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Irreversible Enzyme Inhibitors. LXXV.[1,2] Inhibitors of Thymidine Phosphorylase. I. Mode of Ribofuranose Binding

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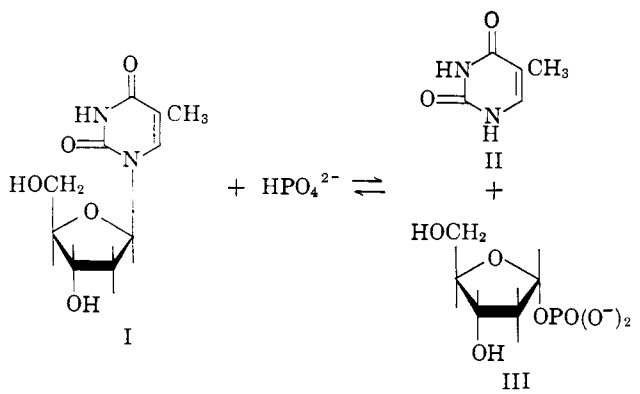
5′-Deoxythymidine (V) was about as good a substrate for thymidine phosphorylase as was thymidine (I); both had about the same apparent K_m. In contrast, 3′-deoxythymidine (IV) was not a substrate and a 20-fold loss in binding, compared to thymidine, was noted; thus the 5′-hydroxyl of thymidine is not necessary for binding, but the 3′-hydroxyl is necessary. Replacement of the furanose oxygen of thymidine (I) with methylene (VI) gave a greater than tenfold loss in binding, but this result could not necessarily be attributed to binding by the furanose oxygen. When the 5′-hydroxyl of thymidine (I) was acylated with the highly polar carbamoyl group (VIII), a 40-fold loss in binding occurred; with less polar but bulkier acyl groups such as dimethylcarbamoyl (IX) or carbophenoxy, only a six- and twofold loss in binding occurred, respectively. The possible biological significance of repulsion of polar groups at 5′ position of thymidine is discussed; also discussed are further modifications of thymidine that could lead to more specific inhibitors of thymidine phosphorylase from different species and tissues.

Thymidine phosphorylase is an enzyme that catalyzes phosphorolysis of the nucleosidic linkage of pyrimidine 2′-deoxynucleosides, such as thymidine (I), with formation of 2-deoxy-α-D-ribofuranose 1-phosphate (III) and the pyrimidine (II). This enzyme has been isolated from a variety of plant, animal, and bacterial sources. Although its main function appears to be catabolic, some bacteria and tumors can utilize the reverse reaction anabolically under stress of certain dietary or genetic deficiencies. A particularly good source for the enzyme is *Escherichia coli* B since thymidine phosphorylase is readily separable from uridine phosphorylase.[3] In contrast, from some mammalian sources these two enzymes are not separable and may be one and the same enzyme;[4,5] however, separation of these two enzymes from some mammalian sources has been achieved.[6]



I

There are two main chemotherapeutic interests in thymidine phosphorylase, namely, (a) its ability to convert 5-fluorouracil (FU)[7] to its 2′-deoxy-β-D-ribofuranoside (FUDR),[7] the first step in the intracellular activation of FU to FUDRP[7] *via* thymidine phosphorylase[8,9] and thymidine kinase,[8,10,11] and (b) the detoxification of preformed FUDR[7] by cleavage to FU,[9] then further catabolism to α-fluoro-β-alanine by other enzymes.[12]

Since some tumors fail to detoxify FU or FUDR due to the genetic deletion[13] of these enzymes,[9,12] it has been proposed[14] that the selective action of FU on certain tumor lines is due to the lack of one of the detoxification enzymes in a susceptible cell line, and its presence in less susceptible normal tissues. If an inhibitor of thymidine phosphorylase could be designed such that it would inhibit phosphorolysis of FUDR in a tumor cell line with little inhibition of the phosphorylase in normal tissues, then such an inhibitor would be a useful

(1) This work was generously supported by grants CA-05845, CA-05867, and CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series, see B. R. Baker and D. V. Santi, *J. Med. Chem.*, **10**, 62 (1967).

(3) W. E. Razzell and H. G. Khorana, *Biochim. Biophys. Acta*, **28**, 562 (1958).

