Journal of Medicinal Chemistry

© *Copyright 1980 by the American Chemical Society*

Volume 23, Number 2 February 1980

Perspective

Perspectives on the Design and Biochemical Pharmacology of Inhibitors of Thymidylate Synthetase

Daniel V. Santi

Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received November 5, 1979*

dTMP[1] synthetase (EC 2.1.1.45) catalyzes the reductive methylation of dUMP to dTMP with concomitant conversion of CH_2-H_4folate to H_2folate. This enzyme is

$$\text{dUMP} + \text{CH}_2\text{-H}_4\text{folate} \rightarrow \text{H}_2\text{folate} + \text{dTMP}$$

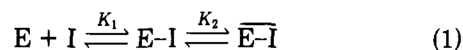
unique among those which utilize H_4folate cofactors in that CH_2-H_4folate serves the dual function of both one-carbon donor and reductant. As a result, the synthesis of dTMP consumes an equivalent amount of H_4folate, and the dihydrofolate reductase catalyzed conversion of H_2folate to H_4folate is required for continued dTMP synthesis, as well as purine biosynthesis. Since dTMP synthetase represents the sole de novo pathway for dTMP synthesis, it is apparent that blockade of its activity would have dramatic effects on proliferating cells. Indeed, inhibition of dTMP production promotes a complex response in cells in which DNA synthesis is impaired in the face of continuing protein and RNA synthesis, a phenomenon termed "thymineless death".[2]

Many workers have been involved in investigations of various aspects of dTMP synthetase, and the biochemistry, mechanism, and inhibition of this enzyme have been extensively reviewed.[3,4] The purpose of this article is to present one investigator's perspective of approaches used to design analogues of dUMP as mechanism-based inhibitors of dTMP synthetase and to possible approaches for modulating the effects of known inhibitors to achieve desired biochemical effects.

Analogues of dUMP as Inhibitors of dTMP Synthetase

In recent years, there has been much interest in the development of mechanism-based inhibitors of enzymatic reactions. These inhibitors reversibly bind to the active site of an enzyme and then undergo events in a manner analogous to one or more steps of the normal catalytic reaction, ultimately leading to formation of a covalent enzyme–inhibitor complex. Although the combined requirements of reversible binding and enzyme-induced covalent bond changes place restrictions on the design of such inhibitors, once these are fulfilled the resultant inhibitors are extremely specific for their target enzymes. Description of approaches used to design mechanism-based inhibitors of dTMP synthetase requires consideration of the binding properties of dUMP analogues to the enzyme and the catalytic mechanism of dTMP synthetase.

For optimal efficacy, mechanism-based inhibitors of dTMP synthetase should be derived from dUMP analogues which form tight, reversible complexes with the enzyme. Tight reversible binding will enhance specificity and, in most cases, decrease the concentration of inhibitors required to obtain a maximal rate of inactivation (viz., formation of a covalent complex). Moreover, since the covalent complex ($\overline{\text{E-I}}$) formed with many mechanism-based inhibitors of dTMP synthetase is reversible (eq 1),



$$\text{E} + \text{I} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{E-I} \overset{K_2}{\rightleftharpoons} \overline{\text{E-I}} \quad (1)$$

the overall dissociation constant is the product of the dissociation constant (K_1) of the reversible complex (E–I) and the equilibrium constant (K_2) which describes the ratio of covalent and noncovalent complexes; as such, the stability of the covalent complex is directly related to that of the noncovalent complex, E–I (eq 2). For such inhib-

$$(\text{E})(\text{I})/(\overline{\text{E-I}}) = K_1K_2 \quad (2)$$

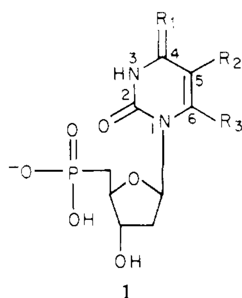
itors, efforts expended in obtaining maximal reversible binding of the parent dUMP analogue are clearly worth-

(1) The following abbreviations are used. Folate derivatives: H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; CH_2-H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; 5-CHO-H_4folate, 5-formyl-5,6,7,8-tetrahydrofolate; MTX, methotrexate. Pyrimidines: Ura, uracil; FUra, 5-fluorouracil; NO_2Ura, 5-nitrouracil; OA, orotic acid. Nucleosides: dUrd, 2′-deoxyuridine; dThd, thymidine; FdUrd, 5-fluoro-2′-deoxyuridine; BrdUrd, 5-bromo-2′-deoxyuridine; NO_2dUrd, 5-nitro-2′-deoxyuridine; CF_3dUrd, 5-(trifluoromethyl)-2′-deoxyuridine; Urd, uridine. Nucleotides: dTMP, 2′-deoxythymidylate; dUMP, 2′-deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; NO_2dUMP, 5-nitro-2′-deoxyuridylate; CF_3dUMP, 5-(trifluoromethyl)-2′-deoxyuridylate; $CF_3CH{=}CH$dUMP, *trans*-5-(3,3,3-trifluoro-1-propenyl)-2′-deoxyridylate; FUMP, 5-fluorouridylate; OMP, orotidylic acid. Miscellaneous: dR-1-P, 2-deoxyribose 1-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate.

(2) Cohen, S. S. *Ann. N.Y. Acad. Sci.* **1971**, *186*, 292.
(3) (a) Pogolotti, A. L., Jr.; Santi, D. V. in "Bioorganic Chemistry", E. E. Van Tamelen, Ed.; Academic Press: New York, 1977; Vol. 1, p 277. (b) Friedkin, M. *Adv. Enzymol.* **1973**, *38*, 235.
(4) Danenberg, P. V. *Biochim. Biophys. Acta* **1977**, *473*, 73.

0022-2623/80/1823-0103$01.00/0 © 1980 American Chemical Society

while. Although a thorough, systematic study of the factors required for optimal reversible binding of dUMP analogues has not been performed, diverse observations permit a number of generalizations to be made. Structure 1 is a



composite of essential binding points and positions on dUMP where substitutions might be made to enhance binding to dTMP synthetase. At the present time, it appears that the 5′-phospho-2′-deoxyribosyl moiety of dUMP is required for optimal binding. Virtually all modifications in this region which have thus far been examined have resulted in substantial decreases in affinity for the enzyme. Perhaps more in-depth studies will reveal permissible alterations of the sugar–phosphate moiety which will confer desirable properties on such analogues, but more substantial immediate findings probably will result from modifications of the pyrimidine base. As indicated in 1, the intact 2-keto and 3-NH groups of the heterocycle appear to be important for binding. Most modifications at the 4 position induce tautomerization to the "enol" form of the heterocycle and loss of the proton from the 3 nitrogen. Although it is not known which of these factors is responsible, large decreases in the affinity for dTMP synthetase results. In contrast, 4-thio-dUMP (1, R_1 = S) is a substrate for the enzyme with K_m = 70 μM,[5] and N^4HOdCMP (1, R_1 = NOH) is a very effective inhibitor.[6] Since the tautomeric form of these analogues may be analogous to the 4-keto form of dUMP, it is reasonable to suggest that modifications at the 4 position may be tolerated provided the proper tautomeric structure and the 3-NH are retained. dUMP's that are 5 substituted have been extensively investigated as inhibitors of dTMP synthetase and have proven to be most effective. Electron-withdrawing substituents at this position greatly enhance binding by increasing the acidity of the 3-NH and/or by affecting the electronic properties of the heterocycle.[7-9] To this author's knowledge, 6-substituted dUMP's have not been examined as inhibitors of this enzyme but represent prime candidates as potential inhibitors. Since much is already known regarding the effects of substitutions at the 5 position of dUMP, it appears that the most relevant directions to pursue at this time would involve systematic studies of the effects of modifications at the 4 and 6 positions. If substituents at the 6 position of dUMP are tolerated by the enzyme, this position could be appropriately modified in search of favorable interactions adjacent to the active site in the manner described by Baker.[10] Moreover, electron-withdrawing groups might

