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Specificity of Pyrimidine Nucleoside Phosphorylases and the Phosphorolysis of 5-Fluoro-2′-deoxyuridine[1]

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ABSTRACT

Isoelectric focusing and studies with 1-(2′-deoxy-β-D-glucopyranosyl)thymine (GPT), a specific inhibitor of uridine phosphorylase activity, were used to determine the substrate specificities of mammalian pyrimidine nucleoside phosphorylases and their cleavage of 5-fluoro-2′-deoxyuridine (FdUrd). Isoelectric focusing profiles for the cytosol fractions from Ehrlich ascites cells and from Novikoff hepatoma cells each consisted essentially of one peak of nucleoside phosphorylase activity [isoelectric points (pI) 5.4 and 5.8, respectively] that cleaved both uridine and thymidine (dThd), as well as FdUrd. By contrast, cytosol fractions from HeLa (S3) cells, mouse liver, and normal human leukocytes each exhibited a major peak of activity (pI 4.6, 6.5, and 4.9, respectively) that cleaved only dThd and FdUrd, while mouse liver exhibited a second peak (pI 5.2) that cleaved primarily uridine. To distinguish clearly between (*a*) uridine phosphorylases that cleave primarily uridine and that are inhibited by GPT and (*b*) dThd phosphorylases that cleave only deoxynucleosides and that are not inhibited by GPT, we propose the term "uridine-deoxyuridine phosphorylases" to define those pyrimidine nucleoside phosphorylases that cleave both uridine and dThd and that are inhibited by GPT. On the basis of this definition and studies with GPT in nonfocused cytosol preparations, we conclude that FdUrd is cleaved to 5-fluorouracil by uridine-deoxyuridine phosphorylase activity in Ehrlich ascites cells and in Novikoff hepatoma cells, and by dThd phosphorylases in mouse liver, in normal human leukocytes, and in HeLa (S3) cells.

INTRODUCTION

Pyrimidine nucleoside phosphorylases are present in many normal and neoplastic cells (5, 8, 17, 20, 24, 28, 31). There are 2 distinct pyrimidine nucleoside phosphorylases: (*a*) dThd[5] phosphorylase (EC 2.4.2.4; dThd:orthophosphate deoxyribosyltransferase), which catalyzes the reversible reaction:

$$\text{dThd} + P_i \rightleftharpoons \text{thymine} + \alpha\text{-D-deoxyribofuranose-1-phosphate}$$

and is highly specific for deoxyribonucleosides, in both eukaryotes and prokaryotes; and (*b*) uridine phosphorylase (EC 2.4.2.3; uridine:orthophosphate ribosyltransferase), which catalyzes the reversible reaction (8):

$$\text{Uridine} + P_i \rightleftharpoons \text{uracil} + \alpha\text{-D-ribofuranose-1-phosphate}$$

In both *Escherichia coli* (17) and the plasma membranes of rat liver cells (6), uridine phosphorylase acts primarily on uridine. In other prokaryotes (24) and eukaryotes (14, 22), however, uridine phosphorylase also cleaves dThd and 2′-deoxyuridine. Consequently, the substrate specificity of this phosphorylase is uncertain.

In humans, pyrimidine nucleoside phosphorylase activity rapidly cleaves FdUrd to FUra and has been implicated as a major reason for why FdUrd is not significantly more effective clinically than is FUra (4, 12, 21). Chemical modifications of FdUrd, designed to render the drug resistant to phosphorolytic cleavage, have not yet produced a derivative that is more active than FdUrd (4, 13). Attempts to inhibit nucleoside phosphorylase activity (1, 4, 16) in order to maintain the level of FdUrd available for conversion to 5-fluoro-2′-deoxyuridine 5′-monophosphate, an active form of the drug (10), have met with relatively little success. Before inhibitors with a higher potency and specificity for these nucleoside phosphorylases can be developed, more information is required on the phosphorylases that cleave FdUrd to FUra.

In this study, isoelectric focusing and GPT (15, 30), a specific inhibitor of uridine phosphorylase that does not inhibit the activity of dThd phosphorylase, were used to characterize the substrate specificites of several mammalian pyrimidine nucleoside phosphorylases. Our objective was to identify specifically those nucleoside phosphorylases in mammalian tissues that cleave FdUrd to FUra.

