Cytoplasmic Uridine Phosphorylase of Rat Liver

CHARACTERIZATION AND KINETICS*

ARTHUR KRATJT$ AND ESTHER W. YAMADA

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

SUMMARY

Uridine phosphorylase (uridine:orthophosphate ribosyl-transferase, EC 2.4.2.3) of rat liver cytoplasm has been purified 1900-fold. Initial velocity patterns of the phosphorolysis of uridine or deoxyuridine and of the synthesis of uridine indicated a sequential mechanism. Product inhibition patterns with uracil or ribose 1-phosphate as inhibitors were consistent with an "ordered Bi Bi" reaction in which inorganic phosphate is the first substrate to bind to the enzyme and ribose 1-phosphate the last product to leave the enzyme.

The most highly purified enzyme fractions still retained uridine-, deoxyuridine-, and thymidine-cleaving activities in the ratio of 10:7:1 at pH 7.4. The fractions separated into a major and a minor band of protein on electrophoresis in phosphate buffer. Aging of the enzyme with loss of activity resulted in an increase in the proportion of the minor band. The pH optimum of the enzyme for uridine cleavage was 8.2, for deoxyuridine cleavage, 6.5, and for uridine synthesis, 8.5. Uridine or uridine synthesis was inhibited 50% in the presence of 1 μM uracil. The enzyme did not catalyze the direct transfer of ribose from uridine to uracil in the absence of phosphate. Uridine protected the enzyme to a greater degree than phosphate against inhibition by o-iodosobenzoate. Thus, at least one sulfhydryl group and possibly three are present at or near the active site.

Uridine cleavage, uridine synthesis, and deoxyuridine cleavage were inhibited by deoxyglucosylthymine. The phosphorolysis of deoxyuridine and thymidine by thymidine phosphorylase, also purified from rat liver cytoplasm, was not inhibited by deoxyglucosylthymine. The molecular weights of both uridine and thymidine phosphorylases were estimated to be 110,000.

EXPERIMENTAL PROCEDURE

Materials

Deoxyuridine, uracil, deoxyribose 1-phosphate (cyclohexylammonium salt), ribose 1-phosphate (dicyclohexylammonium salt), and p-mercuriphenylsulfonate were purchased from Sigma. Iodoacetic acid, molecular weight markers, o-iodosobenzoate, N-ethylmaleimide, thymidine, and uridine were obtained from Mann. Dithiothreitol, 5,5'-dithiobis(2-nitrobenzoic acid), p-mercuribenzoate, and iodoacetamide were products of Calbiochem. Carbowax-20 M is a product of Union Carbide. Uracil-2¹⁴C (58.0 mCi per mmole) was purchased from Schwarz. Deoxyglucosylthymine was a gift from Dr. M. Zimmerman, Merck, Sharp and Dohme.

Enzyme Assays

Phosphorolytic Activity—Enzyme activity was assayed by the spectrophotometric procedures of Yamada (8). Procedure 1

* This work was supported by the Medical Research Council of Canada.

† This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

+ The nomenclature of Cleland (3) is used throughout.
was used to assay the activity of enzyme fractions dissolved in Buffer A (0.05 M potassium phosphate buffer (pH 7.0)-5 mM β-mercaptoethanol-1 mM EDTA (pH 7.0)) and Procedure 2 for those dissolved in Buffer B (0.02 M potassium phosphate buffer (pH 8.0)-10 mM β-mercaptoethanol-1 mM EDTA (pH 8.0)). The final pH of both reaction mixtures was 7.4. To adjust the pH of the incubation medium to 8.2 or 6.5, 150 μmoles of glycylglycine buffer (pH 8.9) or acetate buffer (pH 5.5) were included in the reaction mixture. To obtain graded concentrations of each substrate, phosphate buffer was replaced by appropriate volumes of Tris buffer of the same molarity and pH while uridine was replaced by water buffered to pH 7.0.

In studies of the action of inhibitors phosphate buffer was replaced by Tris buffer. After preliminary incubation of the enzyme with inhibitor at 25° the reaction was started by the addition of phosphate and then the nucleoside; incubation was continued at 37° for 10 min as in the standard assay procedures.

All assays were done in duplicate and in all assays the test solutions were read against controls (8). For every micromole of uracil formed from uridine per tube (1.5 ml) the increase in absorbance at 290 μm was 4.40 and from deoxyuridine at 290 μm was 4.70 in cuvettes of 1-cm light path. Similarly, for every micromole of thymine formed from thymidine, the increase in absorbance at 295 μm was 4.20.