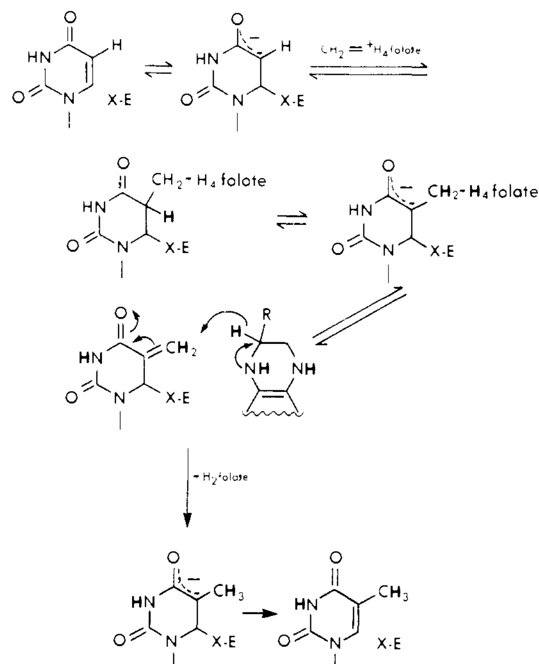


Figure 1. Catalytic mechanism of dTMP synthetase. See ref 3a and 11.

confer similar effects on the heterocycle as do 5 substituents and significantly enhance binding. It also might be fruitful to examine analogues in which the 4-oxo group of dUMP is replaced by other substituents which favor the "keto" form of the heterocycle rather than the "enol" form. Candidate compounds in this class might include *O*-alkyl and *O*-aryl derivatives of N^4-hydroxy-dCMP (1, R_1 = NOH), as well as analogues of N^4-amino-dCMP (R^1 = NNH_2). Here, the objective would be to first ascertain the extent of structural modification tolerated by the enzyme at this position and then to attempt to increase the affinity of such compounds by substituents which favorably interact with regions of the enzyme adjacent to the active site. Once the necessary information is obtained regarding the properties of monosubstituted dUMP's, it should be a simple task to construct analogues which possess a combination of groups at the 4, 5, and 6 positions of dUMP which would optimize reversible binding to dTMP synthetase. With appropriate considerations (see the following discussion), such analogues also could be designed as mechanism-based inhibitors of dTMP synthetase.

A knowledge of catalytic features of the mechanism of an enzyme often provides an approach to the design of mechanism-based inhibitors; likewise, mechanism-based inhibitors are most useful tools in elucidating aspects of enzyme catalysis. The currently accepted minimal mechanism of dTMP synthetase is depicted in Figure 1.[3a,11] It is most relevant to the topic of this paper that an early event in catalysis involves attack of a nucleophile of the enzyme at the 6 position of dUMP; as a consequence, a variety of 5,6-dihydropyrimidine intermediates are formed which remain covalently bound to the enzyme throughout the catalytic sequence and serve to activate moieties of dUMP which normally are inert. This early step of the reaction provides the basis for the design of mechanism-based inhibitors of this enzyme. After formation of a noncovalent reversible complex, a nucleophile of the en-

(5) Kalman, T. I.; Bloch, A.; Szekeres, G. L.; Bardos, T. J. *Biochem. Biophys. Res. Commun.* **1973**, *55*, 210.
(6) Lorenson, M. Y.; Maley, G. F.; Maley, F. *J. Biol. Chem.* **1967**, *242*, 3332.
(7) Wataya, Y.; Santi, D. V.; Hansch, C. *J. Med. Chem.* **1977**, *20*, 1469.
(8) Kalman, T. I.; Bardos, T. J. *Mol. Pharmacol.* **1970**, *6*, 621.
(9) Ellis, P. D.; Dunlap, A. L.; Pollard, K.; Seidman, K.; Cardin, A. D. *J. Am. Chem. Soc.* **1973**, *95*, 4398.
(10) Baker, B. R. "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley: New York, 1967.
(11) Pogolotti, A. L., Jr.; Santi, D. V. *Biochemistry* **1974**, *13*, 456.

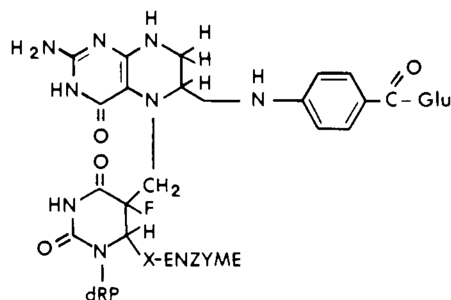


Figure 2. Structure of the FdUMP-CH_2-H_4folate-dTMP synthetase complex.

zyme attacks the 6 position of the heterocycle of the inhibitor in a manner directly analogous to the normal enzymatic reaction. After formation of a noncovalent reversible complex, a nucleophile of the enzyme attacks the 6 position of the heterocycle of the inhibitor in a manner directly analogous to the normal enzymatic reaction. The inhibitors either remain covalently attached to the nucleophile of the enzyme or covalent bond formation may activate a latent chemically reactive group at the 5 position of the heterocycle which subsequently inactivates the enzyme.

The prototype mechanism-based inhibitor of dTMP synthetase is FdUMP.[3a,4,12] In the absence of the cofactor, binding of FdUMP is noncovalent and weak, with $K_i \simeq 10^{-5}$ M. However, in the presence of CH_2-H_4folate, a series of reactions occurs in a manner analogous to the first two steps depicted for the normal enzymatic reaction (Figure 1). At this stage, the C-5 proton of the covalently bound substrate, dUMP, is removed in the normal enzymatic reaction; however, the C–F bond of the analogous intermediate formed with FdUMP cannot be broken, and an analogoue of a steady-state intermediate accumulates. As shown in Figure 2, the 6 position of FdUMP and a nucleophile of the enzyme are covalently bound, and the cofactor, CH_2-H_4folate, is linked to the 5 position of FdUMP. The covalent complex is slowly reversible with a $t_{1/2}$ for dissociation of ca. 10 h, and the K_d has been estimated to be ca. 10^{-12} M, some 10^{-7} lower than that of the binary enzyme–FdUMP complex. Elucidation of the structure of the FdUMP–CH_2-H_4folate–enzyme complex provided strong support for the catalytic mechanism of this enzyme, which previously was implicated only from studies of model chemical counterparts.

