°C. Cumulative dose–response curves for acetylcholine-induced contraction were determined in the absence or in the presence of test compounds (1×10^{-7} to 1×10^{-5} M) or atropine (3×10^{-7} to 1×10^{-4} M).

Histamine H_2 Receptor Antagonistic Activity. The histamine H_2 receptor antagonistic activity was determined by using the guinea pig isolated right atrium preparation suspended in Krebs solution aerated with 95% O_2/5% CO_2 at 32 °C. Cumulative dose–response curves for histamine-induced positive chronotropic action were determined in the absence or in the presence of test compounds (1×10^{-7} to 1×10^{-5} M) or cimetidine (3×10^{-6} to 3×10^{-5} M).

Registry No. **2** ($R^1 = C_6H_5$), 2166-31-6; **2** ($R^1 = CH_3$), 13327-27-0; **2** (R^1 = morpholino), 27464-00-2; **2** [$R^1 = N(CH_3)_2$], 35716-89-3; **2** [$R^1 = C_6H_4$(4-Cl)], 2166-13-4; **2** [$R^1 = C_6H_4(4\text{-}CH_3)$], 2166-32-7; **3** ($R^1 = C_6H_5$; $m = 1$), 32949-37-4; **3** ($R^1 = C_6H_5$; $m = 2$), 23916-77-0; **4** ($R^1 = C_6H_5$; $m = 2$), 74316-74-8; **4** ($R^1 = C_6H_5$, $m = 3$), 23916-79-2; **4** ($R^1 = CH_3$, $m = 1$), 34477-79-7; **4** ($R^1 = C_6H_5$, $m = 1$), 34477-77-5; **5** ($R^1 = CH_3$, $m = 1$, $n = 2$), 85748-96-5; **5** ($R^1 = C_6H_5$, $m = n = 2$), 85748-97-6; **5** ($R^1 = C_6H_5$, $m = 3$, $n = 2$), 85748-98-7; **5** ($R^1 = C_6H_5$, $m = 1$, $n = 2$), 79460-68-7; **6** ($R^1 = C_6H_5$, $n = 2$), 85748-99-8; **6** ($R^1 = C_6H_5$, $n = 3$), 85749-00-4; **6** ($R^1 = C_6H_5$, $n = 4$), 85749-01-5; **7** ($R^1 = C_6H_5$, $m = 1$, $n = 2$), 79460-67-6; **7** ($R^1 = C_6H_5$; $m = 1$; $n = 3$), 85749-02-6; **8a**, 85749-03-7; **8b**, 82023-15-2; **8c**, 82023-14-1; **8d**, 79460-53-0; **8e**, 79460-54-1; **8f**, 79460-55-2; **8g**, 85749-04-8; **8h**, 85749-05-9; **8i**, 79460-49-4; **8j**, 85658-25-9; **8k**, 85658-26-0; **10**, 85748-88-5; **11**, 85748-89-6; **12**, 82022-95-5; **13**, 82033-49-6; **14**, 79460-56-3; **15**, 79460-58-5; **16**, 79460-60-9; **17**, 79460-57-4; **18**, 85748-90-9; **19**, 85748-91-0; **20**, 85748-92-1; **21**, 85748-93-2; **22**, 85748-94-3; **23**, 85748-95-4; **24**, 79460-50-7; **25**, 85658-27-1; **26**, 85658-29-3; $(CH_3S)_2C{=}NCN$, 10191-60-3; ethylene chlorohydrin, 107-07-3; cysteamine hydrochloride, 156-57-0.

