The Phosphorolysis of Nucleosides by Rabbit Bone Marrow*

ESTHER W. YAMADA

From the Department of Biochemistry, University of Manitoba, Winnipeg 8, Canada

(Received for publication, June 12, 1961)

It has been reported that the capacity of rabbit bone marrow to carry out the synthesis de novo of purine ribonucleotides is quite low, and that this tissue requires preformed purine derivatives supplied by the liver (1, 2). Presumably, the salvage enzymes, including nucleoside phosphorylases (3), participate in the reutilization of the preformed derivatives. So far, only pyrimidine nucleoside phosphorylases of rabbit and human bone marrow have been described (4, 5). It was very probable, then, in view of their wide distribution in other tissues (6), that purine nucleoside phosphorylases would also be present in bone marrow cells.

This investigation resulted in the isolation of a nucleoside phosphorylase that appears to be highly specific for guanosine and deoxyguanosine. In this respect, the enzyme is unlike the purine nucleoside phosphorylases isolated from liver (7), erythrocytes (8), and brain (9).

EXPERIMENTAL PROCEDURE

The enzyme activity was assayed spectrophotometrically by measuring the increase in absorption at 290 nm resulting from the conversion of guanosine (or deoxyguanosine) to guanine. For every micromole of guanine formed, the increase in absorption was 2.9. All experiments were done in duplicate, and in all experiments the test solutions were read against incubated controls. Unless otherwise specified, the incubation mixture contained the following (in micromoles) in a final volume of 1.0 ml: potassium phosphate buffer at pH 7.0, 100; mercaptoethanol, 5; enzyme; and substrate, 1.5. The reaction was started by the addition of substrate and the tubes were incubated for 10 minutes at 37°C. The reaction was stopped by heating the reaction mixture for 2 minutes in a boiling water bath. The controls were incubated without substrate, which was added just after the tubes were placed in the boiling water bath. The tubes were cooled in an ice bath and to each was added 2 ml of 1 N potassium hydroxide. Any precipitate that was present was spun down at 10,000 r.p.m., for 10 minutes.

Under the standard conditions of assay, the formation of guanine was a linear function of time for 30 minutes and of protein concentration up to an optical density reading of 0.250. A unit of enzyme activity is defined as the number of units per mg of protein. Protein was determined by the method of Lowry et al. (10).

In some experiments, the phosphorolysis of uridine, thymidine, cytidine, inosine, xanthosine, and adenosine was measured. The enzyme assay with these substrates was essentially that described for guanosine phosphorylase. The absorbancy changes for the conversion of 1 μmole of nucleoside to 1 μmole of base were: uridine, +3.9; xanthosine, read at 295 μm, +3.48; thymidine, read at 295 μm, +3.2; adenosine, read at 280 μm, +3.5; inosine, at pH 7.2, read at 259 μm, +3.16; and cytidine, at pH 7.2, read at 280 μm, −4.17.

In other experiments, the enzyme preparations were tested for guanase activity. The procedure was that described for the assay of guanosine phosphorylase, except that 1.5 μmoles of guanine replaced guanosine. The increase in absorption at 290 μm was 3.3 for every micromole of guanine that was deaminated to xanthine.

Enzyme Purification—The buffers used in these procedures all contained 5 mM mercaptoethanol.

Two rabbits were killed by a blow at the base of the head. The animals were perfused with ice-cold 0.9% sodium chloride solution through the right atrium until the livers were completely blanched. All subsequent operations were carried out at 0°C. The bone marrow was removed from the leg bones and homogenized with 5 volumes of 0.05 M potassium phosphate buffer at pH 7.1. The homogenate (Fraction 1) was centrifuged for 40 minutes at 105,000 × g. After centrifugation, 99% of the enzyme activity was in the supernatant, Fraction 2 (Table I).