Knowledge of the mechanism of inhibition by FdUMP and the features required for reversible binding of dUMP analogues to dTMP synthetase provide the basis for rational design of other mechanism-based inhibitors of this enzyme. This is exemplified by 5-nitro-2′-deoxyuridylate (NO_2dUMP), a compound which was predicted to be both a potent reversible inhibitor of the enzyme and a mechanism-based inhibitor. Studies of the inhibitory properties of a number of 5-substituted 2′-deoxyuridylates revealed that electron-withdrawing substituents greatly increased the reversible affinity of such analogues for dTMP synthetase.[7] From a quantitative structure–activity relationship developed for such compounds, it was predicted that NO_2dUMP would reversibly bind to the enzyme with a very high affinity. Further, since the electron-withdrawing NO_2 moiety also positively polarizes the 6 position of the heterocycle, it was anticipated that nucleophilic attack at that site would be facilitated and that the inhibitor might covalently bind to the nucleophilic catalyst of dTMP synthetase. The reactivity of the 6 position of

(12) Heidelberger, C. *Handb. Exp. Pharmakol.* 1974, *38*, 193.

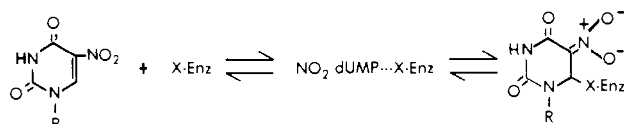
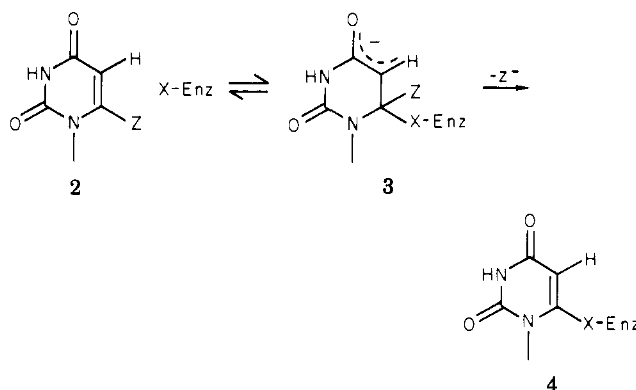


Figure 3. Proposed mechanism of interaction of NO_2dUMP with dTMP synthetase; R = 5-phospho-2-deoxyribosyl.

Scheme I



NO_2dUMP was confirmed by the report that 1,3-dimethyl-5-nitrouracil reacts with a variety of nucleophiles at the 6 position to form the corresponding 5,6-dihydropyrimidines with favorable equilibrium constants.[13] Two research groups have reported that NO_2dUMP is a mechanism-based inhibitor of dTMP synthetase.[14,15] As shown in Figure 3, the interaction involves formation of a reversible noncovalent enzyme–inhibitor complex, with K_i = 23 nM, followed by formation of a thermodynamically favorable reversible covalent bond between a nucleophile of the enzyme and the 6 position of NO_2dUMP. Unlike FdUMP, the interaction of NO_2dUMP with dTMP synthetase neither requires nor is facilitated by the presence of CH_2-H_4folate. This is not surprising since if, as in the ternary FdUMP–CH_2-H_4folate–dTMP synthetase complex, the cofactor were attached to the 5 position of the covalently bound NO_2dUMP the resonance stabilization provided by the 5-*aci*-nitro moiety would be precluded and the stability of the complex would be decreased. NO_2dUMP represents the first mechanism-based inhibitor of dTMP synthetase which was designed on the basis of combined knowledge of binding and mechanistic properties of the enzyme and of chemical properties of the inhibitor. The relative simplicity of the interaction and properties of the NO_2dUMP–enzyme complex indicate that this inhibitor will be a most useful tool for future investigations of this enzyme. Moreover, further insight is provided as to how the design of similar mechanism-based inhibitors of this enzyme might be approached. In general, these should be analogues of dUMP possessing electron-withdrawing substituents at the 5 and/or 6 position(s) which (a) facilitate formation of a reversible binary complex, (b) enhance the susceptibility of the 6 carbon to nucleophilic attack, and (c) stabilize the resultant adduct.

Providing that substitution at the 6 position of dUMP does not prevent reversible binding to the enzyme or nucleophilic attack at the 6 carbon of the heterocycle, a new type of mechanism-based inhibitor should be feasible. As

(13) Pitman, I. H.; Cho, M. J.; Rork, G. S. *J. Am. Chem. Soc.* 1974, *96*, 1840.

(14) Matsuda, A.; Wataya, Y.; Santi, D. V. *Biochem. Biophys. Res. Commun.* 1978, *84*, 654.

(15) Mertes, M. P.; Chang, C. T.-C.; De Clercq, E.; Huang, G.-F.; Torrence, P. F. *Biochem. Biophys. Res. Commun.* 1978, *84*, 1054.

Scheme II

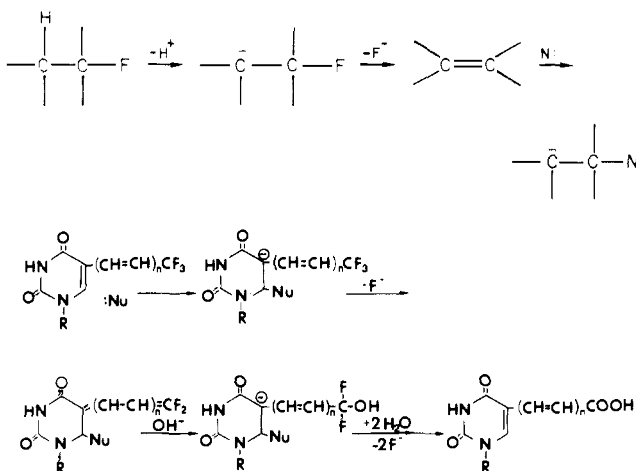
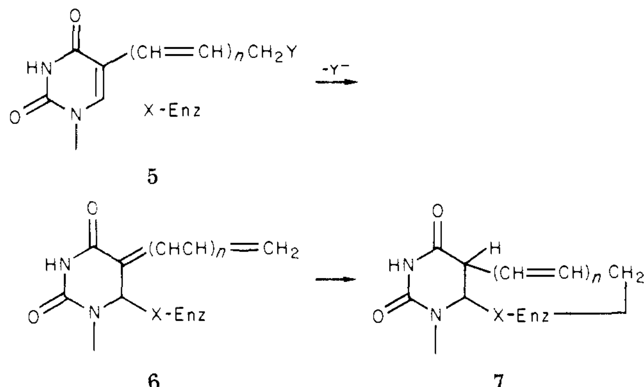


Figure 4. General mechanism of hydrolysis of 5-CF_3 (n = 0) and 5-$CF_3CH{=}CH$dUrd (n = 1); R = 2-deoxyribosyl. See ref 17 and 19.

shown in Scheme I, these would be analogues of dUMP which possess a reasonably good leaving group at C-6. The electronic effect of the substituent should be such that C-6 is sufficiently reactive to form a covalent bond with the juxtaposed nucleophile in the E–I complex, but not so electrophilic that it nonspecifically reacts with solvent or solute nucleophiles. Nucleophilic attack at C-6 by the enzyme would provide the carbanion **3**, which, barring stereochemical restraints, could undergo a β-elimination to provide dUMP covalently bound to the enzyme (**4**). Such covalent enzyme–inhibitor complexes would differ from those formed with FdUMP and NO_2dUMP in that the 6 position of the bound pyrimidine would be sp^2 hybridized and the complex would not be reversible. Judicious use of 5 substituents could also alter the stability of the incipient carbanion at the 5 position and thus modify the reactivity of such inhibitors. Although a number of factors might preclude the effectiveness of such analogues as inhibitors of dTMP synthetase, their potential usefulness as chemotherapeutic agents and as probes for further studies of enzyme mechanism warrants their serious consideration.

