine phosphokinase (10 units per cc.), 0.30 cc. of myokinase, 0.30 cc. of pyruvate phosphokinase (Acetone I), and water to a final volume of 9.0 cc. The control incubation mixture lacked only adenosine. After 30 minutes incubation at 23°, 1.0 cc. of the mixture was added to 1.0 cc. of 6 per cent perchloric acid; 1.0 cc. of crystalline bovine serum albumin (10 mg. per cc.) was then added to facilitate precipitation of the myokinase. The neutralized perchloric acid supernatants were used for enzymatic and phosphate assays. The remainder of the incubation mixture was immersed in a boiling water bath for 3 minutes. The heated sample was chromatographed on a Dowex-1 chloride column (6 cm. X 1 sq. cm.) (5). Adenosine was eluted with 0.003 N HCl (0 to 36 cc.); A-5'-P did not appear even after 700 cc. ADP was eluted with 0.02 M NaCl in 0.01 N HCl (0 to 120 cc.) and ATP with 0.3 M NaCl in 0.01 N HCl (0 to 190 cc.).
The isolation of 2-aminoATP was attempted from an incubation mixture exactly as described in Fig. 2, except that it was 10 times as large (17.7 µM of 2-aminoadenosine). After 3 hours incubation, the mixture was chilled, adsorbed directly on a Dowex-1 chloride column (4 cm. x 1 sq. cm.), washed with 15 cc. of water, and eluted at 2° with 0.01 N HCl containing 0.075 M KCl. Fractions (10.8 cc.) were collected and examined.

\[ \text{TIME-HOURS} \]

**Fig. 2.** Phosphorylation of adenosine to ATP and 2-aminoadenosine to 2-aminoATP. Phosphopyruvate consumption with adenosine (○) and with 2-aminoadenosine (●); 2-aminoadenosine remaining (▲). The incubation mixture contained 0.107 cc. of adenosine (0.167 mM) or 0.25 cc. of 2-aminoadenosine (0.0071 M), 0.20 cc. of phosphopyruvate (0.06 mM), 0.05 cc. of ATP (0.001 M), 0.25 cc. of succinate buffer (0.33 M, pH 6.0), 0.025 cc. of MgCl₂ (0.1 M), 0.025 cc. of MnCl₂ (0.04 M), 0.05 cc. of glutathione (0.15 mM), 0.05 cc. of pyruvate phosphokinase (Acetone 1), adenosine phosphokinase (10 units per cc.), and water to a final volume of 1.5 cc. In the adenosine experiment, 0.25 cc. of adenosine phosphokinase was used and 0.5 cc. was used in the 2-aminoadenosine experiment. 23°. Aliquots (0.1 cc.) were removed into 1.4 cc. of 3 per cent perchloric acid at intervals and the neutralized supernatants were assayed for pyruvate and, in the 2-aminoadenosine experiment, for remaining nucleoside as well. In control incubation mixtures, which lacked only the nucleoside addition, there was a steady decomposition of phosphopyruvate amounting to about 0.9 µM per hour. The plotted values are corrected for these blanks determined at every point.

at 280 and 255 mµ. The chromatogram resembled that of ATP (5, 28) rather closely. Fractions 6 through 37, in which the ratio of densities at 280 and 255 mµ was nearly correct (0.67) and which appeared to contain approximately 90 per cent of the nucleotide, were pooled. The combined eluate was treated with 3.0 cc. of saturated barium acetate and adjusted to pH 8.6 with 1 N KOH. Cold absolute ethanol (197 cc.) was added and after 2 hours at 0° the flocculent precipitate was collected in the centrifuge. The precipitate failed to dissolve completely in cold 1 N HCl and only about one-half of the ultraviolet-absorbing material was recovered. Bar-
ium was removed by treatment with Dowex-50 (H⁺ form) without loss of ultraviolet-absorbing substance and the solution was neutralized. This solution possessed the spectral characteristics of 2-aminoadenosine and represented an overall yield of 47 per cent. Analysis of this solution (with a corrected concentration of 1.00 μM per cc. based on ultraviolet absorption) was as follows: pentose, 0.93, acid-labile phosphate, 1.88, and