To Fraction 2, ammonium sulfate (24.5 g per 100 ml) was added to bring the concentration to 35% of saturation. The precipitate was spun down at 12,000 × g for 20 minutes. To the supernatant, ammonium sulfate (21.0 g per 100 ml) was added to bring the concentration to 65% of saturation. The resulting precipitate was resuspended in 0.05 M potassium phosphate at pH 7.0 (Fraction 3), and contained 57.5% of the enzyme activity.

Fraction 3 was treated with precooled (−30°C) acetone in an alcohol-water-Dry Ice bath at −3°C so that a concentration of 33% was obtained. After centrifugation at −5°C and 12,000 × g for 20 minutes, the supernatant was cooled to −10°C and acetone was again added to a final concentration of 66%. After centrifugation at −10°C, the residue was suspended in 0.05 M Tris at pH 7.2, and 0.1 volume of calcium phosphate gel was added. After centrifugation, 50.1% of the activity was in the supernatant (Fraction 4).

A volume of calcium phosphate gel, 3 times that of Fraction 4, containing 21 mg (dry weight) per ml, was centrifuged for 5 minutes at 5000 × g and the supernatant was discarded. Fraction 4 was adjusted to pH 6.8 with acetic acid, poured onto the calcium phosphate gel, and thoroughly mixed with the gel. Usually about 95% of the activity was adsorbed onto the gel. The gel was eluted successively with 5-ml aliquots of 0.05 M Tris at pH 7.0, 0.05 M potassium phosphate at pH 6.5 (eluate A1), 0.03 M potassium phosphate at pH 6.75 (eluate A2), 0.03 M potassium phosphate at pH 6.0 (eluate A3).
Bone Marrow Guanosine Phosphorylase

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TABLE I

Summary of purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Ratio (guanosine:deoxyguanosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homogenate</td>
<td>52.7</td>
<td>1270</td>
<td>19.5</td>
<td>1.1</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant</td>
<td>44.0</td>
<td>934</td>
<td>15.5</td>
<td>3.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>Ammonium sulfate</td>
<td>3.8</td>
<td>616</td>
<td>19.0</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Acetone</td>
<td>3.7</td>
<td>515</td>
<td>8.8</td>
<td>18.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Gel eluates</td>
<td>2.6</td>
<td>450</td>
<td>2.6</td>
<td>67.0</td>
<td>1.04</td>
</tr>
<tr>
<td>6</td>
<td>Combined DEAE eluates*</td>
<td>1.3</td>
<td>30</td>
<td>0.08</td>
<td>292.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Of 132 units of Fraction 5 actually used, 120 units were recovered in the DEAE eluates. Of these, only 30 units were recovered after ammonium sulfate precipitation of the DEAE eluates.

TABLE II

Phosphorolysis of other substrates

The respective substrate, 1.5 μmoles, was added to the standard incubation medium. The specific activities of the supernatant, gel eluate A₁, and combined eluates were 3.7, 18.4, and 57, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Supernatant</th>
<th>Gel eluate A₁</th>
<th>Combined eluates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Xanthosine</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inosine</td>
<td>1.0</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE III

Phosphorolysis of uridine

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Substrate</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Ratio (guanosine:uridine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supernatant</td>
<td>Guanosine</td>
<td>2165</td>
<td>3.2</td>
<td>2.7:1</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulfate</td>
<td>35 to 60%</td>
<td>Uridine</td>
<td>800</td>
<td>1.18</td>
</tr>
<tr>
<td>3</td>
<td>Supernatant from calcium phosphate gel</td>
<td>Guanosine</td>
<td>1790</td>
<td>9.9</td>
<td>3.7:1</td>
</tr>
<tr>
<td>4</td>
<td>Gel eluate A₁</td>
<td>Uridine</td>
<td>490</td>
<td>2.7</td>
<td>0:1:6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guanosine</td>
<td>660</td>
<td>82.6</td>
<td>83:0</td>
</tr>
</tbody>
</table>

The highest specific activity were combined and the enzyme precipitated with ammonium sulfate and resuspended in phosphate buffer (Fraction 6). The specific activity of Fraction 6 was 292 and represents a 270-fold purification over the initial homogenate (Table I).