MATERIALS AND METHODS

All chemicals were of analytical grade and were purchased from the Sigma Chemical Co., St. Louis, Mo., unless otherwise stated. GPT (NSC 402666) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, and FdUrd was generously provided by Hoffmann-La Roche Inc., Nutley, N. J.

Cell Culture. HeLa (S3) cells and Novikoff hepatoma cells were grown in a suspension culture in minimum essential medium with Spinner's salts (Eagle's) supplemented with 10% heat-inactivated calf serum and with 0.1% pluronic acid (27). Media and sera were purchased from Grand Island Biological Co., Grand Island, N. Y., and pluronic-F68 was a gift from BASF Co., Wyandotte, Mich. The cells were examined routinely for *Mycoplasma* (11, 18, 26), since many of these organisms

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[5] The abbreviations used are: dThd, thymidine; FdUrd, 5-fluoro-2′-deoxyuridine; FUra, fluorouracil; GPT, 1-(2′-deoxy-β-D-glucopyranosyl)thymine; Ura-dUrd phosphorylase, uridine-deoxyuridine phosphorylase.

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have high nucleoside phosphorylase activity (18); in each instance, the results were negative.

Animal Tissues. Ehrlich ascites cells were grown in female Swiss/ICR mice (Sprague Dawley Co., Madison, Wis.), as reported previously (4). Mouse livers were obtained from non-tumor-bearing animals of the same sex and strain; before excision, blood was removed by perfusion of the portal vein with an ice-cold 0.9% NaCl solution.

Isolation of Human Leukocytes. Units of blood were drawn from healthy donors, and the erythrocytes were removed with 3% dextran in 0.9% NaCl solution at 37°, followed by hypertonic shock (7). The leukocytes, which were harvested by centrifugation at 800 × *g* for 10 min at 4°, contained from 55 to 80% mature granulocytes.

Enzyme Isolation and Partial Purification. Cells or tissues were washed twice in ice-cold 0.9% NaCl solution and were homogenized in 2 volumes of 5 mM sodium phosphate buffer (pH 8.0), containing 10 mM β-mercaptoethanol. The homogenates were centrifuged at 105,000 × *g* for 1 hr at 4°, and the cytosol fractions were treated with ammonium sulfate (Schwarz/Mann, Orangeburg, N. Y.). The fractions obtained between 33 and 55% saturation were used for mouse liver, Ehrlich ascites cells, and Novikoff hepatoma cells, while those obtained between 0 and 55% saturation were utilized for human leukocytes and for HeLa (S3) cells; the precipitates were harvested by centrifugation at 16,000 × *g* for 20 min at 4°. The pellets were resuspended in 5 mM sodium phosphate buffer (pH 8.0), containing 10 mM β-mercaptoethanol and were dialyzed against the same buffer for 4 hr with 4 changes of the buffer. Protein concentrations were determined by the method of Lowry *et al.* (19). Freshly prepared extracts were used for all isoelectric focusing experiments, while the inhibition studies with GPT were conducted with preparations that had been stored at −22°. Preparations stored in this manner showed no appreciable loss of dThd or of Ura-dUrd phosphorylase activity (defined in "Discussion") over 1 month, while uridine phosphorylase activity was less stable.

Isoelectric Focusing. Isoelectric focusing was performed in a 110-ml LKB column (LKB Instruments, Inc., Rockville, Md.) with the anode and cathode solutions described previously (23). Dialyzed samples (15 to 100 mg protein) were mixed with the light gradient solution containing 1.25 ml 40% ampholytes (pH 3.5 to 10, or pH 4 to 6) (LKB Instruments), and the volume was adjusted to 60 ml with distilled water. A 0 to 60% sucrose gradient was formed on the column by mixing the light gradient solution with 46 ml of a dense gradient solution containing 3.75 ml of 40% ampholytes and 28 g of sucrose (Schwarz/Mann). Isoelectric focusing was conducted for 20 hr at 4° with the anode located at the bottom of the column; settings were 20 ma and 1600 V; and after 2 hr, the power was increased from 5 to 14 watts.

After electrophoresis, the column was emptied in 2-ml increments, and the linearity of the pH gradient was established using a Radiometer Model 26 pH meter calibrated at 4°. Each fraction was then assayed for pyrimidine nucleoside phosphorylase activity.

