Biochemical Pharmacology, Vol. 33, No. 15, pp. 2383–2395, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

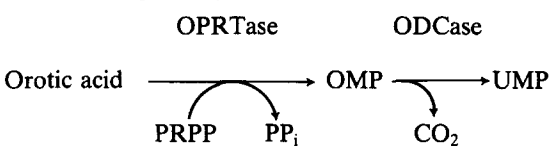
STRUCTURE–ACTIVITY RELATIONSHIP OF PYRIMIDINE BASE ANALOGS AS LIGANDS OF OROTATE PHOSPHORIBOSYLTRANSFERASE*

JOHN G. NIEDZWICKI†, MAX H. ILTZSCH, MAHMOUD H. EL KOUNI and SUNGMAN CHA‡
Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

(*Received* 29*August* 1983; *accepted* 7 *December* 1983)

Abstract—Eighty pyrimidine base analogs were evaluated as inhibitors of mouse liver orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10). Based on these findings and an extensive literature review, a structure–activity relationship has been formulated for the binding of pyrimidine base analogs to OPRTase. This study provides a basis for the rational design of new inhibitors of this enzyme, and several such compounds are proposed. Additionally, 4,6-dihydroxypyrimidine has been found to be a potent OPRTase inhibitor. Eleven OPRTase inhibitors were also evaluated as inhibitors of orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.2.23). 5-Azauracil, 5-azaorotate, and barbituric acid inhibited ODCase significantly only after preincubation with PRPP and $MgCl_2$ in the presence of cytosol.

Orotate phosphoribosyltransferase (OPRTase§, EC 2.4.2.10) and orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.2.23) catalyze the following sequential reactions in the *de novo* pyrimidine biosynthetic pathway:



In mammalian tissues, OPRTase and ODCase are not separable [1–6]; consequently, these two enzymes have been referred to as the single entity "complex U" [7] or "UMP synthetase" [8]. Both OPRTase and ODCase activities in Ehrlich ascites cells are catalyzed by a single polypeptide [8]; however, whether or not this finding is a general characteristic of these enzymes from all mammalian sources remains to be determined.

Inhibitors of OPRTase and ODCase are potentially useful as chemotherapeutic agents that act by interfering with the *de novo* biosynthesis of pyrimidine derivatives. Indeed, the antineoplastic drugs pyrazofuran and 6-azauridine, after intracellular phosphorylation to their respective nucleoside 5′-monophosphate derivatives, are believed to produce their effects through ODCase inhibition [9]. On the other hand, OPRTase has received little attention as a chemotherapeutic "target enzyme" and only a few potent inhibitors of OPRTase, e.g. 5-azaorotate [10], 5-fluoroorotate [11, 12] and barbituric acid [13], are known. A theoretical advantage of OPRTase inhibitors is that such compounds, being analogs of the substrate orotic acid, would not necessarily require metabolic activation as is generally the case with ODCase inhibitors.

OPRTase inhibitors may also have applications as experimental pharmacological tools in identifying the metabolic pathways of pyrimidine base analogs. For example, activation of the antineoplastic drug FUra involves its initial conversion to FUMP. This "lethal synthesis" may be catalyzed either sequentially by uridine phosphorylase (EC 2.4.2.3) and uridine kinase (EC 2.7.1.48) or directly by OPRTase [14]. An OPRTase inhibitor may be useful in assessing the relative importance of these pathways in the activation of FUra in a given tissue.

In the present study, eighty pyrimidine base analogs were evaluated as inhibitors of mouse liver OPRTase. These findings have allowed us to formulate a structure–activity relationship for the binding of pyrimidine base analogs to OPRTase that provides a basis for the rational design of OPRTase inhibitors. During the course of these studies, we discovered a new OPRTase inhibitor, 4,6-dihydroxypyrimidine, which is as potent as the best known ligand of this enzyme, 5-fluoroorotic acid. Additionally, evidence was obtained to support the contention that the OPRTase inhibitors barbituric

* Supported by Grant CH-136 awarded by the American Cancer Society. M. H. I. was supported by Training Grant CA-09204 awarded by the National Cancer Institute, DHHS.

† Present address: The George Washington University School of Medicine, Washington, DC 20037, U.S.A.

‡ Author to whom all correspondence should be addressed.

§ Abbreviations: OPRTase, orotate phosphoribosyltransferase; ODCase, orotidine 5′-monophosphate decarboxylase; PRPP, 5-phosphorylribose-1-pyrophosphate; PP_i, pyrophosphate, OMP, orotidine 5′-monophosphate; FUra, 5-fluorouracil; FUMP, 5-fluorouridine 5′-monophosphate; FUrd, 5-fluorouridine; DTT, dithiothreitol; and PCA, perchloric acid.

acid and 5-azauracil are also substrates for this enzyme and that the products (barbituric acid riboside 5′-monophosphate and 5-azauridine 5′-monophosphate) are inhibitors of ODCase.

MATERIALS AND METHODS

Chemicals. The sources of the pyrimidine bases and analogs used in this study are indicated in Table 5 by the following abbreviations: ALD, Aldrich Chemical Co., Milwaukee, WI; CAL, Calbiochem-Behring Corp., La Jolla, CA; CDC, Chemical Dynamics Corp., South Plainfield, NJ; K&K, K&K Laboratories, Inc., Plainview, NY; MAL, Mallinckrodt Chemical Works, St. Louis, MO; PL, P-L Biochemicals, Milwaukee, WI; SHC, Dr. Shih Hsi Chu, Brown University, Providence, RI; SIGMA, Sigma Chemical Co., St. Louis, MO; and VEGA, Vega Biochemicals, Tucson, AZ. Additionally, FUMP was obtained from Sierra Biochemicals, Tucson, AZ; FUrd was obtained from Vega Biochemicals, Tucson, AZ; [2-^{14}C]FUra (56 mCi/mmole) was obtained from Moravek Biochemicals, Brea, CA; [6-^{14}C]orotic acid (46.9 mCi/mmole), used for the enzymatic synthesis of [6-^{14}C]OMP [15], and Omnifluor were obtained from the New England Nuclear Corp., Boston, MA; silica gel G/UV$_{254}$ and Polyethyleneimine Cellulose 300 PEI/UV$_{254}$ Polygram TLC plates were obtained from Brinkmann, Westbury, NY; Bio-Rad Protein Assay Kit was obtained from Bio-Rad Laboratories, Richmond, CA; and 5-benzylacyclouridine was obtained from Dr. Shih Hsi Chu, Brown University. All other chemicals were obtained from the Sigma Chemical Co.

Cytosol extracts. CD-1 mice (Charles River Laboratories, Wilmington, MA) were killed by cervical dislocation, and the livers were homogenized using a motor-driven Teflon pestle in either 3 vol. for OPRTase or 15 vol. for ODCase of 50 mM Tris–Cl buffer (pH 8.0) which also contained 1 mM DTT and 2 mM $MgCl_2$. The homogenate was then centrifuged for 1 hr at 105,000 *g* at 4°. All cytosol extracts were prepared and used in enzyme assays on the same day.