A different class of mechanism-based inhibitors of dTMP synthetase is based on the principle that nucleophilic attack at the 6 position of dUMP analogues may activate a latent chemically reactive group at the 5 position of the heterocycle. This type of inhibitor is exemplified by CF_3dUMP, which was first reported to irreversibly inactivate the enzyme from Erlich ascites cells.[16] Although carbon–fluorine bonds are generally quite strong, when a transient or stable negative charge exists on the carbon adjacent to a C–F bond, elimination of fluoride ion may occur as depicted in Scheme II.[17] The resultant olefinic carbon, originally bearing the fluorine atom, may undergo attack by a nucleophile, and if additional fluorine atoms are attached to this carbon, successive elimination–addition reactions result in cleavage of all C–F bonds. In addition, C–F bonds which are labilized by this mechanism may be extended by carbon–carbon double bonds and retain their reactivity. The activation of CF_3 moieties of 1-substituted 5-CF_3-[18] and 5-$CF_3CH{=}CH$-uracils[19] has been shown to

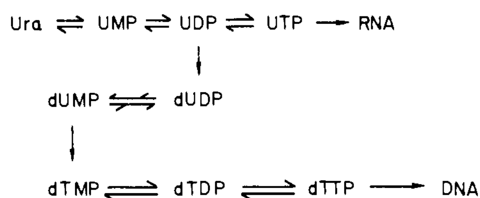
Scheme III



proceed by the aforementioned mechanism; the pathway leading to hydrolysis of the CF_3 moiety of such compounds is depicted in Figure 4. The first step involves addition of a nucleophile to the 6 position to generate an incipient negative charge at C-5. This activates the latently reactive CF_3 group which undergoes a series of three elimination–addition reactions to provide the corresponding carboxylic acid. In the interaction of CF_3dUMP with dTMP synthetase, the normally inert CF_3 group would behave as an acylating agent only when a nucleophile is first added to the 6 position of the heterocycle to produce a transient carbanion at C-5. Using dTMP synthetase from *Lactobacillus casei*, it has been demonstrated that the enzyme catalyzes the release of fluoride ion from CF_3dUMP and produces a product which has a UV spectrum similar to 5-acyl derivatives of dUMP; in the process, the enzyme is inactivated.[20] Although the interaction of CF_3dUMP and dTMP synthetase is much more complicated than originally suspected, it is clear that nucleophilic attack occurs at the 6 position of the inhibitor which activates the 5-CF_3 moiety for subsequent inactivation of the enzyme. As previously mentioned, C–F bonds which are labilized by β-carbanion activation can be extended by vinylogous conjugation and retain their reactivity.[17] From the standpoint of designing mechanism-based irreversible inhibitors, this could be a very important property. An enzyme might have the mechanistic features necessary to activate a C–F bond, but if there is no nucleophile in close proximity, hydrolysis rather than covalent attachment to the enzyme would occur. By vinylogous activation, the C–F moiety could be extended to *reach out* until it is suitably juxtaposed to a nucleophile of the enzyme to react with it; of course, this assumes the enzyme will accept this structural modification. As with CF_3dUMP, $CF_3CH{=}CH$dUMP caused a time-dependent inactivation of dTMP synthetase and the UV spectrum indicated that the CF_3 moiety had been transformed by the enzyme to an acyl derivative.[19] While these results are too preliminary to allow definitive mechanistic conclusions, they clearly demonstrate an irreversible or pseudoirreversible inhibition of dTMP synthetase which is initiated by nucleophilic attack at the 6 position of $CF_3CH{=}CH$dUMP, followed by activation of the CF_3 moiety; current evidence suggests that the mechanism of inhibition by this analogue is similar to that of CF_3dUMP. The principles responsible for the inactivation of dTMP synthetase by 5-CF_3- and 5-$CF_3CH{=}CH$dUMP should be directly applicable to other 5-substituted dUMP's which might be activated by the

(16) Reyes, P.; Heidelberger, C. *Mol. Pharmacol.* **1965**, *1*, 14.
(17) Sakai, T. T.; Santi, D. V. *J. Med. Chem.* **1973**, *16*, 1079.
(18) Santi, D. V.; Sakai, T. T. *Biochemistry* **1971**, *10*, 3598.
(19) Wataya, Y.; Matsuda, A.; Santi, D. V.; Bergstrom, D. E.; Ruth, J. L. *J. Med. Chem.* **1979**, *22*, 339.
(20) Santi, D. V.; Pogolotti, A. L., Jr.; James, T. L.; Wataya, Y.; Ivanetich, K. M.; Lam, S. S. M. *ACS Symp. Ser.* **1976**, *no. 28* 57.

Scheme IV



presence of an adjacent carbanion produced upon nucleophilic attack at the 6 position. For example, as shown in Scheme III, compounds of general structure **5**, possessing an intrinsically unreactive leaving group Y, would upon nucleophilic attack at the 6 position form a reactive alkylating agent **6**; should another nucleophile of the enzyme be close to the electrophilic carbon atom, irreversible inhibition would result. Structure **6**, $n = 0$, is identical with an intermediate formed in the normal enzymatic reaction (Figure 1); as such, **5**, $n = 0$, would be of interest for mechanistic studies of this enzyme but should not be considered as a candidate inhibitor. As with all mechanism-based inhibitors, the reactivity of the leaving group must be properly balanced so it is not reactive toward solvent and solute nucleophiles but sufficiently reactive when activated by an adjacent carbanion so that an electrophilic carbon will be produced on the active site of the enzyme. This should not be a difficult task, since even ethers of 5-(hydroxymethyl)uracils readily eliminate alkoxide ion upon formation of a carbanion at the 5 position of the heterocycle.[11]

Biochemical Pharmacology of Inhibitors of dTMP Synthetase

From the above, it is clear that approaches now exist which permit the rational design of analogues of dUMP as inhibitors of dTMP synthetase. Such inhibitors have proven to be most useful tools for studies of biochemical aspects of this enzyme,[3a] and progress in this area is expected to continue. Of course, one of the primary reasons for the continuing interest in inhibitors of dTMP synthetase is their potential as chemotherapeutic agents. The remainder of this paper presents views on aspects of biochemical pharmacology which are necessary for obtaining and modulating the in vivo effectiveness of such inhibitors.

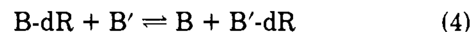
Primary consideration must be given to achieving an intracellular concentration of the analogue sufficient to inhibit dTMP synthetase. Since cells are poorly permeable to nucleotides, the inhibitors as such are unlikely to demonstrate the in vivo effects expected of inhibition of dTMP synthetase. Although carrier systems such as liposomes or prodrugs appear to be attractive approaches for circumventing this problem, it is more practical at this time to rely on the use of pyrimidine bases and nucleosides as precursors of the active 2′-deoxyribonucleotides.