Pyrimidine Nucleoside Phosphorylase Assays. The phosphorolysis of uridine, dThd, and FdUrd was assayed in the catabolic direction by use of a radioisotopic technique. In 100 μl, the incubation mixture contained 0.1 M sodium phosphate buffer (pH 6.4), β-mercaptoethanol (2.5 mM), 0.4 mM unlabeled nucleoside (final concentration), and the appropriate radioactive nucleoside of either 2.5 μCi of [*methyl*-^{3}H]dThd (24 Ci/mmol), 2.5 μCi of [5-^{3}H]uridine (46 Ci/mmol), or 1.5 μCi of [6-^{3}H]FdUrd (2.5 Ci/mmol) (Amersham/Searle Corp., Arlington Heights, Ill.); each nucleoside was shown by thin-layer chromatography to be at least 97% pure. For the isoelectric focusing studies, the above incubation mixture also included 25 μl of the column fraction, while the inhibition studies with GPT utilized 25 μl (20 to 200 μg of protein) of the enzyme extract (nonfocused) and 0 to 0.29 mM GPT. Incubations were at 37° for 20 min (GPT inhibition studies) or for 3 hr (isoelectric focusing), and the reaction was terminated by boiling for 2 min. The tritiated bases (products) and nucleosides (substrates) were separated on Silica Gel UV_{254} Polygram plates (Brinkmann, Instruments Inc., Westbury, N. Y.) with chloroform:methanol (9:1). Methanol eluates of the UV-absorbing spots were assayed for radioactivity in a liquid scintillation counter.

RESULTS

Inhibition of Phosphorolysis by GPT. In preliminary experiments, all 5 enzyme preparations [Ehrlich ascites cells, Novikoff hepatoma cells, HeLa (S3) cells, mouse liver, and human leukocytes] phosphorolytically cleaved dThd and FdUrd (data not shown). Except for the human leukocyte extracts, all preparations cleaved uridine.

GPT reportedly inhibits uridine phosphorylase activity but does not inhibit the activity of dThd phosphorylases (15, 30). In the present study, however, with nonfocused extracts of Ehrlich ascites cells and with Novikoff hepatoma cells, GPT (0.29 mM) inhibited by approximately 80% the phosphorolysis of dThd (Chart 1*C*), as well as FdUrd (Chart 1*A*) and uridine (Chart 1*B*); in addition, as shown in the charts, virtually identical profiles for the inhibition of the phosphorolysis of all 3 nucleosides were obtained with these 2 enzyme preparations. The results obtained with the other enzyme preparations were quite different, however. Thus, with extracts of mouse liver or HeLa (S3) cells, as well as human leukocytes, the phosphorolysis of FdUrd was essentially unaffected by GPT (Chart 1*A*), while that of dThd was inhibited less than 10% (Chart 1*C*). By contrast, GPT inhibited the phosphorolysis of uridine by about 90% at 0.29 mM, in both the mouse liver and the HeLa (S3) cell preparations (Chart 1*B*). In addition, the dose-response profile for the inhibition of uridine phosphorolysis in the mouse liver preparation was different from profiles obtained with the extracts of HeLa (S3) cells, Novikoff hepatoma cells, and Ehrlich ascites cells (Chart 1*B*).

Isoelectric Focusing. The pyrimidine nucleoside phosphorylase activities present in each of the 5 mammalian cytosol preparations were examined with isoelectric focusing. The Ehrlich ascites preparation contained only one peak of activity that phosphorolytically cleaved uridine and 2 smaller peaks of activity that cleaved only dThd, and whose major peak co-migrated with that of the uridine-cleaving activity at an isoelectric point (pI) of 5.4 (Chart 2*A*). The uridine:dThd cleavage ratio was approximately 5.5:1. After refocusing on a pH gradient of 4 to 6, the 2 peaks of dThd phosphorolytic activity were reduced to a single peak (pI 5.4) that again co-migrated with the major peak of uridine phosphorolytic activity. The reduction of the 2 peaks of dThd phosphorolytic activity to a single peak (pI 5.4) on the pH gradient of 4 to 6 was reproducible. This