(4) T. A. Krenitsky, J. W. Mellors, and R. K. Barclay, *J. Biol. Chem.*, **240**, 1281 (1965).

(5) H. Pontis, G. Degerstedt, and P. Reichard, *Biochim. Biophys. Acta*, **51**, 138 (1961).

(6) M. Friedkin and D. Roberts, *J. Biol. Chem.*, **207**, 245 (1954).

(7) Abbreviations used: FU = 5-fluorouracil, FUDR = 5-fluoro-2′-deoxyuridine, and FUDRP = 5-fluoro-2′-deoxy-5′-uridylate.

(8) S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1004 (1958).

(9) G. D. Birnie, H. Kroeger, and C. Heidelberger, *Biochemistry*, **2**, 566 (1963).

(10) R. Okazaki and A. Kornberg, *J. Biol. Chem.*, **239**, 269, 275 (1964).

(11) K.-U. Hartmann and C. Heidelberger, *ibid.*, **236**, 3006 (1961).

(12) E. Harbers, N. K. Chaudhuri, and C. Heidelberger, *ibid.*, **234**, 1255 (1959).

(13) V. P. Potter, *Univ. Mich. Med. Bull.*, **23**, 401 (1957).

(14) B. R. Baker, *Cancer Chemotherapy Rept.*, **4**, 1 (1959).

adjunct to FUDR treatment of a tumor cell line which has thymidine phosphorylase present. Reversible inhibitors rarely show tissue or species specificity unless the inhibitor can also bind to the enzyme in a nonfunctional area as well as the active site; for example, a nonfunctional hydrophobic bonding area of dihydrofolic reductase has been utilized for species specificity;[15-17] however, the tissue differences in the hydrophobic bonding region of dihydrofolic reductase may be too small to exploit for cancer chemotherapy with reversible inhibitors.[15]

In contrast, active-site-directed irreversible enzyme inhibitors can greatly magnify small differences in isozymes, being sufficiently selective to inactivate one lactic dehydrogenase isozyme but not another;[18,19] these irreversible inhibitors operate by initial formation of a reversible complex, followed by a facile neighboring group reaction within the enzyme–inhibitor reversible complex that leads to selective inactivation.[18-21] Therefore studies on the design of active-site-directed irreversible inhibitors of thymidine phosphorylase have been initiated. To design such an inhibitor for a relatively unexplored enzyme, a definite *modus operandi* has been developed, namely, (a) the binding points on the inhibitors to the enzyme should be determined, (b) an area of the inhibitor not in contact with the enzyme in the complex should be found, so-called bulk tolerance, and (c) the placement of a covalent forming group such as bromoacetamido on the noncontact area of the inhibitor which could react with the enzyme within the complex. In this and the accompanying papers, facets a and b of the *modus operandi* for thymidine phosphorylase have been explored. A similar exploration of another enzyme utilizing thymidine as a substrate, namely, thymidine kinase, has been reported from this laboratory;[22] therefore, these same candidate reversible inhibitors could be used for initial exploration of thymidine phosphorylase.

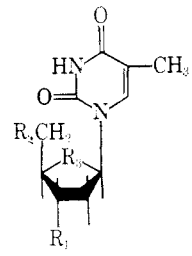
Since thymidine phosphorylase is reversible, it can be assayed in either direction. Anabolically, either inorganic phosphate[5] or the disappearance of the pyrimidine base measured spectrophotometrically[23] can be determined. Catabolically, the rate of formation of pyrimidine, measured spectrophotometrically,[6] or the rate of liberation of the sugar, measured with thiobarbituric acid,[5] can be determined. Since the reaction is reversible, an equilibrium is reached that decreases the amount of reaction that is linear with time. Arsenolysis of thymidine[6] neatly evaded this difficulty since the resultant 2-deoxy-D-ribofuranose 1-arsenate hydrolyzes far more rapidly in water than it back reacts to re-form thymidine. Although the reaction can go to completion and is linear through a greater amount of the reaction, it does eventually become nonlinear due to inhibition by the product, thymine.

