water and then washed with 250 mL of 0.5 N formic acid, followed by 0.5 N formic acid in 50% ethanol. The product was removed with 300 mL of 50% formic acid. Evaporation yielded a residue, which was dissolved in 25 mL of methanol containing 5% water. A large volume of acetone, ca. 500 mL, was added to precipitate the product. This process was then repeated. The filtered material was then dried under vacuum: yield 0.7 g (67%); UV λ_{max} at pH 1, 257 nm (ϵ 15 800), 280 sh; at pH 7.4, 268 (14 500), 295 sh; at pH 13.0, 271 (15 300); TLC R_f (A) 0.15, R_f (B) 0.51. Anal. ($C_{17}H_{20}N_5O_8P \cdot H_2O$) C, H, N.

8-Bromoinosine 5'-Phosphate Dilithium Salt. The pH of a solution of 8-Br-AMP (1.0 g, 2.32 mmol) in 20 mL of H_2O was adjusted to 7.0 with 1 N NaOH, and 1.12 g (20.5 mmol) of $NaNO_2$ was added. After the solution was flushed with nitrogen, 2 mL of AcOH was added, and the loosely covered solution was stirred for 2 days and then evaporated to dryness. The residue was dissolved in 100 mL of H_2O, the pH was adjusted to 7.0 with NaOH, and this solution was applied to a column of Dowex 1 × 8 (Cl^-, 100–200 mesh). The column was washed with water and the product eluted with a 1-L gradient of 0 to 0.5 N LiCl. The appropriate fractions were concentrated to dryness in vacuo and triturated with acetone containing 5% ethanol, and then this residue was dissolved in water and the product was precipitated with acetone: yield 1.05 g (95%); UV λ_{max} at pH 1, 254 nm (ϵ 7030); at pH 7.4, 254 (7190); at pH 13, 259 (6820); TLC R_f (A) 0.12; R_f (B) 0.35. Anal. ($C_{10}H_{10}BrLi_2N_4O_8P \cdot 3.75H_2O$) C, N; H: calcd, 3.48; found, 2.98.

Synthesis of IMP Analogues. Method D. The appropriate aryl or aralkyl mercaptan was added to a deoxygenated solution of 8-Br-IMP·2.5H_2O dilithium salt (200 mg, 0.42 mmol) in 1.2 mL of 1 N NaOH and 15 mL of MeOH. The solution was refluxed for 2 h and stirred at ambient temperature overnight, and the product was purified as described above.

Method E. A solution of the appropriate 8-substituted AMP derivative and 0.40 g of $NaNO_2$ in 6 mL of 0.17 N NaOH was deoxygenated by bubbling with nitrogen, and 1 mL of HOAc was added. This solution was loosely covered, stirred for 2 days at ambient temperature, and then worked up as described above.

Acknowledgment. This work was supported by Grants CA 30157 and GM 29291 from the National Institutes of Health, USPHS. E.B.S. received partial support from Training Grant GM 07175 from the NIH. We gratefully acknowledge the use of the computer facilities at the University of California, San Francisco School of Pharmacy Computer Graphics Laboratory, Robert Langridge, Director, which is supported by USPHS Grant RR 1081.

Interaction of 1-(5-Phospho-β-D-arabinofuranosyl)-5-substituted-uracils with Thymidylate Synthetase: Mechanism-Based Inhibition by 1-(5-Phospho-β-D-arabinosyl)-5-fluorouracil[1]

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A number of 1-(5-phospho-β-D-arabinosyl)-5-substituted-uracils (*ara*-UMP's) have been examined as inhibitors of dTMP synthetase. As reversible inhibitors, all were substantially less potent than their 2'-deoxyribosyl counterparts. In the presence of 5,10-methylenetetrahydrofolate (CH_2-H_4folate), *ara*-FUMP caused a first-order, time-dependent inactivation of the enzyme. At 0 °C, kinetic studies indicated a reversible K_d of 3.6 μM for the *ara*-FUMP–CH_2-H_4folate complex, and $k = 0.22$ min^{-1} for the subsequent inactivation. Spectral studies of the complex and its behavior toward protein denaturants demonstrate that its structure and stoichiometry are directly analogous to those which have previously been described for FdUMP. The significance of this finding with regard to prodrugs of *ara*-FU and the potential of *ara*-FU as a chemotherapeutic agent are discussed.