Under the standard conditions of both procedures, the formation of free base was a linear function of time for 30 min and of protein concentration up to an optical density reading of 0.390. One unit of enzyme activity is defined as that quantity that catalyzes the formation of 1.0 μmole of free base per hour. Specific activity is defined as the number of units per mg of protein. Protein was usually measured by the method of Lowry et al. (9); however, the method of Warburg and Christian (10) was used for fractions separated by column chromatography.

Nucleoside Synthesis—The decrease in absorbance due to the formation of nucleoside from free base was measured (11). The incubation medium contained the following (in micromoles) in a final volume of 1.5 ml: glycylglycine buffer (pH 8.4), 150; ribose 1-phosphate (pH 7.0), 4.2; uracil, 1.0; and enzyme diluted with 0.05 M Tris buffer (pH 7.2). The final pH of the reaction mixture was 8.2. The reaction was started by the addition of uracil and the tubes were incubated for 30 or 65 min at 37°. The rest of the assay was identical with that described for the assay of phosphorolytic activity (8). Linearity between the formation of uridine and time was evident up to 70 min and the maximum velocities (V₁ and V₂) were calculated from these replots.

It will be assumed (and justified later) that the reaction mechanism is of the "ordered Bi Bi" type (3) as represented below:

$$ E \overset{A}{\rightarrow} (EA) \overset{B}{\rightarrow} (EAB) \overset{P}{\rightarrow} (EPQ) $$

where A, B, P, Q, and E represent inorganic phosphate, uridine, uracil, ribose 1-phosphate, and enzyme, respectively.

RESULTS

Enzyme Purification

Approximately 50 male Holtzman rats of 250 to 350 g were used in each purification. The rats were decapitated; their livers were perfused in situ with ice-cold 0.9% sodium chloride, removed, and weighed. All subsequent steps were carried out in a cold room at 3-4° and all solutions were kept at 0° in ice buckets unless specified otherwise. Routinely, all dialyzing tubing was soaked overnight at 3° in 1% EDTA (pH 7.0) and was washed with buffer before use. The procedures are summarized in Table I.

Step 1: Homogenization—Livers were homogenized in a cold room at 3-4° and all solutions were kept at 0° in ice buckets unless specified otherwise. Routinely, all dialyzing tubing was soaked overnight at 3° in 1% EDTA (pH 7.0) and was washed with buffer before use. The procedures are summarized in Table I.

Step 2: Separation of Cytoplasm—The nuclear pellet was sedimented by centrifugation for 20 min at 900 × g in a Sorvall RC-2 refrigerated centrifuge. The cytoplasmic fraction was removed; the sediment was washed with two times its weight of sucrose solution. After centrifugation as before the supernatant was added to the cytoplasmic fraction. This fraction was then dialyzed for 16 hours in 100 volumes of Buffer A in a
of KC1 was started by allowing 0.4 M KCl, dissolved in 400 ml
of 280 mp, to flow into a mixing chamber containing 400 ml of
Buffer B and thence into the column. Sufficient \( \beta \)-mercapto-
ethanol was added to each fraction of eluate to increase the final
concentration by 8 to 10 mM. A typical chromatogram showing the
separation of uridine phosphorylase and thymidine phospho-
phorylase is given in Fig. 1A. Column Fractions 104 to 116
were pooled; the enzyme was precipitated with ammonium sul-
fate at 80% saturation (56 g/100 ml) and resuspended in Buffer
A.

Step 7: \( \text{DEAE-Sephadex (pH 8.0)} \)--Step 6 was repeated. The
fractions with the highest uridine-cleaving activity were com-
bined and concentrated as described in Step 6.

Step 8: \( \text{DEAE-Sephadex (pH 7.2)} \)--The enzyme preparation
was dialyzed and purified by chromatography on a third \( \text{DEAE-
Sephadex} \) column (2.5 \( \times \) 45 cm; bed volume, 240 ml) by a pro-
cedure identical with that of Step 6 except that the pH of Buffer
B was adjusted to pH 7.2 and the KC gradient was linear from
0 to 0.15 M. Again the fractions with the highest uridine-cleav-
ing activity were combined and concentrated as described in
Step 6.

