Specificity of Mouse Uridine Phosphorylase*

CHROMATOGRAPHY, PURIFICATION, AND PROPERTIES†

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Two distinct pyrimidine nucleosidases, isolated from Escherichia coli, show complete specificity for the pentose moiety of the nucleoside. Pyrimidine deoxyribonucleoside phosphorylase (1) shows complete specificity for the deoxyriboyl group, and uridine phosphorylase (2) for the ribosyl group. Mammalian uridine phosphorylase preparations (3, 4), however, have appreciable deoxyuridine phosphorylase activity. Pontis, Dengstedt, and Reichard (3) present data which they interpret as evidence for a distinct enzyme, deoxyuridine phosphorylase, in uridine phosphorylase preparations (3, 4), however, have appreciated deoxyuridine phosphorylase activity. Pontis, Dengstedt, and Reichard (3) present data which they interpret as evidence for a distinct enzyme, deoxyuridine phosphorylase, in uridine phosphorylase preparation purified from Ehrlich ascites cells. A thymidine phosphorylase purified from horse liver (5) possesses the same phosphorolytic activity as the analogous bacterial enzyme but is not as specific for the deoxyribose moiety in the direction of synthesis.

Three hypotheses explaining the pyrimidine deoxyribonucleoside phosphorolytic activity of mammalian uridine phosphorylase preparations are: (a) contamination by thymidine phosphorylase; (b) contamination by a distinct enzyme, deoxyuridine phosphorylase; (c) nonspecificity of some mammalian uridine phosphorylases for the pentose moiety, in contrast to an analogous bacterial enzyme. This investigation provides evidence in support of the last hypothesis.

EXPERIMENTAL PROCEDURE

Chemicals—Uridine and deoxyuridine were obtained from the California Corporation for Biochemical Research; thymidine and ammonium sulfate (special enzyme grade) from the Mann Research Laboratories, and DEAE-cellulose from Eastman Organic Chemicals.

Enzyme Assays—The final assay solution, incubated at 37°, contained 10 μM nucleoside substrate in 0.1 M sodium phosphate buffer at pH 7.4, and an amount of enzyme which kept the cleavage as close to the linear range as possible. At 0 minute and from 5 to 30 minutes, 0.8-ml aliquots were taken from the reaction mixture, pipetted into 0.9 ml of 0.5 N sodium hydroxide, mixed, and read in the Beckman spectrophotometer at 290 μm for uracil and 300 μm for thymine (1). In cases where the reaction had proceeded beyond the linear range, the assay data were corrected to values based on the initial rate of reaction as calculated from Fig. 1.

One unit of pyrimidine nucleosidase activity was defined as that amount of enzyme which would produce 1 μmole of free pyrimidine in 1 hour when incubated at 37° in 10 mM nucleoside and 0.1 M sodium phosphate buffer, pH 7.4.

DEAE-cellulose Chromatography—The exchange agent was washed according to the procedure of Sober, Gutter, Wykoff, and Peterson (6), poured into a column, and packed by suction from a vacuum line. The column was equilibrated by passing 10 times its volume of eluting buffer through it.

Liver and intestinal epithelium were obtained from normal adult female Swiss albino mice. Ehrlich ascites tumor cells, grown in Swiss albino mice, were harvested (10 days after 0.1-ml intraperitoneal inoculation) and added to equal volumes of 0.8% NaCl solution containing 0.2 mg of heparin per 50 ml. An equal volume of redistilled water was added to the harvested volume, lysing most of the erythrocytes present. After centrifugation of the suspension and decanting of the supernatant pellet, the pellet of ascites cells was resuspended in redistilled water.

Homogenates were prepared in ground glass homogenizers with eluting buffer and spun at 22,000 × g for 30 minutes at 1°. The clear supernatant fluids (lipid-containing top layers discarded) were dialyzed against the eluting buffer for 2 hours.

Escherichia coli B was grown in a salts-yeast extract-glucose medium in the Biogen (American Sterilizer Company). The cells were harvested at the end of the log phase by centrifugation, washed twice with 0.5% KCl-0.5% NaCl solution, and resuspended in 400 ml of 0.05 M potassium phosphate, pH 7.15. Washed glass beads were added, and the entire suspension was ruptured in an Eppenbach micromill at 13°. The supernatant fluid, obtained by centrifugation of the suspension in a Servall type SS-1 centrifuge for 1 hour at 14,700 r.p.m. at 10°, was lyophilized and stored at −10°. Before application to the column, the redisolved preparation was dialyzed against the eluting buffer for 2 hours.