Ara-C is one of the most effective drugs available for the treatment of acute myeloblastic leukemia in adults.[4-6] Its usefulness is, however, limited by development of resistance and a short half-life that in part is due to its deamination to the inactive *ara*-U.[7-9] Consequently, a large number of analogues of *ara*-C have been synthesized with the objective of surmounting these difficulties. One such analogue, *ara*-FC, is about as effective as is *ara*-C and appears to have a similar major mode of action (presumably on DNA synthesis); however, in contrast to *ara*-C, deamination provides the cytotoxic *ara*-FU[10] which has been suggested to have a mode of action similar to FUra.[11,12] Because 2,2'-anhydro-*ara*-C is hydrolyzed to *ara*-C, it was reasoned that the corresponding 2,2'-anhydro-*ara*-FC might serve as a "double-barreled" prodrug for both *ara*-FC and *ara*-FU, each of which might exert its effect by different mechanisms.[13] Recently, the

(1) Abbreviations used: *ara*-Py refers to 1-β-D-arabinofuranosylpyrimidines (Py) with cytosine (C), 5-fluorocytosine (FC), uracil (U), 5-fluorouracil (FU); *ara*-PyMP refers to 1-β-D-arabinofuranosylpyrimidine 5'-phosphates; *xylo*-FC, 1-β-D-xylofuranosyl-5-fluorocytosine; CH_2-H_4folate, 5,10-methylene-*dl*,L-tetrahydrofolate; NMM, *N*-methylmorpholine, NaDodSO$_4$, sodium dodecyl sulfate. All other abbreviations used are those recommended by IUPAC.

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same rationale was applied in designing a number of 1-β-D-xylofuranosyl-5-fluorocytosines with good leaving groups in the 3′ position;[14] it was proposed that after a number of intramolecular conversions these *xylo*-FC analogues would give rise to anhydro-*ara*-FC and ultimately be converted to *ara*-FC and *ara*-FU. While many of these prodrugs have significant inhibitory effects on *ara*-C sensitive mouse leukemia systems, what is of most interest are their effects and presumed mechanism on *ara*-C resistant cells.[14,15] As with *ara*-C, the cytotoxicity of *ara*-FC and the aforementioned prodrugs (where tested) toward *ara*-C sensitive lines is reversed by dCyd but not by dThd, implying that all of these drugs share a similar mode of action via *ara*-CTP. In *ara*-C resistant cell lines, many of the *xylo*-FCs with good leaving groups at C-3′ show significant, albeit attenuated, cytotoxicity which is not observed with the corresponding 5-unsubstituted analogues or with *ara*-FC. Moreover, in resistant lines the toxicity of these analogues is reversed by dThd but not dCyd, implicating dTMP synthetase as the target. Thus, consistent with the double-barreled precursor approach, it has been proposed that such compounds provide both *ara*-FC, which behaves as *ara*-C in *ara*-C sensitive cells, and *ara*-FU, which inhibits thymidylate biosynthesis in *ara*-C resistant cells. If this is correct, such compounds have the potential of circumventing major problems of resistance and inactivation by enzymatic deamination which limits the usefulness of *ara*-C.