Step 9: \( \text{Sephadex G-200 (pH 8.0)} \)--Sephadex G-200 was al-
lowed to swell in Buffer B minus \( \beta \)-mercaptoethanol. The
supernatant liquid and fines were decanted and replaced by
fresh buffer, four times. The gel slurry was then poured into a
column of 2.5-cm diameter to a height of 90 cm (bed volume,
421 ml). The column was equilibrated with 2 bed volumes of
Buffer B. The enzyme preparation of Step 8 was concentrated
in Carbowax over a volume of 4.8 ml and was then applied to the
column. The column was developed with Buffer B; 48 ml
fractions were collected at a flow rate of 32 ml per hour. The
fractions with the highest uridine-cleaving activity were pooled,
concentrated in Carbowax over a 6-hour period, and dialyzed
for 2 hours in 2 liters of 0.02 M potassium phosphate buffer
(pH 7.2)-5 mM \( \beta \)-mercaptoethanol.

Step 10: \( \text{Hydroxylapatite (pH 7.2)} \)--Hydroxylapatite was
washed five times with large volumes of 0.02 M potassium phos-
phate buffer (pH 7.2). After each washing the supernatant
and fines were removed. The absorbent was then added to a
column of 0.9-cm diameter to a height of 50 cm (bed volume, 50
ml) and equilibrated with 5 bed volumes of Buffer C (0.02 M
potassium phosphate buffer (pH 7.2)-10 mM \( \beta \)-mercaptoethanol).
Two and one-half milliliters of enzyme fraction from Step 9 were
applied to the column, followed by 3 bed volumes of Buffer C.
The column was then eluted with a linear gradient of phosphate
allowing 300 ml of Buffer C adjusted to a phosphate concen-
tration of 0.25 M to flow into a mixing chamber containing 300 ml
of Buffer C and thence into the column. The fractions con-
taining more than 150 units of uridine-cleaving activity (Fig. 1B)
were combined and concentrated with Carbowax. The concen-
trate was dialyzed for 3 hours against Buffer A, clarified by
centrifugation at 11,000 \( \times \)g for 20 min, and stored frozen at
-40°C. The specific activity of the enzyme preparation from
Step 10 was 144 which represents a 1900-fold purification of the
initial homogenate (Table I).
Fig. 1. A, DEAE-Sephadex chromatography at pH 8.0 of enzyme fraction from Step 6 containing 2781 mg of protein (——) and 1595 units of deoxyuridine-cleaving activity of which 80 and 93%, respectively, were recovered in the eluates. The vertical arrow (1) indicates the point at which elution with a linear gradient of KCl was begun. Deoxyuridine-cleaving activity of uridine phosphorylase (URPase, O) or thymidine phosphorylase (TdrPase, ●) was assayed by Procedure 2. The region of overlap of the two enzymes is indicated by the dotted lines. Column Fractions 164 to 116 (uridine phosphorylase) and 123 to 146 (thymidine phosphorylase) were pooled and concentrated by precipitation with ammonium sulfate. B, hydroxylapatite chromatography at pH 7.2 of enzyme fraction from Step 9. This sample contained 26 mg of protein (——), 832 units of uridine-cleaving activity (O), and 571 units of deoxyuridine-cleaving activity (●) of which 58, 92, and 92%, respectively, were recovered. Specific activity with uridine as the substrate (●) is given. Fractions 34 to 42 were combined and concentrated with Carbowax.

Disc Gel Electrophoresis—The specific activities were not constant across the peak of enzyme activity (Fig. 1B; Step 10), indicating that homogeneity of protein had still not been achieved. Thus, three different enzyme preparations representing purifications of 820-fold (C), 1900-fold (D), and 1100-fold (E) were subjected to electrophoresis and the results are shown in Fig. 2. All three preparations contained two dense bands of protein, the more slowly migrating band always being the widest and most intensely staining band. The banding pattern of Preparation D at pH 7.2 was similar to that found at pH 8.9. After aging at —4° for 10 weeks and thawing and freezing several times the minor band of Preparation C stained more intensely than in the original and four additional minor bands appeared.

Properties of Enzyme

Molecular Weight Estimation—Uridine and thymidine phosphorylases, separated by chromatography on DEAE-Sephadex (Step 6) and purified 36- and 13-fold, respectively, were subjected to gel filtration on Sephadex G-200 in buffer containing 0.05 M potassium phosphate buffer (pH 7.0) -10 mM β-mercaptoethanol. By the procedure of Andrews (23) it was estimated that the molecular weight of each enzyme was 110,000, well within the range estimated for purine (24) and other pyrimidine (25) nucleoside phosphorylases.