Orotate phosphoribosyltransferase assay. The standard assay mixture contained 44 mM Tris–Cl, 0.8 mM DTT, 1.8 mM $MgCl_2$, 0.1 mM [2-^{14}C]FUra (20 mCi/mmole), 5 mM PRPP, 0.1 mM 5-benzylacyclouridine, cytosol extract (0.5 to 1.0 mg protein), and an appropriate amount of a compound to be tested for OPRTase inhibition in a final volume of 160 μl at pH 8.0. Typically, test compounds were screened at concentrations of 0.1, 0.5, 1, 3, and 5 mM; however, lower concentrations had to be used for very potent inhibitors and for compounds having very poor water solubility.

FUra was used as the substrate for OPRTase assay because it is an excellent substrate for this enzyme [1–3], and the reaction generates only two products (FUMP and, indirectly, FUrd) which separate well from FUra on silica gel TLC. In the present assay system, which employed crude enzyme extracts, FUrd must be included in the incubation mixture, because it may be formed from FUMP, the product of the OPRTase reaction, by the subsequent action of various 5′-nucleotidases and phosphatases [3, 16, 17]. In contrast, [6-^{14}C]orotic acid was not considered to be a suitable substrate for the OPRTase assay with crude enzyme extract because the separation of this compound from the product (OMP) and numerous metabolites thereof (orotidine, UMP, and Urd) involves a more laborious process using PEI-cellulose TLC similar to that described below for the ODCase assay. Furthermore, because OPRTase from mouse liver has an extremely low K_m value (significantly lower than 1 μM) for orotic acid [15], a saturating concentration of this substrate is necessary for accurate enzyme assay. Therefore, orotic acid is not an appropriate substrate for the screening of inhibitors which may not compete well with this substrate at a saturating concentration. The uridine phosphorylase inhibitor 5-benzylacyclouridine [18] was included in all reaction mixtures to prevent the interference of uridine phosphorylase because this enzyme can catalyze both the synthesis [17, 19] and cleavage [20, 21] of FUrd. 5-Benzylacyclouridine *per se* has no effect on mouse liver OPRTase.

The reactions were started by the addition of cytosol extract and were carried out for 20 min at 37°. At 0 and 20 min, 50 μl aliquots of each reaction mixture were withdrawn and pipetted into test tubes that contained 5 μl of 40% PCA. After centrifugation, 25 μl of the supernatant fluid was neutralized with 90 μl of 0.2 N KOH which also was 10 mM with respect to the standards (FUra, FUrd, and FUMP); this facilitated visualization of spots on TLC. These samples were then chilled and centrifuged to remove potassium perchlorate, and 10-μl aliquots of the supernatant fluid were spotted on silica gel G/UV$_{254}$ TLC plates. The plates were then developed in a solvent system of chloroform: methanol (9:1, v/v). Spots containing the substrate FUra ($R_f = 0.28$) and products FUMP ($R_f = 0$) and FUrd ($R_f = 0.11$) were then identified under a U.V. lamp, cut out, and placed into vials containing 20 ml of Omnifluor. The radioactivity contained in each spot was then measured in a Packard Tri-Carb 460 scintillation counter.

Orotidine 5′-monophosphate decarboxylase assay. The standard mixture contained 25 mM Tris–Cl, 0.5 mM DTT, 1 mM $MgCl_2$, 0.01 mM [6-^{14}C]OMP (22.1 mCi/mmole), and cytosol extract (approximately 0.02 mg protein) in a final volume of 150 μl at pH 8.0. Compounds were tested as ODCase inhibitors at a concentration of 5 mM with the exceptions of 6-carboxymethyluracil and uracil-6-methylsulfone which were tested at 2.5 mM because of poor water solubility. Assays were carried out both in the presence and absence of 5 mM PRPP; in the former case the cytosol extract, test compound, and PRPP were preincubated for 30 min at 37° and the $MgCl_2$ concentration was increased to 5 mM. Reactions were carried out for 30 min at 37° and stopped by immersion in a boiling water bath for 1 min. The product (UMP, $R_f = 0.25$) and metabolites thereof (Urd, $R_f = 1.0$, and Ura, $R_f = 0.94$) were separated from the substrate (OMP, $R_f = 0.09$) on PEI cellulose TLC plates using 0.2 M LiCl as the developing solvent.

Determination and significance of apparent K_i

values. Apparent K_i values were determined from Dixon plots ($1/v$ vs $[I]$) of the data by a computer program with least-squares fitting. The PRPP concentration used (5 mM) is saturating for OPRTase [2, 3, 5, 6]. Apparent K_i values are related to true K_i values by the following equation:

$$\text{apparent } K_i = \frac{K_{is}(1 + [S]/K_m)}{1 + ([S]/K_m)\,(K_{is}/K_{ii})}$$

where K_{is} and K_{ii} are the inhibition constants that would have been estimated from the replots of slope and intercept, respectively, of a Lineweaver–Burk plot vs $[I]$. If a compound is a competitive inhibitor with respect to the substrate, FUra, $K_{ii} = \infty$ and $K_{is} = K_i$. Therefore:

$$\text{apparent } K_i = K_i(1 + [S]/K_m)$$

In the present study, the concentration of one substrate (FUra) was at its K_m value for OPRTase (0.1 mM) [11] and the other substrate (PRPP) was at a saturating concentration (5 mM) [3, 5, 6]. Therefore, the apparent K_i value of a competitive inhibitor is approximately 2-fold higher than the K_i. It must be stressed, however, that inhibitors were not characterized with regard to the type of inhibition (i.e. competitive, non-competitive, or uncompetitive) or the substrate activity. In the present study, the apparent K_i values of various compounds determined at 0.1 mM 5-fluorouracil were assumed to represent the (reciprocal of) affinity of these compounds for OPRTase. This does not imply, however, that the values presented in Table 5 are indeed dissociation constants of *E-I* complexes. In fact, when a compound serves as a substrate, the apparent K_i value would reflect its K_M value.

Protein determination. Protein concentrations were determined by the method of Bradford [22] using bovine γ-globulin as a standard.

RESULTS

Literature survey. In order to have a broad basis upon which to formulate a structure–activity relationship, an extensive literature survey on mammalian OPRTase has been conducted. It must be emphasized that this study is limited to a consideration of mammalian OPRTase, and that the inhibitor and substrate specificities of bacterial, protozoal, and yeast OPRTase are quite different (see Refs. 23 and 24).

Known substrates and non-substrates of mammalian OPRTase are listed in Tables 1 and 2 respectively. It is noteworthy that OPRTase from bovine erythrocytes appears to have a broader substrate specificity than the enzymes from other mammalian sources since thymine [2], uric acid [2, 28], and xanthine [2, 28] are substrates. Thymine [11, 33], uric acid [33], and xanthine [33, 34] do not inhibit and therefore do not bind to the other mammalian OPRTases tested. Also, it should be noted that uracil, reported as both substrate (Table 1) and non-substrate (Table 2), is a very poor substrate for OPRTase from many different sources, particularly at low pH.

Known inhibitors and non-inhibitors of mammalian OPRTase are listed in Tables 3 and 4 respectively. Other pyrimidine base analogs have been shown to antagonize orotic acid *in vitro* [10, 13, 36, 37] or *in vivo* [38–40]. These compounds are not included in Table 3, however, because it was not clear whether the site of inhibition was indeed OPRTase. Also omitted from Table 4 are thirty-two pyrimidine analogs reported by Handschumacher [10] to inhibit orotate anabolism *in vitro* by "less than fifty per cent" because no quantitative estimate of binding or the lack thereof could be calculated from his report. Additionally, nucleotide analogs of purines and pyrimidines that have been evaluated as OPRTase inhibitors [6, 12, 33, 36] are also not included in Tables 3 and 4. With the exception of dTTP, these nucleotides inhibit OPRTase competitively with respect to PRPP rather than orotic acid [36]. Since the present study is primarily concerned with the inhibition of OPRTase by pyrimidine base analogs, it was not considered essential to summarize nucleotide inhibition data.