One of the unexplained difficulties with this proposal is how *ara*-FU might act to inhibit dTMP synthesis in the *ara*-C resistant lines. Two general possibilities exist. First, *ara*-FU may serve as a precursor of FUra which could then be metabolized to FdUMP, an extremely potent inhibitor of dTMP synthetase.[16,17] If this were the mechanism, conversion of the prodrugs to *ara*-FU would have to be near complete, since in *ara*-C resistant cell lines *ara*-FU and FUra are approximately equitoxic.[14,15] The second possibility is that the cytotoxic metabolite in *ara*-C resistant cells is *ara*-FUMP. The major difficulty here is that dTMP synthetase is generally thought to have stringent specificity for the 2′-deoxy-5′-phosphoribosyl moiety of nucleotide substrates or inhibitors;[18] virtually all modifications of this moiety which have been examined result in large losses in binding and, a priori, one might not expect *ara*-FUMP to be a potent inhibitor of this enzyme. On the other hand, binding of *ara*-UMP analogues to this enzyme has not been extensively investigated, and FdUMP binds so tightly that even a substantial decrease in affinity could still result in effective blockade of this enzyme.

In this paper, we describe the properties of a number of *ara*-UMP analogues as inhibitors of dTMP synthetase. We report results which show that although there is a substantial loss in reversible binding of *ara*-FUMP as compared to FdUMP, *ara*-FUMP causes a time-dependent loss of enzyme activity which could well account for the cytotoxicity of the aforementioned prodrugs in *ara*-C resistant cells. Lastly, experiments are described which suggest that the covalent complex formed between *ara*-FUMP and dTMP synthetase is analogous to that which is formed with FdUMP.

Table I. Inhibition of dTMP Synthetase by 5-Substituted *ara*-UMP's and dUMP's[a]

compd	5-substituent	K_i,[b] μM
ara-UMP	H	30
ara-TMP	CH_3	120
ara-EtUMP	C_2H_5	230
ara-FUMP	F	0.25
dUMP	H	2.0 (K_m)
dTMP	CH_3	16[c]
FdUMP	F	0.01[c]

[a] Kinetic parameters were determined by double-reciprocal plots. [b] K_i values reported were ±10% of those obtained using three or more different inhibitor concentrations. All compounds were competitive inhibitors with respect to dUMP. [c] Data from ref 19.

Results and Discussion

Table I shows the inhibitory properties of a number of 5-substituted *ara*-UMP's, as well as the K_m value for dUMP in the standard assay for dTMP synthetase. Also included for comparison are previously reported reversible K_i values for dTMP and FdUMP.[19] Using a crude preparation of *Escherichia coli* dTMP synthetase, it has been reported that *ara*-UMP has ca. 2% of the substrate activity of dUMP and that *ara*-FUMP is ca. 100-fold less inhibitory than FdUMP.[20] With the *Lactobacillus casei* enzyme we have found that at 25 μM the rate of reaction of *ara*-UMP with CH_2-H_4folate was less than 0.02% that of dUMP. From the data shown in Table I, it can be seen that the 2′ "up" hydroxyl group of the arabinosyl nucleotides tested are indeed detrimental to binding as compared to the corresponding 2′-deoxyribonucleotides. The losses in binding amount to 7.5-fold for *ara*-TMP vs. dTMP, 15-fold for *ara*-UMP vs. dUMP, and 25-fold for *ara*-FUMP vs. FdUMP. There is also a reasonably good cross-correlation between the binding ability of the 5-substituents of *ara*-UMP's and the corresponding dUMP's. In both series it can be seen that the binding affinity decreases as the 5-substituent is changed from fluorine to hydrogen to methyl; the larger 5-ethyl substituent decreases binding only about 2-fold compared to the 5-methyl substituent of *ara*-TMP. We also examined the corresponding 1-β-arabinosyl nucleosides as substrates for the two known mammalian pyrimidine nucleoside phosphorylases. The *ara*-Urd's were all poor or nonsubstrates of dThd phosphorylase; within the limitations of the sensitivity of the assay used,[21] we can say that the rate of phosphorolysis of any of these compounds by this enzyme is less than 1% that of dThd. Urd phosphorylase catalyzed a slow hydrolysis of the *ara*-Urd's examined. Of importance here is the finding that at 0.2 mM, *ara*-FU was converted to FUra by this enzyme at a rate which is 17% that of the natural substrate dUrd.