Substrates—The most highly purified enzyme fractions retained deoxyuridine- and thymidine-cleaving activities (Table 1). Constant ratios of 1.4 (uridine to deoxyuridine) and 10.0 (uridine to thymidine) were obtained after Step 7 for five different enzyme preparations. Nucleoside-cleaving activity was negligible in the absence of phosphate. Preliminary incubation of the enzyme with deoxyuridine for 5 min at 25° and optimal concentrations of uridine or deoxyuridine reduced uridine-cleaving activity at pH 8.2 only by 9% whereas uridine reduced the
phosphorylysis of deoxyuridine at pH 6.5 more than 50%. These results are in agreement with those reported for uridine phosphorylase of Ehrlich ascites cells (26).

**Synthesis of Nucleosides**—The synthesis of uridine was inhibited 50% at pH 8.2 by 1.2 mM uracil and the synthesis of deoxyuridine at pH 6.5, 65%. Others found much higher concentrations of uracil to be required to inhibit the activity of less pure preparations of uridine (27) or thymidine phosphorylase (12, 28).

**pH and Stability**—The phosphorylase of uridine and deoxyuridine proceeds with maximum velocity at pH 8.2 and 6.5, respectively, in agreement with earlier findings (1, 8, 26). At the pH optimum for each substrate ratios of 1.1 (uridine to deoxyuridine) were obtained. The pH optimum for the synthesis of uridine was 8.5 in agreement with the value of 8.2 observed for an enzyme preparation from Ehrlich ascites cells (29).

The most highly purified preparations were fairly stable; samples stored frozen at -40° still retained 85% of their activity after 60 days. Preparations that had lost from 25 to 55% of their activity could be reactivated to give 80% of the original activity by incubation at 25° for 1 to 2 hours in the presence of p-mercuribenzoate. Some reactivation was also found in the presence of dithiothreitol although the results were less reproducible at concentrations greater than 10 mM. Deoxyuridine-cleaving activity increased concomitantly with uridine-cleaving activity in these fractions.

**Transferase Activity**—In the absence of phosphate ribosyl transfer from uridine to uracil was not detected; it became measurable in the presence of 0.83 mM phosphate.

**Inhibitors**

*Deoxyguanosylthymine*—The phosphorylase of uridine catalyzed by Preparation E was inhibited 50% at pH 8.2 or 7.4 by 0.10 mM deoxyguanosylthymine, whereas that of deoxyuridine, at pH 6.5 or 7.4, was inhibited 50% by 0.018 mM deoxyguanosylthymine. Uridine synthesis was inhibited 50% at pH 8.2 by 0.14 mM deoxyguanosylthymine. The degree of inhibition with deoxyguanosylthymine was the same after preliminary incubation for 5 or 20 min. Preliminary incubation with either phosphate or uridine in saturating concentrations at 25° for 5 min did not decrease the inhibition by deoxyguanosylthymine. The phosphorylase of deoxyuridine or thymidine by thymidine phosphorylase (from Step 6) was not inhibited by deoxyguanosylthymine at a concentration of 0.19 mM.

—**SH Reagents**—Of the —SH reagents tested p-mercuriphenylsulfonate reacted most rapidly, maximum inhibition occurred after 3 min. Maximum inhibition with N-ethylmaleimide or o-iodosobenzoate obtained between 20 and 30 min, with 5,5’-dithiobis(2-nitrobenzoic acid) after 30 min, but was not reached even after 60 min with iodoacetamide or 120 min with iodoacetic acid.

For each —SH reagent the concentration-inhibition curves for phosphorolytic activity were sigmoidal and steep as expected for inhibitors that combine tightly with enzyme protein (30). The concentrations at which 50% inhibition occurred are listed in Table II (in increasing order of magnitude). The mercurials were the most effective inhibitors. Others found thymidine phosphorylase of human spleen (31) and purine nucleoside phosphorylase of human erythrocytes (32) to be inhibited by p-mercuribenzoate. There was little if any difference in the concentrations of p-mercuribenzoate or o-iodosobenzoate required to inhibit the phosphorylase of uridine and deoxyuridine at the pH optimum of each.

