Purification and Properties of Nucleoside Hydrolase from Pseudomonas fluorescens*

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SUMMARY

An enzyme which catalyzes the hydrolytic cleavage of various ribonucleosides to free base and ribose components has been obtained from cells of Pseudomonas fluorescens and purified about 300-fold by fractionation with protamine sulfate, ammonium sulfate, alumina Cγ, and diethylaminoethyl cellulose. The preparation thus purified catalyzes the hydrolysis of about 50 μmoles of uridine per min per mg of protein at 37°, and is stable when stored at -15°. The enzyme is highly specific for nucleosides containing the β-D-ribofuranosyl carbon-nitrogen linkage. A broad specificity is shown with respect to the base components: both pyrimidine and purine ribonucleosides are hydrolyzed. Because the maximum rate of hydrolysis of a pyrimidine nucleoside is generally much greater than that of a purine nucleoside, the systematic name N-ribosylpyrimidine ribohydrolase is suggested for this enzyme.

The enzyme which catalyzes the hydrolytic cleavage of nucleosides to free base and ribose components was first shown to be present in bakers' yeast by Carter (1), and in several Lactobacillus species by Kalckar (2). Similar enzymes have later been shown to be present in bacteria (3-11), yeast (12), and fish muscle (13), but none of them has been extensively studied except for that from Lactobacillus delbrueckii (N-ribosylpurine ribohydrolase, EC 3.2.2.1). The latter enzyme was described by Tabor and Hayaishi (6), and was purified about 50-fold by Takagi and Horecker (8), who found that the enzyme was highly specific for the β-ribofuranosyl linkage and that purine ribonucleosides were hydrolyzed much faster than pyrimidine ribonucleosides. The present paper describes the partial purification of a novel nucleoside hydrolase from a strain of Pseudomonas fluorescens and some properties of the enzyme, with particular emphasis on its substrate specificity. In contrast to the hydrolase from L. delbrueckii, this enzyme is unique in that its activity toward pyrimidine ribonucleosides is greater than that toward purine ribonucleosides. The specific activity of the final preparation, with uridine as substrate, is more than 100 times higher than that of any similar enzyme preparation so far described in the literature.

EXPERIMENTAL PROCEDURE

Materials

Chemicals—The following chemicals were obtained from commercial sources: uridine, cytidine, adenosine, guanosine, inosine, deoxyuridine, deoxycytidine, thymidine, cytosine, 6-azauridine, 5-bromouridine, 5'-UMP, 5'-CMP, β-NAD, and uracil from Sigma; deoxyadenosine, deoxyguanosine, and 4-amino-5-imidazolecarboxamide ribonucleoside from Calbiochem; xanthosine from C. F. Boehringer und Soehne, Mannheim; puromycin from Lederle Laboratories, Division of American Cyanamid Company, Pearl River, New York; and β-d-ribose from Fisher Scientific. 1-β-D-Ribofuranosylnicotinamide was generously provided by Dr. T. Deguchi of this department. 6-Dimethylamino-9-(3'β-amino-3'-deoxy-β-D-ribofuranosyl)purine (the aminonucleoside of puromycin) (14) was a gift of Dr. Y. Takeda, Osaka University School of Dentistry. 7-Amino-3-(β-D-ribofuranosyl)-pyrazolo[4,3-d]pyrimidine (formycin) (15) was kindly supplied by Dr. H. Umezawa of the University of Tokyo. Pseudouridine was furnished by Dr. K. Kusama, Shizuoka University, and 9-β-D-3'-deoxyribonucleosyladenine (cordycepin) was provided by Dr. E. Walter, Merck. 4-Amino-7-(β-D-ribofuranosyl)-7H-pyrazolo[2,3-d]pyrimidine (tubercidin) (16) and 1-β-D-arabinofuranosylcytosine were furnished by Dr. C. G. Smith of The Upjohn Company, Kalamazoo, Michigan. Imidazoleacetic acid ribonucleoside was generously provided by Dr. T. Honjo of this department. The following compounds were generously supplied by Dr. G. Sunagawa of the.