Metabolism of Analogues of Uracil. Metabolic conversion of a Ura analogue to its corresponding 2′-deoxyribonucleotide should, in most cases, follow that of Ura (Scheme IV) and require at least four enzyme-catalyzed reactions. Since each of the enzymes involved has a unique substrate specificity, a considerable barrier exists which will prevent activation of all but a few such analogues. Indeed, it appears that a close structural resemblance to natural substrates is required, since 5-FUra is one of the few Ura analogues which can be converted to the corresponding 2′-deoxyribonucleoside via this pathway. The first enzyme in this pathway has been referred to as pyrimidine phosphoribosyltransferase; in mammalian cells this activity is associated with orotate phosphoribosyltransferase (OPRTase) which catalyzes the condensation of orotic acid (OA) and 1-phosphoribosyl 5-pyrophosphate (PRPP) to give OMP. If OPRTase were the only barrier in this pathway, the corresponding ribonucleoside could serve as a precursor, providing it were a substrate for Urd kinase; while this enzyme is probably less specific than the pyrimidine phosphoribosyltransferase, the same number of barriers exist as in the metabolism of pyrimidine bases, and the probability of discovering ribonucleoside analogues which are effective precursors of deoxyribonucleotides must be considered low. Even if pyrimidine bases or ribonucleosides were found which are effectively metabolized by this pathway, the intermediates formed and products of branch points could result in biological effects unrelated to inhibition of dTMP synthetase. This is well illustrated by the extensive incorporation of FUra into RNA which occurs in most cells and has led to much controversy regarding the importance of dTMP synthetase inhibition to the mechanism of action of this drug. Thus, it would appear that the rational design of pyrimidine bases or ribonucleosides which will provide analogues of dUMP by the pathway shown in Scheme IV requires considerable knowledge of the substrate properties of each of the enzymes involved. Until such information is available, trial and error remains the only viable approach for obtaining suitable substrates for this metabolic pathway. In the opinion of this author, a more fruitful approach for developing precursors of dUMP analogues would involve attempts at utilizing metabolic routes other than that which has thus far been described.

Pyrimidine bases may also be directly converted to their 2′-deoxyribonucleosides by the salvage enzyme dThd phosphorylase. As shown in eq 3, the reaction involves a

$$\mathrm{B + dR\text{-}1\text{-}P \rightleftharpoons B\text{-}dR + P_i} \quad (3)$$

reversible reaction of a pyrimidine base (B) and 2-deoxyribose phosphate (dR-1-P) to give the corresponding 2′-deoxyribonucleoside (B-dR). In addition, the enzyme also catalyzes a direct transfer of the sugar moiety of a pyrimidine 2′-deoxyriboside (B-dR-1-P) to a pyrimidine base (B′) (eq 4).[21,22] It is noted that Urd phosphorylase cata-



$$\mathrm{B\text{-}dR + B' \rightleftharpoons B + B'\text{-}dR} \quad (4)$$

lyzes similar reactions, but this enzyme is substantially less tolerant of large substituents at the 5 position of the pyrimidine heterocycle. In constrast, a wide variety of 5-substituted Ura's and dUrd's are excellent substrates for dThd phosphorylase (unpublished results). Moreover, the catalytic efficiency of this enzyme is enhanced by electron-withdrawing groups at the 5 position, which is an important factor in the design of analogues of dUMP as inhibitors of dTMP synthetase. Thus, pyrimidine precursors of 5-substituted dUMP's can be converted to the corresponding 2′-deoxynucleoside via this reaction, and if the nucleoside is phosphorylated by dThd kinase the active dUMP analogues would be produced in a sequence of only two enzymatic reactions. (The substrate properties of 4- and 6-substituted Ura's for dThd phosphorylase remain to be determined.) This pathway of activation is also worthy of consideration because different tissues possess varying amounts of dThd phosphorylase in a manner unrelated to the dTMP synthetase levels or the proliferative activity of cells;[23] as a result, tissue selectivity might be achieved in the activation of pyrimidine analogues to the active dUMP analogues. It is clear that the intracellular

(21) Bose, R.; Yamada, E. W. *Biochemistry* **1974**, *13*, 2051.
(22) Gallo, R. C.; Breitman, T. R. *J. Biol. Chem.* **1968**, *243*, 4936.
(23) Zimmerman, M.; Seidenberg, J. *J. Biol. Chem.* **1964**, *239*, 2618.

levels of dR-1-P are so low that, under normal circumstances, dThd phosphorylase cannot be very important in the synthesis of 2′-deoxyribonucleosides in most cells; rather, the major role of this enzyme in drug metabolism probably involves catabolism of 2′-deoxynucleoside substrates. However, if cells possessing this activity could be provided with a sufficient supply of a suitable donor of the 2′-deoxyribose moiety, a facile conversion of Ura analogues to the corresponding 2′-deoxyribonucleosides might be induced. Indeed, a number of reports have appeared which support the feasibility of this approach.[24-26] One source of a 2-deoxyribosyl moiety is the naturally occurring purine 2′-deoxyribonucleosides, which are effective substrates for purine nucleoside phosphorylase and would provide intracellular dR-1-P. Although it may be difficult to obtain or maintain a sufficiently high intracellular level of dR-1-P to effectively serve as a substrate for dThd phosphorylase, this approach certainly warrants further experimentation. The other source of the 2′-deoxyribosyl moiety is dUrd or appropriate 5-substituted analogues of dUrd which are themselves substrates of dThd phosphorylase. These have the advantage that a direct transfer of the sugar moiety to the pyrimidine analogue could occur (eq 4),[21,22] avoiding the necessity of maintaining a high intracellular concentration of the labile dR-1-P. Further, high concentrations of the donor (B-dR-1-P), would favor the formation of the desired dUrd analogue (B′-dR-1-P) and protect it from degradation (eq 4). The caveat here is that suitable donors might also compete with the nucleoside analogue for dThd kinase or the phosphorylated donor might compete with the active dUMP analogue for inhibition of dTMP synthetase. Clearly, many complications can be conjured which might discourage attempts at using dThd phosphorylase for activation of Ura analogues; however, since the in vivo behavior of enzymes cannot be predicted with certainty, it would be judicious to ascertain whether this approach is feasible through experimentation. Here, the objective should be to obtain donors of 2′-deoxyribose which induce rather than prevent formation of the desired dUMP analogue. For this purpose, tissue culture cells possessing dThd phosphorylase could be treated with radioactive FUra and a variety of candidate donors of the deoxyribosyl moiety (FUra is an excellent substrate for dThd phosphorylase and the product FdUrd is readily converted to FdUMP by dThd kinase). Quantitation of FdUMP and/or the FdUMP–dTMP synthetase complex formed in vivo[27] would permit direct assessment of the efficiency of various deoxyribosyl donors in affecting the net conversion of FUra to FdUMP.

