Cytoplasmic Uridine Phosphorylase of Rat Liver

CHARACTERIZATION AND KINETICS*

ARTHUR KRAUT‡ 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 deoxyuridine synthesis was inhibited 50% in the presence of 1 mM 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-iodosobenzolate. 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-iodosobenzolate, 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-2-14C (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.

2021
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 gly-
cylglycine buffer (pH 8.9) or acetate buffer (pH 5.5) were
included in the reaction mixture. To obtain graded concentra-
tions of each substrate, phosphate buffer was replaced by ap-
propriate 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
enzymes with inhibitor at 25°C the reaction was started by the
addition of phosphate and then the nucleoside; incubation was
continued at 37°C 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 each 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 forma-
tion 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
decomposition of free base was measured by an optical density
reader at 290 μm for 30 min at a constant pH of 8.2. The
reaction was started by the addition of uracil and the tubes were
incubated for 30 or 65 min at 37°C. 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 protein concentration up to an
optical density reading of 0.350.

Ribonuclease Type Activity—A modification of the methods
of Gallo, Perry, and Breitman (12) and deVerdier and Potter
(13) was used. 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°C.
The rest of the assay was identical with that described for the
assay of phosphorolytic activity (8). Linearity between the forma-
tion of uridine and time was evident up to 70 min and protein
concentration up to an optical density reading of 0.350.

Concentration of Protein with Carbowax-20 M

This procedure is a modification of the methods of Setlow and
Lowenstein (14) and Kohn (15). Enzyme fractions were placed in
dialyzing tubing, tied at one end, and immersed in a 500-ml beaker containing Carbowax in a cold room at 2°C. After every
45 min the wet Carbowax was replaced by dry over a 4- to 6-hour
period. The surface of the tubing was then washed with buffer.

Disc Gel Electrophoresis

Analytical disc gel electrophoresis was performed with 7%
polyacrylamide gel at pH 8.9 (16) or pH 7.5 (17) at 25°C for 90
min with a current of 2 to 2.5 ma per tube. Protein bands were
located with Coomassie blue (18). Enzyme preparations con-
taining 0.3 to 13.0 mg of protein per ml were diluted 3- to 20-fold
with spacer gel. Usually the buffers of the upper and lower
reservoirs contained 5 mM β-mercaptoethanol.

Data Processing

Reciprocals of initial velocities were plotted against reciprocals
of substrate concentration (10). Any points that deviated
greatly from a straight line relationship were discarded as recom-
mended by Watten and Cleland (20). The remaining data
were fitted, as described by Wilkinson (21), to the equation:

\[
V = \frac{V_S}{K + S}
\]

Values of apparent \(K_m\) and apparent \(V\) were calculated by
computer (Olivetti-Underwood Programma 101). From these
data slopes and intercepts plotted against the reciprocals of
nonvariable substrate or inhibitor concentration were fitted by
the method of least squares to the lines of best fit (22). The
Michaelis constants \(K_a, K_b, K_p, \) and \(K_j\), the inhibition con-
stants \(K_i\) for uracil and ribose 1-phosphate, the dissociation
constants \(K_a\) and \(K_b\), and the maximum velocities \(V_1\) and \(V_2\) 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:

\[
E \rightarrow (EA) \rightarrow (EAB) \rightarrow (EPQ) \rightarrow E
\]

where \(A, B, P, Q, E\) 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°C and all solutions were kept at 0°C in ice
buckets unless specified otherwise. Routinely, all dialyzing
tubing was soaked overnight at 3°C in 1% EDTA (pH 7.0) and
was washed with buffer before use. The procedures are sum-
marized in Table I.

Step 1: Homogenization—Livers were homogenized in 3 volumes
of sucrose solution (0.25 M sucrose-5 mM β-mercaptoethanol).
The homogenate was filtered through four layers of cotton gauze.

Step 2: Separation of Cytoplasm—The nuclear pellet was sedi-
mented 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 super-
natant 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 280 mp, to low, constant levels. At this point a linear gradient and the optical density of the effluents was reduced, at 260 and of 0.4 ml per min. Washing of the column with Buffer B was

A typical chromatogram showing the separation of uridine phosphorylase and thymidine phosphorylase is given in Fig. 1A. Column Fractions 104 to 116 were pooled; the enzyme was precipitated with ammonium sulfate at 80% saturation (56 g/100 ml) and resuspended in Buffer A.