**Protection by Substrates**

Neither uridine nor phosphate at saturating concentrations protected the enzyme against inhibition by p-mercuribenzoate or p-mercuriphenylsulfonate (Table III). Both gave substantial protection against the other —SH reagents. Uridine afforded more protection than did phosphate, the preferential protection

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor at 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
</tr>
<tr>
<td>Uridine</td>
<td></td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td></td>
</tr>
<tr>
<td>p-Mercuriphenylsulfonate</td>
<td>5</td>
</tr>
<tr>
<td>p-Mercuribenzoate</td>
<td>5</td>
</tr>
<tr>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
<td>30</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>30</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>5</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>60</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>120</td>
</tr>
</tbody>
</table>

Table III

**Protection by substrates against —SH reagents**

Either 3.3 mM uridine or deoxyuridine or 10 mM phosphate was first incubated at 25° with enzyme Preparation E (7.2 μg of protein, 0.7 unit) for 5 min in the incubation mixture of Procedure 1 adjusted to pH 8.2 or 6.5 for deoxyuridine cleavage. Tris buffer replaced phosphate in the medium. The —SH reagent was added and preliminary incubation was continued for the time periods given in Table II. The second substrate was then added and enzyme activity was assayed as described for Procedure 1. The averages of two experiments are given.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor after preliminary incubation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uridine</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>p-Mercuriphenylsulfonate</td>
<td>60</td>
</tr>
<tr>
<td>p-Mercuribenzoate</td>
<td>50</td>
</tr>
<tr>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
<td>69</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>44</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>63</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>74</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>60</td>
</tr>
</tbody>
</table>
Fig. 3. Phosphorolysis of uridine (UR) at pH 8.2 with P$_1$ as the variable substrate. Double reciprocal plot of initial velocity (micromoles of uracil formed per 10 min per ml) with respect to P$_1$ concentration (millimolar). Uridine was held constant at concentrations of 1.670 mM (O), 0.500 mM (A), 0.333 mM ( ), 0.167 mM ( ), and 0.089 mM ( ). Enzyme Preparation E (7 to 57 μg of protein) was used. Kinetic parameters estimated from this plot and replots (insets) are $K_a = 0.250$ mM; $V_I = 0.257$ pmole per ml per 10 min; $K_{i1} = 0.360$ mM; $K_{i2} = 2.450$ mM.

Fig. 4. Phosphorolysis of deoxyuridine (DUR) at pH 6.5 with deoxyuridine as the variable substrate. Plot of the reciprocal of initial velocity (micromoles of uracil formed per 10 min per ml) with respect to the reciprocal of deoxyuridine concentration (millimolar). $P_1$ was held constant at 117 mM (O), 3.980 mM ( ), 1.670 mM ( ), and 0.835 mM ( ). Enzyme Preparation E (13 μg of protein) was used. Kinetic parameters estimated from this plot and replots (insets) are $K_a = 0.582$ mM; $V_I = 0.094$ pmole per ml per 10 min; $K_{i1} = 2.530$ mM; $K_{i2} = 0.278$ mM.

by uridine being most apparent with o-iodosobenzoate. Results with deoxyuridine were similar to those with uridine at the pH optimum of each nucleoside.

**Initial Velocity Patterns**

**Phosphorolysis of Uridine at pH 8.2**—Fig. 3 shows the double reciprocal plot of initial velocity with respect to inorganic phosphate concentration when uridine was the nonvariable substrate. A series of intersecting lines in which both the slopes and intercepts change can be seen. The point of intersection is above the horizontal axis, indicating that the ratio of $K_{i2}/K_a$ is greater than 1.

A similar pattern was obtained when uridine was the variable substrate and phosphate the nonvariable substrate. In both

**Phosphorolysis of Deoxyuridine at pH 6.5**—The data from initial velocity studies with phosphate as the variable substrate and deoxyuridine as the nonvariable substrate give a pattern of crossing lines resembling that observed for uridine cleavage (Fig. 3). Similar data were obtained with deoxyuridine as the variable substrate and phosphate as the nonvariable substrate (Fig. 4).

**Uridine Synthesis at pH 8.2**—Initial velocities of uridine synthesis with uracil as the variable substrate and ribose 1-phosphate as the nonvariable substrate are shown in Fig. 5. Those with ribose 1-phosphate as the variable substrate and uracil as the nonvariable substrate gave a series of similarly

**Table IV**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phosphorolysis</th>
<th>Nucleoside synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$ (mM)</td>
<td>$0.349$</td>
<td>$0.071$</td>
</tr>
<tr>
<td>$K_{i2}$ (mM)</td>
<td>$3.215$</td>
<td>$0.085$</td>
</tr>
<tr>
<td>$K_{i1}$ (mM)</td>
<td>$0.240$</td>
<td>$0.286$</td>
</tr>
</tbody>
</table>

studies the replots of the intercepts or slopes against the reciprocals of uridine or $P_1$ concentrations showed linear relationships. These results indicate that the predominant mechanism is sequential (3).