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§ To whom reprint requests should be directed.
Sankyo Research Laboratories, Tokyo: 1-β-D-ribofuranosylthymine, 1-β-D-glucopyranosylthymine, 3-β-D-ribofuranosyladenine, 9-β-D-glucopyranosyladenine, 9-β-D-glucopyranosylhypoxanthine, 9-β-L-ribofuranosylhypoxanthine, 9-β-L-ribofuranosyluracil, 1-α-D-ribofuranosyluracil, 1-α-D-ribofuranosylthymine, 9-α-D-glucopyranosyladenine, and 9-α-D-glucopyranosylhypoxanthine. 1-α-D-Ribofuranosylnicotinamide was prepared enzymatically from α-NAD, by successive digestion by phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) from snake venom and prostatic acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2). The final product was purified on a Dowex 1-formate column (17). 35P-labeled ribose 1-phosphate was enzymatically prepared from inosine and 35P1 (18) with the use of purified nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) from human erythrocytes. Hemolysates were treated with DEAE-cellulose to obtain nonhemoglobin protein fractions according to the procedures used by Stansell and Deutsch for the purification of crystalline catalase and erythrocyte enolase (19). A yellowish brown protein fraction thus obtained was further fractionated on a DEAE-cellulose column previously equilibrated with 0.01 M collidine-HCl, pH 7.5. Elution was carried out by a linear gradient of increasing concentrations of NaCl, from 0.01 M to 0.09 M, in the same buffer. The enzyme recovered from the column was found to be purified about 100-fold, with a yield of 30% over the initial hemolysates, and was practically free from acid phosphatase activity. The activity of nucleoside phosphorylase was measured with guanosine as substrate (20, 21) throughout the purification. Ribose 1-phosphate was identified by its hydrolysis constant in an acid-molybdate solution (22) and by an equimolar formation of ribose and P1 upon hydrolysis. The compound was also found to be active as substrate in the reverse reaction of nucleoside phosphorylase from human erythrocytes (20, 21). Charcoal, Shira-sagi, was the product of Takeda Pharmaceutical Industries. Protamine sulfate was purchased from Sigma, and alumina C by as described (23), and aged for at least 3 months prior to use. Ammonium sulfate was recrystallized twice. DEAE-cellulose was the product of Serva Company, West Germany, and was prepared for use (24). Sephadex G-25 and Sephadex G-50 were obtained from Pharmacia, Uppsala, Sweden. Carrier-free 35P was the product of the Radiochemical Centre, Amersham, England. The radioisotope was purified prior to use by treatment with 1 N HCl at 100°, followed by passage through a Dowex 50 column. Uridine-2-14C and uracil-2-14C were obtained from Schwarz BioResearch and Dai-ichi-Kagaku, Tokyo, respectively. All other reagents were of analytical grade.

**Biological Material—Pseudomonas fluorescens**, strain N, which was used throughout this work, was originally isolated from aged autolysates of baker's yeast. A pure culture of an aerobic gram-negative rod was obtained by successive transfers with serial dilution on a solid medium. The organism was identified as a strain of Pseudomonas fluorescens by Dr. R. Y. Stanier, University of California. This strain was maintained by monthly transfers and stored at 4° on peptone-bouillon agar slants.

**Methods**

**Enzyme Assay**—The activity of the enzyme was routinely followed by determination of uracil from uridine as substrate. The standard assay system contained 2.5 μmoles of uridine, 20 μmoles of Tris-acetate buffer, pH 8.5, and enzyme, in a final volume of 0.4 ml. Incubation was at 37° for 30 min, and the reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The absorbance at 290 μm was determined and corrected for a zero time control. The method is based on the fact that uracil has a higher molar extinction at 290 μm in an alkaline solution than does uridine (1, 8). For enzyme assay with other substrates, free pentose formed was determined by the orcinol reaction (25), after unutilized nucleosides were removed by charcoal treatment (26). The reaction mixture usually contained 1.25 μmoles of a substrate, 10 μmoles of Tris-acetate buffer, pH 8.5, and enzyme, in a volume of 0.2 ml. The reaction was terminated by the addition of 1.8 ml of 0.025 N perchloric acid containing about 8 mg of activated charcoal as a suspension, and the mixture was left for approximately 10 min at room temperature with occasional shaking, followed by centrifugation. An aliquot of the supernatant solution was used for the assay of free ribose. For the control run, the enzyme was replaced by water or by a boiled enzyme preparation. The two methods described above gave nearly identical results with uridine as substrate. Nicotinamide ribonucleoside cleavage was measured spectrophotometrically by means of the cyanide method of Colowick, Kaplan, and Gotti (27). In each assay system mentioned above, the concentration of enzyme was adjusted so that not more than 15% of the substrate was hydrolyzed during the incubation. The hydrolysis of uridine was proportional both to time and to protein concentration under the assay conditions. One unit of enzyme activity was defined as that amount of enzyme which produces 1 μmole of free uracil in 1 min at 37°. Protein was determined according to the method of Lowry et al. (28), with crystalline bovine serum albumin as a standard.