Mechanism of Action of 2′,5-Difluoro-1-arabinosyluracil

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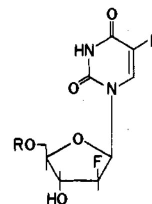
Results are described which demonstrate that the cytotoxic action of 2′,5-difluoro-1-arabinosyluracil (FFara-Ura) involves conversion to the corresponding 5′-phosphate, FFara-UMP, and subsequent inhibition of thymidylate synthetase. The evidence for this is as follows: (a) cells lacking thymidine kinase are 120-fold more resistant to FFara-Ura; (b) FFara-Ura markedly inhibits the incorporation of 2′-deoxyuridine (dUrd) into DNA with little or no effect on 2′-deoxythymidine (dThd) incorporation; (c) FFara-Ura causes changes in deoxynucleoside triphosphate pool sizes, which are characteristic of specific inhibition of dTMP synthetase. Binding and spectroscopic studies demonstrate that FFara-UMP inactivates dTMP synthetase from *Lactobacillus casei* in a manner analogous to that described for FdUMP. Furthermore, FFara-Ura is not a substrate for the pyrimidine phosphorylases; the significance of this finding with regard to the possible chemotherapeutic utility of FFara-Ura is discussed.

FdUrd[1] is a potent cytotoxic agent toward most tissue culture cells because of its direct conversion to FdUMP, which is a potent and specific mechanism-based inhibitor of dTMP synthetase.[2,3] With in vivo systems the anticipated direct effect of FdUrd is altered by its conversion to FUra, which, in addition to inhibition of dTMP synthetase, undergoes extensive metabolism and is incorporated into RNA.[4,5] As a result, it is not yet known whether specific inhibition of dTMP synthetase would be of chemotherapeutic benefit in the treatment of neoplastic diseases.

One approach that has been used in an attempt to overcome this problem has been the construction of analogues that (a) are not substrates for the pyrimidine nucleoside phosphorylases and, hence, are metabolically stable, (b) are converted to the corresponding 5′-nucleotides by a nucleoside kinase, and (c) provide specific and potent inhibitors of dTMP synthetase. Examples of compounds that fulfill, or partially fulfill, these requirements include the carbocyclic analogue of FdUrd,[6] 5-fluoro-2′-deoxycytidine,[7] and 1-β-D-arabinofuranosyl-5-fluorouracil.[8]

Recently, 2′,5-difluoro-1-arabinosyluracil (FFara-Ura) has been synthesized and shown to be moderately cytotoxic toward tissue culture cells.[9] In this report, we describe experiments which demonstrate that FFara-Ura is not a substrate for mammalian pyrimidine phosphorylases and that the cytotoxic action of FFara-Ura involves conversion to the corresponding nucleotide, FFara-UMP, and subsequent inhibition of dTMP synthetase. Experiments are described which demonstrate that FFara-Ura forms a covalent complex with dTMP synthetase in a manner analogous to FdUMP.



R = H , FFara-Ura
R = PO_3H_2 , FFara-UMP

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(1) The following abbreviations are used. FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; FFara-Ura, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-fluorouracil or 2′,5-difluoro-1-arabinosyluracil; FFara-UMP, 2′,5-difluoro-1-arabinosyluridylate; H_2folate, 7,8-dihydrofolate; CH_2-H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; NMM, *N*-methylmorpholine; RV, retention volume. All other abbreviations used are those recommended by IUPAC.