Inhibition studies. The inhibitory potencies of the compounds examined in this study as OPRTase inhibitors are expressed in terms of apparent K_i values in Table 5. Roman numerals cited in the following text refer to compounds listed in Table 5.

Some of the pyrimidine base analogs that we found to be OPRTase inhibitors were also evaluated as ODCase inhibitors (see Table 6). When no PRPP was added to the reaction mixtures, these analogs produced minimal inhibition of ODCase. However, significant inhibition of this enzyme occurred when 5-azaorotic acid (L), 5-azauracil (XVIII), or barbituric acid (XXXIII) was preincubated with PRPP and $MgCl_2$, suggesting that these compounds might serve as substrates for OPRTase to form their respective nucleotide 5′-monophosphate derivatives which inhibit ODCase.

DISCUSSION

For the purpose of evaluating the binding of various compounds to OPRTase, uracil (I) was chosen as the reference compound because it is a simple pyrimidine that binds to OPRTase. The general approach was to determine how various functional groups at each position of uracil affect binding to this enzyme. It must be realized, however, that a substituent at one position of the uracil molecule may affect the properties of other positions as well. For example, an electron-withdrawing functional group (e.g. chloro, nitro, etc.) at the 5-position of uracil may affect binding not only by the direct interaction of the substituent group with the binding site on the enzyme, but also indirectly by increasing the acidity of the pyrimidine ring system, as will be discussed below. Another factor that complicates evaluation of substituent effects "position-by-position" is the possibility that certain analogs may bind to OPRTase in different orientations than uracil. While it seems reasonable to assume that most uracil analogs bind to the enzyme in a uracil-like orientation, this may not be the case when one of the ring nitrogen atoms or oxo groups of uracil is replaced by carbon or hydrogen atoms respectively. For ex-

Table 1. Substrates of mammalian orotate phosphoribosyltransferase

Compound	Enzyme source	pH	K_m (mM)	Ref.
5-Azaorotic acid	Mouse liver	7.4		25
6-Azathymine	Bovine erythrocytes	8.0		2
6-Azauracil	Bovine erythrocytes	8.0		2
2,4-Dihydroxy-pteridine	Bovine erythrocytes	8 0		2
5-fluoroorotic acid	Bovine thymus	9.0	0.075	11
	Mouse erythroleukemia	7.4	0.033	26
	P388 mouse leukemia	9.0	0.082	11
5-Fluorouracil	Bovine erythrocytes	8.0		2
	Bovine thymus	8.0		3
		9.0	0.080	11
	Gardner (mouse) lymphosarcoma	8.0		1
	CEM human leukemia	8.0		19
	LAZ human leukemia	8.0		19
	P1534J mouse leukemia	8.0		5
	P388 mouse leukemia	9.0	0.100	11
	S-49 mouse lymphoma	7.0	0.385	27
5-Iodouracil	Bovine erythrocytes	8.0		2
Orotic acid	Bovine erythrocytes	8.0		2
		8.0		28
	Bovine thymus	8.0	0.032	3
		9.0	0.050	11
	Ehrlich ascites cells	7.0	0.002	29
	Human colorectal adenocarcinoma	8.0		30
	CEM human leukemia	8.0		19
	LAZ human leukemia	8.0		19
	Mouse erythroleukemia	7.4	0.002	26
	P338 mouse leukemia	9.0	0.050	11
	P1534J mouse leukemia	8.0		5
	Mouse liver	7.4		25
	S-49 mouse lymphoma	7.0	0.002	27
Thymine	Bovine erythrocytes	8.0		2
Uracil	Bovine erythrocytes	8.0		2
	Bovine thymus	9.0	4.0	11
	P1534J mouse leukemia	10.0		5
	P388 mouse leukemia	9.0	5.0	11
Uric acid*	Bovine erythrocytes	8.0		2
		8.0		28
Xanthine†	Bovine erythrocytes	8.0		2
		8.0		28

* The product of the reaction was (3-ribosyluric acid) 5′-phosphate.
† The product of the reaction was (3-ribosylxanthine) 5′-phosphate.

Table 2. Non-substrates of mammalian orotate phosphoribosyltransferase

Compound	Enzyme source	pH	Ref.
5-Azauracil	L5178Y mouse leukemia	7.0	10
Cytosine	Bovine erythrocytes	8.0	2
2-Hydroxypyrimidine	Bovine erythrocytes	8.0	2
4-Hydroxypyrimidine	Bovine erythrocytes	8.0	2
2-Thiouracil	Bovine thymus	8.0	31
Uracil	Ehrlich ascites cells	7.4	32
	P1534J mouse leukemia	8.0	5
	Mouse liver	7.4	25

Table 3. Inhibitors of mammalian orotate phosphoribosyltransferase

Compound	Enzyme source	pH	K_i* (mM)	Inhibition				
				Substrate, conc. (mM)		Inhibitor (mM)	% Inhibition	Ref.
5-Aminoorotic acid	P388 mouse leukemia	9.0	1.3					11
5-Aminouracil	P388 mouse leukemia	9.0	0.85					11
5-Azaorotate	Mouse liver	7.4		Orotic acid,	0.1	0.0006	57	10
5-Azauracil	P388 mouse leukemia	9.0	4.0					11
6-Azauracil	Ehrlich ascites cells	7.4		Orotic acid,	0.005	1.0	13	12
	P388 mouse leukemia	9.0	5.0					11
Barbituric acid	Ehrlich ascites cells	7.4		Orotic acid,	0.005	0.5	50	12
	Rat brain	7.8	0.018†					13
5-Bromouracil	Ehrlich ascites cells	7.4		Orotic acid,	0.005	5.0	24	12
5-Carboxyuracil	P388 mouse leukemia	9.0	8.0					11
5-Chlorouracil	Ehrlich ascites cells	7.4		Orotic acid,	0.005	1.0	41	12
Cytosine	Ehrlich ascites cells	7.4		Orotic acid,	0.005	10.0	36	12
5-Diazouracil	P338 mouse leukemia	9.0	10.0					11
D-Dihydroorotate	P338 mouse leukemia	9.0	3.3					11
L-Dihydroorotate	p388 mouse leukemia	9.0	0.8					11
D,L-Dihydroorotate	Ehrlich ascites cells	7.4		Orotic acid,	0.005	10.0	56	12
2-Ethylmercapto-5-fluoro-orotic aldehyde	P388 mouse leukemia	9.0	2.0					11
5-Fluoroorotic acid	Ehrlich ascites cells	7.4		Orotic acid,	0.005	0.05	74	12
	Mouse erythroleukemia	7.4	0.11†					26
	P388 mouse leukemia	9.0	0.085					11
5-Fluorouracil	Ehrlich ascites cells	7.4		Orotic acid,	0.005	5.0	41	12
	P388 mouse leukemia	9.0	0.1					11
2-Hydroxypyrimidine	P388 mouse leukemia	9.0	8.0					11
5-Methylorotic acid	P388 mouse leukemia	9.0	10.0					11
5-Nitroorotic acid	P388 mouse leukemia	9.0	0.3					11
Orotic acid	Mouse erythroleukemia	7.4	0.042‡					26
	P1534J mouse leukemia	8.0	0.046					5
		10.0		5-Fluorouracil,	0.5	4.0	99	33
	P388 mouse leukemia	9.0	0.5					11
6-(2-Thio-4-oxy-6-pyrimidylmethylidine)-5′-oxo-2′-phenyloxazoline	P388 mouse leukemia	9.0	5.0					11
5-Trifluoromethyluracil	P388 mouse leukemia	9.0	10.0					11
Uracil	Ehrlich ascites cells	7.4		Orotic acid,	0.005	1.0	29	12
	P1534 mouse leukemia	8.0	1.8					5
		10.0		5-Fluorouracil,	0.5	4.0	86	33
	P388 mouse leukemia	9.0	5.0					11