**Procedures for Separation of Uridine and Uracil—Uridine was separated from uracil by chromatography, either on ion exchange resin or on paper. A sample solution was made 0.01 M with respect to potassium borate and applied on a Dowex 1-X2-chloride column (200 to 400 mesh, 0.6 × 7.0 cm), followed by washing of the column with 0.01 M potassium borate and by elution with 0.02 M potassium borate containing 0.03 M KCl. Uric acid appeared as the first peak and uracil as the second when they were assayed by determination of the ultraviolet absorption.**

**Paper chromatography was carried out on Toyo-Roshi No. 53 paper by the ascending method with the following three solvent systems: n-butyl alcohol saturated with 3 N NH4OH (9), n-butyl alcohol-water (86:14) (20), and isopropyl ether- n-butyl alcohol-98% formic acid (30:30:20) (30). Spots of nucleosides and bases were detected under ultraviolet light, and those of free sugars were detected by the spray of a p-anisidine reagent (31).**

**Solvent Systems of Paper Chromatographic Separation of Nucleosides and Base Components**—The following solvent systems were used for qualitative examination of the cleavage of natural and synthetic nucleosides: isopropyl ether-n-butyl alcohol-98% formic acid (30:30:20) (30) for deoxyuridine, 1-α-D-ribofuranosyluracil, pseudouridine, deoxyxycytidine, thymidine, 1-β-D-glucopyranosylthymine, 1-β-D-ribofuranosyluracil, 1-α-D-ribofuran- nosylthymine, and orotidine; n-butyl alcohol-water (86:14) (29) for cytidine, adenosine, guanosine, inosine, xanthosine, 4-amino-β-imidazolecarboxamidine ribonucleoside, 9-β-D-glucopyranosyladenine, 9-α-D-glucopyranosyladenine, 9-β-D-glucopyranosyladenine, 9-β-D-glucopyranosyladenine, 9-β-D-glucopyranosyladenine, 9-β-D-glucopyranosyladenine, 9-β-D-glucopyranosyladenine.
pyranosylhypoxanthine, 9-α-D-glucopyranosylhypoxanthine, 9-β-L-ribofuranosyladenine, 9-β-L-ribofuranosylhypoxanthine, 6-azauridine, 5-bromouridine, formycin, tubercidin, 3-β-d-ribofuranosyladenine, 1-β-d-arabinofuranosylcytosine, the aminonucleoside of puromycin, and puromycin; n-butyl alcohol-n-propyl alcohol-ethanol-25% NH₄OH-water (40:40:10:45:15) (30) for deoxyadenosine, deoxyguanosine, and coressyopin; 1 x ammonium acetate-ethanol (3:2) (32) for 1-β-ribofuranosylnicotinamide, 2.0 ml of isobutyl alcohol, and 0.5 ml of water, and the mixture was shaken vigorously by hand for 10 sec immediately after the addition of the sample. When the two phases were well separated, an aliquot from each layer was assayed for radioactivity without drying in a Geiger-Müller counter. When the two phases were well separated, an aliquot from each layer was assayed for radioactivity without drying in a Geiger-Müller counter.

Measurement of Radioactive Organic and Inorganic Phosphate—32Pi was extracted with isobutyl alcohol in the presence of molyblic acid, by a slight modification of the method of Berenblum and Chain (35). A sample solution (0.05 ml) containing 32Pi, was transferred to a glass-stoppered tube which contained 0.5 ml of 2 N H₄SO₄, 0.5 ml of 0.5 m ammonium molybdate, 2.0 ml of isobutyl alcohol, and 0.5 ml of water, and the mixture was shaken vigorously by hand for 10 sec immediately after the addition of the sample. When the two phases were well separated, an aliquot from each layer was assayed for radioactivity without drying in a Geiger-Müller counter. When purified 32Pi was submitted to the extraction procedure, the radioactivity that remained in the aqueous layer was less than 0.2% of that extracted in the organic layer.

RESULTS

Purification of Enzyme

Growth of Organism—The medium consisted of 0.15% KH₂PO₄, 0.05% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.2% NH₄Cl, 0.4% peptone, and 0.03% yeast extract. An overnight culture of Pseudomonas fluorescens, strain N, was grown for 17 hours at 25°C with mechanical shaking in 1.5 liters of the medium in 5-liter Erlenmeyer flasks. Cells were harvested by a Sharples centrifuge at 4°C at 28,000 rpm, and about 5 g of cells were obtained per 1.5 liters of the medium. Subsequent steps were carried out at 4°C unless otherwise stated.

Preparation of Extract—The harvested cells were washed three times with 0.85% KCl, weighed, suspended in 4 volumes of 0.02 M Tris-acetate, pH 7.0, and disrupted in a Kabota 10-kc sonic disintegrator for 20 min. The sonically treated suspension was centrifuged at 20,000 × g for 20 min in a Lourdes centrifuge. The supernatant solution was designated as the crude extract.

Purification of Enzyme—The crude extract was diluted with 0.02 M Tris-acetate buffer, pH 7.0, to a protein concentration of 10 mg per ml. To this solution, 2% protamine solution, pH 6.8, was added over a period of 20 min, and the precipitate was removed by centrifugation at 10,000 × g for 10 min. The appropriate amount of protamine sulfate was determined by a pilot test prior to each run of the treatment so that more than 90% of the activity would be recovered in the resulting supernatant; usually about 0.45 mg of protamine sulfate was necessary for every mg of protein, and the As₄₉₀/A₄₄₀ ratio increased from 0.65 for the crude extract to more than 0.80.