The effect of inhibitors on thymidine phosphorylase varies with the source of the enzyme[4] much more than is commonly observed;[20] therefore, the data to be presented here on the *E. coli* B enzyme would probably not carry over completely to inhibitors of this enzyme from other sources such as mammalian sources. For example, 1-(2-deoxy-β-D-glucopyranosyl)thymine (XXI) inhibits the enzyme from mouse tissue, but not the enzyme from horse liver.[4]

With the *E. coli* B enzyme, 1-(β-D-xylofuranosyl)-thymine,[24] 1-(β-D-arabinofuranosyl)uracil,[25] 3′-deoxythymidine,[24] and uridine[3] were not substrates; therefore, the enzyme from this source will not tolerate a 2′-hydroxyl of either configuration and apparently needs a 3′-hydroxyl for substrate properties; that the β configuration is needed for either substrate or inhibitor properties is shown by the lack of effect of the α anomer of thymidine.[24]

In Table I is listed a group of compounds that give an estimate of the relative contribution of binding by the oxygen functions of the 2′-deoxy-D-ribose moiety of thymidine to the phosphorylase; it was noted that thymidine and FUDR bind about equally as substrates; their apparent K_m's differing by only a factor of about 1.5.

TABLE I
MODE OF RIBOFURANOSE BINDING TO THYMIDINE PHOSPHORYLASE[a] BY



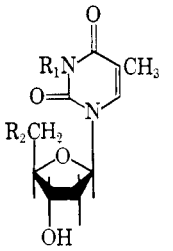
Compd	R_1	R_2	R_3	mM concn	% inhib	Estd[b] $([I],/[S])_{0.5}$	Substrate velocity[c]	K_m × 10^4 M[d]
I	OH	OH	O	0.4		1.5[e]	0.6	13
IV[f]	H	OH	O	6	33	30	0	
V[f]	OH	H	O	0.4		2.0[e]	0.4	17
VI[g]	OH	OH	CH_2	1.5	0	>15[h]		

[a] Thymidine phosphorylase from *E. coli* B was prepared and assayed with 0.4 m*M* FUDR[7] as described in the Experimental Section; FUDR[7] had $K_m = 8 \times 10^{-4}$ *M* (determined by reciprocal plot). [b] Ratio of concentration of inhibitor to 0.4 m*M* FUDR[7] giving 50% inhibition. [c] Relative to 0.4 m*M* FUDR. [d] Determined by the reciprocal plot method. [e] Ratio of K_m of compound to K_m of FUDR. [f] See ref 22a for preparation. [g] We wish to thank Dr. R. B. Angier of Lederle Laboratories for a sample of this compound: K. C. Murdock and R. B. Angier, *J. Am. Chem. Soc.*, **84**, 3758 (1962). [h] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is greater than four times the concentration measured.

Since 5′-deoxythymidine (V) is nearly as good a substrate as thymidine (I) and has about the same apparent K_m, the 5′-hydroxyl group of thymidine is unnecessary for binding or substrate properties. In contrast, the 3′-hydroxyl group is necessary for both substrate and binding properties; note that 3′-deoxythymidine (IV) is not a substrate and requires a 30-fold higher concentration than the substrate, FUDR, for

(15) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **55**, 470 (1966).
(16) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965).
(17) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **53**, 1137 (1964).
(18) B. R. Baker and R. P. Patel, *ibid.*, **53**, 714 (1964).
(19) B. R. Baker, *Biochem. Pharmacol.*, **12**, 293 (1963).
(20) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.
(21) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).
(22) (a) B. R. Baker, T. J. Schwan, and D. V. Santi, *J. Med. Chem.*, **9**, 66 (1966); (b) B. R. Baker and T. J. Schwan, *ibid.*, **9**, 73 (1966).
(23) M. Friedkin and D. Roberts, *J. Biol. Chem.*, **207**, 257 (1954).
(24) W. E. Razzell and P. Casshyap, *ibid.*, **239**, 1789 (1964).
(25) H. Tono and S. S. Cohen, *ibid.*, **237**, 1271 (1962).