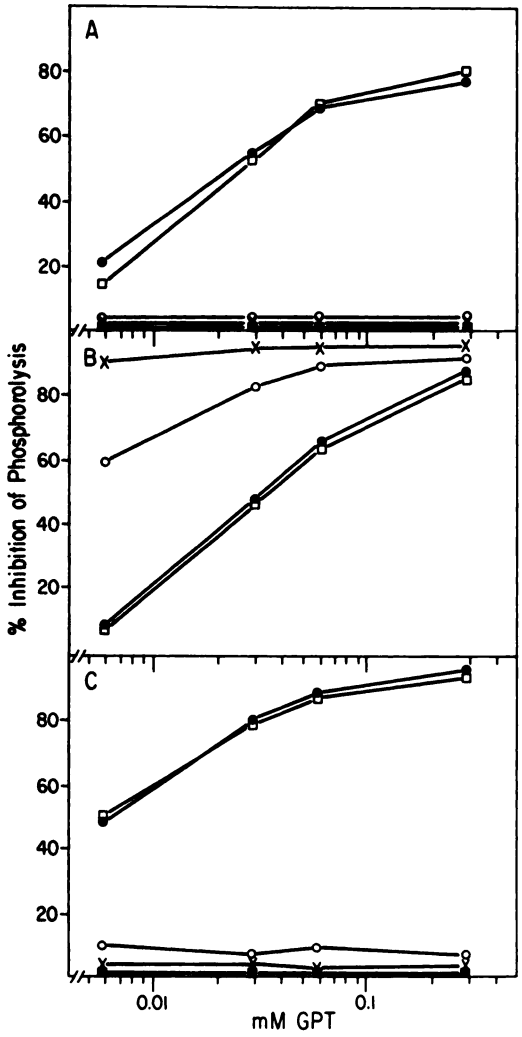

Chart 1. Percentage of inhibition of the phosphorolysis of FdUrd (*A*), uridine (*B*), and dThd (*C*) by GPT. The nucleoside (0.4 mM) was incubated with GPT and either extracts of HeLa (S3) cells (×), mouse liver (○), Ehrlich ascites cells (●), Novikoff hepatoma cells (□), or human leukocytes (■). The values are the mean of 2 experiments (S.E., <2%).

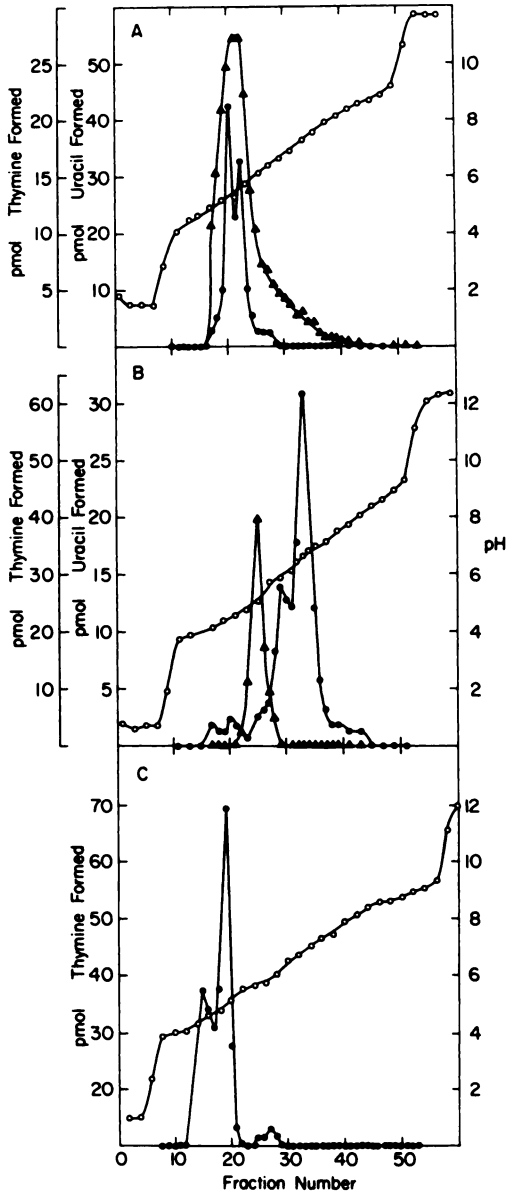

Chart 2. Isoelectric focusing profiles on pH (○) gradients of 3.5 to 10 of dThd (●) and uridine (▲) phosphorolytic activity in cytosol preparations from Ehrlich ascites cells (*A*), mouse liver (*B*), and human leukocytes (*C*).

phenomenon may represent either protein aggregation or the presence of an unstable isozyme of dThd phosphorolytic activity at pI 5.6.

The cytosol preparations from Novikoff hepatoma cells also contained only one peak (pI 5.8) of phosphorolytic activity for uridine, which co-migrated with a single peak of activity that cleaved only dThd (data not shown). The uridine:dThd cleavage ratio was approximately 8.5:1.

