Articles

Synthesis and Biological Activity of 5-Fluoro-2′,3′-dideoxy-3′-fluorouridine and Its 5′-Phosphate

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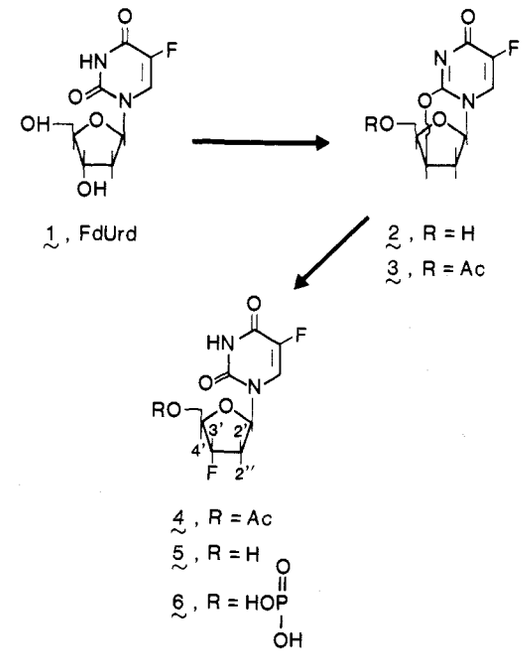
5-Fluoro-2′,3′-dideoxy-3′-fluorouridine (3′-FFdUrd) and 5-fluoro-2′,3′-dideoxy-3′-fluorouridine 5′-phosphate (3′-FFdUMP) have been synthesized, and their interactions with thymidine (dThd) phosphorylase and thymidylate (dTMP) synthetase, respectively, have been examined. 3′-FFdUrd is not a substrate for dThd phosphorylase, but is a weak, noncompetitive inhibitor (K_i = 1.7 mM). 3′-FFdUMP inhibits dTMP synthetase competitively with deoxyuridylate (K_i = 0.13 mM) when both the substrate and inhibitor are present simultaneously. However, in the presence of 5,10-methylenetetrahydrofolate, the inhibition increases with time in a first-order manner (k_{on}^{obsd} = 0.029 s^{-1}). A complex is formed between [6-^{3}H]3′-FFdUMP and dTMP synthetase, which is isolable on nitrocellulose filters, and has a dissociation rate (k_{off}^{obsd} = 1.4 × 10^{-2} min^{-1}) similar to that of the potent inhibitor 5-fluoro-2′-deoxyuridylate (k_{off}^{obsd} = 1.3 × 10^{-2} min^{-1}) from its ternary complex with dTMP synthetase. These results are explained in terms of a two-stage model involving the initial formation of a reversible adsorption complex, followed by a slow conversion to a tight-binding catalytic complex.

The deoxyribonucleoside antimetabolite FdUrd[1] is a very potent cytotoxic agent in cell culture systems,[2] but when used in vivo, it suffers substantial degradation by dThd phosphorylase, a ubiquitous salvage enzyme, to the pyrimidine base FUra and deoxyribose 1-phosphate before the drug reaches the target tissue.[2] The cleavage product FUra is itself a cytotoxic agent, but much less potent on a molar basis than FdUrd; also, FUra has a different mechanism of action. In addition to inhibiting DNA synthesis, FUra also becomes extensively incorporated into RNA with concomitant effects on the functioning of some species of RNA.[3] In contrast, the cytotoxic action of FdUrd is quite DNA specific; intracellularly it rapidly becomes converted by dThd kinase to the deoxyribonucleotide form, FdUMP, which in the presence of 5,10-CH_2H_4folate rapidly forms a tight-binding covalent complex with dTMP synthetase.[4] It is likely, therefore, that the antitumor effect of FdUrd in patients would be substantially different from that of FUra, provided that the cleavage of the deoxyribonucleoside could be prevented.