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Results and Discussion

FFara-Ura inhibits the growth of S-49 mouse lymphoma cells with an EC_{50} value of 3.3 μM. This is in good agreement with values reported in L5178Y (EC_{50} = 3.8 μM) and P815 (EC_{50} = 2.6 μM) cell lines.[9] With a dThd kinase deficient line of S-49 cells, the EC_{50} was increased 120-fold (EC_{50} = 400 μM), indicating the necessity for conversion to the corresponding 5′-nucleotide for manifestation of cytotoxicity. Two lines of evidence were obtained which suggested that FFara-Ura caused the inhibition of dTMP synthetase in S-49 cells. (1) Intracellular inhibition of dTMP synthetase can be demonstrated by the marked inhibition of dUrd incorporation into DNA with little or no effect on dThd incorporation.[10] A 1 h exposure of S-49 cells (9×10^5 cells/mL) to 33 μM FFara-Ura resulted in a 90% reduction in the rate of [6-^{3}H]dUrd incorporation into acid-insoluble material compared to controls, whereas the rate of incorporation of [Me-^{3}H]dThd showed an increase of 4%. (2) Intracellular inhibition of dTMP synthetase results in a depletion of dTTP and perturbations of other deoxyribonucleoside pool sizes, which result from consequent allosteric effects on ribonucleotide reductase.[11,12] When S-49 cells (2×10^5 cells/mL) were exposed to 16.5 μM FFara-Ura, dTTP pools decreased to 80 and 40% of the original value at 1 and 4 h, respectively; further, there was an expansion of the dCTP pool (172% at 1 h), a large decrease in the dGTP pool (<20% by 1 h), and a slight decrease in the dATP pool (90% at 1 h). The pattern of these changes is similar to that observed with FdUrd and 5-nitro-2′-deoxyuridine,[13] which are known to be specific inhibitors of dTMP synthetase in S-49 cells.[13,14]

Having evidence that FFara-Ura inhibits dTMP synthetase in S-49 cells, it was of interest to determine whether FFara-UMP would inhibit the enzyme from *L. casei* in a manner similar to that described for FdUMP.[2,3] dTMP synthetase catalyzes the dehalogenation of 5-bromo-2′-deoxyuridylate (BrdUMP) in the absence of CH_2-H_4folate,[15] and this assay provides a convenient method for evaluating noncovalent binary complexes between the enzyme and analogues of dUMP.[16] FFara-UMP is a competitive inhibitor in this reaction and shows a K_i value (3.3 μM) similar to that of dUMP (2.3 μM)[16] in this assay. In the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate, FFara-UMP caused a concentration-dependent inhibition of both the initial velocity (I/S_{50} = 0.2) and completion of reaction. This behavior is characteristic of time-dependent enzyme inactivation,[17] which, if rapid, precludes accurate evaluation of reversible inhibition constants by initial velocity experiments.[16] Indeed, when excess FFara-UMP and CH_2-H_4folate were incubated with dTMP synthetase, about 70% of the activity was lost within 30 s (Figure 1), which corresponds to an apparent rate constant of about 2.4 min^{-1}. In the absence of the cofactor, no inactivation was observed over a 90-min incubation. 5-Chloro-2′-deoxyuridylate (CldUMP), a competitive inhibitor of the normal enzymic reaction (K_i = 0.19 μM),[16] caused a concentration-related protection of the enzyme against inactivation by FFara-UMP (Figure 1).

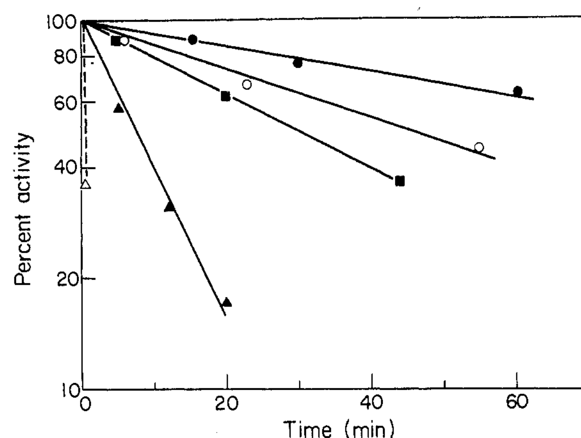

Figure 1. Time-dependnet inactivation of dTMP synthetase by FFara-UMP in the presence and absence of CldUMP. Incubation mixtures contained 1.0 μM FFara-UMP, 0.1 mM CH_2-H_4folate, and 0 (—△—), 1 (-▲-), 5 (-■-), 10 (-○-), and 50 μM (-●-) CldUMP. Reaction conditions are provided under Experimental Section.