* Except where noted otherwise, the substrate used for K_i determination was 5-fluorouracil.
† The substrate used for K_i determination was orotic acid.
‡ The substrate used for K_i determination was 5-fluoroorotic acid.

Table 4. Non-inhibitors of mammalian orotate phosphoribosyltransferase.

Compound*	Enzyme source	pH	Substrate, conc. (mM)		Highest inhibitor conc. tested (mM)	Ref.
Adenine	P1543J mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33
Adenosine	Human erythrocytes	7.4	Orotic acid,	0.000015	1.0	35
Allopurinol	Human erythrocytes	7.4	Orotic acid,	0.1	1.0	34
	Human erythrocytes	7.4	Orotic acid,	0.1	1.0	35
Allopurinol ribonucleoside	Human erythrocytes	7.4	Orotic acid,	0.04	1.0	34
Bemegride	Rat brain	7.4	Orotic acid,	0.1	1.0	13
5-Bromouracil	P388 mouse leukemia	9.0			†	11
5-Cyclohexylethyl-5-ethylbarbituric acid	Rat brain	7.4	Orotic acid,	0.1	1.0	13
Cytosine	P1534J mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33
Guanine	P1534J mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33
Guanosine	Human erythrocytes	7.4	Orotic acid,	0.000015	1.0	35
erytho-9-(2-Hydroxyl-3-nonyl)adenine	Human erythrocytes	7.4	Orotic acid,	0.000015	0.03	35
5-Hydroxyuridine	Rat brain	7.4	Orotic acid,	0.1	1.0	13
Inosine	Human erythrocytes	7.4	Orotic acid,	0.0015	1.0	34
Isobarbituric acid	Rat brain	7.4	Orotic acid,	0.1	4.0	13
Methimazole	Bovine thymus	8.0	Orotic acid,	0.025	0.25	37
6-Methylmercaptopurine riboside	Human erythrocytes	7.4	Orotic acid,	0.000015	1.0	35
Methylthiouracil	Bovine thymus	8.0	Orotic acid,	0.025	0.25	37
Oxypurinol	Human erythrocytes	7.4	Orotic acid,	0.04	1.0	34
Phenobarbital	Rat brain	7.4	Orotic acid,	0.1	4.0	13
Propylthiouracil	Bovine thymus	8.0	Orotic acid,	0.025	0.25	37
2-Thioorotic acid	Bovine thymus	8.0	Orotic acid,	‡	‡	37
2-Thiouracil	Bovine thymus	8.0	Orotic acid,	0.025	0.25	37
	P388 mouse leukemia	9.0			†	11
Thymine	P388 mouse leukemia	9.0			†	11
	P1534J mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33
Uric acid	P1534J mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33
Xanthine	Human erythrocytes	7.4	Orotic acid,	0.04	1.0	34
	P1534 mouse leukemia	10.0	5-Fluorouracil,	0.5	4.0	33

* Nucleotide analogs [6, 12, 33, 36] and certain other compounds [10, 13, 36–38] were not included in this table for reasons explained in the text.

† $K_i > 10$ mM.

‡ 2-Thioorotic acid produced no inhibition of OPRTase at ten times the concentration of the substrate (orotic acid). Specific concentrations were not given.

ample, it is not clear whether 2-hydroxypyrimidine (XVII), which exists in solution largely as the keto tautomer "4-deoxyuracil" [40–42] binds to OPRTase as 4-deoxyuracil or in an orientation rotated 180° about the *N*3–*C*6 axis as "1-deaza-2-deoxy-5-azauracil".

Prior to a detailed analysis of the data, some discussion of the tautomerism of uracil and its analogs is appropriate. The oxo groups at the 2- and 4-positions of uracil can theoretically undergo keto-enol tautomerism, and thereby six tautomeric forms are possible. As reviewed by Kwiatkowski and Pullman [43], the diketo (also called the "dilactam") tautomer of uracil is the only species that has been detected in aqueous solution at any pH. The same holds true for 1- and/or 3-position alkyl-substituted uracils [43–45] and uracils substituted at the 5- and/or 6-positions [43]. However, it is essential to realize that the sensitivity of current techniques is such that rare tautomers cannot be detected at ratios less than 1:10,000 [46]. Thus, while it will be assumed that the dilactam form of the foregoing uracils are the only species present, one cannot rule out the possibility that the monolactim or dilactim tautomers of these compounds bind to OPRTase.

Since considerable discussion will be devoted to the binding of the anionic species of uracil derivatives to OPRTase, some preliminary comments on the structures of these compounds are also appropriate. In general, two types of such monoanions exist: those formed by the dissociation of a proton from the pyrimidine ring system (*N*1 or *N*3 proton) and those formed by the dissociation of a proton from an acidic exocyclic functional group. Examples of the former case include the monoanions of uracil and 5-fluorouracil which have been shown to exist in solution as mixtures of tautomers with a proton dissociated from either the *N*1 or *N*3 position [43–45]. The negative charge of these monoanions does not remain localized, but is spread over the π-electron system of the pyrimidine ring, and the pattern of dispersion differs depending upon which proton (*N*1 or *N*3) is dissociated (Fig. 1). On the other hand, the negative charge generated by the dissociation of a

Table 5. Inhibitory potencies of pyrimidine base analogs as orotate phosphoribosyltransferase inhibitors, using 5-fluorouracil as a substrate