The chromatograms were developed at 0-4° with 0.02 M sodium phosphate-0.01 M mercaptoethanol buffer, pH 8.0 (Buffer A).
and then centrifuged for 45 minutes at 44,000 g, redissolved in a minimal volume of Buffer A, and dialyzed for 10 hours against two changes of this buffer before addition to an equilibrated DEAE-cellulose column (3.5 × 50 cm). After 140 ml of effluent had been collected by elution with Buffer A, a NaCl gradient was initiated with a 1 m NaCl-Buffer A solution. The eluent containing the highest specific activity was promptly applied to a smaller, similarly equilibrated column (60 × 1 cm). A NaCl gradient was initiated at the start of the chromatogram with a NaCl molarity of 0.75. The eluted active peak was immediately centrifuged for 2 hours at 114,000 g. The top half of the supernatant fluid was considered the end product of the procedure. One milliliter of this end product was chromatographed without delay on a DEAE-cellulose column (50 × 1 cm), equilibrated and developed with 0.02 m sodium phosphate-0.01 m mercaptoethanol, pH 7.0.

RESULTS AND DISCUSSION

DEAE-cellulose Chromatography—From the chromatograms shown in Figs. 2 to 4, it is apparent that at least two distinct pyrimidine nucleoside phosphorylases occurred in the liver and intestinal epithelium of Swiss albino mice. The specificity of the first enzyme eluted agreed with that of the thymidine phosphorylase isolated from horse liver by Friedkin and Roberts (5). Thymidine was cleaved but not uridine. The chromatograms show that the level of thymidine phosphorylase was high in liver (Fig. 2), low in intestinal epithelium (Fig. 3), and not detectable in Ehrlich ascites tumor (Fig. 5).

The specificity of the second enzyme eluted agreed with that of the uridine phosphorylase isolated from Ehrlich ascites cells by Pontis et al. (3). This enzyme cleaved uridine, deoxyuridine, and thymidine in ratios shown in Table I. Its occurrence was the converse of that of thymidine phosphorylase, e.g. a high level in intestinal epithelium and in Ehrlich ascites cells, and a low level in liver.

Razzell and Khorana (1) reported that the ribosyl and deoxyribosyl specific pyrimidine nucleoside phosphorylases of E. coli were not separated on a Dowex 1 column. The first chromatograms of the E. coli extract (Fig. 6) showed a discernible separation of two distinct pyrimidine nucleoside phosphorylases, both present in high levels. The active fractions of this chromatogram were applied to a column and eluted with a more gradual NaCl gradient (Fig. 7). The wider separation of the two enzymes enabled the determination of the specificity of the uridine phosphorylase of E. coli (Table I). The specificity of the E. coli thymidine phosphorylase could not be determined because of uridine phosphorylase trailing. The greatly different rate of elution of the thymidine phosphorylase from the E. coli and the mouse extracts is difficult to interpret, since the possibility of enzyme-protein complexes which would alter the rate of elution of the enzymes, cannot be eliminated in whole cell or tissue extracts.

The wide chromatographic separation of the uridine and thymidine phosphorylases from mouse intestinal epithelium and a saturated ammoniacal ammonium sulfate solution, and allowed to settle overnight. Most of the supernatant fluid was decanted and discarded. The precipitate was centrifuged from the remaining liquid for 20 minutes at 44,000 × g, redissolved in a minimal volume of Buffer 1, and dialyzed for 10 hours against two changes of this buffer before addition to an equilibrated DEAE-cellulose column (3.5 × 50 cm). After 140 ml of effluent had been collected by elution with Buffer 1, a NaCl gradient was initiated with a 1 m NaCl-Buffer 1 solution. The eluent containing the highest specific activity was promptly applied to a smaller, similarly equilibrated column (60 × 1 cm). A NaCl gradient was initiated at the start of the chromatogram with a NaCl molarity of 0.75. The eluted active peak was immediately centrifuged for 2 hours at 114,000 × g. The top half of the supernatant fluid was considered the end product of the procedure. One milliliter of this end product was chromatographed without delay on a DEAE-cellulose column (50 × 1 cm), equilibrated and developed with 0.02 m sodium phosphate-0.01 m mercaptoethanol, pH 7.0.

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All water was glass-redistilled, boiled to remove dissolved gases, and rapidly cooled shortly before use.