TABLE II
INHIBITION OF THYMIDINE PHOSPHORYLASE[a] BY



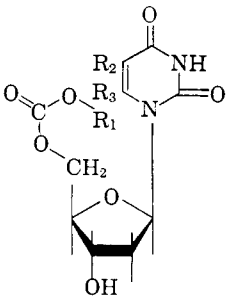
Compd[b]	R_1	R_2	mM conen	% inhib	Estd[c] $([I]/[S])_{0.5}$	Compd/ thymidine[d]
I	OH	OH	0.4	Substrate	1.5[e]	
V	H	H	0.4	Substrate	2.0	1.3
VII	n-C_5H_{11}	OH	9[f]	36	64	43
VIII[g]	H	NH_2CO_2	4[f]	14	60	40
IX	H	Me_2NCO_2	3.5	50	8.7	5.8
X	H	$C_2H_5OCO_2$	2.0	50	5.0	3.3
XI[g]	H	$C_6H_5OCO_2$	1.1	50	2.7	1.8

[a] The thymidine phosphorylase from *E. coli* B was prepared and assayed with 0.4 m*M* FUDR[7] as described in the Experimental Section. [b] See ref 22a for synthesis of these compounds unless otherwise indicated. [c] Ratio of concentration of inhibitor to 0.4 m*M* FUDR estimated to give 50% inhibition. [d] Ratio of concentration of compound to thymidine giving equal binding to the enzyme. [e] Ratio of K_m of compound to the K_m of FUDR. [f] No substrate properties detectable at 0.4 m*M*. [g] For synthesis see B. R. Baker, P. M. Tanna, and G. D. F. Jackson, *J. Pharm. Sci.*, **54**, 987 (1965).

50% inhibition. Replacement of the furanose oxygen by methylene results in a compound (VI) that shows no inhibition at a relatively high concentration, that is, a greater than tenfold loss in binding occurs compared to thymidine. This result is similar to that observed with VI as an inhibitor of thymidine kinase[22a] and can be interpreted as either (a) the ground-state conformation of the five-membered rings I and VI are different, (b) the furanose oxygen is necessary for binding, or (c) the enzyme cannot tolerate the larger CH_2 group in place of –O–, or a combination of these factors.

The 3 and 5′ positions of thymidine (I) were then studied for bulk tolerance within the inhibitor–thymidine phosphorylase complex; the results are presented in Table II. Introduction of the *n*-amyl group on the 3 position (VII) of thymidine gave a 43-fold loss in binding compared to thymidine. This result could be due to either the loss of the 3-hydrogen as a binding point to the enzyme or a lack of bulk tolerance at this position within the enzyme–inhibitor complex, or both; in either case, the loss in binding is too great to warrant further investigation at the 3 position for design of an active-site-directed irreversible inhibitor.[20,21] It had been previously reported that 3-methylthymidine at a concentration equal to thymidine was neither a substrate nor an inhibitor.[24]

The 5′-carbamate (VIII) of thymidine suffered a 40-fold loss in binding compared to thymidine (Table II). That this loss in binding was not primarily due to a lack of bulk tolerance was indicated by the sevenfold better binding of the 5′-dimethylcarbamate (IX) than the 5′-carbamate (VIII). Since the dimethylcarbamate is bulkier than the carbamate, but the former is much less polar, the solvated carbamate could well be repulsed from a hydrophobic area on the enzyme. This suggestion was further substantiated by the even better binding of the less polar 5′-carbethoxy (X) and 5′-carbophenoxy (XI) derivatives of thymidine; XI was complexed about half as well as thymidine. The presence and nature of a hydrophobic region on thymidine phosphorylase that can be complexed by alkyl of aralkyl groups at the 1,[26] 5,[27,28] or 6 position[27,28] or uracil will be presented in the accompanying papers. If these 5′-carbonates were complexed to the enzyme in a conformation such as XII, it is possible that the R_1 group of the 5′-carbonate is



XII

actually complexed hydrophobically to the region on the enzyme that can complex alkyl or aralkyl groups at the R_2 and R_3 positions. Further studies on the possible hydrophobic bonding of the R_1 group is warranted; furthermore, additional studies on candidate active-site-directed irreversible inhibitors[20,21] of thymidine phosphorylase by appropriate substitution of a leaving group on R_1 would be worthwhile.

If such a hydrophobic region were further verified to be near R_1, it could have a biological role; note that thymidylate is not a substrate or inhibitor of this phosphorylase.[24] Therefore, this enzyme could have evolved with a hydrophobic region to repulse the 5′-phosphate group of thymidylate, as noted with the 5′-carbamate (VIII), in order to gain the needed substrate specificity.