This problem could be approached in two ways: (1) inhibition of dThd phosphorylase or (2) modification of the structure of the nucleoside in such a way that the analogue fulfills the same biochemical function as the parent drug, but is not cleaved as readily by the phosphorylase. The first approach has more general applicability and, in fact, has been explored extensively in the past,[5] but no inhibitors of dThd phosphorylase have emerged as yet that have been considered effective enough to be used in combination chemotherapy with FdUrd. We are examining the feasibility of the second approach with analogues of FdUrd substituted with fluorine atoms in various parts of the deoxyribose moiety. In addition to a substantially reduced substrate interaction with dThd phosphorylase, chemotherapeutically effective FdUrd derivatives would have to be good substrates for phosphorylation by intracellular kinases and also strongly inhibit dTMP synthetase via the resulting deoxyribonucleotides.

Scheme I



1, FdUrd

2, R = H

3, R = Ac

4, R = Ac

5, R = H

6, R = HOP(O)OH

In this paper, we describe the synthesis of 3′-FFdUrd and its deoxyribonucleotide, 3′-FFdUMP, and their interactions with dThd phosphorylase and dTMP synthetase, respectively.

Chemistry. 3′-FFdUrd was synthesized as shown in Scheme I. FdUrd (1) was used as the starting material for the synthesis. The method of Fox and Miller[6] was followed to synthesize 2. Treatment of 2 with acetic anhydride in pyridine gave 3. By the procedure of Kowollik and Langen,[7] 3 was heated with hydrogen fluoride in the

(1) Abbreviations used are: FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine 5′-phosphate; dThd, thymidine; dTMP, thymidylate acid; 3′-FFdUrd, 5-fluoro-2′,3′-dideoxy-3′-fluorouridine; 3′-FFdUMP, 5-fluoro-2′,3′-dideoxy-3′-fluorouridine 5′-phosphate.

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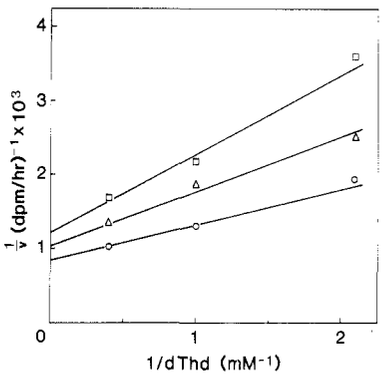

Figure 1. Inhibition of dThd phosphorylase by 3′-FFdUrd. The substrate dThd was varied at the following concentrations of 3′-FFdUrd: 0 (○), 1.0 mM (△), 2.5 mM (□).

presence of aluminum trifluoride as a catalyst to give protected nucleoside **4**. This compound was deblocked with an ammoniacal solution of methanol to give 3′-FFdUrd in 30% yield. An alternate synthesis of 3′-FFdUrd has been reported involving the direct fluorination of 2′,3′-dideoxy-3′-fluorouridine; however, this procedure gave only an 8% yield of the product.[8] An examination of the NMR spectrum of 3′-FFdUrd revealed a multiplet at δ 2.54 ($J_{2',F}$ = 40.5 Hz) and 2.37 ($J_{2'',F}$ 21.5 Hz), which is due to $C_{2'}$ H and $C_{2''}$ H, respectively. The signal of the $C_{3'}$ H appeared at δ 5.38 ($J_{3',F}$ = 53.5 Hz) and the $C_{4'}$ H at δ 4.29 ($J_{4',F}$ = 28.5 Hz). The chemical shifts and the magnitude of the coupling constants are consistent with the assignments previously made for 3′-deoxy-3′-fluorothymidine.[7] 3′-FFdUrd was treated with $POCl_3$ in triethyl phosphate to give the 5′-phosphate (3′-FFdUMP).[9]

Results

Interaction of 3′-FFdUrd with dThd Phosphorylase. 3′-FFdUrd was not a substrate for dThd phosphorylase isolated from Lewis lung carcinoma. There was no detectable FUra generated from a 10-mM solution of 3′-FFdUrd over a period of 24 h. However, 3′-FFdUrd was a linear noncompetitive inhibitor of the dThd cleavage reaction, with an estimated K_i value of 1.7 mM (Figure 1).