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4 Brought to pH 7.5 to 8.0 with concentrated ammonium hydroxide.
FIG. 2. DEAE-cellulose chromatogram of supernatant fluid from mouse liver homogenate. Column dimensions were 1 x 52 cm; flow rate, 0.82 ml per minute. Fluid, 11 ml, containing 1424 mg of protein and 38.9 units of uridine-cleaving activity and 281.6 units of thymidine-cleaving activity, was applied. Activities recovered were 75% uridine- and 88% thymidine-cleaving activity.

FIG. 3. DEAE-cellulose chromatogram of supernatant fluid from mouse intestinal epithelium homogenate. Column dimensions were 1 x 52 cm; flow rate, 0.75 ml per minute. Fluid, 9.5 ml, containing 312 mg of protein and 278.2 units of uridine-cleaving activity and 88.7 units of thymidine-cleaving activity, was applied. Activities recovered were 101% uridine-cleaving activity and 61% thymidine-cleaving activity.

Liver extracts, with the retention by the uridine phosphorylase of the characteristic deoxyuridine- and thymidine-cleaving activities, eliminated the hypothesis that the deoxyribonucleoside phosphorylase activity of mammalian uridine phosphorylase preparations results from contamination with thymidine phosphorylase. In addition, the ratios of thymidine-cleaving activity to deoxyuridine-cleaving activity of the uridine and thymidine phosphorylases were so different (Table I) that the deoxyuridine-
Fig. 4. DEAE-cellulose chromatogram of combined active effluents of previously chromatographed supernatant fluids from mouse liver and intestinal epithelium homogenates. Column dimensions were $1 \times 41 \text{ cm}$; flow rate, 0.91 ml per minute. Fluid, 15 ml, containing 300 mg of protein and 30.5 units of uridine-cleaving activity and 51.6 units of thymidine-cleaving activity, was applied. Activities recovered were 138% uridine-cleaving activity and 89% thymidine-cleaving activity. Liver and intestinal epithelium homogenate supernatant fluids were first chromatographed separately as in Figs. 2 and 3. The uridine-cleaving activity peak of the intestinal epithelium chromatogram and the thymidine-cleaving activity peak of the liver chromatogram were pooled and added to the column.

Fig. 5. DEAE-cellulose chromatogram of supernatant fluid from Ehrlich ascites tumor homogenate. Column dimensions were $1 \times 52 \text{ cm}$; flow rate, 0.90 ml per minute. Fluid, 8.5 ml, containing 222 mg of protein and 280.5 units of uridine-cleaving activity and 44.7 units of thymidine-cleaving activity, was applied. Activities recovered were 100% uridine-cleaving activity and 76% thymidine-cleaving activity.
**FIG. 6.** First DEAE-cellulose chromatogram of *E. coli* extract. Column dimensions were 1 X 46 cm; flow rate, 0.80 ml per minute. Fluid, 7.6 ml, containing 341 mg of protein and 839.7 units of uridine-cleaving activity and 833.0 units of thymidine-cleaving activity, was applied. Activities recovered were 78% uridine-cleaving activity and 66% thymidine-cleaving activity.

**FIG. 7.** Second DEAE-cellulose chromatogram of *E. coli* extract. Column dimensions were 1 X 57 cm; flow rate, 0.70 ml per minute. Fluid, 15 ml, containing 12.6 mg of protein and 127.3 units of uridine-cleaving activity and 129.4 units of thymidine-cleaving activity, was applied. Activities recovered were 100% uridine-cleaving activity and 15% thymidine-cleaving activity. The overlapping uridine- and thymidine-cleaving activity peaks of the first *E. coli* chromatogram (Fig. 6) were pooled and added to the column. The NaCl gradient was initiated at the start of the chromatogram with a NaCl molarity of 0.75.
TABLE I

Ratios of activities of eluted enzymes on freezing. Its activity can be maintained with moderate success, if stored in 10 times its volume of saturated ammonium sulfate-0.04 M mercaptoethanol, pH 7.5 to 8.0, at 0-2°C. The chromatograms of the purification procedure are shown in Fig. 8. Chromatogram 3 shows the complete overlap of eluted protein with uridine- and thymidine-cleaving activities. This indicated the chromatographic homogeneity of the uridine phosphorylase preparation. The constancy of the ratios of uridine-cleaving activity to deoxyuridine-cleaving activity during purification is shown in Table II.

Electrophoresis—Fig. 9 shows the results of moving boundary electrophoresis of the purified preparation. These patterns suggest the electrophoretic homogeneity of the preparation.

Kinetics—Michaelis-Menten constants in Table III were obtained by plotting kinetic data according to the method of Lineweaver and Burk (10). The reactions were run at 0.1 M peak of thymidine phosphorystase.

Peak of uridine phosphorylase.