	Compound	Source	Apparent K_i (mM)
I	Uracil	SIGMA	4.43 ± 0.89
1-Position			
II	Acyclouridine [1-(2'-Hydroxyethoxymethyl)uracil]	SIGMA	24.5 ± 0.07
III	1-Cyclohexyluracil	VEGA	*
IV	1-Cyclohexylthymine	VEGA	*
V	1-Ethyluracil	VEGA	†
VI	Ftorafur [5-fluoro-1-(tetrahydro-2-furyl)uracil]	CAL	†
VII	1-Methyluracil	VEGA	†
VIII	2,6-Pyridinediol ("1-Deazauracil")	ALD	2.64 ± 1.30
2-Position			
IX	2-Amino-4-hydroxypyrimidine	SIGMA	28.8 ± 1.0
X	4-Hydroxypyrimidine ("2-Deoxyuracil")	VEGA	†
XI	2-Thiouracil	SIGMA	17.7 ± 7.9
3-Position			
XII	3-Deazauracil	SIGMA	0.261 ± 0.082
XIII	3-Methyluracil	SIGMA	†
XIV	3-Oxauracil	SIGMA	0.409 ± 0.127
4-Position			
XV	Cytosine	SIGMA	†
XVI	2-Hydroxy-4-methylpyrimidine	CDC	†
XVII	2-Hydroxypyrimidine ("4-Deoxyuracil")	VEGA	24.3 ± 12.0
5-Position			
XVIII	5-Azauracil	SIGMA	0.201 ± 0.056
XIX	5-Aminouracil	SIGMA	‡
XX	5-Bromouracil	SIGMA	4.01 ± 0.92
XXI	5-Carboxyuracil (Isoorotic acid)	SIGMA	5.30 ± 1.99
XXII	5-Chlorouracil	SIGMA	0.854 ± 0.161
XXIII	5-Diazouracil	SIGMA	10.8 ± 5.7
XXIV	5-Fluorouracil	SIGMA	0.544 ± 0.086
XXV	5-Hydroxymethyluracil	VEGA	3.23 ± 0.42
XXVI	5-Hydroxyuracil (Isobarbituric acid)	SIGMA	8.56 ± 1.51
XXVII	5-Iodouracil	SIGMA	7.21 ± 1.86
XXVIII	5-Mercaptouracil	CDC	7.71 ± 2.75
XXIX	5-Nitrouracil	SIGMA	0.867 ± 0.155
XXX	Thymine (5-Methyluracil)	SIGMA	35.7 ± 13.6
6-Position			
XXXI	6-Aminouracil	SIGMA	0.771 ± 0.164
XXXII	6Azauracil	SIGMA	1.12 ± 0.29
XXXIII	Barbituric acid (6-Hydroxyuracil)	SIGMA	0.023 ± 0.003
XXXIV	6-Benzyluracil	SHC	*
XXXV	6-Carboxymethyluracil	SIGMA	0.156 ± 0.028
XXXVI	6-Chloromethyluracil	ALD	4.41 ± 0.59
XXXVII	6-Chlorouracil	VEGA	0.363 ± 0.45
XXXVIII	6-Iodouracil	VEGA	5.41 ± 1.09
XXXIX	Methylorotate (Orotic acid methyl ester)	ALD	0.027 ± 0.007
XL	6-Methyluracil	SIGMA	13.4 ± 4.9
XLI	Orotic acid	SIGMA	0.048 ± 0.009
XLII	Uracil-6-methylsulfone	SIGMA	0.138 ± 0.036
More than one position substituted			
XLIII	5-Aminobarbituric acid (5-Amino-6-hydroxy-uracil or Uramil	SIGMA	0.522 ± 0.061
XLIV	4-Amino-5-bromo-2-hydroxypyrimidine	VEGA	‡
XLV	2-Amino-4,6-dihydroxypyrimidine	VEGA	1.94 ± 0.36
XLVI	4-Amino-6-hydroxy-2-mercaptopyrimidine	ALD	‡
XLVII	5-Aminoorotic acid	SIGMA	4.48 ± 0.91
XLVIII	5-Azabarbituric acid (Cyanuric acid)	ALD	0.212 ± 0.034
XLIX	5-Aza-1-methyluracil	CDC	13.4 ± 3.0
L	5-Azaorotic acid (Oxonic acid)	ALD	0.045 ± 0.016
LI	6-Azathymine	SIGMA	6.69 ± 2.05
LII	6-Benzyl-2-thiouracil	SIGMA	*
LIII	5-Bromo-2,4-dimethoxypyrimidine	VEGA	*
LIV	5-Bromo-1-methyluracil	SIGMA	†

Table 5. *(continued)*

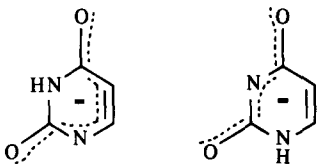
	Compound	Source	Apparent K_i (mM)
LV	5-Bromoorotic acid	CDC	1.00 ± 0.18
LVI	5,6-Diaminouracil	SIGMA	1.19 ± 0.50
LVII	4,5-Dihydroxy-2-methylpyrimidine	VEGA	7.34 ± 1.63
LVIII	4,6-Dihydroxy-2-methylpyrimidine	CDC	8.95 ± 2.77
LIX	4,6-Dihydroxypyrimidine ("5-Aza-3-deazauracil")	ALD	0.015 ± 0.001
LX	1,3-Dimethylbarbituric acid	VEGA	2.99 ± 0.53
LXI	1,3-Dimethyluracil	SIGMA	†
LXII	2,4-Dithiouracil	SIGMA	11.3 ± 3.2
LXIII	5-fluoroacyclouridine [5-Fluoro-1-(2'-hydroxyethoxy-methyl)uracil]	SHC	29.3 ± 16.4
LXIV	5-Fluoro-1-methyluracil	VEGA	†
LXV	5-Fluoroorotic acid	PL	0.016 ± 0.002
LXVI	5-Iodoorotic acid	SIGMA	0.970 ± 0.129
LXVII	1-Methylbarbituric acid	VEGA	1.06 ± 0.09
LXVIII	2-Methylthioorotate	VEGA	6.01 ± 1.52
LXIX	5-Nitroorotic acid	ALD	0.039 ± 0.005
LXX	4-Oxo-2,6-dicarboxypyridine (Chelidamic acid)	ALD	‡
LXXI	6-*n*-Propyl-2-thiouracil	VEGA	27.8 ± 18.7
LXXII	2-Thiobarbituric acid	CDC	0.210 ± 0.036
LXXIII	2-Thioorotic acid	VEGA	1.24 ± 0.14
LXXIV	4-Thiothymine	VEGA	‡
Miscellaneous			
LXXV	DL-Dihydroorotate	SIGMA	1.22 ± 0.11
LXXVI	L-Dihydroorotate	VEGA	0.90 ± 0.08
LXXVII	Dihydrouracil	SIGMA	†
LXXVIII	Hydantoin	SIGMA	†
LXXIX	Pentobarbital	K&K	§
LXXX	Phenobarbital	MAL	‡

* Less than 5% inhibition of OPRTase at the maximal inhibitor concentration of 0.125 mM.
† Less than 5% inhibition of OPRTase at the maximal inhibitor concentration of 5.0 mM.
‡ Less than 5% inhibition of OPRTase at the maximal inhibitor concentration of 2.5 mM.
§ Less than 5% inhibition of OPRTase at the maximal inhibitor concentration of 1.25 mM.

Table 6. Inhibition of orotidine 5'-monophosphate decarboxylase by pyrimidine base analogs

Compound*	% Inhibition	
	No PRPP, no preincubation	5 mM PRPP, preincubated†
5-Azabarbituric acid (XLVIII)	25	29
5-Azaorotic acid (L)	11	69
5-Azauracil (XVIII)	13	96
Barbituric acid (XXXIII)	13	88
6-Carboxymethyluracil (XXXV)	16	11
3-Deazauracil (XII)	14	23
4,6-Dihydroxypyrimidine (LIX)	18	27
5-Fluoroorotic acid (LXV)	28	26
5-Nitroorotic acid (LXIX)	25	9
2-Thiobarbituric acid (LXXII)	35	31
Uracil-6-methylsulfone (XLII)	16	19

* All compounds were tested as ODCase inhibitors at a concentration of 5 mM, except for 6-carboxymethyluracil and uracil-6-methylsulfone which were tested at a concentration of 2.5 mM because of poor solubility.
† Inhibitor, cytosol extract, and PRPP were preincubated for 30 min at 37°.