Fractionation with Ammonium Sulfate—Solid ammonium sulfate (313 mg per ml) was slowly added to the protamine fraction, and the precipitate was removed by centrifugation at 10,000 × g for 10 min. Solid ammonium sulfate (137 mg per ml) was added to the supernatant solution, and the precipitate was collected and dissolved in a minimum volume of 0.005 M Tris-acetate, pH 7.0, and dialyzed against several changes of the same buffer solution for 12 hours. The insoluble material formed during dialysis was removed by centrifugation.

Alumina Cy Eluate—The dialyzed solution was diluted with 0.005 M Tris-acetate, pH 7.0, to a protein concentration of 10 mg per ml. To 1 volume of the solution was added, with stirring, 0.5 volume of 2% alumina Cy suspension. The gel was collected by centrifugation and washed once with 1 volume of 0.01 M potassium phosphate, pH 7.0. The enzyme was eluted with a volume of 0.1 m potassium phosphate, pH 7.0, equal to 0.5 volume of the initial enzyme solution subjected to the gel treatment, and the elution was repeated in the same manner. The eluates were combined.

DEAE-cellulose Chromatography—The alumina Cy fraction was dialyzed against 0.005 M potassium phosphate, pH 7.3, for about 12 hours with frequent changes of the external solution. Then the Pi concentration of the enzyme solution was determined (36) and adjusted, when necessary, to 0.005 M. The solution was applied to a DEAE-cellulose column with a packed volume of about 0.2 ml per mg of protein, previously equilibrated with 0.005 M potassium phosphate, pH 7.3. The column was washed with 5 bed volumes of the same buffer. Desorption of the enzyme was carried out with a linear gradient from 0.005 M to 0.02 M potassium phosphate, pH 7.3. The total volume of the eluent was 4 times the column bed volume. The enzyme began to appear in the eluate after approximately 1.3 bed volumes of the eluting solution passed through the column. Active fractions with specific activity higher than 40 were pooled and concentrated with the use of Sephadex G-50; the enzyme solution was placed in cellophane tubing and the bag was embedded in dry powder of Sephadex G-50, followed by application of a moderate continuous pressure on it. The preparation at this step of purification was used for all the studies described in this paper unless otherwise stated. The procedure gave a fairly reproducible result in the four trials carried out, and the resulting specific activities ranged from 43 to 54 units per mg of protein. Table 1 gives the result of a purification procedure in which 100 g of packed cells, wet weight, were used as the starting material.

Alternate Method for Purification by Repeated DEAE-cellulose
Properties of Enzyme

Effect of pH—The enzyme exhibited maximal activity at pH 8.5 in Tris buffer with uridine, cytidine, or adenosine as substrate, and the activity toward inosine was maximal at pH 6.0 in sodium acetate or potassium phosphate buffer (Fig. 1).

Stability—When an alumina C\textsubscript{7} or DEAE fraction containing 1 to 3 mg of protein per ml was kept frozen at -15\textdegree C, no detectable loss in activity was observed over a period of 6 months. The enzyme was fairly stable even in a very dilute solution: for example, a DEAE fraction, containing 32 \textmu g of protein per ml, maintained over 70\% of its initial activity after storage at 4\textdegree C for 1 month. Stabilities at higher temperatures were tested at different pH values. After incubation at 45\textdegree C for 10 min at pH 7.0 to 9.2, more than 90\% of the enzyme activity was recovered. When incubation was at pH 5.0, the remaining activity was only 50\%. Heating at 70\textdegree C for 5 min at pH 8.5, however, completely inactivated the enzyme.

Chromatography—Further purification of the enzyme was carried out in the following way. Alumina C\textsubscript{7} eluates, containing 580 mg of protein in a volume of 200 ml, were thoroughly dialyzed against 0.005 M potassium phosphate, pH 7.0. The enzyme solution was then applied to a DEAE-cellulose column (2.5 × 20 cm) previously equilibrated with 0.005 M potassium phosphate, pH 7.0. After the column was washed with 400 ml of the same buffer, the enzyme was eluted with a 400-ml linear gradient of 0.005 M to 0.05 M potassium phosphate, pH 7.0. The active fractions were pooled, dialyzed against 0.005 M potassium phosphate, pH 7.6, and subjected to rechromatography on a DEAE-cellulose column (2.5 × 10 cm) previously equilibrated with 0.005 M potassium phosphate, pH 7.6. The bulk of the inactive protein was removed by washing the column with 300 ml of the same buffer solution. The enzyme was then eluted by a 600-ml linear gradient elution from 0.005 M to 0.05 M potassium phosphate, pH 7.6. Only a single protein peak was observed, coinciding with a single peak of activity in the middle part of the eluates from the column. The specific activity of the final preparation thus obtained was 272 units per mg of protein.