Table II

Purification of uridine phosphorylase from Ehrlich ascites tumor

Protein was estimated in the first four steps according to the method of Kalckar (8), and in the last three steps according to Lowry et al. (9).

<table>
<thead>
<tr>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Ratio of uridine-cleaving activity to deoxyuridine-cleaving activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>umoles uracil formed/hr/mg protein</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>48,530</td>
<td>0.40</td>
<td>100</td>
</tr>
<tr>
<td>Clear fraction of the homogenate supernatant fluid</td>
<td>4,734</td>
<td>3.50</td>
<td>85</td>
</tr>
<tr>
<td>Dialed ammonium sulfate fraction</td>
<td>1,242</td>
<td>8.60</td>
<td>65</td>
</tr>
<tr>
<td>Active peak of Chromatogram 1</td>
<td>62.4</td>
<td>45.13</td>
<td>14.4</td>
</tr>
<tr>
<td>Active peak of Chromatogram 2</td>
<td>18.2</td>
<td>81.87</td>
<td>7.6</td>
</tr>
<tr>
<td>Top half of ultracentrifuged supernatant fluid</td>
<td>4.77</td>
<td>108.40</td>
<td>2.65</td>
</tr>
<tr>
<td>Active peak of Chromatogram 3</td>
<td>0.909</td>
<td>168.69</td>
<td>1.98</td>
</tr>
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</table>

cleaving activity of uridine phosphorylase cannot be due to thymidine phosphorylase contamination. If the hypothesis were valid, the thymidine-cleaving activity of the contaminated uridine phosphorylase would be higher than its deoxyuridine-cleaving activity.

The difference between the specificities of the eluted E. coli and mouse uridine phosphorylases was only quantitative. Under the assay conditions employed, the chromatographed uridine phosphorylase of E. coli was not completely specific for the ribose moiety (Table I) but was much more specific than the uridine phosphorylase from mouse tissues.

Purification—A typical uridine phosphorylase purification is summarized in Table II. The procedure is not geared to obtain high yields, but rather the most active monodisperse preparation. The dialyzed ammonium sulfate fraction can be frozen and stored for several months at -10°C. The most highly purified preparation is extremely labile and loses almost all its activity on freezing. Its activity can be maintained with moderate success, if stored in 10 times its volume of saturated ammonium sulfate-0.04 M mercaptoethanol, pH 7.5 to 8.0, at 0-2°C.
phosphate concentration to give first order kinetics. Because of the early nonlinearity of the phosphorylase reaction (Fig. 1), great care was taken to keep the extent of reaction as low as possible. A final reaction mixture concentration of 0.01 to 0.1 mM free pyrimidine was the reaction range employed. If the reaction was allowed to proceed further, nonlinearity being more pronounced at low substrate concentration than at high substrate concentration, the $K_m$ values obtained were higher. For example, with uridine at pH 7.4 the $K_m$ value determined with a final concentration of uracil in the reaction mixture at 0.05 to 0.07 mM was $1.29 \times 10^{-4}$ M; with the final uracil concentration at 0.23 to 0.36 mM; $3.6 \times 10^{-4}$ M. Table III shows that uridine phosphorylase had a 3- to 14-fold greater affinity for uridine than for deoxyuridine over a pH range of 5.5 to 8.0.

$\textbf{pH Optima}$—The pH optima of the purified enzyme for uridine and deoxyuridine (8.1 and 6.4, respectively) were similar to those reported by Pontis et al. (3). The pH optimum for thymidine cleavage was 6.8.

$\textbf{Comparison of Assay Procedures}$—To examine more directly data from various sources, assay conditions were compared with the purified mouse uridine phosphorylase (Fig. 10). Spectrophotometric determination of free pyrimidine in alkalinized aliquots, described above, was used to follow the reactions.

With arsenolysis, the uridine phosphorylase showed a marked decrease in uridine-cleaving activity and an increase in the deoxyribonucleoside-cleaving activity. This arsenate sensitivity is consistent with the results of deVerdier and Potter (11) with transfer activities, and gives indirect evidence for the identity of pyrimidine ribosyl transferase and uridine phosphorylase.