Under the standard conditions of assay, guanase activity was not detected in any of the enzyme fractions and did not interfere with the assay of guanosine phosphorylase.

Properties of the Enzyme

Substrates—Under the conditions of the standard assay, the enzyme appeared to be specific for guanosine and deoxyguanosine. Constant ratios of 1 (guanosine:deoxyguanosine) were obtained for all fractions isolated during the purification procedure (Table I). The supernatant had minimal activity with xanthosine, inosine, and thymidine. No phosphorolysis of cytidine was detected and adenosine was rapidly deaminated. Highly purified preparations also showed minimal or no activity with xanthosine and inosine (Table II). When the combined eluate fraction was allowed to age, the specific activity with guanosine decreased 8.2 units but remained constant with xanthosine.

When both guanosine and xanthosine were added to the incubation medium containing the purified enzyme preparation, there was no inhibition of the phosphorolysis of guanosine.

The crude enzyme preparations also showed considerable activity with uridine. During subsequent purification procedures, the uridine phosphorylase was isolated (Table III). Thus, Fraction 3 had no measurable activity with guanosine and Fraction 4 none with uridine. Dihydrouracil dehydrogenase, assayed by measuring the decrease in absorbancy at 340 mg due to the oxidation of TPNH, was not detected during a 20 minute incubation period and did not interfere with the assay of uridine phosphorylase.

The enzyme preparations were tested for activity in the formation of nicotinamide ribonucleoside when incubated with 2 μmoles of ribose 1-phosphate, 2 μmoles of nicotinamide, and 10 μmoles of sodium acetate at pH 6.05, in a final volume of 1 ml. After 15- to 30-minute incubation periods, aliquots were removed and tested by the cyamide addition reaction for the appearance of nicotinamide ribonucleoside (8). In all of the tests, no nicotinamide ribonucleoside could be detected.

pH and Stability—The phosphorolysis of guanosine proceeds with maximal velocity over a pH range of 7.0 to 7.4, whereas the phosphorolysis of deoxyguanosine was maximal at a pH of 7.0 (Fig. 1). The enzyme showed no noticeable loss of activity after storage for a month at −10°C. After 2 months of storage, there was loss of activity.

Inhibitors and Activators—The partially purified enzyme requires 2-mercaptoethanol for optimal activity. It was completely inhibited by p-chloromercuribenzenesulfonic acid (2 × 10⁻⁴ M). Arsenite at a concentration of 10⁻⁴ M, and EDTA at a concentration of 0.003 M, had no effect on the enzyme. Tris buffer (0.1 M at pH 7.5) inhibited the enzyme 22%.

Effect of Substrate Concentration—A Lineweaver-Burk plot (12) of the rate of phosphorolysis as a function of guanosine concentration is shown in Fig. 2. The Kₘ (13) was 2.16 × 10⁻⁴ M. A similar plot was obtained with increasing deoxyguanosine concentrations. The Kₘ values obtained indicate equal affinities for guanosine and deoxyguanosine, and are additional evidence that the same enzyme is present.

Potassium phosphate at pH 6.04 (eluate A₁), and 0.05 M potassium phosphate at pH 7.4 (eluate A₂). Most of the activity was in the last three eluates (Table I), which were combined. The enzyme was precipitated from these eluates with ammonium sulfate and resuspended in 0.05 M potassium phosphate at pH 7.0 (Fraction 5). This fraction was further purified by chromatography on a DEAE-cellulose column (11). The fractions containing 1 Obtained from the Brown Company, Berlin, New Hampshire.
FIG. 1. Rate of phosphorolysis of guanosine (O---O) and deoxyguanosine (□---□) as a function of pH. The former was determined with a preparation of specific activity of 17 in the presence of 0.1 M sodium acetate and 0.1 M potassium phosphate (□) or 0.1 M Tris (O---O). The latter was determined with a preparation of specific activity of 27 in the presence of 0.1 M potassium phosphate (□).