Cell Growth Inhibition. 3′-FFdUrd was tested as an inhibitor of the growth of L1210 mouse leukemia cells in culture. The IC_{50} value (concentration required for 50% inhibition of growth) for the compound against L1210 cells was 3.4×10^{-6} M compared to a value of 5×10^{-10} M for FdUrd.

Interaction of 3′-FFdUMP with dTMP Synthetase. Evaluation of the mode of inhibition of dTMP synthetase by 3′-FFdUMP using the conventional procedure of initiating the reaction by addition of enzyme and measuring apparent initial velocities showed inhibition competitive with the substrate dUMP (Figure 2A). The K_i value for 3′-FFdUMP obtained from this plot was 0.13 mM, compared to a K_i value for FdUMP of about 0.4 μM,[10] indicating a sizeable loss in binding resulting from substitution of the 3′-hydroxyl group with a fluorine atom. It was noted, however, that progress curves for the enzyme reaction (not shown) were not linear in the presence of the inhibitor but showed a rapid decrease in slope over the same period of time when the uninhibited control reaction was still linear. Assuming the absence of substances that denature the enzyme, this rate behavior indicates that inhibition does not remain constant but increases with time. This phenomenon was further confirmed by preincubation experiments. When the enzyme was preincubated with 3′-FFdUMP and the reaction was initiated by adding various concentrations of dUMP, the resulting reciprocal plot of the initial velocities against dUMP concentration resembled a noncompetitive inhibition pattern (Figure 2B). Exposure of dTMP synthetase to 1 mM 3′-FFdUMP for various periods of time before initiating the enzyme reaction with dUMP resulted in a progressive decline in the initial velocity of the enzyme reaction (Figure 3A). This time-dependent inactivation was

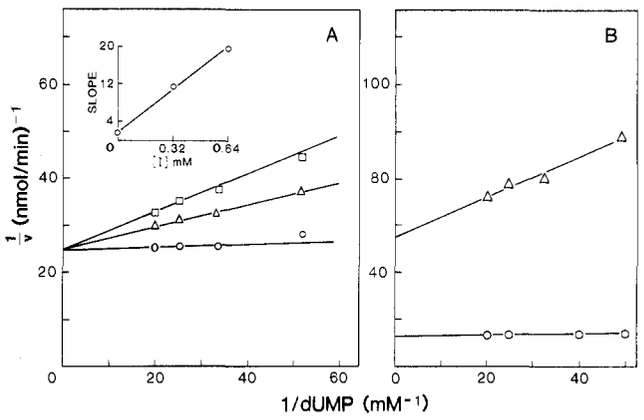

Figure 2. Inhibition of dTMP synthetase by 3′-FFdUMP. (A) Double-reciprocal plot of initial velocities in the presence of no inhibitor (○), 0.13 mM 3′-FFdUMP (△), and 0.26 mM 3′-FFdUMP (□) when the reaction was initiated by addition of the enzyme. (B) The enzyme was preincubated for 10 min with 3′-FFdUMP before the addition of enzyme: no inhibtion (○), 0.13 mM 3′-FFdUMP (△). The reactions contained 0.1 mM 5,10-CH_2H_4 folate, variable concentrations of dUMP, 10 mM β-mercaptoethanol, and dTMP synthetase purified to homogeneity from *L. casei* in 1 mL of 0.1 M Tris buffer, pH 7.5.