Step 7: DEAE-Sephadex (pH 8.0)—Step 6 was repeated. The fractions with the highest uridine-cleaving activity were combined and concentrated as described in Step 6.

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

Step 9: Sephadex G-200 (pH 8.0)—Sephadex G-200 was allowed to swell in Buffer B minus β-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 to a volume of 4.8 ml and was then applied to the column. The column was developed with Buffer B; 45 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 β-mercaptoethanol.

Step 10: Hydroxylapatite (pH 7.2)—Hydroxylapatite was washed five times with large volumes of 0.02 M potassium phosphate 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 β-mercaptoethanol). Two and one-half milliliters of enzyme fraction from Step 9 were applied to the column, followed by 1/3 bed volume of Buffer C. The column was eluted with a linear gradient of phosphate allowing 300 ml of Buffer C adjusted to a phosphate concentration of 0.25 M to flow into a mixing chamber containing 300 ml of Buffer C and thence into the column. The fractions containing more than 150 units of uridine-cleaving activity (Fig. 1B) were combined and concentrated with Carbowax. The concentrate was dialyzed for 3 hours against Buffer A, clarified by centrifugation at 11,000 x g for 20 min, and stored frozen at -40°. The specific activity of the enzyme preparation from Step 10 was 144 which represents a 1900-fold purification of the initial homogenate (Table I).

TABLE I

<table>
<thead>
<tr>
<th>Step and treatment</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity (uridine)</th>
<th>Ratio (uridine to deoxuryidine)</th>
<th>Ratio (uridine to thymidine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>3,673</td>
<td>7,139</td>
<td>94,197</td>
<td>0.076</td>
<td>0.40</td>
<td>0.47</td>
</tr>
<tr>
<td>2. Cytoplasm</td>
<td>3,753</td>
<td>6,425</td>
<td>48,832</td>
<td>0.12</td>
<td>0.42</td>
<td>0.51</td>
</tr>
<tr>
<td>3. 151,000 × g supernatant</td>
<td>3,357</td>
<td>5,608</td>
<td>23,698</td>
<td>0.23</td>
<td>0.45</td>
<td>0.32</td>
</tr>
<tr>
<td>4. Ammonium sulfate</td>
<td>172</td>
<td>4,472</td>
<td>11,988</td>
<td>0.37</td>
<td>0.39</td>
<td>0.76</td>
</tr>
<tr>
<td>5. Heat</td>
<td>170</td>
<td>8,650</td>
<td>8,202</td>
<td>0.44</td>
<td>0.45</td>
<td>0.69</td>
</tr>
<tr>
<td>6. DEAE-Sephadex (pH 8.0) No. 1</td>
<td>47</td>
<td>5,149</td>
<td>1,968</td>
<td>2.01</td>
<td>1.50</td>
<td>6.40</td>
</tr>
<tr>
<td>7. DEAE-Sephadex (pH 8.0) No. 2</td>
<td>23</td>
<td>2,014</td>
<td>488</td>
<td>4.10</td>
<td>1.50</td>
<td>11.0</td>
</tr>
<tr>
<td>8. DEAE-Sephadex (pH 7.2)</td>
<td>6</td>
<td>1,226</td>
<td>193</td>
<td>6.30</td>
<td>1.40</td>
<td>11.0</td>
</tr>
<tr>
<td>9. Sephadex G-200 (pH 8.0)</td>
<td>3</td>
<td>832</td>
<td>26</td>
<td>32.0</td>
<td>1.40</td>
<td>10.0</td>
</tr>
<tr>
<td>10. Hydroxylapatite (pH 7.2)</td>
<td>4</td>
<td>576</td>
<td>4</td>
<td>144.0</td>
<td>1.40</td>
<td>10.0</td>
</tr>
</tbody>
</table>
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 (↓) indicates the point at which elution with a linear gradient of KCl was begun. Deoxyuridine-cleaving activity of uridine phosphorylase (URPase, ○) 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.