Metabolism of Analogues of dUrd. Deoxyribonucleoside analogues of dUrd are excellent precursors of mechanism-based inhibitors of dTMP synthetase, since they only require phosphorylation by a single enzyme, dThd kinase, for activation to the dUMP analogue. Mammalian dThd kinase specifically phosphorylates the 5′ position of 2′-deoxyribonucleosides of pyrimidine-2,4-diones and will tolerate a variety of substituents at the 5 position, providing they are not too bulky. As a result, analogues such as BrdUrd, FdUrd, NO_2dUrd, and CF_3dUrd are readily converted to their 5′-monophosphates in tissue culture cells. In contrast to their effects on tissue culture cells, such 2′-deoxyribonucleoside derivatives have not found popular use as anticancer agents. In the treatment of animal or human neoplasia, these nucleosides appear to be hydrolyzed to the corresponding pyrimidine bases more rapidly than they are converted to the active dUMP analogues. For example, FUra is cytotoxic to tissue culture cells with the concentration required for 50% inhibition of cell growth (EC_{50}) being about 1 μM. Although FUra does serve as a precursor of FdUMP, it is also extensively incorporated into RNA[12] and the mechanism of action of this drug probably involves both effects. FdUrd is about 10^3-fold more cytotoxic in most tissue culture cell systems than is FUra, showing EC_{50} values of about 1 nM.[28] At these concentrations there is no significant incorporation of FUra into RNA, and the cytotoxic effect of FdUrd is due solely to the inhibition of dTMP synthetase.[27] From the results obtained in tissue culture systems, one would expect that FUra and FdUrd might show different properties as antitumor agents. However, in treatment of animal and human neoplasia, FUra and FdUrd show similar effects at approximately equimolar concentrations. It appears that FdUrd is metabolized to FUra by dThd and Urd phosphorylase more rapidly than it is converted to FdUMP in animals and humans and thus simply serves as a depot for FUra. A similar situation appears to occur with NO_2dUrd.[28] In tissue culture cells which possess dThd kinase, this compound is converted to NO_2dUMP, which results in a potent and specific inhibition of dTMP synthetase; as a result, NO_2dUrd shows an EC_{50} of about 30 nM in a variety of cells in culture. The corresponding pyrimidine base, NO_2Ura, is not cytotoxic to tissue culture cells at concentrations exceeding 0.1 mM, suggesting that, unlike FUra, it is not effectively metabolized to cytotoxic nucleotides. When tested in L1210 bearing mice, NO_2dUrd did not increase survival time, nor was it toxic at doses as high as 150 mg/kg (unpublished results). As with FdUrd, it appears that in animals the dThd phosphorylase catalyzed conversion of NO_2dUrd to NO_2Ura is more rapid than the dThd kinase catalyzed phosphorylation to provide the active inhibitor, NO_2dUMP.

Clearly the catabolism of analogues of dUrd by dThd and Urd phosphorylases has been a major impediment in the development of this class of compounds as useful chemotherapeutic agents. If methods could be found to prevent this catabolism, new compounds with interesting in vivo properties might be found, and many nucleosides which have been discarded as ineffective might reemerge as useful chemotherapeutic agents. The necessary structural modifications of a particular compound could probably be found to provide a derivative inert to these catabolic enzymes. However, these structural changes would also have to be compatible with the desired biological effect of the parent compound and, even if found, would probably not be generally applicable to other 2′-deoxyribonucleosides. It would appear more reasonable to direct efforts at developing procedures which would inhibit the in vivo activity of the phosphorylases; with success, this would provide a general method for unmasking the biological activity of *all* 2′-deoxyribonucleosides which are catabolized by these enzymes. Since Urd and dThd phosphorylase are not uniformly present in all cells,[23] it may be argued that they are not critical to cell life; thus, inhibition of these enzymes should be without untoward toxic effects. Further, since the activities of the phosphorylases vary in different tissues,[23] their inhibition could result in altered tissue response to cytotoxic analogues of dUrd.

(24) Yagil, E.; Rosner, A. *J. Bacteriol.* **1970**, *103*, 417.
(25) Yagil, E.; Rosner, A. *J. Bacteriol.* **1971**, *108*, 760.
(26) Gotto, Am. M.; Belkhode, M. L.; Touster, O. *Cancer Res.* **1969**, *29*, 807.
(27) Washtien W. L.; Santi, D. V. *Cancer Res.* **1979**, *39*, 3397.
(28) Washtien, W.; Matsuda, A.; Wataya, Y.; Santi, D. V. *Biochem. Pharmacol.* 1978, *27*, 2663.

Baker and co-workers embarked on a program directed toward the design of nonclassical inhibitors of dThd and Urd phosphorylases. Through these investigations it was found that 5-benzyluracil was a potent in vitro inhibitor ($K_i \simeq 10^{-7}$ M) of mammalian Urd phosphorylase.[29] A number of 6-substituted uracil derivatives were also found to be potent inhibitors of the dThd phosphorylase from *Escherichia coli*[30] but, unfortunately, were ineffective as inhibitors of the mammalian enzyme.[31] Although these studies were never completed, they demonstrated that these enzymes were quite amenable to specific inhibition by taking advantage of the properties of regions adjacent to the active site. As such, they provide a firm basis and direction for future design of nonclassical inhibitors of these enzymes. 2′-Deoxyribonucleoside substrates of the phosphorylases could also be used to protect cytotoxic analogues of dUrd from degradation. The protection would be afforded both by direct competition for the enzyme, as well as by mass-action transfer of the sugar moiety from the 2′-deoxyribosyl substrates to the Ura analogue, should degradation of the cytotoxic nucleoside occur. However, as previously described, difficulties may be encountered in that many of the 2′-deoxyribosides which are effective substrates for the pyrimidine nucleoside phosphorylases could also inhibit the dThd kinase catalyzed phosphorylation of cytotoxic analogues of dUrd and thus prevent their activation. If Urd phosphorylase were the major enzyme responsible for degradation of the 2′-deoxynucleoside analogue (as is the case in the catabolism of FdUrd by Walker 256 rat tumor[29]), pyrimidine ribonucleoside substrates, such as Urd, should serve as effective protecting agents. Here it is noted that 5-benzyluracil binds much more tightly ($K_i \simeq 10^{-7}$ M) than Urd ($K_m \simeq 10^{-4}$ M)[29] and currently appears to be the candidate of choice for in vivo inhibition of this activity. At the present time, it would be appropriate to attempt to ascertain whether inhibition of these enzymes will, as speculated, increase the in vivo efficacy of 2′-deoxynucleosides as precursors of dUMP analogues. Using a model system which possesses high activity of one of the phosphorylases (e.g., Walker 256, rich in Urd phosphorylase), a potent inhibitor of this enzyme (e.g., 5-benzyluracil), and a cytotoxic 2′-deoxynucleoside which is well understood and whose metabolic fate can easily be monitored (e.g., FdUrd), it should be a straightforward task to ascertain whether this objective can be met.

Metabolic Manipulation of FUra Action. In recent years, much interest has centered about the use of FUra in combination with methotrexate (MTX) for the treatment of certain neoplasms. With few exceptions, clinical protocols for multiple drug cancer chemotherapy have of necessity been empirically derived and often yield inconclusive data as to the efficacy of such agents. Indeed, while the combination of cyclophosphamide, MTX, and FUra has been acclaimed a significant breakthrough in adjuvant treatment of human breast cancer,[32] studies on experimental systems have yielded conflicting reports as to whether MTX and FUra are additive, synergistic,[33-37] or

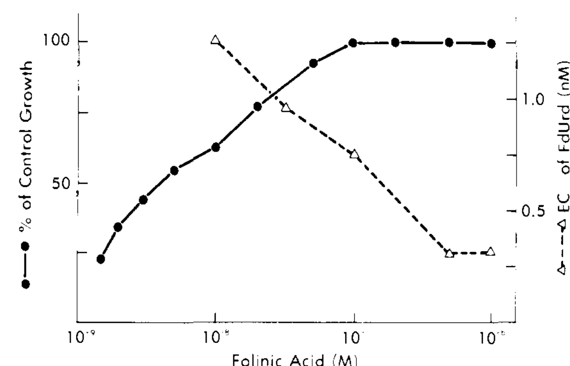


Figure 5. Effect of folinic acid concentration in media on growth of L1210 cells and EC_{50} values of FdUrd. The experimental procedure is described in ref 40.

antagonistic.[37-40] In the ensuing discussion, the MTX–FUra combination is used as a paradigm to illustrate how a knowledge of the mechanism of action of individual drugs and their effects on cellular metabolism may serve as a basis for altering their disposition and provide rational approaches for design of schedules and combinations.

