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Thymidylate Synthetase: Interaction with 5-Fluoro and 5-Trifluoromethyl-2′-Deoxyuridylic Acid

DANIEL V. SANTI, ALFONSO L. POGOLOTTI, THOMAS L. JAMES, YUSUKE WATAYA, KATHRYN M. IVANETICH, and STELLA S. M. LAM

Department of Biochemistry and Biophysics, and Department of Pharmaceutical Chemistry, University of California, San Francisco, Calif. 94143

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to thymidylate (TMP) with the concomitant conversion of 5, 10-methylene tetrahydrofolate (CH_2FAH_4) to 7, 8-dihydrofolate (FAH_2) as depicted in Figure 1 (for a recent review, see reference 1). In this process the hydrogen at C-6 of FAH_4 is directly transferred to the methyl group of TMP (2).

One of our objectives over the past few years has been to establish the mechanism of catalysis of thymidylate synthetase. Extensive investigations of chemical counterparts (3-6) have indicated that the reaction is initiated by attack of a nucleophile at the 6-position of dUMP and that many, if not all, reactions along the pathway are facilitated by analogous nucleophilic catalysis.

The proposed mechanism of this enzyme, as derived from investigations of chemical models is illustrated in Figure 2. It is proposed that the reaction is initiated by attack of a nucleophilic group of the enzyme to the 6-position of dUMP. In this manner, the 5-position of dUMP could be made sufficiently nucleophilic (viz I, Figure 2) to react with CH_2FAH_4 or an equivalent reactive species of formaldehyde. Thus, the initial condensation product between dUMP and CH_2FAH_4 is now generally accepted (1, 7) to be one which is covalently bound to the enzyme and saturated across the 5, 6-double bond of dUMP (II). Proton abstraction from II would give the intermediate enolate III. As with the chemical models, III should readily undergo a β-elimination to produce the highly reactive exocyclic methylene intermediate IV and FAH_4, bound to the enzyme in close proximity. Intermolecular hydride transfer from FAH_4 to IV would yield dTMP, FAH_2, and the native enzyme. It should be emphasized that all of the aforementioned reactions and intermediates have direct chemical counterparts, and are in complete accord with all available biochemical data.

With the availability of a stable enzyme from an amethopterin resistant strain of Lactobacillus casei (8, 9) and facile methods for its purification (9-11), we undertook studies which

In Biochemistry Involving Carbon-Fluorine Bonds; Filler, Robert;
ACS Symposium Series; American Chemical Society: Washington, DC, 1976.

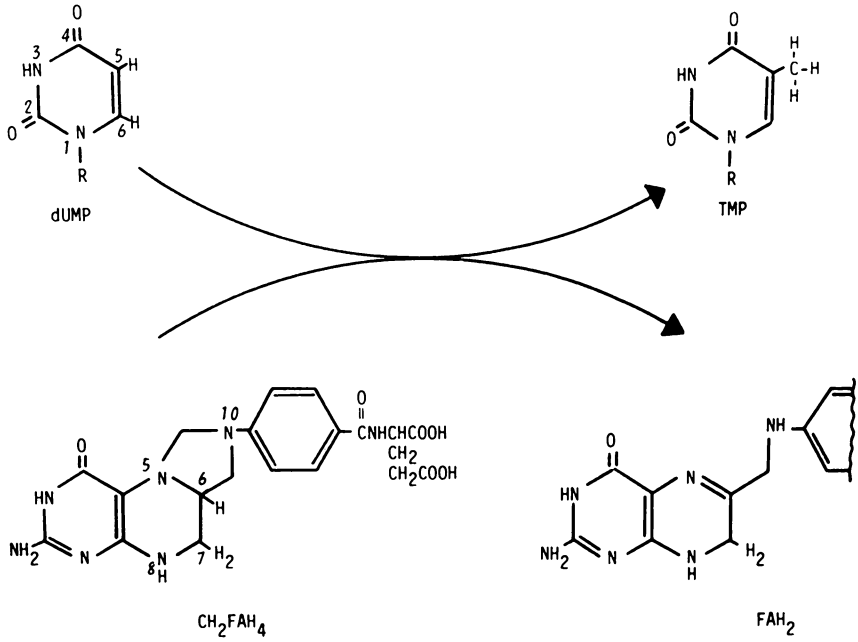

Figure 1. Reaction catalyzed by thymidylate synthetase; R = 5-phospho-2′-deoxyribosyl