Knowing that *ara*-FUMP is a reasonably good competitive inhibitor of dTMP synthetase, it was of interest to determine whether it would covalently interact with the enzyme as has previously been described for FdUMP.[22-24]

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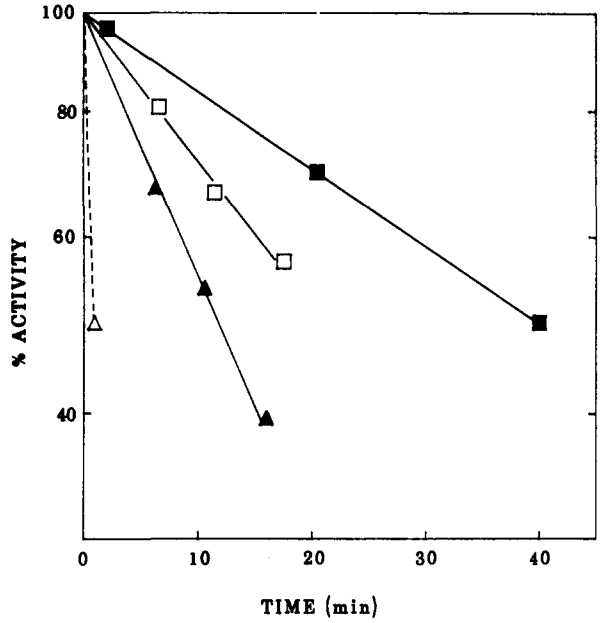

Figure 1. Time-dependent inactivation of dTMP synthetase by 0.32 (■), 0.58 (□), and 1.4 μM (▲) *ara*-FUMP and 0.32 μM (△) FdUMP with 50 μM (±) CH_2-H_4folate at 0 °C. Conditions are provided under Experimental Section.

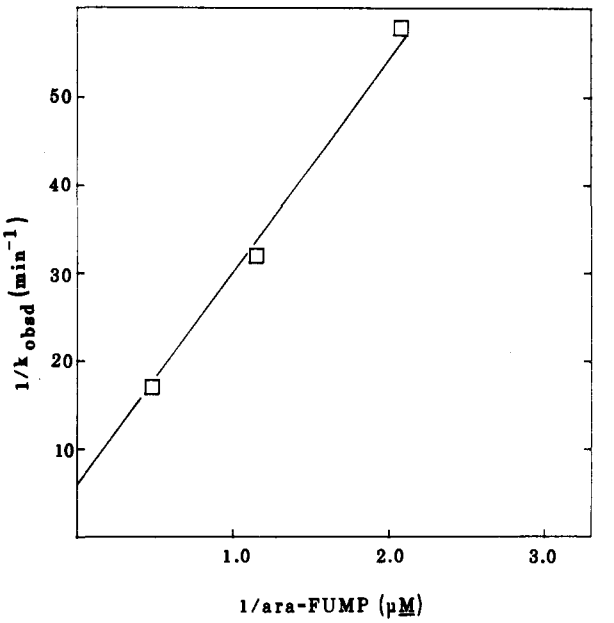

Figure 2. Double-reciprocal plot of $1/k_{obsd}$ for the inactivation of dTMP synthetase vs. 1/*ara*-FUMP. Data are replots of those obtained in Figure 1.

In initial experiments, dTMP synthetase was incubated with FdUMP (0.47 μM) or *ara*-FUMP (0.58 μM) in the presence and absence of the cofactor, CH_2-H_4folate, at 30 °C. As previously reported, FdUMP caused almost complete loss of activity after a 1-min incubation with CH_2-H_4folate. In the presence of CH_2-H_4folate, *ara*-FUMP caused a 70% reduction in activity after a 10-min incubation but no inactivation when the cofactor was omitted. Because the loss of activity was too rapid to conveniently monitor at 30 °C, more complete investigation of the time-dependent inhibition by *ara*-FUMP was performed at lower temperature. Figure 1 shows the time-dependent inactivation of dTMP synthetase caused by *ara*-FUMP in the presence of CH_2-H_4folate at 0 °C (ice bath). As shown, there is a first-order loss of enzyme activity, the rate of which increases with increasing concentration of the inhibitor. It is also noted that at similar concentrations the rate of inactivation by *ara*-FUMP is approximately 40-fold slower than that caused by FdUMP. The simplest model which explains time-dependent inactivation of an enzyme by an irreversible inhibitor is