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 (---), 82 units of uridine-cleaving activity (○), and 571 units of deoxyuridine-cleaving activity (●) of which 58, 52, 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 520-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 7.5 was similar to that found at pH 8.9. After aging at −40° 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 I). 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
phosphorolysis of deoxyuridine at pH 6.5 more than 50%. These results are in agreement with those reported for uridine phosphorolylase 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 phosphorolylase (12, 28).

**pH and Stability**—The phosphorolysis 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 83% 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 50 to 100 mM β-mercaptoethanol. 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**

**Deoxyglucosylthymine**—The phosphorolysis of uridine catalyzed by Preparation E was inhibited 50% at pH 8.2 or 7.4 by 0.10 mM deoxyglucosylthymine, whereas that of deoxyuridine, at pH 6.5 or 7.4, was inhibited 50% by 0.018 mM deoxyglucosylthymine. Uridine synthesis was inhibited 50% at pH 8.2 by 0.14 mM deoxyglucosylthymine. The degree of inhibition with deoxyglucosylthymine 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 deoxyglucosylthymine. The phosphorolysis of deoxyuridine or thymidine by thymidine phosphorolylase (from Step 6) was not inhibited by deoxyglucosylthymine 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 phosphorolase of human spleen (31) and purine nucleoside phosphorolase 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 phosphorolysis of uridine and deoxyuridine at the pH optimum of each.

### Table II

**—SH reagents and phosphorolysis of nucleosides**

Enzyme Preparation E (7.2 μg of protein, 0.7 unit) was first incubated at 25° with —SH reagent. Tris buffer replaced phosphate in the reaction mixture of Procedure 1 adjusted to pH 8.2 or 6.5 for the phosphorolysis of uridine or deoxyuridine, respectively. The substrates were then added and activity was measured according to Procedure 1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pre-liminary incubation time</th>
<th>Concentration of inhibitor at 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>μM</td>
</tr>
<tr>
<td>p-Mercuriphenylsulfonate</td>
<td>5</td>
<td>4.0 × 10⁻²</td>
</tr>
<tr>
<td>p-Mercuribenzoate</td>
<td>5</td>
<td>6.2 × 10⁻⁵</td>
</tr>
<tr>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
<td>30</td>
<td>1.8 × 10⁻¹</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>30</td>
<td>3.5 × 10⁻²</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>5</td>
<td>1.6 × 10⁻⁴</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>60</td>
<td>1.8 × 10⁻⁴</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>120</td>
<td>5.6 × 10⁻⁶</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 for uridine cleavage and 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>Inhibition after preliminary incubation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uridine</td>
</tr>
<tr>
<td>p-Mercuriphenylsulfonate</td>
<td>00</td>
</tr>
<tr>
<td>p-Mercuribenzoate</td>
<td>50</td>
</tr>
<tr>
<td>5,5'-Dithiobis(2-nitrobenzoic acid)</td>
<td>44</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>42</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>63</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>40</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
</tbody>
</table>
Cytoplasmic Uridine Phosphorylase of Rat Liver

Vol. 246, No. 7

FIG. 3. Phosphorolysis of uridine (UR) at pH 8.2 with Pi as the variable substrate. Double reciprocal plot of initial velocity (micromoles of uracil formed per 10 min per ml) with respect to Pi concentration (millimolar). Uridine was held constant at concentrations of 1.670 mM (○), 0.500 mM (△), 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; \( K_{i_a} = 0.360 \) mM; \( K_{i_p} = 2.450 \) 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 uracil concentration (millimolar). Pi was held constant at concentrations of 117 mM (○), 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 \( K_a = 0.074 \) mM; \( V_I = 0.094 \) pmole per ml per 10 min; \( K_{i_u} = 0.074 \) mM; \( K_{i_p} = 0.278 \) 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 \( K_{i_a}/K_a \) is greater than 1.