**TABLE III**

**Specificity of Adenosine Phosphokinase**

The test conditions were as described for Experiments I and II, Table II. 0.8 μM of test compound was used. The values are expressed as micromoles of pyruvate formed, corrected for the blank. Results with the test compounds are compared with those obtained with adenosine in the same experiment.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxy-9-β-d-ribofuranosyladenine (crotosine)</td>
<td>0.02, 0.00</td>
</tr>
<tr>
<td>9-β-d-2-Desoxyribofuranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>9-β-d-Ribopyranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>9-β-d-Glucoopyranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>9-α-d-Arabofuranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>9-α-L-Arabofuranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>2,6-Diamino-9-β-d-ribofuranosylpurine</td>
<td>0.22, 0.26</td>
</tr>
<tr>
<td>2,6-Diamino-9-β-d-xylofuranosylpurine</td>
<td>0.00</td>
</tr>
<tr>
<td>2-Chloro-9-β-d-ribofuranosyladenine</td>
<td>0.05, 0.05*</td>
</tr>
<tr>
<td>2,8-Dichloro-9-β-d-ribofuranosyladenine</td>
<td>0.03, 0.01</td>
</tr>
<tr>
<td>2-Methylthio-9-β-d-ribofuranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>2-Acetamido-9-β-d-ribofuranosyladenine</td>
<td>0.00</td>
</tr>
<tr>
<td>6-Oxy-9-β-d-ribofuranosylpurine (inosine)</td>
<td>0.00</td>
</tr>
<tr>
<td>2-Amino-6-oxy-9-β-d-ribofuranosylpurine (guanosine)</td>
<td>0.00</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.00</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.02</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>0.00</td>
</tr>
<tr>
<td>Yeast adenyllic acid</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* This value may be attributed to an impurity of adenosine; prolonged incubation resulted in no increase of this value.

total organic phosphate, 3.72 μM per cc. This analysis is approximately correct for 2-aminoATP except for the high total organic phosphate value, which is unexplained.

The isolated 2-aminoATP was active as phosphate donor to 2-aminoadenosine and to glucose. The 2-aminoATP (0.1 cc., containing 0.188 μM of acid-labile phosphate) transferred 0.112 μM of phosphate to glucose in the presence of hexokinase and myokinase and 0.058 μM when myokinase was omitted. The phosphorylation of 2-aminoadenosine (under conditions analogous to those described in Experiment I, Table I) as indicated
by the removal of kinase-labile phosphate was 0.04, 0.07, and 0.11 μM after incubation periods of 1, 2, and 3 hours respectively.

**Specificity of Adenosine Phosphokinase**—A large number of related nucleosides were found inactive under conditions which resulted in the phosphorylation of adenosine and 2-aminoadenosine (Table III). These compounds when tested at equimolar levels in the presence of adenosine showed only slight inhibitory effects (13 to 30 per cent) on the rate of adenosine phosphorylation.

**Other Properties of Enzyme**—Rates of adenosine phosphorylation by ATP, estimated by adenosine removal, were maximal at the lowest substrate concentrations which could be conveniently tested. The levels were $5 \times 10^{-4}$ M for ATP and $2 \times 10^{-4}$ M for adenosine. It is noteworthy that, when adenosine phosphokinase is coupled with pyruvate phosphokinase and myokinase (Table II, Fig. 2), the removal of adenosine or phosphopyruvate is readily completed, depending upon which substance is limiting.

<table>
<thead>
<tr>
<th>Mg++, $\times 10^{-3}$</th>
<th>0.33</th>
<th>1.7</th>
<th>3.3</th>
<th>0.33</th>
<th>1.7</th>
<th>3.3</th>
<th>0.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn++, $\times 10^{-3}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table IV**

Mg$^{++}$ Requirement of Adenosine Phosphokinase

Test conditions as in Experiment I, Table I. The results are in micromoles.