$$E + I \underset{}{\overset{K_d}{\rightleftharpoons}} E\text{-}I \xrightarrow{k} \overline{E\text{-}I}$$

where K_d is the dissociation constant of the reversible E–I complex and k is the unimolecular rate constant describing inactivation of the E–I complex.[25] This model is described by the expression $1/k_{obsd} = 1/k + K_d/k[I]$, where k_{obsd} is the first-order loss of enzyme activity. Using the data in Figure 1, a plot of $1/k_{obsd}$ vs. $1/[ara\text{-FUMP}]$ (Figure 2) permits calculation of $K_d = 3.6$ μM and $k = 0.22$ min^{-1} at 0 °C. The difference in this K_d value and the K_i determined by initial velocity experiments is attributable to the 30 °C difference in temperature at which these experiments were performed.

The aforementioned experiments clearly demonstrate that, as previously shown for FdUMP,[22-24] *ara*-FUMP causes a time-dependent inactivation of dTMP synthetase

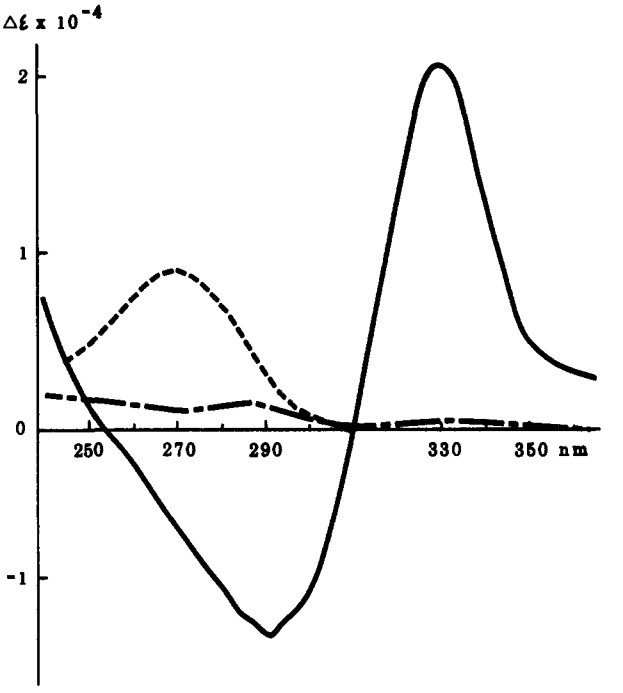

Figure 3. UV difference spectra of the *ara*-FUMP-CH_2-H_4folate–dTMP synthetase complex: (- - -) spectrum of *ara*-FUMP; (—) difference spectrum of *ara*-FUMP-CH_2-H_4folate–dTMP synthetase complex vs. CH_2-H_4folate and enzyme after 30 min; (- - -) difference spectrum of complex after treatment with Na-DodSO_4 for 2 h.

in the presence but not in the absence of the cofactor, CH_2-H_4folate. It was most reasonable at the outset to suspect that the mechanism of inhibition by these two compounds was analogous, and this was demonstrated by UV-difference spectroscopy. Figure 3 shows the difference spectrum obtained when limiting *ara*-FUMP is added to the sample cuvette of two previously balanced cuvettes containing equal amounts of dTMP synthetase. The spectrum obtained is that of *ara*-FUMP (λ_{max} 270 nm) and shows no change for at least 10 min. When equal amounts of CH_2-H_4folate are included in both the sample and reference cuvettes, there is a complete loss of the pyri-

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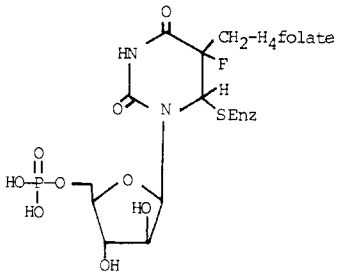