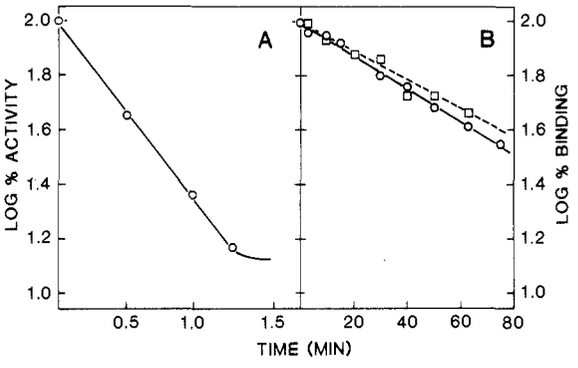

Figure 3. (A) Decrease in the initial velocity of the dTMP synthetase reaction upon preincubation of 1.2 mM 3′-FFdUMP with the enzyme for various periods of time before the addition of dUMP. Reaction components are as described in the legend to Figure 2. (B) Rate of dissociation of [6-^{3}H]3′-FFdUMP from the dTMP synthetase–[6^3-H]3′-FFdUMP–5,10-CH_2H_2folate (○), and rate of dissociation of [^{14}C]FdUMP from its corresponding ternary complex (□). The complexes were preformed by incubation of dTMP synthetase, 0.1 mM 5,10-CH_2H_4folate, and the labeled nucleotide. The complexes were then separated from unbound components by Sephadex G-25 chromatography and mixed in one container. FdUMP (0.1 mM) was added to initiate the exchange reaction. Aliquots were removed at intervals and filtered through nitrocellulose filters, which were dissolved and counted as previously described[12] to determine radioactivity remaining bound to the protein.

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first order up to a loss of about 85% of the original enzyme activity. From these data, the apparent rate constant (k_{on}^{obsd}) for inactivation of dTMP synthetase by 3′-FFdUMP at 30 °C was calculated to be 0.029 s^{-1}. In order to determine whether 3′-FFdUMP and 5,10-CH_2H_4 folate form a tight-binding ternary complex with dTMP synthetase analogously to FdUMP, we prepared [6-^{3}H]-3′-FFdUMP and incubated it with the enzyme in the presence 5,10-CH_2H_4folate. The resulting complex was isolable by the nitrocellulose filtration technique for separating protein-bound from unbound ligands,[11] indicating a much tighter binding than would be expected from the K_i value obtained from the competitive plot in Figure 2A. However, in the absence of 5,10-CH_2H_4folate, no filtrable complex was formed. The rate of dissociation of [6-^{3}H]-FFdUMP from this ternary complex was measured, as previously described for [6-^{3}H]FdUMP,[12] by an isotope exchange reaction initiated by the addition of a large excess of a competing nucleotide. In the presence of 0.1 mM FdUMP, the dissociation of [6-^{3}H]3′-FFdUMP was first order (Figure 3B) with an apparent rate constant (k_{off}^{obsd}) of 1.3×10^{-2} min^{-1} ($t_{1/2}$ = 53 min). The k_{off}^{obsd} value for [6-^{3}H]FdUMP from its ternary complex with dTMP synthetase measured at the same time was 1.4×10^{-2} min^{-1} ($t_{1/2}$ = 49 min) (Figure 3B).

Discussion

The data for the interaction of 3′-FFdUMP with dTMP synthetase are readily interpretable in terms of a two-stage model, which involves the rapid formation of a reversible complex (E·I), followed by a relatively slow, first-order conversion of this complex to a more tightly bound complex (E–I) (eq 1).

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}I \qquad (1)$$

In terms of this model, the competitive plot (Figure 2A) represents initial velocity conditions in which no appreciable amount of the E–I complex has yet formed, and thus the K_i value obtained from the plot should be equal to the dissociation constant of the initial E·I complex (i.e., the ratio of k_2/k_1). The change in the $1/v$ intercept of the reciprocal plot when the enzyme is preincubated with the inhibitor (Figure 2B) does not, of course, indicate actual noncompetitive inhibition but shows that the portion of the enzyme diverted into the E–I complex prior to addition of dUMP is not available for reaction with dUMP even when the latter is present at high levels. This behavior would result when k_4 is appreciably smaller than k_2, thus causing the inhibition to appear irreversible during the initial velocity phase of the enzyme reaction. The constants k_3 and k_4 can be evaluated from the kinetics of formation and dissociation of the E–I complex. The first-order rate of inactivation of the enzyme by 3′-FFdUMP, k_{on}^{obsd}, which represents the rate of the "tightening up" reaction, is related to k_3 by eq 2.[13]

$$\frac{1}{k_{on}^{obsd}} = \left(\frac{1}{k_3}\right)\left(1 + \frac{K_i}{[I]}\right) \qquad (2)$$