A B

Fig. 1. Tautomeric forms of uracil monoanion.

proton from an acidic exocyclic functional group of a pyrimidine analog may or may not remain localized to the functional group. In the case of orotic acid, the negative charge appears to be localized to the exocyclic carboxyl group because the pK_a values for the pyrimidine ring protons (i.e. *N*1 or *N*3) are identical to those of uracil [47]. Whereas the monoanions of other analogs, e.g. 5-azabarbituric acid [48], may exist with the negative charge partially dispersed over the pyrimidine ring system.

Binding of anionic uracil analogs to OPRTase. The present study indicates that the monoanionic species of uracils formed by the dissociation of either the *N*1 or *N*3 proton bind more tightly to OPRTase than the corresponding unionized compounds. The validity of this hypothesis may be illustrated by a comparison of the binding of uracil and 5-fluorouracil (XXIV). The fluorine atom of 5-fluorouracil (XXIV) resembles the 5-position hydrogen of uracil in both hydrophobic character [49] and van der Waals radius [50] and thus it is unlikely that a direct interaction of the fluorine atom of XXIV can affect binding significantly. However, the presence of the electron-withdrawing fluorine atom at this position considerably increases acidity of the ring system, as may be seen from pK_a values and per cent ionization under the conditions of the present assay system for these and other compounds given in Table 7. Thus, the 9-fold increase in binding of 5-fluorouracil (XXIV) as compared to uracil is attributable to the greater prevalence of the monoanion of XXIV in solution (i.e. 51 vs 3%). Likewise, the poor substrate activity of uracil, as compared to 5-fluorouracil, may be attributed to this phenomenon (see Tables 1 and 2). Indeed, the K_m of uracil (6.6 mM) is nearly 40-fold higher than that of 5-fluorouracil (0.13 mM), whereas the V_{max} values are not significantly different [15]. A similar argument may apply, at least in part, to the tight binding of certain pyrimidine base analogs having acidic ring systems listed in Table 7. These compounds (compound name, percent ionization at pH 8.0, apparent K_i) are: 5-azauracil (XVIII, 97%, 0.2 mM), 5-bromouracil (XX, 60%, 4.0 mM), 5-chlorouracil (XXII, 53%, 0.9 mM), 6-chlorouracil (XXXVII, ~100%, 0.4 mM), 5-nitrouracil (XXIX, ~100%, 0.9 mM) and uracil 6-methylsulfone (XLII, ~100%, 0.1 mM). Whether or not one of the two possible tautomers of these uracil monoanions (i.e. *N*1 or *N*3 proton dissociated) binds preferentially to OPRTase cannot be determined with any certainity.

The monoanionic species of uracils substituted with acidic groups (e.g. carboxyl) are formed by the dissociation of a proton from the functional group, and the resulting charge usually remains localized. Discussion of the effects of such substituents on binding to OPRTase is included in the following position-by-position analysis.

Substitution at the 1-position. No *N*1 substituted uracil included in this study bound better to OPRTase than uracil. Substitution of a methyl group at the 1-position of uracil or 5-fluorouracil (XXIV) yielded compounds, 1-methyluracil (VII) and 5-fluoro-1-methyluracil (LXIV), which did not bind to OPRTase. Likewise, 1-methylbarbituric acid (LXVII) bound 46-fold less tightly than barbituric acid (XXXIII). The larger functional groups of acyclouridine (II), 1-cyclohexyuracil (III), 1-cyclohexylthymine (IV), 1-ethyluracil (V), and ftorafur (VI) evidently prevented binding to OPRTase completely.

2,6-Pyridinediol (VIII), which exists in aqueous solution as 1-deazauracil and the mono-keto tautomer thereof in a 1:4 molar ratio [65], bound to OPRTase nearly 2-fold better than uracil. This finding may or may not be attributed to the substitution of the 1-position, however, because this compound theoretically may bind to the enzyme in more than one orientation (i.e. rotated 180° about the *C*2–*C*5 axis as "3-deaza-4-deoxybarbituric acid").

Substitution at the 2-position. Present findings indicate that the 2-position oxo group is required for the binding of uracil analogs to OPRTase. 2-Aminouracil (2-amino-4-hydroxypyrimidine, IX) bound much less tightly to OPRTase than uracil. The same was true for 2-thiouracil (XI), although the unique ionic structure of this compound (see §§ footnote to Table 7) makes it difficult to attribute this finding strictly to 2-position effects. Likewise, 4-hydroxypyrimidine (which exists in solution predominantly as the 4-position keto tautomer and thus is analogous to "2-deoxyuracil" [40–42]) did not bind to OPRTase.

Substitution at the 3-position. 3-Oxauracil (XIV) (a uracil analog in which an endocyclic oxygen atom replaces *N*3) bound nearly 11-fold better to OPRTase than uracil. Since 3-oxauracil does not have a 3-position proton, this finding suggests that an undissociated *N*3 proton is not required for the binding of uracils to OPRTase. On the other hand, replacement of the *N*3 proton of uracil with a methyl group, i.e. 3-methyluracil (XIII), abolished binding. Therefore, steric factors appear to preclude the binding of XIII.

It is of interest that 3-deazauracil (XII) bound nearly 17-fold better to OPRTase than uracil. This fact is consistent with the speculation that the proton at N3 of uracil analogs is not essential for binding. It must be stressed that 3-deazauracil is a pyridine derivative that undergoes keto-enol tautomerism at the 4-position [58] and is also highly acidic (pK_a 6.50) [53]. Neither the relative proportions of the monoketo and diketo tautomers nor the structure of the anion of 3-deazauracil have been reported, and thus it is difficult to draw conclusions about structure–activity relationship from this compound.

Substitution at the 4-position. Replacement of the

Table 7. Ionization of pyrimidine analogs

Compound*	pK_a	% Ionized at pH 8.0	Ref.
5-Aminoorotic acid (XLVII)	2.63, 8.72	16†	51
5-Azabarbituric acid (XLVIII)‡	4.75	100	48
5-Aza-1-methyluracil (XLIX)	8.15	41	52
5-Azaorotic acid (L)	6.7	95†	10
5-Azauracil (XVIII)	6.5	97	53
6-Azauracil (XXXII)	7.0	91	54
Barbituric acid (XXXIII)§	3.9	100	53
5-Bromouracil (XX)	7.83	60	51
5-Carboxyuracil (XXI)	4.16, 8.89	11†	51
5-Chlorouracil (XXII)	7.95	53	51
6-Chlorouracil (XXXVII)	5.67	100	51
3-Deazauracil (XII)‖	6.50	97	53
4,6-Dihydroxypyrimidine (LIX)¶	5.4	100	53
2,4-Dithiouracil (LXII)	6.35	98	51
5-Fluorouracil (XXIV)	7.98	51	51
2-Hydroxypyrimidine (XVII)	9.17	6	53
4-Hydroxypyrimidine (X)	8.59	20	53
5-Hydroxyuracil (XXVI)**	8.11	44	53
5-Iodoorotic acid (LXVI)	1.88, 7.63	70†	51
5-Iodouracil (XXVII)	8.25	36	51
5-Mercaptouracil (XXVIII)††	5.3	100	63
Methylorotate (XXXIX)	7.93	54	51
5-Nitrouracil (XXIX)	5.56	100	51
Orotic acid (XLI)	2.07, 9.45	3†	51
2-Thiobarbituric acid (LXXII)‡‡	3.7, 7.89	56	51
2-Thiouracil (XI)§§	7.65	69	51
Uracil (I)	9.50	3	51
Uracil-6-methylsulfone (XLII)	4.68	100	51

* Unless otherwise indicated, the anions of these compounds are formed by dissociation of either the *N*1 or *N*3 proton.