Figure 4. Proposed structure of the *ara*-FUMP–CH_2-H_4folate–dTMP synthetase complex.

midine absorbance at 270 nm, as well as spectral changes attributable to perturbations of chromophores of the cofactor which result from reversible interactions with the enzyme;[23,26] notably, there is a minimum at 292 nm and the appearance of a peak at 330 nm with $\Delta\epsilon$ = 21 000. When this complex is treated with the protein denaturant $NaDodSO_4$ (final concentration = 2%), there is a complete loss of the absorbance differences due to the cofactor (k = 0.09 min^{-1}), without reappearance of the pyrimidine chromophore of *ara*-FUMP. The difference spectrum shown in Figure 3 and its behavior toward $NaDodSO_4$ are exactly analogous to that which has previously been reported for FdUMP[23] and, by analogy, confirms that the *ara*-FUMP–CH_2-H_4folate–dTMP synthetase complex has the structure depicted in Figure 4. As shown, the nucleophilic catalyst of the enzyme is covalently bound to carbon-6 of the pyrimidine, and the 5 position is linked to the one-carbon unit of the cofactor. Using the increase in absorbance at 330 and 340 nm which accompanies formation of this complex, the enzyme (13 nmol/mL) was titrated with *ara*-FUMP in the presence of 0.23 mM 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 0.94 mol of *ara*-FdUMP is bound per site of the dimeric enzyme, with $\Delta\epsilon_{330}$ = 19 800 and $\Delta\epsilon_{340}$ = 13 200 (calculated for each binding site of the enzyme). Thus, the stoichiometry of binding of *ara*-FUMP to dTMP synthetase is also analogous to that reported for FdUMP.[23]

The results described here demonstrate that *ara*-FUMP is a mechanism-based inhibitor of dTMP synthetase which differs from FdUMP only in the reversible K_d values, the kinetics of inactivation, and perhaps in the dissociation constants of the ultimate complexes formed. As such, they are in accord with the proposal that the cytotoxicity of certain prodrugs of *ara*-FC toward *ara*-C resistant cell lines is due to the inhibition of dTMP synthesis by a metabolite of *ara*-FU.[13,14] What remains unclear is why prodrugs of *ara*-FC are effective cytotoxic agents in such cells but *ara*-FC itself is not. The answer to this requires a more precise definition of the enzymatic lesions in *ara*-C resistant lines and identification of the intracellular metabolic products of these prodrugs.