Therefore, since the concentration of 3′-FFdUMP that we used in determining k_{on}^{obsd} was substantially above K_i it can be assumed that $k_{on}^{obsd} \cong k_3$. Similarly, if $k_4 \ll k_2$, the experimentally measured k_{off} value for the dissociation of [6-^{3}H]FFdUMP from the ternary complex should be equal to k_4. These constants and the corresponding ones for FdUMP[12] are listed in Table I.

Table I. Comparison of Rate Constants for Formation and Dissociation of dTMP Synthetase-Nucleotide-5,10-CH_2H_4folate Ternary Complexes

kinetic constant[a]	3′-FFdUMP	FdUMP
$K_i(k_2/k_1)$	1.3×10^{-4} M	4×10^{-7} M[b]
$k_3(k_{on}^{obsd})$	2.9×10^{-2} s^{-1}	2.2×10^{-5} s^{-1}[c]
$k_4(k_{off}^{obsd})$	1.4×10^{-2} min^{-1}	1.3×10^{-2} min^{-1}

[a] As defined in eq 1. [b] From previously published data.[10] [c] From previously published data.[12]

It should be made clear at this point that Scheme I is simplified in that it does not show the participation of 5,10-CH_2H_4folate in the complex. Since we observed no tight binding of 3′-FFdUMP in the absence of the cofactor, it can be assumed that the complexes are actually ternary ones that include a molecule of 5,10-CH_2H_4folate. However, we kept the concentration of this component constant at all times, and, therefore, terms for its interaction with the enzyme would be included in the values of the apparent rate constants in the above scheme.

Previous studies of the kinetic isotope effects involved in the formation and dissociation of the covalent FdUMP–5,10-CH_2H_4folate–dTMP synthetase complex have indicated the presence of a rate-determining step preceding the formation of (and therefore following the cleavage of) the covalent bonds joining the ligands to the enzyme.[14] This rate-limiting event may well be the extensive protein conformational change that has been shown to accompany ternary complex formation.[15] On the basis of this model, the almost identical rates of dissociation (k_{off}^{obsd} values, Table I) for 3′-FFdUMP and FdUMP from their respective ternary complexes suggest that these complexes have very similar conformations. That is, the ultimate extent of the conformational change in the enzyme induced by 3′-FFdUMP appears to be about the same as it is with FdUMP; the difference between the interactions of these two nucleotides with the enzyme lies in the rates at which these transitions occur. The first-order rate constant for FdUMP analogous to k_3 (eq 1) is about 10^3-fold greater than the k_3 value for 3′-FFdUMP.[12] Thus, 3′-FFdUMP not only has a much weaker interaction (higher K_i value) with the enzyme in the binary adsorption complex than does FdUMP but also, because formation of the ternary complex is considered to be equivalent to a portion of the catalytic pathway, has a substantially lower "V_{max}" for this partial enzyme reaction. This situation may be an illustration of an "induced fit" phenomenon,[16] whereby 3′-FFdUMP, because of its less than optimal fit into a binding site designed for a nucleotide bearing a 3′-hydroxyl group, is able to trigger the transition to the catalytically active (tight-binding) conformational form at only a much slower rate than FdUMP or dUMP.