As discussed earlier, biochemical studies have revealed that CH_2-H_4folate is required for the potent inhibition of dTMP synthetase by FdUMP. In the absence of this cofactor, FdUMP binds poorly to the enzyme ($K_d \simeq 10$ μM), whereas the presence of CH_2-H_4folate results in a ternary complex in which FdUMP is bound some 7 to 8 orders of magnitude more tightly. This dramatic increase in affinity results, in part, from two-ligand synergism in formation of reversible complexes[7,41] but, for the most part, is due to the formation of covalent bonds that link the enzyme to FdUMP and CH_2-H_4folate, as shown in Figure 2. From this, it can be surmised that intracellular levels of CH_2-H_4folate should be a critical determinant of FUra and FdUrd cytotoxicity and that depletion of this cofactor could antagonize the cytotoxic effects of these fluorinated pyrimidine analogues. To test these hypotheses, the ability of FdUrd to inhibit growth of L1210 leukemia cells in culture was examined under conditions that would result in various concentrations of intracellular H_4folates.[40] A typical experiment using folinic acid (5-CHO-H_4folate) as the H_4folate source is shown in Figure 5. In the absence of FdUrd, half-maximal growth is achieved at 4 nM folinic acid, and maximal growth rate occurs at concentations greater than 100 nM. The concentration of FdUrd necessary to inhibit growth by 50% (EC_{50}) is 0.3 nM at high folinic acid levels but increases as the folinic acid is decreased below 500 nM. Interestingly, the concentration of folinic acid required for optimal cell growth was significantly lower than that required for maximal cytotoxicity of FdUrd. To prove the molecular mechanism of the antagonism of FdUrd by MTX, the FdUMP–CH_2-H_4folate–dTMP synthetase complex formed in L1210 cells upon treatment with [6-^{3}H]FdUrd was directly quantitated under conditions of H_4folate excess and deprivation. As

(29) Baker, B. R.; Kelley, J. L. *J. Med. Chem.* **1970**, *13*, 461.
(30) Baker, B. R.; Hopkins, S. E. *J. Med. Chem.* **1970**, *1*, 87.
(31) Baker, B. R.; Kelley, J. L. *J. Med. Chem.* **1970**, *13*, 456.
(32) Bonadonna, G.; Brusamoline, E.; Valagussa, P.; Rossi, A.; Brugnatelli, L.; Brambilla, C.; De Lena, M.; Tancini, G.; Bajetta, E.; Musumeci, R.; Veronsei, U. *N. Engl. J. Med.* **1976**, *294*, 405.
(33) Kline, I.; Vendetti, J. M.; Mead, J. A. R.; Tyrer, D. D.; Goldin, A. *Cancer Res.* **1966**, *26*, 848.
(34) Bareham, C. R.; Griswold, D. E.; Calabresi, P. *Cancer Res.* **1974**, *34*, 571.
(35) Martin, D. S.; Hayworth, P.; Davin, E.; Stolfi, R.; Fugmann, R.; *Proc. Am. Assoc. Cancer Res.* **1976**, *17*, 130, abstract 518.
(36) Bertino, J. R.; Sawicki, W. L.; Lindquist, C. A.; Gupta, V. S. *Cancer Res.* **1977**, *37*, 327.
(37) Cadman, E.; Heimer, R.; Davis, L. *Science* **1979**, *205*, 1135.
(38) Bowen, D.; White, J.; Goldman, I. *Cancer Res.* **1978**, *38*, 219.
(39) Tattersal, M. H. N.; Jackson, R. C.; Connors, T. A.; Harrup, D. R. *Eur. J. Cancer*, **1973**, *9*, 733.
(40) Ullman, B.; Lee, M.; Martin, D. W.; Santi, D. V. *Proc. Natl. Acad. Sci.* **1978**, *75*, 980.
(41) Galivan, J. H.; Maley, G. F.; Maley, F. *Biochemistry* **1976**, *15*, 356.

expected, the complex formed in H_4folate-deprived cells was less than 8% of that formed in media containing folate. In toto, these results clearly demonstrate that CH_2-H_4folate is necessary for FdUMP inhibition of dTMP synthetase in vivo and the cytotoxicity of FdUrd. Likewise, MTX, which depletes intracellular H_4folates by inhibition of H_2folate reductase, antagonizes the interaction of FdUMP with dTMP synthetase and the cytotoxicity of FdUrd in tissue culture cells.

In view of these results, how does one explain the reports that MTX and FUra are synergistic in some systems? One explanation is that MTX might also stabilize the FdUMP–dTMP synthetase complex. In vitro studies have shown that at sufficiently high concentrations a number of folate analogues synergize binding of FdUMP to thymidylate synthetase, but covalent bonds are not formed and the complexes are not as stable as those formed with CH_2-H_4folate.[42] Bertino et al.[36] have recently applied this rationale in designing an MTX–FUra schedule for treatment of the Sarcoma 180 murine tumor model. These workers surmised that, although MTX would deplete H_4folates, sufficiently high intracellular concentrations of the drug might, in addition, synergize binding of FdUMP to thymidylate synthetase, as it does in vitro, and override the partial antagonism observed at low MTX concentrations. Indeed, significant therapeutic enhancement was observed when tumor-bearing animals were treated with *high* doses of MTX 2 h prior to FUra administration. While the basis for the success of this schedule remains unproven, a salient point is that its design was based on biochemical reasoning rather than the empiricism frequently used in developing such regimens. Another explanation of the synergism of the MTX–FUra combination is that the mechanism of FUra action is more complex than that of FdUrd. In L1210 cells and other tissue culture cells examined, FdUrd is metabolized almost exclusively to FdUMP with resultant specific inhibition of dTMP synthetase.[27] In contrast, FUra is both metabolized to FdUMP and is incorporated into RNA. The effect of FUra in L1210 cells cannot be exclusively due to inhibition of dTMP synthetase, since cytotoxicity is only partially reversed by dThd. Thus, if FUra–RNA is an important component of FUra cytotoxicity, MTX should not show the antagonistic effect it does toward FdUrd. In a recent report, Cadman et al.[37] demonstrated that a 3-h exposure of L1210 cells to 0.1 to 100 μM MTX followed by 10 μM FUra resulted in a significant synergistic effect on the number of cells killed in culture. This contrasts with the aforementioned antagonistic effect of MTX on FdUrd in the same cell line. These workers found that MTX caused an increase in the intracellular concentrations of FUra nucleotides and the incorporation of FUra into RNA. These changes were correlated with increased concentrations of phosphoribosyl pyrophosphate (PRPP). It was reasoned that MTX induced a state of H_4folate depletion sufficient to inhibit purine biosynthesis; the resultant increase in PRPP levels stimulated conversion of Ura to FUMP via the pyrimidine phosphoribosyl transferase (i.e., OPRtase, eq 5). Supporting the role of increased PRPP

$$\underset{(OA)}{\text{FUra}} + \text{PRPP} \rightleftharpoons \underset{(OMP)}{\text{FUMP}} + \text{PP}_i \quad (5)$$