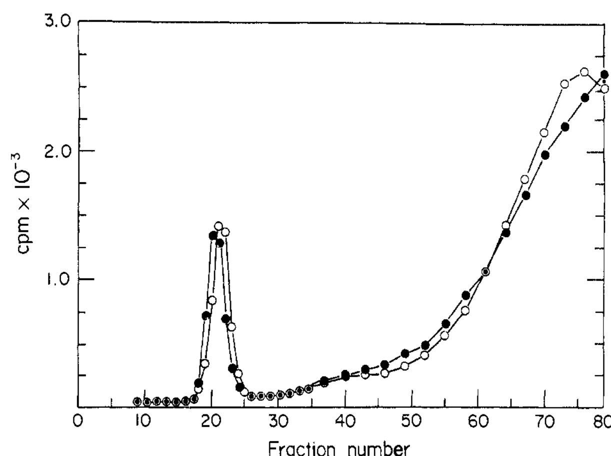

Figure 2. Sephadex G-25 chromatography of the FFara-UMP–CH_2-H_4folate–dTMP synthetase synthetase complex. dTMP synthetase (3.16 μM) was incubated with *l*,L-CH_2-[6-^{3}H]H_4folate (78 μM) and FFara-UMP (78 μM) at 25 °C for 20 min. Aliquots were chromatographed on a Sephadex G-25 column (0.75 × 27 cm), equilibrated and eluted with 20 mM Na_2HPO_4 (pH 7.4) and 10 mM mercaptoethanol before (-○-) and after (-●-) $NaDodSO_4$ denaturation (2% $NaDodSO_4$, 25 °C for 2 h).

The above experiments show that FFara-UMP reversibly binds to the nucleotide binding site of dTMP synthetase and, in the presence of CH_2-H_4folate, causes a time-dependent inactivation of the enzyme. These properties are similar to those observed with FdUMP, and it was considered likely that the mechanism of inactivation by these inhibitors was analogous. This was substantiated by the binding and UV spectroscopy studies described below.

When dTMP synthetase was treated with excess FFara-UMP and CH_2-[6-^{3}H]H_4folate and filtered through a Sephadex G-25 column, macromolecular-bound radioactivity elutes in the void volume (Figure 2) and corresponds to 2.04 mol of cofactor bound per mole of enzyme. This stoichiometry is in accord with the fact that the enzyme consists of two identical subunits, each of which is capable of forming complexes with inhibitors. The UV spectrum of the complex freed from excess ligands shows

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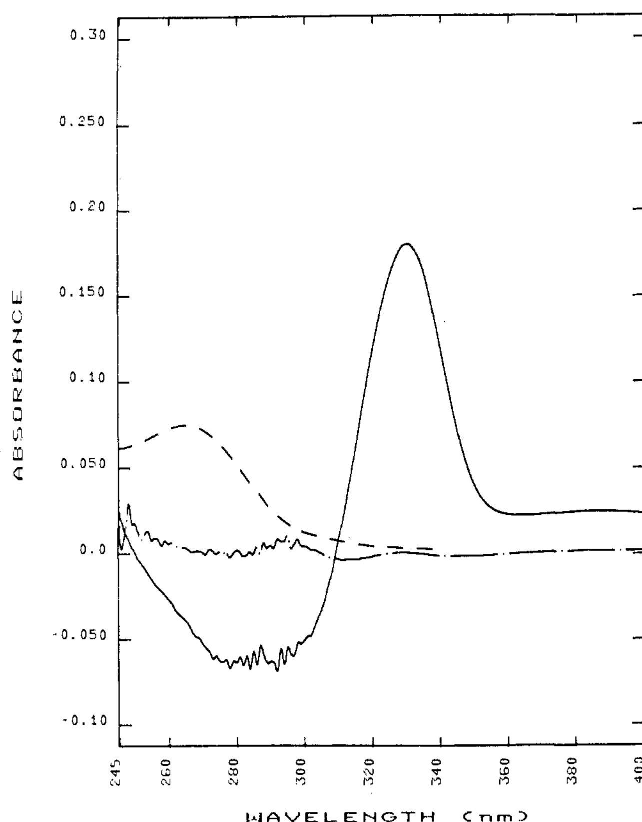