To determine possible simulation of the ribofuranose

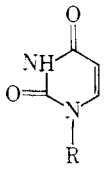
(26) B. R. Baker and M. Kawazu, *J. Med. Chem.*, **10**, 302 (1967); paper LXXVI of this series.

(27) B. R. Baker, M. Kawazu, D. V. Santi, and T. J. Schwan, *ibid.*, **10**, 304 (1967); paper LXXVII of this series.

(28) B. R. Baker and M. Kawazu, *ibid.*, **10**, 311 (1967); paper LXXVIII of this series.

group, a series of 1-(ω-hydroxyalkyl)uracils were investigated as inhibitors of thymidine phosphorylase; the results are presented in Table III. These compounds should be compared with 1-methyluracil (XIII) as a base line; XIII was complexed 200-fold less effectively than FUDR. The four hydroxyalkyluracils (XIV–XVII) were complexed 2–3-fold better than 1-methyluracil, but 60–80-fold less effectively than FUDR. That this 2–3-fold increment is due to hydroxyl binding to the enzyme is highly improbable since (a) only the 3′-hydroxyl and possibly the furanose oxygen of thymidine are complexed to the enzyme (Table I), and (b) it is unlikely that all four hydroxyalkyluracils could complex to one or the other binding point due to the energetically unfavorable conformational changes from their ground-state conformations that would have to take place. A more logical explanation arises from a comparison of 1-methyl- (XIII) and 1-butyluracil (XX); the latter is a ninefold better inhibitor of the enzyme than 1-methyluracil (XIII), a result that can only be accounted for by hydrophobic bonding and the accompanying van der Waals forces.[20] Furthermore, the amount of hydrophobic binding is not increased by higher alkyl groups.[26] Therefore, it is quite plausible that the 2–3-fold additional bonding by the four hydroxyalkyluracils (XIV–XVII) is due to hydrophobic bonding by their 2′-methylene group.

Table III
Inhibition of Thymidine Phosphorylase[a] by

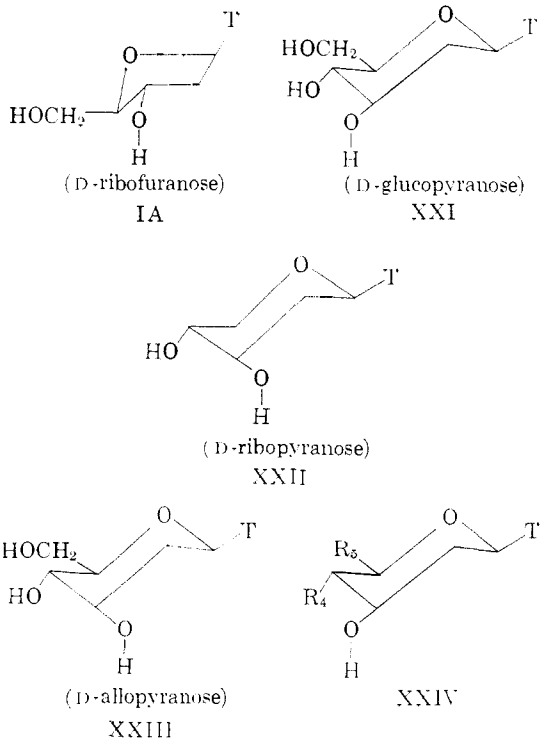


Compd[b]	R	mM concn	% inhib	Estd[c] ([I]/[S])$_{0.5}$
XIII	CH_3	50	50	200
XIV	$(CH_2)_2OH$	12	30	70
XV	$(CH_2)_3OH$	12	24	80
XVI	$(CH_2)_4OH$	12	33	60
XVII	$(CH_2)_5OH$	12	30	70
XVIII	$(CH_2)_4OCH_3$	6	0	>60[d]
XIX	$(CH_2)_2CHOH(CH_2)_2OH$	5.4	0	>54
XX	n-C_4H_9	7.5	50	22

[a] Thymidine phosphorylase from *E. coli* B was prepared and assayed with 0.4 m*M* FUDR[7] as described in the Experimental Section. [b] See ref 22b for synthesis of these compounds. [c] Ratio of concentration of inhibitor to 0.4 m*M* FUDR estimated to give 50% inhibition. [d] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is at least four times greater than the concentration measured.