† The carboxyl group is completely ionized at pH 8.0; the percent ionization figure refers to the dianion formed by dissociation of either the N1 or N3 proton.

‡ The predominant species of 5-azabarbituric acid monoanion is apparently the diketo, monoenol tautomer in which the proton of the enolic hydroxyl group is dissociated [48]. It is uncertain whether or not the negative charge remains localized to this functional group.

§ The neutral species of barbituric acid has been clearly shown to be the 2,4,6-triketo tautomer [55]. The monoanion of barbituric acid is formed by loss of a proton from the 5-position methylene group with subsequent enolization involving the 6-position oxo group [55–57]. Localization of the negative charge occurs on the exocyclic 6-position oxygen [55, 56].

‖ The structure of the monoanion of 3-deazauracil is unknown. Theoretically this species may be formed either by loss of the *N*1 proton or a proton from the 4-position hydroxyl group of the *C*3-*C*4 enol tautomer [58].

¶ The predominant species of the monoanion of 4,6-dihydroxypyrimidine is most likely a symmetrical diketo structure formed by the loss of a proton from a ring nitrogen [59–61].

** No data on the ionic structure of 5-hydroxyuracil *per se* are available. However, by analogy to 2-methyl-4,5-dihydroxypyrimidine [62] it may be reasonably assumed that the monoanion is formed by loss of the proton of the 5-position hydroxyl group.

†† The anion of 5-mercaptouracil is formed by loss of the proton of the 5-position sulfhydryl group [63].

‡‡ The structure of the monoanionic or dianionic form of 2-thiobarbituric acid is presently unknown.

§§ The predominant species of the monoanion of 2-thiouracil is the 2-keto-4-enol tautomer in which the proton of the enolic hydroxyl group is dissociated [64]. The negative charge appears to be localized to the exocyclic 4-position oxygen [64].

4-position oxo group of uracil with either an amino group, i.e. cytosine (XV), or a methyl group, i.e. 2-hydroxy-4-methylpyrimidine (XVI), precluded binding to OPRTase. The same was true when the 4-position oxo group of thymine (XXX) was replaced by a thio group, i.e. 4-thiothymine (LXXIV).

2-Hydroxypyrimidine (XVII), which exists predominantly as the 2-keto tautomer in solution [40–42] and thus may be regarded as "4-deoxyuracil", bound weakly to OPRTase. A similar finding has been reported by Kessel *et al.* [11]. However, several orientations of binding are possible for 2-hydroxypyrimidine (i.e. analogous to uracil or rotated 180° about the *N*3-*C*6 axis as "2-deoxy-1-deaza-5-azauracil"), and thus it is difficult to limit discussion of this compound to effects at the 4-position.

Substitution at the 5-position. Despite the limited number of uracil analogs substituted at the 5-position that were available for study, the following observations can be made. Substitution of *C*5 of uracil with either a methyl group, i.e. thymine (XXX), or an amino group, i.e. 5-aminouracil (XIX), greatly diminished binding to OPRTase. Similar findings have been reported by Kessel *et al.* [11]. 5-Carboxyuracil (XXI), 5-hydroxyuracil (XXVI), and 5-mercaptouracil (XXVIII), which all have functional groups that are largely negatively charged (see Table 7), bind less tightly to this enzyme than does uracil. On the other hand, 5-hydroxymethyluracil (XXV) bound slightly better to OPRTase than uracil.

Uracils substituted with electron-withdrawing groups at the 5-position, i.e. 5-bromouracil (XX), 5-chlorouracil (XXII), 5-fluorouracil (XXIV), 5-iodouracil (XXVII), and 5-nitrouracil (XXIX), bind well to OPRTase. While the greater acidity of the pyrimidine ring system, relative to uracil, certainly enhances binding (see above), local substituent effects are also involved. For example, both 5-fluorouracil (XXIV) and 5-bromouracil (XX) are approximately 50% ionized under the present assay conditions (see Table 7), yet 5-bromouracil (XX) bound over 9-fold less tightly to the enzyme than 5-fluorouracil (XXIV). As mentioned above, the 5-position hydrogen of uracil and the fluorine atom of 5-fluorouracil are almost identical in terms of hydrophobic character [49] and van der Waals radius [50]. The decrease in binding of 5-bromouracil (XX) may be due to the similarity between a bromine atom and methyl group in both hydrophobic character [49] and van der Waals radius [50]. Since an iodine atom also resembles a methyl group in both hydrophobic character [49] and van der Waals radius [50], a similar argument may be applied to the poor binding of 5-iodouracil (XXVII) despite the acidity of this compound (see Table 7). Likewise, enhanced ring acidity and local 5-position effects appear to have cancelled out in the binding of 5-chlorouracil (XXII) and 5-nitrouracil (XXIX) (see Table 7).

5-Azauracil (XVIII), in which *C*5 of uracil is replaced with an endocyclic imino group, bound nearly 22-fold better to OPRTase than uracil. We attribute the tight binding of XVIII primarily to the high acidity of the ring system (see Table 7), because the structural differences (i.e. bond angles and bond lengths) between 5-azauracil and uracil are minimal [66]. It is possible, however, that other unknown factors are involved in the binding of 5-azauracil (XVIII), because this compound evidently is not a good substrate for OPRTase [10] despite the fact that it apparently binds well. The acidity of the ring system alone does not preclude substrate activity for 5-fluorouracil (XXIV) and 5-fluoroorotic acid (LXV) (see Table 1), and this is probably also true for 5-azauracil.

Substitution at the 6-position. The present study indicates preferential binding of uracils that are substituted at the 6-position with polar or negatively charged functional groups. The nonpolar 6-position methyl group of 6-methyluracil (XL) caused nearly a 3-fold decrease in binding relative to uracil. On the other hand, better than a 6-fold increase in binding occurred when the substituent was a polar amino group, i.e. 6-aminouracil (XXXI). Binding is further enhanced (i.e. better than 10-fold) by the negatively charged hydroxyl group of barbituric acid (XXXIII) and carboxyl group of orotic acid (XLI) (see Table 7).

Electron-withdrawing groups substituted at the 6-position of uracil enhance binding to OPRTase by virtue of increasing the acidity of the pyrimidine ring system. The tight binding of 6-chlorouracil (XXXVII) can be attributed to such an effect because this compound is essentially 100% ionized under the present assay conditions (Table 7). Likewise, the 4-fold increase in binding of 6-azauracil (XXXII) as compared to uracil is probably attributable to the greater acidity of this compound (see Table 7), because there are only minimal structural differences (i.e. bond lengths and bond angles) between these compounds [66]. However, 6-azauracil (XXXII) (91% ionized) bound much less tightly to OPRTase than 5-fluorouracil (XXIV) (51% ionized) and thus other factors must be involved in the binding of XXXII. It is interesting to note that monoanion of 6-azauracil is formed largely by the loss of a proton from *N*3 [67], in contrast to 5-fluorouracil (XXIV) in which the monoanion is formed predominantly (but not exclusively) by loss of a proton from *N*1 [44, 68]. Thus, the poorer binding of XXXII relative to XXIV suggests, although does not positively prove, the preferential binding of uracil monoanions formed by loss of a proton from *N*1 rather than *N*3.