Comparison of these assay conditions with chromatographed mouse liver thymidine phosphorylase showed a slight increase over the phosphorolysis rate of thymidine- and deoxyuridine-cleaving activities with arsenolysis.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m \times 10^4$</th>
<th>pH</th>
<th>$K_m \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.15</td>
<td>0.60</td>
<td>8.10</td>
<td>4.02</td>
</tr>
<tr>
<td>7.75</td>
<td>1.50</td>
<td>7.58</td>
<td>4.43</td>
</tr>
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<td>7.58</td>
<td>1.50</td>
<td>7.40</td>
<td>5.85</td>
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<td>1.07</td>
<td>7.00</td>
<td>8.62</td>
</tr>
<tr>
<td>7.25</td>
<td>0.59</td>
<td>6.65</td>
<td>6.03</td>
</tr>
<tr>
<td>6.85</td>
<td>0.56</td>
<td>5.90</td>
<td>4.10</td>
</tr>
<tr>
<td>6.73</td>
<td>1.12</td>
<td>5.50</td>
<td>4.07</td>
</tr>
<tr>
<td>5.90</td>
<td>1.33</td>
<td>5.50</td>
<td>4.45</td>
</tr>
<tr>
<td>5.50</td>
<td>1.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE III**

$\text{Apparent Michaelis-Menten constants for the phosphorolysis of uridine and deoxyuridine by uridine phosphorylase purified from Ehrlich ascites tumor}$

Fig. 10. Comparison of assay procedures with uridine phosphorylase purified from mouse Ehrlich ascites cells.
The specificity, pH optimum, and arsenate sensitivity of uridine phosphorylase must be considered before interpreting any data in the literature concerning the thymidine phosphorylase levels of tissues. For example, Zimmerman’s observation of the presence of thymidine-cleaving activity in a number of tissues, in the absence of deoxyribosyl transfer activity, prevented him from making a conclusion about the identity of the deoxyribosyl transferase and phosphorylase, despite his inability to separate the two activities on purification (12, 13). At least one of these tissues, Novikoff hepatoma, contains a high level of uridine phosphorylase and virtually no thymidine phosphorylase; its significant thymidine-cleaving activity results from uridine phosphorylase (14). If this is the case for the other tissues with this enzyme pattern, one can hypothesize that the deoxyribosyl transfer activity of mammalian uridine phosphorylase is very limited and that the pyrimidine deoxyribosyl transfer and phosphorolytic activities are the properties of one enzyme, thymidine phosphorylase.

SUMMARY

Three hypotheses explaining the pyrimidine deoxyribonucleoside-cleaving activity of mammalian uridine phosphorylase preparations are proposed and investigated: Hypothesis 1, contamination by thymidine phosphorylase; Hypothesis 2, contamination by a deoxyuridine phosphorylase; and Hypothesis 3, nonspecificity of some mammalian uridine phosphorylases for the pentose moiety.

The following evidence, obtained with enzymes from mouse tissue extracts, is presented against Hypothesis 1: (a) the wide diethylaminoethyl cellulose chromatographic separation of thymidine and uridine phosphorylases, with the retention by the uridine phosphorylase of the characteristic deoxyribonucleoside-cleaving activity; (b) under the assay conditions, the deoxyuridine-cleaving activity of the thymidine phosphorylase is lower than the thymidine-cleaving activity, while the inverse ratio of activities exists for the pentose moiety.

To investigate Hypothesis 2, uridine phosphorylase from Ehrlich ascites cells was purified in a form whose homogeneity was suggested by diethylaminoethyl cellulose chromatography and moving boundary electrophoresis. The characteristic deoxyribonucleoside-cleaving activity was completely retained by the purified enzyme.

By elimination of Hypotheses 1 and 2, it is concluded that the uridine phosphorylase from mouse Ehrlich ascites cells is not as specific for the ribose moiety of the nucleoside as is an analogous bacterial enzyme.

In comparing assay conditions with the purified mouse uridine phosphorylase, the uridine-cleaving activity is markedly decreased by arsenolysis, while the deoxyuridine- and thymidine-cleaving activities are enhanced. Uridine phosphorylase has a pH optimum for the phosphorolyis of uridine at 8.1, for deoxyuridine at 6.4, and for thymidine at 6.8. From kinetic studies, it is shown that uridine phosphorylase has a 3- to 14-fold greater affinity for uridine than for deoxyuridine over a pH range of 5.5 to 8.0.

Diethylaminoethyl cellulose chromatography with the two analogous phosphorylases from Escherichia coli suggests a much greater, but not complete, specificity for the ribose moiety by the bacterial uridine phosphorylase. The rate of elution of the E. coli pyrimidine deoxyribonucleoside phosphorylase is not very different from that of the uridine phosphorylase, in contrast to the widely different rates of elution of the corresponding mouse enzymes.

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Specificity of Mouse Uridine Phosphorylase: CHROMATOGRAPHY, PURIFICATION, AND PROPERTIES
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