Pertinent to the binding of the 2′-deoxy-D-ribofuranosyl moiety of thymidine (I) (Table I) are the inhibition results with some nucleoside analogs (XXI–XXIII); of these three analogs, only the 2′-deoxy-D-glucopyranosyl analog (XXI) showed inhibition of the thymidine phosphorylase from Dunning hepatoma.[29] It was previously proposed[22a] that thymidine had conformation IA when complexed to thymidine kinase (Chart I). The most stable conformation for the glucosylthymine would appear to be XXI. This conformation of XXI places its 3′-hydroxyl in about the same position as the 3′-hydroxyl of thymidine (IA) with respect to the thymine moiety, thus allowing for similar binding to thymidine phosphorylase. That XXI is not a substrate could be due to the fact that a furanosyl derivative hydrolyzes about 50–200 times as fast as the corresponding pyranosyl derivative[30] or that when XXI is complexed to the enzyme, the catalytic site is not properly aligned for cleavage to occur.

Chart I
Conformation of Thymine Nucleosides (T = 1-Thyminyl)



The lack of inhibition by the 3′-epimer (XXIII) of the glucosyl nucleoside (XXI) substantiates that the 3′-hydroxyl of XXI is involved in binding to the enzyme. Similarly, the 2′-deoxy-D-ribopyranosyl analog (XXII), which has its 3′-hydroxyl group in an axial configuration rather than the equatorial configuration of IA and XXI, does not inhibit at a concentration equal to substrate. Therefore, a general structure for inhibition of thymidine phosphorylase could be XXIV, where the 3′-hydroxyl and the pyrimidine are equatorial. Since R_4 and R_5 are most probably not involved in binding (Table I), R_4 and R_5 could be H, CH_3, $HOCH_2$, or other substituents. The species difference noted between the enzyme from horse liver and mouse tissue[4] may well be due to a difference in bulk tolerance for the R_5 group, since such differences in bulk tolerance between two enzymes[31] or two isozymes[15,16] have been

(29) (a) W. W. Zorbach, H. R. Munson, and K. V. Rhat, *J. Org. Chem.*, **30**, 3955 (1965); (b) M. Zimmerman, *Biochem. Biophys. Res. Commun.*, **16**, 600 (1964).

(30) W. N. Haworth, *Ber.*, **65A**, 43 (1932).

(31) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

previously noted.[20] Such comparative studies on thymidine phosphorylase from different species or tissues would be of considerable interest with thymine nucleosides derived from 2,6-dideoxy-D-glucopyranose, 2-deoxy-D-xylopyranose, and 6′ derivatives of XXIV for use as an adjunct to chemotherapy with FUDR.

Experimental Section[32]

Thymidine Phosphorylase.—*E. coli* B cells were broken in a French pressure cell at 1400 kg/cm². The 45–90% $(NH_4)_2SO_4$ fraction used for thymidylate synthetase[22a,33] also contained thymidine phosphorylase.[3] The final volume from 40 g of frozen cells was 47 ml.

Solutions. The buffer employed[23] was 0.2 *M* in succinate and arsenate adjusted to pH 5.9. A 4 m*M* solution of FUDR[7,34] in water was stable indefinitely and was used as the substrate.[9] Dimethyl sulfoxide (DMSO) was a reagent grade purchased from J. T. Baker Chemical Co.

Enzyme Standardization.—An assay mix was prepared with 1.50 ml of buffer, 75 μl of enzyme solution, 0.30 ml of DMSO, and 0.83 ml of water; total volume = 2.70 ml. If more or less enzyme solution was employed, the water was adjusted accordingly to give the same total volume.

In each of five test tubes was placed 0.45 ml of assay mix. To tube 5 was added 300 μl of 1 *M* aqueous KOH; this tube served as the zero-time tube. To each of the tubes was added 50 μl of 4 m*M* FUDR[7] at 30-sec intervals, noting the starting time. The optical density of tube 5 was then read in a 1-ml cuvette on a Beckman DU spectrophotometer.