Figure 3. UV difference spectra of the FFara-UMP–CH_2-H_4-folate–dTMP synthetase complex: (---) spectrum of FFara-UMP; (—) difference spectrum of FFara-UMP–CH_2-H_4folate–dTMP synthetase complex vs. CH_2-H_4folate and enzyme after 20 min; (-·-) difference spectrum of the complex after treatment with 2% $NaDodSO_4$ for 40 min. Appropriate corrections were made for dilution.

the characteristic protein absorbance at 282 nm and a prominent peak at 322 nm with A_{280}/A_{322} = 3.0. This spectrum is analogous to that reported for the FdUMP–CH_2-H_4folate–dTMP synthetase complex, which, after gel filtration, shows A_{280}/A_{322} = 2.8.[18] Denaturation of the complex with $NaDodSO_4$ did not result in the loss of macromolecular-bound radioactivity (Figure 2), demonstrating that the cofactor is covalently attached to the protein.

When limiting FFara-UMP was added to the sample cuvette of two previosuly balanced cuvettes containing enzyme and CH_2-H_4folate, the UV-difference spectrum shows a loss of the absorbance due to the pyrimidine chromophore at 268 nm, a minimum at 292 nm, and a peak at 330 nm with $\Delta\epsilon$ = 17 800 (Figure 3). Addition of $NaDodSO_4$ to each cuvette results in a time-dependent (k_{obsd} = 0.14 min^{-1}) loss of all differential absorbance; the fact that the denatured complex does not regain the absorbance at 268 nm is indicative that the pyrimidine had undergone irreversible, covalent bond changes, which destroyed its chromophore. The difference spectra in Figure 3 and the behavior of the complex toward $NaDodSO_4$ denaturation are exactly analogous to that which was previously been reported for FdUMP[19] and 1-β-D-arabinofuranosyl-5-fluorouridylate.[8] Using the increase in absorbance at 330 nm which accompanies formation of the FFara-UMP ternary complex, we titrated the enzyme (13.7 nmol/mL)

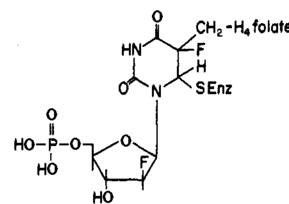

Figure 4. Proposed structure of the FFara-UMP–CH_2-H_4-folate–dTMP synthetase complex.

with FFara-UMP in the presence of 27.4 μM CH_2-H_4folate. The increase in absorbance is linear with respect to the amount of complex present until the enzyme is saturated; from this, it was calculated that 1.88 mol of FFara-UMP is bound per mol of the dimeric enzyme, with $\Delta\epsilon_{330}$ = 17 800 and $\Delta\epsilon_{340}$ = 12 700 (calculated for each binding site of the enzyme). This stoichiometry is in good agreement with that determined for binding of CH_2-H_4folate, and we conclude that, as with the FdUMP–complex, 2 mol of the nucleotide and 2 mol of the cofactor are covalently bound to the dimeric enzyme.

The aforementioned properties of the FFara-UMP–CH_2-H_4folate–dTMP synthetase complex, together with analogy to the ternary FdUMP complex, provide convincing evidence for the structure depicted in Figure 4. As shown, the thiol nucleophilic catalyst is covalently bound to the 6-position of the pyrimidine, and the 5-position is linked to the one carbon unit of the cofactor. It is of interest to note that the analogue of FdUMP with a fluorine in the 2′-"down" position (i.e., 2′,5-difluoro-2′-deoxyribosyluridylic acid) has also been reported to form a similar complex.[20]