Isoelectric focusing of the mouse liver preparation disclosed 2 major peaks of pyrimidine nucleoside phosphorylase activity: (*a*) one that cleaved primarily uridine (pI 5.2); and (*b*) the other that cleaved dThd (pI 6.6) (Chart 2*B*). There were also minor peaks of dThd phosphorolytic activity at pI 4.2, 4.5, 5.8, 7.4, and 8. The uridine:dThd cleavage ratio was approximately 1: 3.25, markedly different from that for the Ehrlich ascites and for the Novikoff hepatoma preparations. When pooled fractions from each of the 2 major peaks of activity were refocused on a pH gradient of 3.5 to 10, the same pI values were obtained.

Human leukocyte preparations obtained from several donors each yielded only one major peak of pyrimidine nucleoside phosphorylase activity that cleaved dThd (pI 4.9), with a minor peak of activity between pI 4.4 and 4.6. A representative profile is shown in chart 2*C*. After refocusing on a pH gradient of 4 to 6, however, the minor peak at pI 4.4 disappeared, while the major peak (pI 4.9) remained. Peaks of uridine phosphorolytic activity were not detected in our cytosol preparations of human leukocytes.

Isoelectric focusing of HeLa (S3) cytosol preparations disclosed only one peak of dThd phosphorolytic activity (pI 4.6), which refocused at the same pI on a pH gradient of 4 to 6 (data not shown). No peak of uridine phosphorolytic activity was detected. This was surprising since our nonfocused cytosol extracts phosphorolytically cleaved uridine. Even electrofocusing of a dialyzed fraction obtained at 40 to 60% ammonium sulfate saturation, which was enriched 5-fold for uridine phosphorolytic activity, failed to disclose any discernible peak of uridine cleavage.

Table 1

pI of pyrimidine nucleoside phosphorylases

Dialyzed fractions of freshly prepared cytosols were electrofocused on ampholine gradients, pH 3.5 to 10, in a 110-ml LKB Isoelectric Focusing column as described in "Materials and Methods." Column fractions were assayed for pyrimidine nucleoside phosphorylase activity, and the pI's were established for the major peaks of dThd and uridine phosphorolytic activity.

	Major peaks of phosphorolytic activity	
Enzyme source	dThd	Uridine
Ehrlich ascites cells	5.4	5.4
Novikoff hepatoma cells	5.8	5.8
Mouse liver	6.6	5.2
Human leukocytes	4.9	
HeLa (S3) cells	4.6	

The phosphorolysis of FdUrd by the various peaks of pyrimidine nucleoside phosphorylase activity was also investigated. The profiles of FdUrd cleavage paralleled closely those of both dThd and uridine in the Ehrlich ascites and Novikoff hepatoma preparations but only those of dThd phosphorolysis in mouse liver, human leukocytes, and HeLa (S3) extracts (data not shown).

The pI values obtained for the major peaks of phosphorolytic activity are summarized in Table 1. Similar pI values were obtained for all 3 peaks of uridine phosphorolytic activity in the 3 murine tissues studied (Ehrlich ascites, Novikoff hepatoma, and mouse liver). Moreover, the pI values for the peaks of dThd and uridine phosphorolytic activities in the Ehrlich ascites extracts were identical as were those of the Novikoff hepatoma preparations. The 2 human cytosol preparations [leukocytes and HeLa (S3) cells] had similar pI values for the peaks of dThd phosphorolysis, but these were more acidic than the value for the peak of dThd phosphorolytic activity in mouse liver (Table 1).

DISCUSSION

GPT is a specific inhibitor of uridine phosphorylase activity (15, 30). In the present study, GPT inhibited the phosphorolysis of FdUrd (Chart 1*A*), uridine (Chart 1*B*), and dThd (Chart 1*C*) by extracts of Ehrlich ascites and Novikoff hepatoma cells. Since GPT does not inhibit dThd phosphorylase activity (15, 30), the data indicate that FdUrd, uridine, and dThd were cleaved by a uridine phosphorylase activity that could also cleave deoxyribonucleosides. Because of the broad substrate specificity of uridine phosphorylase activity (6, 14, 17, 22, 24), we wish to propose the term Ura-dUrd phosphorylase" (EC 2.4.2.3) to describe those pyrimidine nucleoside phosphorylases that cleave both uridine and dThd and that are inhibited by GPT. This term, although not defined as above, has been used by other workers to describe pyrimidine nucleoside phosphorylase activity in several murine neoplasms (2, 3, 22, 25). Our proposed definition clearly distinguishes between (*a*) uridine phosphorylase activity that cleaves primarily uridine (6, 17), but not dThd, and is inhibited by GPT (15, 30) and (*b*) dThd phosphorylase activity that cleaves only deoxyribonucleosides (8) and is not inhibited by GPT.