**Phosphorolysis of Deoxyuridine at pH 6.5**—The data from initial velocity studies with phosphate as the variable substrate and deoxyuridine as the nonvariable substrate give a pattern of crossing lines resembling that observed for uridine cleavage (Fig. 3). Similar data were obtained with deoxyuridine as the variable substrate and phosphate as the nonvariable substrate (Fig. 4).

**Uridine Synthesis at pH 8.2**—Initial velocities of uridine synthesis with uracil as the variable substrate and ribose 1-phosphate as the nonvariable substrate are shown in Fig. 5. Those with ribose 1-phosphate as the variable substrate and uracil as the nonvariable substrate gave a series of similarly
Product Inhibition by Uracl of Phosphorolysis at pH 8.2 with $P_i$ as the Variable Substrate at Low Uridine Concentration (0.133 mM). Plot of reciprocal of the initial velocity (micromoles of uracil formed per 10 min per ml) with respect to the reciprocal of $P_i$ concentration (millimolar). Enzyme Preparation C was used (20 to 40 µg of protein). Uracl concentrations: 0 (O), 0.061 mM (A), 0.121 mM (C), and 0.242 mM (O). Kinetic parameters estimated from replots (insets) are $K_{eq} = 0.102$ mM; $K_e$ (uracil) = 0.064 mM.

Intersecting lines. In both cases linear replots of the slopes or intercepts with respect to the concentration of nonvariable substrate were obtained. Thus, the mechanism for the release of products from the enzyme is predominantly sequential (3).

**Kinetic Constants**—The Michaelis constants obtained from analysis of initial velocities are summarized in Table IV. It is apparent from this table that the $K_a$ for phosphate is greater at pH 6.5 than at 8.2. At both pH 6.5 and 8.2 the $K_{ia}$ values are greater, by factors of 4 and 9, respectively, than the corresponding values for $K_a$. In contrast, in uridine synthesis the ratio of $K_{eq}/K_a$ for ribose 1-phosphate is close to unity and as would be predicted (3) the lines in the Lineweaver-Burk plot intersect at the horizontal axis. From a comparison of the $K_{ia}$ and $K_{eq}$ values it is apparent that the affinity of ribose 1-phosphate for the enzyme is at least 38 times greater than that of phosphate at pH 8.2.

Table IV also shows that the $K'_b$ value for the alternate substrate, deoxyuridine, is higher than the $K_b$ for uridine, measured at the pH optimum of each nucleoside.

**Product Inhibition Studies**

_Inhibition of Phosphorolysis of Uridine at pH 8.2 by Uracl—_A pattern of noncompetitive inhibition was obtained with phosphate as the variable substrate, uridine as the nonvariable substrate, at low, non-saturating levels, and uracil as the product inhibitor (Fig. 6). $K_{eq}$ and $K_e$ (uracil) were calculated as described in Table V. A 4-fold increase in the concentration of uridine with phosphate as the variable substrate resulted in a pattern approaching uncompetitive inhibition by uracil (Fig. 7).
The kinetic parameters were estimated from the apparent \(K_i\) constants, \(K_{ii}\) and \(K_{ii}^\prime\), the points of intersection of the horizontal axis of the intercept and slope replots, respectively, as presented in the insets of Figs. 6 to 10. The \(K_a, K_b, K_{ia}\), and \(K_{ie}\) constants used in the calculations are those given in Table IV.