Although we did not demonstrate it directly, intracellular phosphorylation of 3′-FFdUrd to 3′-FFdUMP is strongly indicated by its moderately good cytostatic activity against cells in culture, which is about the same as

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that of FUra. The rather weak initial interaction of 3′-FFdUMP with dTMP synthetase and slow rate of conversion to the tight-binding complex will probably limit the actual chemotherapeutic usefulness of this particular compound, but it may still be useful as a mechanistic tool for studying dTMP synthetase specific effects in vivo. Nevertheless, the results described here for 3′-FFdUrd (and in an earlier study with *ara*-FUrd)[13] demonstrate the principle that deoxyribonucleosides can be constructed that have glycosidic linkages resistant to dThd phosphorylase and yet retain, at least to a partial degree, the ability to interact with the target enzyme and thereby exert a cytotoxic effect.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was done with 0.2-mm thickness silica gel F plates obtained from E. Merck. The preparative separations were carried out on Analtech 1 or 2-mm (20 × 20 cm) silica gel F glass plates. Du Pont 836 high-pressure liquid chromatography (HPLC) equipped with Hewlett Packard 3380A integrator was used to purify the sample. UV spectra were obtained with a Beckman 25 spectrophotometer, NMR spectra were obtained with a Bruker WM 500 spectrophotometer and mass spectral data were taken with a Hewlett Packard GC/MS 5985A equipped with dual EI/CI source of 70 EV. The elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and were within ±0.4% of the theoretical values.

2,3′-Anhydro-1-(2′-deoxy-β-D-lyxofuranosyl)-5-fluorouracil (2). The method of Fox and Miller[6] was followed to synthesize **2** from 5-fluoro-2′-deoxyuridine (**1**), mp 195–198 °C (lit.[6] mp 197–198 °C).

2,3′-Anhydro-1-(2′-deoxy-5′-*O*-acetyl-β-D-xylofuranosyl)-5-fluorouracil (3). A solution of **2** (2.28 g, 10 mmol) in dry pyridine (100 mL) was heated at 50 °C for 15 min to dissolve the contents. The solution was cooled, and acetic anhydride (125 mL) was added. The reaction mixture was stirred at ambient temperature for 2 h and then evaporated. After several additions–evaporations of ethanol, **3** was crystallized from ethanol (2.5 g, 92%): mp 198–201 °C; MS, *m/e* 270.1 (M^+); NMR (acetone-d_6) δ 8.11 (d, 1, J = 6.2 Hz, CHCF), 5.86 (d, 1, J = 4 Hz, $C_{1'}$ H), 5.35 (d, 1, $C_{3'}$ H), 4.43 (m, 1, $C_{4'}$ H), 4.1 (m, 2, 5′-CH_2), 2.62 (m, 2, $C_{2'}$ H), 2.5 (s, 3, $COCH_3$). Anal. ($C_{11}H_{11}FN_2O_5$) C, H, N.

5′-*O*-Acetyl-3′-deoxy-3′,5-difluoro-2′-deoxyuridine (4) and 3′-Deoxy-3′,5-difluoro-2′-deoxyuridine (5). A mixture of **3** (1.3 g, 4.8 mmol), aluminum trifluoride (2 g), and a solution of hydrogen fluoride in 1,4-dioxane (300 mL, containing 4 mL of anhydrous hydrogen fluoride) was heated at 200 °C in a stainless-steel container for 1 h. The mixture was rapidly cooled to room temperature, and water (100 mL) was added. Calcium carbonate (15 g) was added with stirring, the mixture was filtered, and the filtrate was washed with hot acetone and evaporated to dryness. Preparative TLC ($CHCl_3$–acetone, 3:1) afforded **4** in the form of a syrup, which was placed in a saturated ammonical solution of methanol (100 mL) at 4 °C for 48 h and evaporated to dryness. The residue was recrystallized from ethanol to give **5** (0.35 g, 30%): mp 161–163 °C (lit.[2] mp 159–160 °C); MS, *m/e* 248.2 (M^+); NMR (acetone-d_6 + D_2O) δ 8.20 (d, 1, J = 7.2 Hz, CHCF), 6.34 (d, 1, $J_{1',2'}$ = 9.2 Hz, $C_{1'}$ H), 5.38 (m, 1, $J_{3',F}$ = 43.5 Hz, $C_{3'}$ H), 4.29 (m, 1, $J_{4',F}$ = 28.5 Hz, $C_{4'}$ H), 3.83 (m, 2, J = 3 Hz, 5′-CH_2), 2.54 (m, 1, $J_{2'F}$ = 40.5 Hz, $C_{2'}$ H), 2.37 (m, 1, $J_{2'',F}$ = 21.5 Hz, $C_{2''}$ H). Anal. ($C_9H_{10}F_2N_2O_4$), C, H, N.