FIG. 2. On the left is a plot of data obtained from the rates of guanine formation with increasing guanosine concentration. On the right is a plot of data obtained from the rates of guanine formation with increasing potassium phosphate concentration. Involved. Fig. 2 also shows the reaction velocity as a function of phosphate concentration. The $K_m$ was 3.76 x 10^{-4} M. There was no enzyme activity when phosphate was absent and 0.05 M arsenate completely replaced 0.05 M phosphate in the reaction.

**Equilibrium Constant**—In the presence of 0.47 enzyme unit, 0.75 μmole of guanosine, and 0.75 μmole of potassium phosphate at pH 7.0, 0.10 μmole of guanine was formed after incubation for 40 minutes at 37°C. On further incubation, no additional guanine was formed. From these figures the equilibrium constant was calculated to be 0.019.

**Stoichiometry of Reaction**—The enzyme preparation was incubated with guanosine and inorganic phosphate for periods of 20 to 40 minutes. Aliquots were then removed for the estimation of guanine by the standard assay procedure, and inorganic phosphate and acid-labile phosphate by the method of Lowry and Lopez (14). For every micromole of guanine formed in the reaction, 1 μmole of inorganic phosphate disappeared and 1 μmole of acid-labile phosphate appeared (Table IV). When arsenate replaced phosphate (Experiment 4), 1 μmole of reducing sugar was formed per micromole of guanine. Reducing sugar was determined by the method outlined by Colowick, Kaplan, and Ciotti (15). Under the conditions of this assay, neither ribose 1-phosphate nor guanosine was hydrolyzed, and both gave negative reducing sugar tests.

**Chromatography**—Ascending chromatograms with Whatman No. 1 filter paper were employed. The solvent used was 5% disodium hydrogen phosphate in isoamyl alcohol (16). Aliquots of the boiled reaction mixture were spotted on the paper. The developed chromatogram was viewed under ultraviolet light. Two ultraviolet-absorbing spots with $R_F$'s of 0.45 and 0.64 appeared, and were comparable to those of guanosine ($R_F = 0.64$) and guanine ($R_F = 0.44$).

**Nucleoside Phosphorylase of Other Tissue**—Despite the extensive perfusion of the animals, a few contaminating red blood cells were usually included in the bone marrow preparations. Therefore, homogenates of red blood cells were tested and showed very low uridine and guanosine phosphorylase activities, compared to bone marrow homogenates (Table V). Intestinal mucosa supernatants were more active with the four substrates tested than bone marrow supernatants, and liver supernatants were less active.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Guanine appearance</th>
<th>Inorganic phosphate disappearance</th>
<th>Acid-labile phosphate appearance</th>
<th>Reducing sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.53</td>
<td>0.60</td>
<td>0.60</td>
<td>0.5*</td>
</tr>
<tr>
<td>2</td>
<td>0.60</td>
<td>0.50</td>
<td>0.60</td>
<td>0.0†</td>
</tr>
<tr>
<td>3</td>
<td>0.70</td>
<td>0.61</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.81</td>
<td>0.50</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

* Incubation with 0.1 M arsenate.
† Incubation with 0.1 M phosphate.

**Table IV**

**Stoichiometry of reaction**

Mercaptoethanol was omitted from the reaction mixture. Guanosine, 7.5 μmoles, and potassium phosphate, 10 μmoles, pH 7.0, were added to tubes containing 4.8 units of dialyzed enzyme in a volume of 1 ml. The reaction was terminated by boiling the reaction mixture for 2 minutes.