Introduction of a 2′-fluorine in 2′-deoxyribonucleosides of purines provides analogues that are resistant to phosphorolysis by purine nucleoside phosphorylase.[21] Likewise, we have found that FFara-Ura is not a substrate for Urd or dThD phosphorylase. This result is consistent with the report of Chou et al.,[22] who found that the 2′-fluoro substituent in the *arabino* ("up") configuration in 2′-fluoroarabinofuranosylpyrimidine nucleosides confers more resistance to in vivo catabolism, presumably through the increased metabolic stability of the *N*-glycosyl linkage. Under conditions which catalyzed cleavage of 1.5 μM Urd/min and 2.3 μM dThd/min by the corresponding pyrimidine nucleoside phosphorylases, there was no detectable reaction with FFara-Ura at concentrations of 0.1 to 0.2 mM. Thus, although the cytotoxicity of FFara-Ura is moderate, the stability of its glycosyl linkage may offer an approach to the specific in vivo inhibition of dTMP synthetase without the RNA effects so prominant with FUra. This could result in a different spectrum of in vivo activity than that found with FUra and FdUrd and suggests that FFara-Ura warrants further investigation.

Experimental Section

General. dTMP synthetase from a methotrexate-resistant strain of *L. casei* was the homogeneous preparation previously described.[23] The concentration of dTMP synthetase binding sites was determined as the [6-^{3}H]FdUMP–CH_2-H_4folate–enzyme complex isolated by Sephadex G-25 chromatography[14] or by

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spectrophotometric titration of the ternary complex.[19] dThd phosphorylase from horse liver was obtained from Gipep Co. Ltd. (Paris, France). Urd phosphorylase, purified from rat liver, was a gift from J. Prior. *E. coli* dThd kinase was the preparation previously described.[23] dUMP and *dl*-L-H_4folate were purchased from Sigma Chemical Co., l,L-CH_2-H_4folate and *l*,L-CH_2-[6-^3H]H_4folate (30.0 mCi/mmol) were gifts from T. W. Bruice. FFara-Ura was prepared by the reported procedure.[9] [6-^3H]-FdUMP (18 Ci/mmol), [^3H]dThd (50 Ci/mmol), and [^3H]dUrd (18 Ci/mmol) were obtained from Moravek Biochemicals. All other chemicals were of analytical grade. Measurements of the rate of incorporation of [6-^3H]dUrd and [^3H]dThd into DNA were performed as previously described,[13] as were determinations of deoxyribonucleoside triphosphate pool sizes.[24] Radioactivity was measured in Aquasol (New England Nuclear Corp.), and disintegrations per minute calculations were made by using the external standard ratio method.

Preparation of FFara-UMP. FFara-UMP was prepared from FFara-Ura using dThd kinase by a modification of a previously reported procedure.[23] The reaction mixture (2.5 mL) contained 4.5 mM FFara-Ura, 15 mM ATP, 40 mM Tris-HCl (pH 7.8), 7.5 mM $MgCl_2$, 30 mM KF, 0.25 mg/mL bovine serum albumin, and 0.7 mg of the 20–40% NH_4SO_4 fraction from *E. coli* extract containing dThd kinase. After incubation at 37 °C for 2 h, 2 vol of ice-cold MeOH was added, and after 30 min at 0 °C, protein was removed by centrifugation. The supernatant was diluted to 50 mL with H_2O and loaded onto a DEAE-Sephadex column (1.5 × 5 cm, HCO_3^- form). The column was eluted with an 80-mL linear gradient to 0.25 M triethylammonium bicarbonate (pH 8.0), followed by isocratic elution with 100 mL of 0.25 M triethylammonium bicarbonate (pH 8.0); 2.5-mL fractions were collected and analyzed by HPLC on Lichrosorb C_{18} by using 0.25 M NH_4OAc (pH 6.0, 2 mL/min) as eluant. Unreacted FFara-Ura (RV = 40.8 mL on HPLC) eluted in fractions 20–29, and FFara-UMP (RV = 7.0 mL on HPLC) eluted in fractions 43–60 of the ion-exchange column, contaminated with ATP (RV = 10.6 mL). Fractions 43–60 were pooled, lyophilized, and redissolved in 0.25 M NH_4OAc, pH 8.8. The solution was applied to an Affi-Gel 601 boronate column (1.5 × 3 cm) previously equilibrated with 0.25 M NH_4OAc (pH 8.8) to adsorb the contaminating ATP; the column was washed with the same buffer until all absorbance at 264 nm had eluted. The eluate containing FFara-UMP was evaporated to dryness, twice lyophilized from H_2O (1 mL), and dissolved in 1 mL of H_2O. HPLC showed a single peak corresponding to FFara-UMP (RV = 7.0 mL), and the UV spectrum was essentially identical with that of FFara-Ura: λ_{max} 264, λ_{min} 238 nm (pH 1); λ_{max} 263, λ_{min} 252 nm (pH 13). The yield was 8.2 mmol (72%).