### Table V

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculated value</th>
<th>Fig.</th>
<th>Method of calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_i) (uracil)</td>
<td>0.094</td>
<td>6</td>
<td>(K_{ii} = \frac{B + K_b}{(B/K_{ia}) + (K_aK_b/K_{ia}K_{ie})})</td>
</tr>
<tr>
<td></td>
<td>0.064</td>
<td>7</td>
<td>(K_{ii} = K_i \left(1 + \frac{A}{K_a}\right))</td>
</tr>
<tr>
<td>(K_i) (ribose-1-P)</td>
<td>0.622</td>
<td>10</td>
<td>(K_{ia} = K_i)</td>
</tr>
<tr>
<td></td>
<td>0.167</td>
<td>9</td>
<td>(K_{ia} = K_i)</td>
</tr>
<tr>
<td>(K_{ie}) (ribose-1-P)</td>
<td>0.102</td>
<td>6</td>
<td>(K_{ia} = \frac{K_pK_{ie}}{K_e} \left(1 + \frac{K_eB}{K_{ie}K_e}\right))</td>
</tr>
<tr>
<td></td>
<td>0.231</td>
<td>7</td>
<td>(K_{ia} = K_i \left(1 + \frac{A}{K_{ie}}\right))</td>
</tr>
<tr>
<td></td>
<td>0.144</td>
<td>8</td>
<td>(K_{ia} = K_i)</td>
</tr>
<tr>
<td></td>
<td>0.042</td>
<td>9</td>
<td>(K_{ia} = K_i)</td>
</tr>
</tbody>
</table>

* From studies of product inhibition by uracil at pH 8.2 with uridine as the variable substrate at high Pi concentration (0.667 mM).

* From studies of product inhibition by ribose-1-P at pH 8.2 with Pi as variable substrate at low uridine concentration (0.266 mM).

At a low, fixed concentration of phosphate and uridine as the variable substrate a pattern of noncompetitive inhibition was obtained in the presence of uracil (Fig. 8). The same type of inhibition obtained with a 10-fold increase in phosphate concentration.

In all of the experiments the replots of slopes or intercepts were linear with respect to the concentration of uracil (Figs. 6 to 8), indicating that linear inhibition obtained with either phosphate or uracil as the variable substrate.

### Inhibition of Phosphorolysis of Uridine at pH 8.2 by Ribose 1-Phosphate—At a low, fixed concentration of uridine and with phosphate as the variable substrate a pattern of competitive inhibition by ribose 1-phosphate was found. A pattern of competitive inhibition was also found when the concentration of uridine was increased 12-fold to saturating levels (Fig. 9). These results suggest that ribose 1-phosphate and phosphate compete for the same site or the same enzyme form.

Inhibition by ribose 1-phosphate with uridine as the variable substrate at nonsaturating levels of phosphate was noncompetitive (Fig. 10). In other experiments there was slight inhibition by ribose 1-phosphate (0.73 mM) when the phosphate concentration was increased to 66.7 mM, close to saturating levels, but little or none when the phosphate concentration was increased further to 166.8 mM.

### Inhibition Constants and Dissociation Constants—Table V shows that there was a 6-fold variation in the estimates of \(K_{ie}\), much more than those calculated from initial velocity studies (Table IV), to which, however, they still compare favorably. There was a 4-fold variation in the values for \(K_i\) with ribose-1-phosphate as the product inhibitor. Similarly, the values for \(K_i\) (uracil) varied over a 16-fold range. Since the enzyme catalyzes a reversible reaction it is probable that in product inhibition studies the inhibition constants are influenced by the rate constants of both the forward and reverse reactions, thus leading to variations in their measurement.

### Order of Addition of Reactants—The data from product inhibi-

---

**Fig. 10.** Product inhibition by ribose-1-P (R-1-P) at pH 8.2 with uridine (UR) as the variable substrate at low concentration of Pi (0.667 mM). Enzyme Preparation C (20 to 40 pg of protein) was used. Plot of reciprocal of the initial velocity (micromoles of uracil formed per 10 min per ml) with respect to the reciprocal of Pi concentration (millimolar). Ribose-1-P concentrations: 0 (○), 0.799 mM (△), 0.438 mM (□), and 0.730 mM (●). The kinetic parameters estimated from the replots (insets) are: \(K_i\) (ribose-1-P) = 0.622 mM; \(K_i\) (ribose-1-P) = 0.401 mM.