An interesting substituent effect occurs in the case of methylorotate (orotic acid methyl ester, XXXIX). This compound is an analog of orotic acid in which the acidic 6-position carboxyl group is esterified. This modification causes a shift in the site of ionization from the 6-position functional group to the pyrimidine ring system and lowers the pK_a to 7.93 (see Table 7). It is noteworthy that methylorotate (XXXIX) bound nearly 2-fold better to OPRTase than orotic acid (XLI); however, it is uncertain whether this effect is primarily due to ring ionization or local 6-position effects of the esterified carboxyl group of XXXIX.

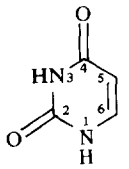
Substitution at more than one position. The present study indicates that the binding of multisubstituted uracils to OPRTase is, in many cases, an approximate summation of individual substituent interactions. For example, 5-aminobarbituric acid (XLIII) and 5-aminoorotic acid (XLVII) bound 20- to 100-fold less

tightly to OPRTase than barbituric acid (XXXIII) and orotic acid (XLI) respectively. These findings are consistent with the fact that the 5-position amino group of 5-aminouracil (XIX) abolishes binding to this enzyme. Likewise, uracil analogs substituted with a methyl group at the 1-position, i.e. 5-aza-1-methyluracil (XLIX), 5-bromo-1-methyluracil (LIV), 5-fluoro-1-methyluracil (LXIV) and 1-methylbarbituric acid (LXVII), either did not bind or bound much less tightly than the corresponding parent compounds 5-azauracil (XVIII), 5-bromouracil (XX), 5-fluorouracil (XXIV), and barbituric acid (XXXIII) respectively. These facts are consistent with the negative effect of substitution at the 1-position on binding. Summation of individual substituent effects may also explain the enhanced binding of 5-fluoroorotic acid (LXV) and 5-nitroorotic acid (LXIX) as compared to orotic acid (XLI).

Of all the multisubstituted uracil analogs studied here, strict additivity of substituent effects was most closely approximated with 4,6-dihydroxypyrimidine (LIX). The fact that 3-deazauracil (XII) and 5-azauracil (XVIII) were both good inhibitors of OPRTase led us to test 4,6-dihydroxypyrimidine (LIX) since it may be considered as "5-aza-3-deazauracil" when rotated 180° about the N1-C4 axis. This compound exists primarily as the diketo tautomer in solution [60, 61] and is also quite acidic (see Table 7). 4,6-Dihydroxypyrimidine (apparent $K_i = 15\ \mu M$) bound to OPRTase as tightly as 5-fluoroorotic acid (LXV) (apparent $K_i = 16\ \mu M$), the most potent inhibitor of this enzyme previously known (see Table 3). The 295-fold increase in binding of 4,6-dihydroxypyrimidine (LIX) relative to uracil represents almost the theoretical limit of an additive effect ($17 \times 22 = 374$-fold) of the 17-fold increase in binding of 3-deazauracil (XII) and the 22-fold increase in binding of 5-azauracil (XVIII).

Design of new orotate phosphoribosyltransferase inhibitors. A structure–activity relationship for ligands of OPRTase is summarized in Fig. 2. Based on these findings and the assumption that multisubstitution of uracil with the appropriate functional groups will enhance binding in an additive manner, we propose that 2-carboxy-4,6-dihydroxypyrimidine (i.e. "5-aza-3-deazaorotate") and its methyl ester, 3-oxaorotic acid and its methyl ester, 3-oxabarbituric acid, 5-fluoroorotic acid methyl ester, 3-deazabarbituric acid (2,4,6-pyridinetriol), 3-deazaorotic acid (4,6-dihydroxypicolinic acid) and its methyl ester, 5-fluorobarbituric acid, and 5-nitrobarbituric acid are worthwhile to synthesize and evaluate as inhibitors of OPRTase. It is interesting to note that Chelbova *et al.* [39] have shown that one of these compounds, 3-deazaorotic acid, inhibits the incorporation of orotic acid into uridine necleotides by a soluble rat liver enzyme system, although these authors did not study the effects of this compound on the activity of OPRTase *per se*.

Orotidine 5'-monophosphate decarboxylase inhibition studies. Although we had no reason to suspect that pyrimidine base analogs that bind well to OPRTase might also bind well to ODCase, we thought that some of these compounds might be OPRTase substrates and are converted to nucleoside 5'-monophosphate derivatives which then inhibit ODCase. This appears to be the case with 5-azaorotic acid (L), 5-azauracil (XVIII), and barbituric acid (XXXIII) which produced significant ODCase inhibition *only* after preincubation with PRPP, $MgCl_2$ and cytosol extract (Table 6). Similar findings have been reported by others for 5-azaorotate [10] and barbituric acid [13]. Consistent with this speculation are the findings that 5-azaorotidine 5'-monophosphate has been shown to be synthesized from 5-azaorotic acid by OPRTase [25] and then decarboxylated by ODCase [25], and that barbituric acid riboside 5'-monophosphate is known to be a very potent inhibitor of ODCase [13, 69]. Although no nucleoside 5'-monophosphate formation has been detected with 5-azauracil [10] or [2-^{14}C]barbituric acid*, it is possible that minute quantities of 5-azauridine 5'-monophosphate and barbituric acid riboside 5'-monophosphate were synthesized and then "channeled" to the ODCase active site as has been proposed to occur with OMP [12]. Because many other inhibitors of pyrimidine *de novo* biosynthesis are highly toxic [9], the finding that barbituric acid is not toxic to mammalian cells [70] is incongruous with the potent inhibitory effects this compound has on OPRTase and ODCase. As proposed by Potvin *et al.* [13], it is possible that barbituric acid may not enter cells readily. Clearly, further study is required to explain the non-toxicity of barbituric acid.



POSITION	SUBSTITUENT EFFECT
N1	Substitution generally diminishes binding
C2	2-Position oxo group required
N3	Replacement of N3 with an endocyclic oxygen enhances binding 11-fold
	Replacement of N3 with a methylene group enhances binding 17-fold
	Substitution with a methyl group abolishes binding
C4	4-Position oxo group required
C5	Electron-withdrawing functional groups (i.e. fluoro, chloro, nitro) enhance binding by increasing the acidity of the pyrimidine ring system
	Replacement of C5 with an endocyclic imino group enhances binding 22-fold
C6	Substitution with a carboxyl group enhances binding 92-fold
	Substitution with the methyl ester of a carboxyl group enhances binding 164-fold
	Substitution with a hydroxyl group (acidic) enhances binding 193-fold
	Substitution with a methylsulfone group enhances binding 32-fold

Fig. 2. Structure–activity relationship for the binding of pyrimidine base analogs to orotate phosphoribosyltransferase.

* J. G. Niedzwicki, M. H. el Kouni and S. Cha, unpublished observations.

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