5-Fluoro-2′,3′-dideoxy-3′-fluorouridine 5′-Phosphate (6). 5-Fluoro-2′,3′-dideoxy-3′-fluorouridine (**5**; 3.0 mg, 1.2 μmol) was purified by HPLC and dissolved in 0.2 mL of triethyl phosphate. To this solution was added 20 μL of $POCl_3$, and the mixture was allowed to stand at ambient temperature. After 2 h, silica gel TLC in $CHCl_3$–MeOH (9:1) showed that all of the nucleoside had become converted to a product that did not migrate. Hexane (5 mL) was added to the reaction mixture. The gummy residue that precipitated was repeatedly washed with hexane and then dissolved in 1 mL of H_2O. The solution was applied to a small DEAE-cellulose column (1 × 5 cm). The column was washed with H_2O (10 mL), 20 mM ammonium acetate, pH 7.5 (10 mL), and finally 200 mM ammonium acetate (10 mL). The last fraction contained the material that had a UV spectrum identical with that of 3′-FFdUrd and a R_f value of 0.1 on a cellulose plate in *i*-PrOH–NH_4OH–H_2O (7:1:2). Anal. calcd base/phosphorus ratio, 1:1; found, 1:0.9.

Radiolabeled [6-^{3}H]3′-FFdUMP (sp act. 10 mCi/mmol) was prepared according to the method of Rabi and Fox.[17]

dTMP Synthetase Assay. The activity of dTMP synthetase purified to homogeneity from *Lactobacillus casei* was assayed according to the procedure of Wahba and Friedkin.[18] Binding assays on nitrocellulose filters were performed as previously described.[11,12]

dThd Phosphorylase Isolation and Assay. Lewis lung carcinoma tissue obtained as tumor growth of rats was thawed in Tris buffer (50 mM, pH 7.4) containing 2-mercaptoethanol (5 mM). The tissue was cut into small pieces, homogenized, and sonicated (5 × 10 s bursts). The sonicate was centrifuged (30 min at 10000 rpm), and the crude free extract was used for ammonium sulfate precipitation. The 0–30% ammonium sulfate precipitate did not have any thymidine phosphorylase activity, but the 30–65% fraction contained enzyme activity. This precipitate was dissolved in the above-mentioned buffer and then dialyzed against the same buffer containing 2-mercaptoethanol for 24 h. The dialyzed solution was used for the kinetic experiments.

The reaction mixture for assaying enzyme activity typically contained [5-C^3H_3]dThd (sp act. 0.9 Ci/mmol) at various concentrations, arsenate (17 mM), 3′-FFdUrd at various concentrations, and the enzyme-containing extract. Buffer (Tris-Cl, 50 mM, pH 7.4) containing 2-mercaptoethanol was added to make the final volume of 150 μL. The reaction was initiated by adding the enzyme solution. Aliquots (20 μL) were taken at 0, 2, 4, 6, and 8 h and applied to silica gel plates. Unlabeled thymine was used as a fluorescent marker. The plates were eluted with methylene chloride/ethanol (85:15). Thymine spots were scraped from the plates, and the product was eluted with water. The radioactivity of the thymine was measured in a liquid scintillation counter. The velocity of the reaction was calculated as the amount of [5-$^3H_3$]thymine generated as a function of time.

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Registry No. 1, 50-91-9; 2, 34393-72-1; 3, 87395-47-9; 4, 87412-13-3; 5, 41107-55-5; 6, 87395-48-0; FdUMP, 134-46-3; 5,10-CH_2H_4folate, 3432-99-3; thymidine phosphorylase, 9030-23-3; thymidylate synthetase, 9031-61-2.

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