At the end of 5 min, 300 μl of 1 *M* KOH was added to tube 1, then the optical density was read in the *same* cuvette used for tube 5. At 10, 20, and 30 min from zero time, tubes 2–4 were treated with 300 μl of 1 *M* KOH and the optical density was read in each case with the original cuvette.

The optical density reading of the zero-time tube 5 was subtracted from the readings from tubes 1–4. The change in optical density was then plotted against time; the reaction was linear through 0.5 optical density unit change and was complete at 0.6 unit. An enzyme concentration and time were selected to give 0.3–0.5 optical density unit change in 15–20 min. For example, 75 μl of enzyme in the assay mix usually gives about 0.4 optical density unit change in 20 min. The incubation concentration of FUDR was 0.4 m*M* and DMSO was 10%.

Inhibitors and Other Substrates.—Solutions of the inhibitors in DMSO or in water were employed at ten times the concentration needed in the assay; other substrates such as I and V were dissolved in water. If the substrate or inhibitor was dissolved in water, then DMSO was added to the assay mix (as in the standardization) in such an amount that the incubation concentration was 10%.

Inhibition of Thymidine Phosphorylase.—An assay mix of 3.00 ml of buffer, 0.15 ml of enzyme solution, and 1.65 ml of water was prepared; if the substrate or inhibitor was dissolved in water then the assay mix contained 0.60 ml of DMSO and 1.05 ml of water. Five pairs of tubes were placed in a rack so that the back tubes could serve as zero-time tubes. In each tube of the pairs 1 and 5 were placed 50 μl of DMSO and in pairs 2–4 were placed 50 μl of DMSO containing varying concentrations of inhibitors. The biggest error in the assay could occur if the DMSO solution was not placed near the bottom of the tube: if any droplets were above the half-way point of the tube, the tube was replaced.

In each of the ten tubes was placed 400 μl of assay mix and the contents were then mixed with a Vibro Jr. Mixer[35] after the addition to each tube. In each of the back zero-time tubes 1–5 was placed 300 μl of 1 *M* KOH, then the contents were mixed. To the five front tubes were added 50 μl of 4 m*M* FUDR at 30-sec intervals, the starting time being noted; care must be taken to get no droplets on the upper half of the tube and the contents of each tube were mixed after each addition. Then 50 μl of 4 m*M* FUDR was added to each back (zero-time) tube; when the additions were complete, the tube contents were mixed.

Assay.—Five 1-ml cuvettes were placed in front of each pair of tubes. The optical density of the back tubes was then read in each of the cuvettes, the cuvettes being rinsed, dried, and replaced at the respective position in front of each pair of tubes.

At 15 min from zero time, 300 μl of 3 *M* KOH was added to the front tubes 1–5 at 30-sec intervals, the contents being mixed after each addition. The optical density of the front tubes was then read in the respective cuvettes. The optical density change (front minus back reading) was noted for each pair of tubes.

Tubes 1 and 5 were enzyme controls, that is, the velocity without inhibitor, V_0; these V_0 values usually agreed within 3% and, if the difference was greater than 6%, the experiment was rejected. Tubes 2–4 represented the rates, V_I, in the presence of inhibitor. The inhibitor concentration, [I], was plotted against V_0/V_I, using the average V_0; a sufficient number of concentrations and sets were run to give sufficient V_0/V_I values between 1.3 and 2.5 to draw a reasonable line. Where $V_0/V_I = 2$, then [I] = the concentration for 50% inhibition.[31]

For determination of apparent K_m values for substrates, six pairs of tubes were employed so that three different concentrations of substrate could be employed in duplicate; the K_m was determined by the reciprocal plot method.

In some cases,[25–28] the concentration for 50% inhibition could not be reached due to insolubility; the maximum solubility was then checked by 1:10 dilution of a DMSO solution of inhibitor with water. Three to six duplicates at the highest attainable concentration were then run. In other cases,[25–28] the optical density of the blank or reaction was above 3.0 and could not be read; in these cases 50 μl of DMSO solution of inhibitor was diluted with 0.45 ml of water and 0.30 ml of 1 *M* KOH, and the optical density was read; the inhibitor solution was diluted until a concentration was found giving a reading no more than 2.3 optical density units.

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(33) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964).

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