Effect of Substrate Concentration—The effect of varying the concentration of uridine on the initial rate of hydrolysis is shown in Fig. 2, and the \textit{K}_m value for uridine, calculated according to the method of Lineweaver and Burk (37), was 0.8 mM. The \textit{K}_m values for cytidine, adenosine, and inosine at pH 8.5 were found to be 1.0 mM, 0.5 mM, and 0.2 mM, respectively.

Evidence against Participation of Phosphorolytic Cleavage—Nucleoside phosphorylase has been reported to be widely distributed in various organisms, and the phosphorolytic cleavage of nucleosides seems to be the ubiquitous pathway of nucleoside metabolism (38, 39). Therefore, it was necessary to investigate the possibility that the hydrolysis of a nucleoside catalyzed by the present enzyme may proceed in two successive steps, i.e., phosphorolysis catalyzed by nucleoside phosphorylase and subsequent decomposition of ribose 1-phosphate by contaminating phosphatase activity. The following several lines of evidence, however, excluded the above possibility. Firstly, careful elimination of \textit{P}_i from the enzyme preparation as well as from the other components of the assay mixture did not cause any decrease in the reaction rate, and the addition of \textit{P}_i to such a system failed to enhance the activity to any extent. The enzyme used was previously treated with a column of Sephadex G-25, and the final concentration of \textit{P}_i in the reaction mixture was less than 1 \mu M (36). Secondly, there was no indication for the formation of ribose-1-P as an intermediate in the reaction catalyzed by the hydrolyase. The enzymatic hydrolysis of uridine was allowed to proceed in the presence of \textsuperscript{32}P\textsubscript{i}, and its esterification was followed. As shown in Table II, no formation of organic phosphate esters was detectable during the course of the reaction. Since this enzyme preparation was free from phosphatase activity toward ribose-1-P (Experiment 1 in Table III), the decomposition of ribose-1-P during the reaction was not likely. Thirdly, an attempt to detect a phospholysis activity by its reverse reaction gave only negative results; when the enzyme was incubated with uracil and ribose-1-\textsuperscript{32}P\textsubscript{i}, no significant amount of \textsuperscript{32}P\textsubscript{i} was released (Experiment 2 in Table III).

![Fig. 1. pH-activity curve. The reaction was followed by determination of free ribose formed, \(A\), from \(-\), uridine or \(-\), cytidine, and \(B\), from \(--\), adenosine or \(--\), inosine, under the standard assay conditions as described under "Experimental Procedure,"
except that the pH of the incubation mixture was varied as indicated. The following buffers were used, at a concentration of 50 mM: \(\circ\), sodium acetate; \(\circ\), potassium phosphate; and \(\times\), Tris-acetate. Activity is expressed as micromoles of ribose formed per mg of protein per min.](image-url)
Evidence against phosphorylolytic cleavage: absence of formation of ribose 1-phosphate

The reaction mixture (0.2 ml) contained Tris-acetate, pH 8.5, 10 μmoles; uridine, 1.25 μmoles; and enzyme, previously passed through a Sephadex G-25 column, 0.1 μg (0.005 unit). At 0, 15, and 30 min at 37°, a 50-μl sample was removed and the formation of phosphoric acid esters was measured by the use of the solvent extraction method, as described under “Experimental Procedure.” Radioactivity was calculated on the basis that the total volume of the aqueous layer was 1.35 ml after shaking, while that of the organic layer was 2.20 ml. In the control run, enzyme was replaced by the heat-inactivated enzyme solution.

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<td>Organic phosphate</td>
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<td>min</td>
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<td>15</td>
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<td>30</td>
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<td>0.4</td>
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<tr>
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have a ribosyl transferring activity. However, when the cleavage of uridine was allowed to proceed in the presence of radioactive uracil, there was no incorporation of radioactivity into uridine (Table IV). This excluded the presence of a ribosyl transferring activity with uracil as an acceptor. Since Tris buffer was described as having an adverse effect on the deoxy-ribosyl transfer reaction (10), the presence of ribosyl transferring activity in the enzyme was also tested in 50 mM glycylglycine, pH 8.5, or 50 mM potassium phosphate, pH 7.5. No ribosyl transferring activity was detected, even under these conditions.

Stoichiometry of Reaction—Stoichiometric data with uridine as substrate are presented in Table V. When the enzyme and uridine were incubated together, essentially equal quantities of uracil and d-ribose were formed, with a concomitant and equivalent decrease in the amount of uridine.