levels in the enhanced formation of FUra nucleotides in MTX-treated cells, it was observed that hypoxanthine, which readily accepts the phosphoribosyl group of PRPP, decreased the intracellular PRPP levels and diminished the increase of FUra nucleotides in cells exposed to MTX. These results confirm that MTX administered before FUra can increase the formation of FUra nucleotides in cells which use PRPP as a phosphoribosyl donor. Further, as the MTX-induced depletion of intracellular CH_2-H_4folate decreases the FdUMP inhibition of dTMP synthetase,[40] the results demonstrate that FUra incorporation into RNA is also an important determinant of the cytotoxicity of this agent. It is possible to increase intracellular PRPP levels without using MTX. This would avoid depleting cells of H_4folate and antagonizing the FdUMP inhibition of dTMP synthetase. For example, direct inhibition of purine biosynthesis at an early site which does not result in feedback inhibition of PRPP synthetase should produce a similar accumulation of intracellular PRPP. It would be interesting to examine the effects of glutamine analogues (e.g., azaserine, DON[43]) which inhibit two enzymes of purine biosynthesis and inhibitors of IMP dehydrogenase (e.g., myocophenolic acid[44]) on the metabolism and cytotoxicity of FUra. As previously noted, the pyrimidine phosphoribosyltransferase primarily responsible for the conversion of FUra to FUMP is, in fact, OPRTase. An alternate approach to increasing the net conversion of FUra to FUMP would be to deplete cells of natural pyrimidine substrate, orotic acid, which competes with FUra for OPRTase. This can be accomplished with the drug *N*-(phosphonoacetyl)-L-aspartate (PALA), a potent inhibitor of aspartate transcarbamoylase,[45,46] the first enzyme of pyrimidine biosynthesis. Indeed, treatment of susceptible cells with PALA blocks the formation of orotic acid, as well as uracil nucleotides which would compete in the subsequent steps of FUra nucleotide metabolism. Interestingly, PALA is most cytotoxic toward cells which have low proliferative activity and has little effect on gastrointestinal epithelium and hematopoetic cells. Since the latter cells represent major sites of FUra toxicity and since FUra appears to be most effective toward solid tumors, the PALA–FUra combination may provide an additional degree of selective toxicity. From the above it may be anticipated that proper scheduling of a combination of PALA, MTX, and FUra would decrease orotic acid and increase PRPP; as a result, conversion of FUra to FUra nucleotides and incorporation of FUra into RNA would increase, and the effect of FdUMP inhibition of dTMP synthetase would be depressed as a result of H_4folate depletion. This combination might prove effective for treatment of solid tumors in which the FUra-RNA is primarily responsible for the cytotoxic effect of FUra and spare cells in which FdUMP inhibition of dTMP synthetase is the initiating cytotoxic event.

Situations may also be envisioned where it would be desirable to reduce the conversion of FUra to FUMP. For example, if qualitative differences in pyrimidine phosphoribosyltransferase activities exist in normal and tumor cells, depression of FUMP formation in normal cells might reduce untoward toxic effects. Further, if, as previously described, FUra may be converted to FdUMP via dThd phosphorylase, the effects of FUra incorporation into RNA might be reduced. A logical approach toward reducing the

(42) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471.

(43) Bennett, L. L. Jr. In "Antineoplastic and Immunosuppressive Agents, Part II", Sartorelli, A. C.; Johns, D. G., Eds.; Springer-Verlag: New York, 1975; p 484.

(44) Nichol, C. A. In "Antineoplastic and Immunosuppressive Agents, Part II", Sartorelli, A. C.; Johns, D. G., Eds.; Springer-Verlag: New York, 1975; p 434.

(45) Swyryd, E. A.; Seaver, S. S.; Stark, G. R. *J. Biol. Chem.* **1974**, *249*, 6945.

(46) Johnson, R. K.; Swyryd, E. A.; Stark, G. R. *Cancer Res.* **1978**, *38*, 371.

metabolism of FUra to FUMP would simply involve inducing intracellular effects which are essentially the opposite of those described above which increase this conversion; that is, reducing intracellular PRPP and increasing the orotic acid levels should depress the conversion of FUra to FUMP. Reduction of PRPP could be achieved by administering a purine base which is a substrate for hypoxanthine–guanine phosphoribosyltransferase (HGPRTase). For example, guanine and hypoxanthine rapidly utilize PRPP in the HGPRTase-catalyzed conversion to their corresponding 5′-mononucleotides, and these further depress synthesis of PRPP by feedback inhibition of PRPP synthetase. In addition, as PRPP levels are depressed, orotic acid should accumulate and further inhibit the conversion of FUra to FUMP. A similar effect (i.e., decreased PRPP, increased orotate) has been achieved by administering adenosine and an adenosine deaminase inhibitor to cells possessing adenosine kinase.[47] Adenosine itself is cytotoxic, but this effect can be reversed by Urd which might also have the desirable effect of depressing further metabolism of FUra ribonucleotides. An alternative approach to achieving high intracellular orotic acid levels would be to inhibit OMP decarboxylase, the last enzyme of de novo pyrimidine biosynthesis, and thereby induce the accumulation of precursors of OMP. Recently, Chen and Jones[48] have demonstrated that treatment of Ehrlich ascites cells with 6-azauridine (6-aza-UMP is a potent inhibitor of OMP decarboxylase) results in the accumulation of large amounts of orotic acid. It would be interesting to ascertain the effect of this inhibitor on the metabolism of FUra.

Quite different approaches might be taken to amplify the action of FUra in cells in which FdUMP inhibition of dTMP synthetase is responsible for cytotoxicity. Most relevant to current chemotherapeutic modalities is the fact that inhibition of dTMP synthetase by FdUMP requires CH_2-H_4folate. Clearly, MTX will antagonize this aspect of FUra action, and it is tempting to suggest that in such situations a folate cofactor (e.g., folinic acid) be administered with FUra instead of the folate antagonist MTX. It may also be relevant that, as shown for L1210 cells, intracellular H_4folates can be sufficient for optimal growth but not for optimal inhibition of thymidylate synthetase by FdUMP.[40] This relationship may vary in different cells for a number of reasons; for example, cells with higher levels of thymidylate synthetase would require higher concentrations of CH_2-H_4folate to achieve optimal inhibition of this enzyme by FdUMP. Thus, the possibility exists that intracellular folate cofactors in tumors that are marginally responsive toward FUra may be sufficient for their optimal growth but not for effective inhibition of thymidylate synthetase by FdUMP. Should this be the case, administration of a reduced folate, such as folinic acid, might increase the effectiveness of thymidylate synthetase inhibition by FdUMP while H_4folate-independent effects of FUra would remain unchanged; it is possible that such tumors could be made more responsive toward FUra or FdUrd, with a resultant increase in the therapeutic index.

In the latter part of this paper, I have attempted to illustrate what I believe to be a most important area for future cancer research. Many drugs of known utility might be made more effective by subtle manipulations in cell metabolism which amplify or alter the action of such drugs. The desired metabolic manipulation is often subtle and, with a knowledge of cell metabolism, may be rationally approached by the use of other drugs or nutrients. Further, since the selectivity which might be achieved by this approach depends on quantitative rather than qualitative differences in the metabolism of normal and neoplastic cells, there is hope that the chemotherapeutic efficacy of many anticancer drugs might be greatly enhanced.

Acknowledgment. This study was supported by Grant CA 14394, awarded by the National Cancer Institute, DHEW. D.V.S. is the recipient of a NIH Career Development Award.

(47) Gudas, L. J.; Cohen, A.; Ullman, B.; Martin, D. W., Jr. *Somat. Cell Genet.* **1978**, *4*, 201.
(48) Chen, J.-J.; Jones, M. E. *J. Biol. Chem.* **1979**, *254*, 4908.