---

**Table VI**

<table>
<thead>
<tr>
<th>Product inhibitor</th>
<th>Variable substrate</th>
<th>Phosphate (A)</th>
<th>Uridine (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unsaturated with B</td>
<td>Saturated with B</td>
</tr>
<tr>
<td>Uracil (P)</td>
<td></td>
<td>Noncompetitive</td>
<td>Competitive</td>
</tr>
<tr>
<td>Ribose 1-phosphate (Q)</td>
<td></td>
<td>Competitive</td>
<td>Uncompetitive</td>
</tr>
</tbody>
</table>

---

**Downloaded from http://www.jbc.org/ by guest on September 21, 2017**
tion studies, summarized in Table VI, are consistent with an ordered Bi Bi mechanism (3) in which inorganic phosphate is the first substrate to bind to the free enzyme and ribose 1-phosphate is the last product to dissociate from the enzyme. The affinity of ribose 1-phosphate for cytoplasmic uridine phosphorylase of rat liver cytoplasm is much greater than that of phosphate (Table IV). This finding takes together with the fact that the equilibrium of the reaction is in favor of nucleoside synthesis (26) suggests a means by which the enzyme may function in an anabolic role (viz. in the salvage pathway) even if ribose 1-phosphate is present in smaller concentrations in the cell than the ubiquitous inorganic phosphate.

**DISCUSSION**

Results from present studies cannot be reconciled with the reverse order of addition of substrates to uridine phosphorylase arrived at by Krenitsky, Barclay, and Jacques (1). In the latter studies the order of addition of substrates was based on the finding that close to stoichiometric amounts of phosphate were required in the transfer of uracil to uridine in the absence of exogenous ribose 1-phosphate. High concentrations of phosphate or arsenate inhibited ribosyl transferase activity. These studies were done at pH 6.5, far from the pH optimum of 8.2 for uridine cleavage or synthesis found for rat liver uridine phosphorylase; no studies of deoxyribosyl transferase activity were done. In addition, enzyme preparations of different purity and from different sources were used in these and present studies.

Apart from the differences in experimental procedures, the transferase and phosphorylase reactions, even though mediated by the same enzyme protein, need not proceed by way of the same enzyme-bound intermediates or at the same enzyme site. That this may well be true for thymidine phosphorylase of human leukocytes was shown by the results of Gallo and Breitman (7). Further investigations of the pentosyl transferase activity of uridine phosphorylase from rat liver cytoplasm, now underway in this laboratory, should help to clarify this question.

**The Cytoplastic uridine phosphorylase of rat liver cells, despite extensive purification, was not homogeneous on electrophoresis. Electrophoresis was performed in phosphate-containing buffers and there is the possibility that conversion of the enzyme to a less active form, such as was reported for purine nucleosidc phosphorylase of Bacillus cereus (33), occurs. The finding that the amount of protein in the minor band increased on aging concomitantly with loss of enzyme activity supports this hypothesis.**

There is no indication from present studies that rat liver contains a uridine cleaving enzyme with a pH optimum of 6.5 such as was described for chicken, human, guinea pig, E. coli, and frog tissues and which may be a cytoplasmic enzyme (34).

The most highly purified preparations of the rat liver enzyme, like uridine phosphorylase of ascites tumor cells (26), still had low thymidine-cleaving activity. Like the rat liver enzyme uridine phosphorylase of ascites tumor cells is also inhibited by deoxyguanosine (35). Thus, both could be mistaken for thymidine phosphorylases that have negligible activity in the transfer of deoxyribose from thymidine to thymine (36) as was suggested earlier by Krenitsky et al. (1) for uridine phosphorylase of Novikoff hepatoma.

In view of the results with --SH reagents it is reasonable to conclude that uridine phosphorylase of rat liver cytoplasm has at least one --SH group at or near the active site of the enzyme. More than one --SH group may very well be involved because of the strong inhibition that occurred in the presence of o-iodosobenzoate which reacts with vicinal --SH groups. The conditions used in present experiments favor the formation of intramolecular rather than intermolecular disulfide bonds in the presence of o-iodosobenzoate (30, 37, 38). With this inhibitor uridine protected the enzyme significantly more than did phosphate. Possibly there are three --SH groups in the near vicinity of the active center, uridine binding with two of these and phosphate with the third.

**Acknowledgments—**The authors are indebted to Norbert Grif, Sheila Grant, Spencer Silver, Allison Loomdarn, Arif Hasanain, and Alex Leung for skilful technical assistance. Appreciation is extended to Dr. T. Pritchard, University of Manitoba, for the use of his radiochromatogram scanner and to Dr. M. Zimmerman of Merek, Sharp and Dohme for his generous gift of deoxyguanosine (35).
Cytoplasmic Uridine Phosphorylase of Rat Liver: CHARACTERIZATION AND KINETICS
Arthur Kraut and Esther W. Yamada


Access the most updated version of this article at http://www.jbc.org/content/246/7/2021

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/7/2021.full.html#ref-list-1