Effect of Metal Ions and Other Reagents—The enzyme did not require the addition of any metal ions for full activity, and various metals were inhibitory when assayed under the standard assay conditions. MnSO4, FeCl3, HgCl2, CoCl2, CdCl2, and ZnCl2, each at a final concentration of 1 mM, caused considerable inhibition, ranging from 40% to 95%. At 0.01 mM, the inhibition was less than 10%. In contrast, the inhibition by MgCl2, CuCl2, CuCl2, FeSO4, AgCl2, and NH4Cl at 1 mM was less than 10%. The enzyme activity was not affected by preliminary incubation for 10 min at 37° with p-chloromercuribenzoic acid, iodoacetate, glutathione, l-cysteine, or mercaptoethanol, each at a final concentration of 1 mM. Although the enzyme did not require the addition of any metal ions for full activity, and various metals were inhibitory when assayed under the standard assay conditions.

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>Absence of P1 formation from ribose 1-phosphate</th>
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<tr>
<td>Experiment 1</td>
<td>The incubation mixture contained 10 μmoles of Tris-acetate buffer, pH 8.5, 0.2 μmole of ribose-1-32P (specific activity, 18,000 cpm per μmole), and 0.1 μg of enzyme (0.005 unit), in a final volume of 0.2 ml. In Experiment 2, the reaction mixture contained 7.5 μmoles of Tris-acetate, pH 8.5, 0.75 μmole of uracil, 0.75 μmole of ribose-1-32P (specific activity, 18,000 cpm per μmole), and 0.8 μg of enzyme (0.04 unit), in a final volume of 0.15 ml. For the control run, enzyme was replaced by the heat-inactivated enzyme solution. The formation of P1 from ribose-1-32P was analyzed as described under “Experimental Procedure.”</td>
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<th>Stoichiometric data with uridine</th>
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<tr>
<td>30</td>
<td>-8.0</td>
</tr>
<tr>
<td>60</td>
<td>-13.4</td>
</tr>
</tbody>
</table>
not seem to require the addition of any metal for activity. 1 mM EDTA inhibited uridine hydrolysis by 50%. No further experiments were carried out to elucidate the nature of the effect of EDTA.

**Participation of Single Enzyme in Hydrolysis of Different Nucleosides**—From the results of the following experiments, it was concluded that at least four representative nucleosides so far examined, i.e. uridine, cytidine, adenosine, and inosine, were cleaved by a single enzyme.

1. Identical behaviors of the four activities through enzyme purification: the ratios of the activities toward these four different substrates remained constant throughout the steps of the enzyme purification. Similarly, nearly identical ratios were obtained with different enzyme fractions belonging to the same peak of the highly purified preparation obtained by repeated DEAE-cellulose column chromatography.

2. Heat denaturation studies of the four activities: a solution of the enzyme (32 μg per ml) in 0.02 M Tris-acetate buffer, pH 8.5, was brought to 60°, and aliquots were removed at 0, 5, 15, and 30 min, cooled at once, and then assayed for activity toward the four substrates. The ratios of the activities were nearly constant throughout the course of the enzyme denaturation (60% denaturation after 15 min).

3. Inhibition of uridine hydrolysis by other substrates: when cytidine, adenosine, or inosine was included at 0.25 mM in the standard assay system containing an equimolar concentration of uridine, the quantity of ribose formed was less than additive (41). Further kinetic studies on the nature of the inhibition by adenosine or inosine were performed according to the method of Lineweaver and Burk (37), and the results are depicted in Fig. 3. The inhibition was found to be competitive for the uridine hydrolase reaction; the Kᵢ values of adenosine and inosine were calculated to be 0.5 mM and 0.07 mM, respectively (37).

**Substrate Specificity**—The activities toward various natural and synthetic nucleosides were tested at a final substrate concentration of 0.25 mM at pH 8.5 (Table VI). Uridine, cytidine, 1-β-ribofuranosylthymine, and 5-bromouridine were the most active substrates and, therefore, uridine has been used as a representative substrate for most of the studies reported in this paper. When the activity toward uridine was taken as 100, the relative activities toward cytidine, 1-β-ribofuranosylthymine, and 5-bromouridine were 109, 76, and 72, respectively. In contrast to pyrimidine ribonucleosides, purine ribonucleosides were much less active: the relative activities toward adenosine, guanosine, inosine, and xanthosine were 30, 6, 5, and 7, respectively. 3-β-D-Ribofuranosyladenine appeared to offer an interesting case. This unusual purine nucleoside, in which the ribofuranosyl group is linked to N-3 of the purine ring instead of to N-9, was split as rapidly as a pyrimidine nucleoside. This compound might be considered as a 5,6-substituted derivative of cytidine.