Cell Culture. S-49 and S-49/TK^- mouse lymphoma cells were grown as suspension cultures at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum as previously described.[14] EC_{50} values refer to the concentration of inhibitor necessary to inhibit the rate of cell growth by 50%, compared to controls grown under identical conditions. Cells were suspended in 1 mL of growth medium, containing specified concentrations of inhibitor and incubated at 37 °C in 10% CO_2 for 72 h. Cell density was determined daily with a Coulter Counter Model ZBI.

Enzyme Assays. Initial velocity measurements of dTMP synthetase activity were performed spectrophotometrically at 25 °C as previously described.[16] The assay mixture (1.0 mL) contained 50 mM NMM·HCl (pH 7.4), 75 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 1 mM EDTA, 7.5 mM H_2CO, 0.1 mM *dl*,L-CH_2-H_4folate, 35 μM dUMP, and ca. 20 nM dTMP synthetase; reactions were initiated by the addition of enzyme and controls omitted dUMP. For experiments on the time-dependent inactivation of dTMP synthetase by FFara-UMP, a solution (ca. 1 mL) containing 0.2 μM dTMP synthetase, 0.1 mM *dl*,L-CH_2-H_4folate, 50 mM NMM·HCl (pH 7.4), 75 mM 2-mercaptoethanol, 25 mM $MgCl_2$, and specified amounts of FFara-UMP and CldUMP were incubated at 25 °C. At specified intervals, 0.1-mL aliquots were added to 0.9 mL of the standard assay mixture, and the initial velocity was determined. The inhibition of the thymidylate synthetase catalyzed BrdUMP dehalogenation in the absence of CH_2-H_4folate was performed spectrophotometrically as previously described.[15,16] Assays of dThd and Urd phosphorylase were performed by using the standard assay conditions and spectrophotometric assay reported for dThd phosphorylase.[25]

Difference Spectra. Ultraviolet difference spectra were recorded on a Hewlett Packard 8450A spectrophotometer by using a modification of a previously described procedure.[19] Two cuvettes containing, in 1.0 mL, 50 mM NMM·HCl (pH 7.4), 8 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 6.85 μM dTMP synthetase, and 33.3 μM *l*,L-CH_2-H_4folate in the sample and reference beams were balanced. To the sample cell was added 25 μL of a 3.65 mM solution of FFara-UMP; 25 μL of H_2O was added to the reference cuvette, and the difference spectrum was recorded. For the difference spectrum of the denatured complex, 265 μL of 10% $NaDodSO_4$ was added to each cuvette (final concentration = 2% $NaDodSO_4$), and spectra were recorded until no further changes were observed (40 min).

Acknowledgment. This work was supported by USPHS Grants CA 14394, 18601, and 18856 from the National Cancer Institute. J.C. received support from NIH Institutional Training Grant 5 T32 CA09270.

Registry No. FFara-Ura, 69123-95-1; FFara-UMP, 85894-46-8; dUrd, 951-78-0; dTMP synthetase, 9031-61-2; pyrimidine nucleoside phosphorylase, 9055-35-0.

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