**Table V**

**Nucleoside phosphorylase activity of rabbit tissues**

Homogenates were prepared in 5 volumes of 0.05 M phosphate and 5 μm 2 mercaptoethanol at pH 7.0. Supernatants were prepared by centrifuging the homogenates for 30 minutes at 105,000 X g.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Guanosine</th>
<th>Deoxy-guanosine</th>
<th>Uridine</th>
<th>Deoxy-uridine</th>
<th>Specific activity, units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow homogenates</td>
<td>1.07</td>
<td>1.16</td>
<td>0.35</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>Red blood cell homogenates</td>
<td>0.17</td>
<td>0.13</td>
<td>0.05</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>Intestinal mucosa supernatant</td>
<td>5.2*</td>
<td>4.3*</td>
<td>1.32</td>
<td>0.67</td>
<td>0.05</td>
</tr>
<tr>
<td>Bone marrow supernatant</td>
<td>4.3</td>
<td>3.9</td>
<td>1.4</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver supernatant</td>
<td>3.3*</td>
<td>3.06*</td>
<td>0.08</td>
<td>0.19</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Corrected for guanase activity.
DISCUSSION

Rabbit bone marrow cells contain several nucleoside phosphorylases in addition to the thymidine phosphorylase described by Friedkin and Roberts (5). Of these enzymes, guanosine phosphorylase is the most active. Although the bone marrow extracts also show low phosphorylase activity toward inosine and xanthosine, the results presented above indicate that this activity is probably not associated with guanosine phosphorylase.

The guanosine phosphorylase of bone marrow differs markedly from the nucleoside phosphorylase of rat liver (7, 17), calf liver (18, 19), brain (9), and yeast (20) in its lack of activity toward inosine and xanthosine. It resembles nicotinamide nucleoside phosphorylase of human erythrocytes (8) in its preference for guanosine, but does not catalyze the formation of nicotinamide ribonucleoside from nicotinamide and ribose 1-phosphate. In its specificity for the deoxyribonucleoside and ribonucleoside of guanine, it resembles the calf liver enzyme which was reported to be equally as active with inosine as with deoxyhypoxanthine (21).

The absolute specificity of the pyrimidine deoxyribonucleoside phosphorylase of Escherichia coli for the deoxyribose moiety (22) is in sharp contrast to the wider specificity of the bone marrow enzyme. It may be, however, that only pyrimidine phosphorylases (23, 24) have this kind of specificity. As yet, it is not certain whether the bone marrow phosphorylase that is active with both uridine and deoxyuridine is one enzyme or more. This point is under investigation.

The high activities of guanosine and uridine phosphorylases of bone marrow suggest that they may play an important role in the utilization by bone marrow of the corresponding deformed bases and nucleosides reaching this tissue via the blood.

SUMMARY

A guanosine phosphorylase from rabbit bone marrow has been purified 270-fold. The enzyme is as active with deoxyguanosine as with guanosine, and exhibits equal affinities for the two substrates (Km values, 2.10 × 10⁻⁴ M and 2.16 × 10⁻⁴ M). With both substrates, the enzyme was most active at pH 7.0. The activities at arsenate concentrations of 0.05 M and 0.1 M were 100% and 88% of the activities at comparable phosphate concentrations. Tris buffer and p-chloromercuribenzoate inhibited the enzyme, but ethylenediaminetetraacetic acid and arsenite had no effect.

The purified preparations showed minimal or no activity with the purine ribonucleosides, adenosine, inosine, and xanthosine. Neither xanthosine nor inosine inhibited the phosphorylase of guanosine. No enzymic formation of nicotinamide ribonucleoside from nicotinamide and ribose 1-phosphate was detectable.

The crude bone marrow preparations also catalyzed the phosphorylization of uridine and thymidine. The rate of phosphorylization of uridine was one-third that of guanosine and one and one-half times that of thymidine. During subsequent purification procedures, it was possible to separate the uridine and guanosine phosphorylases.

The phosphorolysis of guanosine, deoxyguanosine, uridine, and deoxyuridine by other rabbit tissues was investigated. Red blood cell preparations catalyzed very little phosphorolysis of the four substrates. Liver preparations showed more activity and bone marrow still more. Intestinal mucosa extracts were the most active.

Acknowledgment—The author wishes to thank Mr. Mark V. Fortunanase for valuable technical assistance.

REFERENCES