The effect of pH on the hydrolysis of some of these substrates was studied, and some of the results have already been described. To summarize, the pH optimum was around 6.0 with inosine, 0.5 with guanosine, 7.0 with xanthosine, 8.0 with 1-β-ribofuranosylthymine, and 8.5 with cytidine, adenosine, 5-bromouridine, or uridine. When the Vᵢmax value for uridine at pH 8.5 was taken as 100, those for cytidine, 5-bromouridine, and 1-β-ribofuranosylthymine, determined at their respective optimum pH values, were 115, 90, and 90, respectively. In contrast, the Vᵢmax values for adenosine, guanosine, inosine, and xanthosine were 28, 18, 14, and 20, respectively. The Kᵢ values, as determined at the optimum pH values by the Lineweaver-Burk plot (37), were 1.0 mM for cytidine, 2.5 mM for 5-bromouridine, 7.5 mM for 1-β-d-ribofuranosylthymine, 0.8 mM for uridine, 0.5 mM for adenosine, 0.5 mM for guanosine, 0.5 mM for inosine, and 6.5 mM for xanthosine. Deaminase activity toward cytidine or adenosine was not detected in the final preparation of the enzyme.

In Table VII are listed those compounds which were inactive as substrates when tested at a final concentration of 6.25 mM both at pH 8.5 and at pH 6.0. These include five 2'-deoxyribonucleosides and a 3'-deoxyribonucleoside. Synthetic nucleoside analogues possessing a D-glucopyranosyl group as the sugar moiety were not hydrolyzed, whether the glycosidic linkage was of the α or the β type of configuration. 1-β-D-Arabinofuranosylytosine was not cleaved. 5'-UMP and 5'-CMP were also inactive. β-NAD, puromycin, and the amino-nucleoside of puromycin (14) were not split by this enzyme. 9-β-L-Ribofuranosyladenine and 9-β-L-ribofuranosylhypoxanthine were not hydrolyzed. Furthermore, none of the three nucleosides with α-D-ribofuranosyl groups, i.e. 1-α-D-ribofuranosyluracil, 1-α-D-ribofuranosylthymine, and 1-α-D-ribofuranosylcytosine, were cleaved. Neither pseudouridine, in which the ribofuranosyl moiety is linked to uracil with a carbon to carbon linkage of β configuration, nor formycin, in which the ribofuranosyl moiety is linked to 7-aminopyrazolo[4,3-d]pyrimidine with a carbon to carbon linkage (15), served...
The reaction mixture contained 20 μmoles of Tris-acetate, pH 8.5, 2.5 μmoles of a substrate, and various amounts of enzyme, in a final volume of 0.4 ml. The incubation was for 30 min at 37°C. An aliquot of the mixture was removed and the formation of free ribose was determined by the method described under “Experimental Procedure.” Another aliquot was chromatographed on paper with various solvent systems to confirm the cleavage of each nucleoside. For the control run, the heat-inactivated enzyme preparations were used.

### Table VI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-β-D-Ribofuranosyluracil (uridine)</td>
<td>100</td>
</tr>
<tr>
<td>1-β-D-Ribofuranosyletosine (cytidine)</td>
<td>109</td>
</tr>
<tr>
<td>1-β-D-Ribofuranosylthymine</td>
<td>76</td>
</tr>
<tr>
<td>1 β-D Ribofuranosyl 5 bromouracil (5-bromouridine)</td>
<td>72</td>
</tr>
<tr>
<td>3-β-D-Ribofuranosyladenine (3-isoadenosine)</td>
<td>88</td>
</tr>
<tr>
<td>9-β-D-Ribofuranosyladenine (adenosine)</td>
<td>30</td>
</tr>
<tr>
<td>9-β-D-Ribofuranosylguanine (guanosine)</td>
<td>6</td>
</tr>
<tr>
<td>9-β-D-Ribofuranosylhypoxanthine (inosine)</td>
<td>5</td>
</tr>
<tr>
<td>9-β-D-Ribofuranosylxanthine (xanthosine)</td>
<td>7</td>
</tr>
<tr>
<td>1-β-D-Ribofuranosylnicotinamide</td>
<td>87.0</td>
</tr>
<tr>
<td>4-Amino-5-imidazolecarboxamide ribonucleoside</td>
<td>0.9</td>
</tr>
<tr>
<td>Imidazoleacetic acid ribonucleoside</td>
<td>0.2</td>
</tr>
<tr>
<td>1-β-D-Ribofuranosyl-6-azauracil (6-azauridine)</td>
<td>0.1</td>
</tr>
<tr>
<td>4-Amino-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]-pyrimidine (tubercidin)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Activity toward uridine is taken as 100.

The hydrolysis was followed spectrophotometrically by means of the cyanide method (27).

### Discussion

Some of the compounds shown to be inactive as substrates were tested for a possible inhibitory action toward the enzymatic hydrolysis of uridine. In these experiments, the enzyme was incubated with uridine at 0.8 mM, which corresponds to its Km value, and one of the following compounds at a concentration of 0.25 mM: deoxyuridine, cordycepin, 9-β-L-ribofuranosyladenine, 9-β-D-glucopyranosylthymine, pseudouridine, oroticidin, formycin, uracil, and α-ribose. None of these compounds showed more than 5% inhibition of the enzymatic hydrolysis of uridine, indicating no significant interaction of these compounds with the enzyme protein.