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

Michaelis constants of substrates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phosphorolysis</th>
<th>Nucleoside synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. pH 8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate, ( K_a )</td>
<td>0.349</td>
<td></td>
</tr>
<tr>
<td>Phosphate, ( K_{i_a} )</td>
<td>3.218</td>
<td></td>
</tr>
<tr>
<td>Uridine, ( K_b )</td>
<td>0.240</td>
<td></td>
</tr>
<tr>
<td>Ribose 1-phosphate, ( K_q )</td>
<td>0.071</td>
<td>0.071</td>
</tr>
<tr>
<td>Ribose 1-phosphate, ( K_{i_q} )</td>
<td>0.065</td>
<td>0.065</td>
</tr>
<tr>
<td>Uracil, ( K_p )</td>
<td></td>
<td>0.280</td>
</tr>
<tr>
<td>II. pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate, ( K_a )</td>
<td>0.550</td>
<td></td>
</tr>
<tr>
<td>Phosphate, ( K_{i_a} )</td>
<td>2.340</td>
<td></td>
</tr>
<tr>
<td>Deoxyuridine, ( K_b )</td>
<td>0.300</td>
<td></td>
</tr>
</tbody>
</table>

studies the replots of the intercepts or slopes against the reciprocals of uridine or Pi 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 gave 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 Studies

Inhibition of Phosphorolysis of Uridine—A pattern of noncompetitive inhibition was obtained with uracil as the variable substrate, uridine as the nonvariable substrate, at low, nonsaturating levels, and uracil as the product inhibitor (Fig. 6). \(K_{ie}\) and \(K_i\) (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).

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 \(K_i\) for uridine, measured at the pH optimum of each nucleoside.

Product Inhibition Studies

Inhibition of Phosphorolysis of Uridine at pH 8.2 by Uracil—A pattern of noncompetitive inhibition was obtained with phosphate as the variable substrate, uridine as the nonvariable substrate, at low, nonsaturating levels, and uracil as the product inhibitor (Fig. 6). \(K_{ie}\) and \(K_i\) (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).
Table V

Dissociation and inhibition constants derived from product inhibition studies

The kinetic parameters were estimated from the apparent $K_i$, $K_{ii}$, and $K_{i\alpha}$, 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 $K_{ii}$, $K_{i\alpha}$, $K_{i\alpha}$, and $K_{i\alpha}$ constants used in the calculations are those given in Table IV.

<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>$K_{ii} = B + K_3 (B/K_3) + (K_3K_4/K_4K_3)$</td>
</tr>
<tr>
<td></td>
<td>0.064</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.315</td>
<td>8</td>
<td>$K_{i\alpha} = K_i + 1/A$</td>
</tr>
<tr>
<td></td>
<td>1.015</td>
<td>$-$</td>
<td>$K_{i\alpha} = K_i$</td>
</tr>
<tr>
<td>$K_i$ (ribose-1-P)</td>
<td>0.622</td>
<td>10</td>
<td>$K_{i\alpha} = K_i + A/K_3$</td>
</tr>
<tr>
<td></td>
<td>1.067</td>
<td>$-$</td>
<td>$K_{i\alpha} = K_i$</td>
</tr>
<tr>
<td></td>
<td>0.251</td>
<td>9</td>
<td>$K_{i\alpha} = K_i + A/K_3$</td>
</tr>
<tr>
<td></td>
<td>0.401</td>
<td>10</td>
<td>$K_{i\alpha} = K_i + A/K_3$</td>
</tr>
<tr>
<td>$K_{i\alpha}$ (ribose-1-P)</td>
<td>0.192</td>
<td>6</td>
<td>$K_{i\alpha} = K_{i\alpha} B/K_3 + K_{i\alpha} B/K_3$</td>
</tr>
<tr>
<td></td>
<td>0.231</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.144</td>
<td>8</td>
<td>$K_{i\alpha} = K_{i\alpha} B/K_3 + K_{i\alpha} B/K_3$</td>
</tr>
<tr>
<td></td>
<td>0.042</td>
<td>$-$</td>
<td>$K_{i\alpha} = K_{i\alpha} B/K_3 + K_{i\alpha} B/K_3$</td>
</tr>
</tbody>
</table>

* From studies of product inhibition by uracil at pH 8.2 with uridine as the variable substrate at high P$_i$ concentration (0.067 mM).
* From studies of product inhibition by ribose-1-P at pH 8.2 with P$_i$ as variable substrate at low uridine concentration (0.0265 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 uridine 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 10-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 $K_{i\alpha}$ 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 inhibition studies...
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 Gritt, Sheila Grant, Spencer Silver, Allison Loadman, Afif Hasnain, and Alex Leung for skillful 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 thymine.

REFERENCES

10. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1942).
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