The finding that *ara*-FUMP is a potent, mechanism-based inhibitor of dTMP synthetase may also warrant renewed consideration of possible chemotherapeutic uses for *ara*-FU. *Ara*-FU was originally designed and synthesized as a potential antitumor agent;[27] it was cytotoxic toward murine leukemia tissue culture cells[15] and demonstrated reasonable antitumor activity against transplanted mouse leukemias.[27] However, since it was no more effective in animal models than FUra or FdUrd and required higher doses to achieve the same chemotherapeutic effect, interest in *ara*-FU as a neoplastic agent has diminished. Although *ara*-FU is ca. 50-fold less potent than FUra and ca. 10^4-fold less potent than FdUrd as a growth inhibitor of most tissue culture cells, the mechanism of its cytotoxicity is unknown. The major question to be answered is whether *ara*-FU exerts its cytotoxic effect as a precursor to *ara*-FUMP or FUra. *Ara*-U analogues are poor substrates for mammalian dThd kinase,[28] but minimal conversion of *ara*-FU to *ara*-FUMP by this or another kinase would result in potent inhibition of dTMP synthetase and resultant cytotoxicity. If so, the spectrum of activity of *ara*-FU in animals and humans should be quite different than FUra or FdUrd, which exert at least part of their chemotherapeutic/cytotoxic action via effects of incorporation of FUra into RNA.[29] Alternatively, or perhaps concomitantly, *ara*-FU could simply serve as a depot for FUra. As shown here, *ara*-FU is not a substrate for mammalian dThd phosphorylase but is slowly converted to FUra by Urd phosphorylase at a rate which is sufficient to explain its cytotoxic effects. This is interesting from a chemotherapeutic standpoint because different tissues and tumor cells possess different levels of Urd phosphorylase[30] and thus show different sensitivities toward prodrugs of FUra which require this enzyme for activation. Indeed, this is currently believed to be the reason for the selective cytotoxicity of the new antitumor agent 5′-deoxy-FUrd, a good substrate of Urd phosphorylase.[30] If this is the mechanism responsible for the cytotoxicity of *ara*-FU, a more extensive screening directed toward tumor lines with high Urd phosphorylase activity is clearly warranted. A possible use of *ara*-FU is that of a selective antiviral agent. As previously cited, *ara*-U's are poor or nonsubstrates for mammalian dThd kinase and are thus relatively nontoxic. In contrast, dThd kinase from certain viruses, notably herpes simplex virus, show much less stringent substrate specificity;[28,31] as a result, a number of 5-substituted *ara*-U's are substrates for this enzyme and show promise as potentially selective antiherpetic agents.[28,31,32,35] These differences in substrate specificities of mammalian vs. viral dThd kinase toward *ara*-U's, together with our current finding that *ara*-FUMP is a potent inhibitor of dTMP synthetase, portend the possible usefulness of *ara*-FU as an antiviral agent. In view of the aforementioned, it would clearly be worthwhile to define the biochemical mode of action of *ara*-FU; with modern analytical techniques and the availability of mammalian cells with specific enzyme deficiencies, this should be a straightforward task and is currently in progress in this laboratory.

Experimental Section

dTMP synthetase from a MTX-resistant strain of *L. casei* was the homogeneous preparation previously described.[33] dThd phosphorylase from horse liver was obtained from Gipep Co., Ltd. (Paris, France). Urd phosphorylase, purified from rat liver,[34] was

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a gift from J. Prior. dUMP, FdUMP, and *dl*,L-H_4folate were the preparations previously described.[18,23] *Ara*-U, *ara*-T, *ara*-5-EtU, and corresponding 5′-monophosphates were prepared according to the method of Nakayama et al.[32] *Ara*-FU and 2,2′-anhydro-*ara*-FU were prepared as reported.[36] *Ara*-FUMP was prepared by phosphorylation and hydrolysis of 2,2-anhydro-*ara*-FUMP:[32] 2,2′-anhydro-5-fluorouridine (20 mg) in 1 mL of acetonitrile was treated with tetrachloropyrophosphate (66 μL) at room temperature for 10 h. The acetonitrile was evaporated in vacuo and water (ca. 1 mL) was added to the residue. The aqueous solution was heated at 90 °C for 3 h and was concentrated by evaporation under reduced pressure. The concentrated solution was applied to Toyo filter paper (Toyo 52) and developed with the solvent system 1-propanol–concentrated NH_4OH–0.2 M boric acid, 6:1:3. The UV-absorbing band (R_f 0.25) corresponding to *ara*-FUMP was excised and eluted by water: yield 300 ODU (41%); UV λ_{max} (0.1 N HCl) 270 nm [ϵ(P) = 9400]; paper chromatography R_f 0.28 (isobutyric acid–0.5 M ammonia water, 66:34), 0.25 (1-propanol–concentrated NH_4OH–0.2 M boric acid, 6:1:3); paper electrophoresis, mobility of UMP, 6.5 cm; *ara*-FUMP, 6.5 cm (600 V, 40 min, 30 mM sodium acetate, pH 4.0); CD (molecular ellipticity) +22600 (271 nm). The product was converted to *ara*-FU upon treatment with alkaline phosphatase, and was resistant to P_1 nuclease–3′-nucleotidase. HPLC using Lichrosorb C_{18} (4.0 × 250 mm) verified the purity of all compounds used in this study. For nucleosides, the eluant was 1% CH_3CN in water; for nucleotides, 5 mM $Bu_4N^+HSO_4^-$, 5 mM $KHPO_4$ (pH 7.1) containing 1% MeOH was used. Using these systems, arabinofuranosyl analogues are well separated from their ribo and 2′-deoxyribosyl counterparts.