### Table VII

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1β-α-L-2′-Deoxyribofuranosyluracil (deoxyuridine)</td>
<td>100</td>
</tr>
<tr>
<td>1β-α-L-2′-Deoxyribofuranosyletosine (deoxycytidine)</td>
<td>100</td>
</tr>
<tr>
<td>1β-α-L-2′-Deoxyribofuranosylthymine (thymidine)</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-L-Deoxyribofuranosyladenine (deoxyadenosine)</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-2′-Deoxyribofuranosylguanine (deoxyguanosine)</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-3′-Deoxyribofuranosyladenine (cordycepin)</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-Glucopyranosylthymine</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-Glucopyranosyladenine</td>
<td>100</td>
</tr>
<tr>
<td>9-β-α-Glucopyranosylhypoxanthine</td>
<td>100</td>
</tr>
<tr>
<td>1β-β-L-Deoxyribofuranosyluracil</td>
<td>100</td>
</tr>
<tr>
<td>9β-β-L-Deoxyribofuranosyladenine</td>
<td>100</td>
</tr>
<tr>
<td>5′-UMP</td>
<td>100</td>
</tr>
<tr>
<td>5′-CMP</td>
<td>100</td>
</tr>
<tr>
<td>Aminonucleoside of puromycin</td>
<td>100</td>
</tr>
<tr>
<td>Puromycin</td>
<td>100</td>
</tr>
<tr>
<td>β-NAD</td>
<td>100</td>
</tr>
<tr>
<td>1α-α-L-Ribofuranosyluracil</td>
<td>100</td>
</tr>
<tr>
<td>1α-α-L-Ribofuranosylthymine</td>
<td>100</td>
</tr>
<tr>
<td>1α-α-L-Ribofuranosyladenine</td>
<td>100</td>
</tr>
<tr>
<td>9α-α-Glucopyranosylthymine</td>
<td>100</td>
</tr>
<tr>
<td>9α-α-Glucopyranosyladenine</td>
<td>100</td>
</tr>
<tr>
<td>Pseudouridine</td>
<td>100</td>
</tr>
<tr>
<td>Formycin</td>
<td>100</td>
</tr>
<tr>
<td>Orotidine</td>
<td>100</td>
</tr>
</tbody>
</table>

* When the uridine cleaving activity is taken as 100, “inactive” means that the initial velocity of hydrolysis is less than 0.05 on the basis of an observation that at least 20 mmoles of a base per each spot on paper could be detected under ultraviolet light. The absence of free ribose formation was also confirmed by the spray of a p-anisidine reagent on paper.
Pullman and Pullman (42) presented a hypothesis that the rate of hydrolysis of ribonucleosides is related to the value of the net positive charge on N-9 of purine or on N-1 of pyrimidine bases: when the positive charge is greater, more rapid hydrolysis is supposed to take place, and the rate of hydrolysis of purine ribonucleosides, whether enzymatic or chemical, is generally greater than that of pyrimidine ribonucleosides. The hypothesis was supported by experimental observations with enzymes from 

L. delbrueckii (8), yeast (12), and fish muscle (13). The Pseudomonas enzyme, however, hydrolyzes pyrimidine ribonucleosides more rapidly than purine ribonucleosides. Thus, the nature of the enzyme protein as a catalyst can be a dominant factor in determining the rates of hydrolysis of ribonucleosides.

Chemical hydrolysis of a pyrimidine ribonucleoside requires drastic conditions and leads to some destruction of the sugar and base moieties. The nucleoside hydrolase described in this paper thus offers a very useful biochemical means for pyrimidine ribonucleoside cleavage. The stable nature of the enzyme can be cited as another merit for practical use.

Nucleoside phosphorylase is a common species of enzyme involved in nucleoside catabolism, and is widely distributed in nature (20, 26, 38, 39, 43-47). Phosphorylase, however, is not suitable for the enzymatic cleavage of nucleoside because the equilibria of phosphorylase reactions are favorable for the synthesis of nucleosides. The data presented in this paper excluded any participation of a phosphorolytic mechanism, even in part, in the reaction catalyzed by this enzyme.

While nucleoside deoxyribonucleoside transferase was shown to catalyse the hydrolysis of deoxyribonucleosides under appropriate conditions (9, 10), the present hydrolase preparation was found to be free from a ribosyl transferring activity. This observation, as well as the exclusion of a phosphorolytic mechanism, may offer an additional confirmation of the existence of nucleoside hydrolyase in nature as a separate entity.

Although it would be unwise at present to postulate any mechanism of interaction of the enzyme protein with its substrate, the results of substrate specificity studies suggest that the ribofuranose residue plays a primary role in the formation of an enzyme-substrate complex. This enzyme seems to provide a good system for the investigation of a type of interaction between a nucleic acid derivative and an enzyme protein.

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Masaaki Terada, Masamiti Tatibana and Osamu Hayaishi


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