The pH optimum of the reaction determined either with yeast autolysates (phosphopyruvate as donor) or with purified adenosine phosphokinase (ATP as donor) was approximately 6.0; about 50 per cent of the activity was observed at pH 5.0 and 7.0. Citrate buffer was inhibitory.

Mg$^{++}$ was required and was partially replaced by Mn$^{++}$ (Table IV).

**DISCUSSION**

The conversion of adenosine to muscle adenylic acid (A-5'-P) observed by Ostern et al. (3) has now been established as a simple and direct transfer from ATP. An objection to this pathway of A-5'-P synthesis was offered by Canzanelli et al. (29). They observed that sugar phosphates rather than mononucleotides were formed when adenosine was incubated with rabbit kidney homogenate. However, this particular objection cannot be regarded as serious in view of the very potent adenosine deaminase and inosine phosphorylase activities which are present in such homogenates. Alternative pathways of biosynthesis of A-5'-P deserve consideration. The formation by pigeon liver of inosine-5'-phosphate with an imidazole carboxamide ribitide as a possible intermediate was reported by Greenberg.
NTCLEOSIDE PHOSPHORYLATION

(30) and Buchanan and Schulman (31). The amination of inosinic acid to A-5'-P is suggested by Johnson and Bloch (32), who observed that inosinic acid had some effectiveness in replacing adenine nucleotides for activation of glutathione synthesis by pigeon liver extracts. The origin of adenosine is still unsettled. The conversion of adenosine to adenine reported by Wang (33), while stimulated by phosphate and arsenate, has not been demonstrated to be reversible.

The transfer of phosphate to adenosine was limited to the 5' carbon of the nucleoside. Whether adenosine or other nucleotides are under any circumstances directly phosphorylated at the 2' or 3' carbon remains undetermined. The specificity of adenosine phosphokinase also extends to the configuration of the pentose and to substitutions on the purine ring with the single exception of 2,6-diaminopurine riboside. The phosphorylation of this nucleoside and the additional fact that it is further acted upon by myokinase and pyruvate phosphokinase to yield 2-aminoATP suggest that these activities may have some physiologic significance. Brown (34) has reported that adenine is the only purine which is incorporated into rat nucleic acid adenine but that both adenine and 2,6-diaminopurine serve as precursors of nucleic acid guanine. It is conceivable that a 2-aminoadenine nucleotide leads to the formation of a guanosine nucleotide, which is incorporated into nucleic acid.

The availability of a partially purified adenosine phosphokinase of high specificity provides a new means of identifying and estimating adenosine. When coupled with phosphopyruvate, pyruvate phosphokinase, and myokinase, the determination of pyruvate (spectrophotometrically) becomes a sensitive and convenient assay method for adenosine.

SUMMARY

1. An enzyme was partially purified from autolysates of brewers' yeast and shown to catalyze the direct phosphorylation by ATP of adenosine and 2,6-diaminopurine riboside (2-aminoadenosine) according to the equations

\[
\text{Adenosine} + \text{ATP} \rightarrow \text{A-5'-P} + \text{ADP}
\]

\[
2\text{-Aminoadenosine} + \text{ATP} \rightarrow 2\text{-aminoadenosine-5'-phosphate} + \text{ADP}
\]

2. This adenosine phosphokinase when coupled with phosphopyruvate, pyruvate phosphokinase, and myokinase converts adenosine to ATP and 2-aminoadenosine to 2-aminoATP.

3. Adenosine phosphokinase is strictly specific for adenosine and 2-aminoadenosine among a group of seventeen nucleosides tested and the transfer of phosphate is limited to the 5' carbon of the nucleoside.

4. Myokinase activity with acid- and heat-resistant properties like those of muscle myokinase was identified in yeast fractions.
5. Methods are described for the purification of lactic dehydrogenase and pyruvate phosphokinase from rabbit muscle.

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ENZYMATIC PHOSPHORYLATION OF ADENOSINE AND 2,6-DIAMINOPURINE RIBOSIDE

Arthur Kornberg and W. E. Pricer, Jr.


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