We have demonstrated that FdUrd is cleaved by Ura-dUrd phosphorylase activity in the Novikoff hepatoma and Ehrlich ascites preparations. This finding, in light of our proposed definition for Ura-dUrd phosphorylase activity, is consistent with the isoelectric focusing data for the 2 preparations. Both the Ehrlich ascites and the Novikoff hepatoma extracts yielded 2 major peaks of pyrimidine nucleoside phosporylase activity, each with the same pI (Table 1); these cleaved both uridine and dThd, as well as FdUrd, which suggests strongly that only one nucleoside phosphorylase activity, Ura-dUrd phosphorylase, was responsible for the phosphorolysis of all 3 nucleosides. This contention is supported by the work of Krenitsky *et al.* (14), in which the homogeneity of the pyrimidine nucleoside phosphorylase activity in Ehrlich ascites was established by both DEAE-cellulose chromatography and moving-boundary electrophoresis. On purification, the phosphorylase retained both ribonucleoside and deoxyribonucleoside cleavage activity (14).

GPT did not markedly inhibit the phosphorolysis of FdUrd (Chart 1*A*) and dThd (Chart 1*C*) by the extracts of mouse liver, HeLa (S3) cells, and human leukocytes but did inhibit the phosphorolysis of uridine by the preparations of mouse liver and HeLa (S3) cells (Chart 1*B*). It should be noted that uridine was not cleaved by our partially purified preparations of human leukocytes, although Gallo and Perry (9) have reported the cleavage of uridine by a crude leukocyte extract. Since GPT does not inhibit dThd phosphorylase activity (15, 30), our data indicate that FdUrd is cleaved primarily by dThd phosphorylase activity in mouse liver, HeLa (S3) cells, and human leukocytes. These observations are consistent with the isoelectric focusing data that demonstrated only one peak of phosphorolytic activity that cleaved both dThd and FdUrd, but not uridine (Charts 2*A*, 2*B*, and Table 1), in all 3 preparations. Moreover, our data for mouse liver support those of Krenitsky *et al.* (14), who utilized DEAE-cellulose chromatography to separate dThd phosphorylase activity from a pyrimidine nucleoside phosphorylase activity that cleaved both uridine and dThd. The broader substrate specificity of the latter phosphorylase is consistent with our observation that GPT produced a slight inhibition (approximately 10%) of the phosphorolysis of dThd in the mouse liver preparations (Chart 1*C*); this suggests that the peak of uridine phosphorolytic activity present in our isoelectric focusing studies could also cleave dThd (Chart 2*B*). Thus, by our proposed definition, this enzyme activity is Ura-dUrd phosphorylase.

To the best of our knowledge, this is the first time that evidence has been presented to suggest that HeLa (S3) cells contain phosphorylases that act upon uridine, as well as upon dThd and other deoxyribonucleosides. Isoelectric focusing of this preparation disclosed only a single peak of dThd phosphorylase activity (pI 4.6) (data not shown). The 5-fold enrichment of the cytosol by precipitation with ammonium sulfate (40 to 60% fraction) for uridine phosphorolytic activity (data not shown) and the inhibition of this activity by GPT (Chart 1*B*) suggest strongly the presence of a second pyrimidine nucleoside phosphorylase. Studies are underway to stabilize the uridine phosphorolytic activity for further characterization. Since GPT did not inhibit the phosphorolysis of dThd in the HeLa (S3) preparation (Chart 1*C*), the data suggest that this second phosphorolytic activity is uridine phosphorylase.

The proposed definition for Ura-dUrd phosphorylase activity should help to clarify the nomenclature, and hence the substrate specificities, of pyrimidine nucleoside phosphorylases. Identifying the phosphorylases primarily responsible for the cleavage of FdUrd in 5 mammalian tissues has enabled us to evaluate potential inhibitors of these enzymes, with a view

toward enhancing the chemotherapeutic efficacy of FdUrd (29).

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