Kinetic assays of dTMP synthetase activity were performed spectrophotometrically at 30 °C under conditions previously described.[19] The assay mixture (1.2 mL) contained 50 mM *N*-methylmorpholine hydrochloride (pH 7.4), 25 mM $MgCl_2$, 1.0 mM EDTA, 75 mM 2-mercaptoethanol, 0.20 mM *dl*,L-CH_2-H_4folate, 2.5 mM H_2CO, 3.5–20 μM dUMP, specified amounts of the inhibitor (0.01 to 0.2 mM), and ca. 2 nM dTMP synthetase; reactions were initiated by the addition of enzyme and initial velocities were determined over the first 1–3 min. For experiments on time-dependent inactivation of dTMP synthetase, a solution (ca. 1 mL) containing 0.1 μM dTMP synthetase, 50 mM NMM·HCl (pH 7.4), 0.1 mM CH_2-H_4folate, and specified amounts of the nucleotide to be tested was incubated at 0 or 30 °C. At specified intervals, 100 μL aliquots of this solution were added to the standard assay mixture, and the initial velocity was determined. Assays of dThd and Urd phosphorylase were performed using the standard assay conditions and spectrophotometric assay previously reported for dThd phosphorylase.[21]

Ultraviolet difference spectra were obtained by a modification of the procedure described previously.[23] Two cuvettes containing in 0.9 mL 50 μmol of NMM·HCl (pH 7.4), 10 μmol of $MgCl_2$, 1 μmol of EDTA, 5 μmol of DTT, 26 nmol of dTMP synthetase, 30 nmol of (±)-H_4folate, and 0.5 μmol of H_2CO were placed in the reference and sample compartments (30 °C) of a Cary 118 recording spectrophotometer. After the base line was recorded, 100 μL of 10 μM *ara*-FUMP (10 nmol) was added to the sample cuvette and 100 μL of water to the reference cuvette, and the difference spectra of the native complex was recorded. For difference spectra of the denatured complex, 75 μL of 30% Na$DodSO_4$ was added to each of the above cuvettes, and repetitive scans of the spectrum were obtained until no further changes occurred. For the titration of the enzyme, an analogous procedure was used, except that aliquots of the enzyme and CH_2-H_4folate were 13 nmol and 0.23 μmol, respectively; spectra were recorded after the addition of 5-nmol increments of *ara*-FUMP from 5 to 60 μmol, and appropriate corrections were made for dilutions.

Acknowledgment. This investigation was supported by Grant CA 14394 from the National Cancer Institute, DHEW.

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Pyrazolopyrimidine Nucleosides. 12. Synthesis and Biological Activity of Certain Pyrazolo[3,4-*d*]pyrimidine Nucleosides Related to Adenosine[1]

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Received April 24, 1981

The chemical synthesis of certain 4-substituted pyrazolo[3,4-*d*]pyrimidine nucleosides is described. Using 1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidin-4-one (1) as the starting material, the reactive intermediate 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (2) was prepared in excellent yield. Compound 2 served as a versatile precursor for the synthesis of a number of 4-substituted pyrazolo[3,4-*d*]pyrimidine nucleosides. In antitumor studies of these nucleosides, in vitro and in vivo, it was found that any alteration of the 4-amino substituent of 4-amino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine (3) was accompanied by a significant decrease or loss of antitumor activity. On the other hand, introduction of certain substituents at the 3 position of 3 (synthesis reported previously) led to a dramatic increase in antitumor activity in comparison to the parent compound.

Several 6-substituted adenosine derivatives have been reported[2] to possess antitumor activity. On the other hand, there has been a paucity of studies[3] involving the synthesis and biological evaluation of the structurally related 4-substituted derivatives of 4-amino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine (4-APPR). The adenosine

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