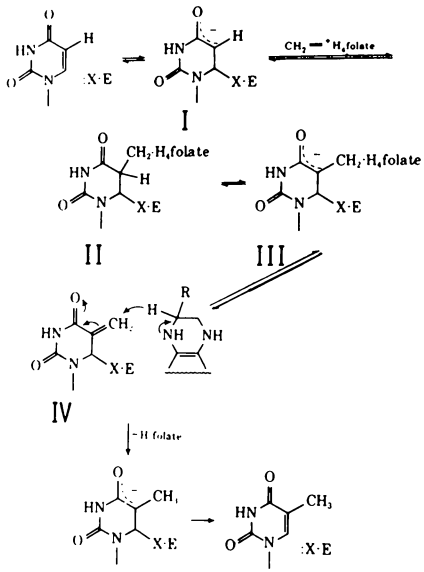

Figure 2. Suggested sequence for the thymidylate synthetase reaction. All pyrimidine structures have a 1-(5-phospho-2′-deoxyribosyl) substituent and R = $CH_2NHC_6H_4COGlu$.

might provide direct support for proposals which were based on the aforementioned nonenzymic models. Although a number of directions have been pursued towards this objective, the following summarizes our investigations of the interaction of thymidylate synthetase with 5-fluoro-2'-deoxyuridylate (FdUMP) and 5-trifluoromethyl-2'-deoxyuridylate (CF_3 dUMP). Studies of these two fluorinated nucleotides have provided convincing evidence for the mechanism of this enzyme; in addition, they have provided insight into how these drugs act, and how the reactivity of fluorinated molecules might be utilized in the design of enzyme inhibitors. We emphasize that a number of other laboratories have been engaged in similar investigations, and regret that space does not permit complete citation of all the excellent studies performed in this area.

5-Fluoro-2'-deoxyuridylate (FdUMP). It has been known for some time (12, 13) that FdUMP is an extremely potent inhibitor of thymidylate synthetase, but the nature of inhibition has been the topic of considerable controversy (14). Since the 6-position of 1-substituted 5-fluorouracils is quite susceptible toward nucleophilic attack (15-17), we suspected that FdUMP might exert its inhibitory effect by reaction with the proposed nucleophilic catalyst of thymidylate synthetase. Studies from this (18, 19) and other (20, 21) laboratories have since demonstrated this to be the case.

A simplified depiction of the interactions of FdUMP and CH_2FAH_4 is given below in Figure 3.

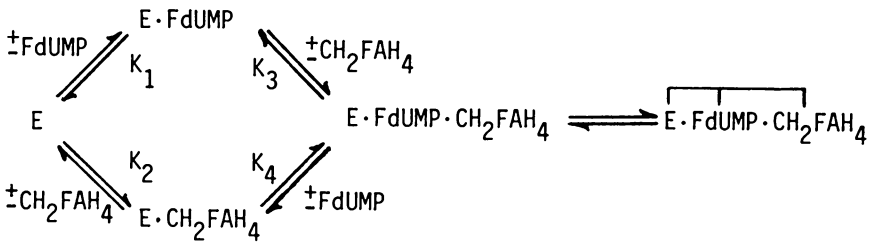

Figure 3

Using the isotope trapping method (22), we have found that the two ligands, FdUMP and CH_2FAH_4, interact with the enzyme in a random fashion, and that formation of the initial ternary complex (E·FdUMP·CH_2FAH_4) is at least partially rate determining. From equilibrium binding techniques we have ascertained that the dissociation constants of both binary complexes (K_1 and K_2) are approximately 10^{-5} M. The first ternary complex which is formed with FdUMP, CH_2FAH_4 and enzyme is depicted as E·FdUMP·CH_2FAH_4 and does not involve covalent bonds. Interestingly, analogous reversible ternary complexes may be formed using analogs of the cofactor. Through studies of the interaction of FdUMP and analogs of CH_2FAH_4 we have ascertained that there is a striking synergism in binding of ligands to this protein. That is, the affinity of either ligand for the cognate binary complex is ca. two orders of magnitude greater than the affinity for the free enzyme. With many analogs of CH_2FAH_4, this ternary complex is sufficiently stable that it may be physically separated from other species (19). These ternary complexes show interesting ultraviolet difference spectra which may be used for their quantitation and characterization. Shown in Figure 4 are difference spectra obtained with CH_2FAH_4 and 5,8-deazafolic acid; similar difference spectra have also been obtained with a number of other analogs of folic acid. Characteristic of these difference spectra is a peak at ca. 330 nm and, usually, a trough at ca. 290 nm. Although the exact reason for the spectral changes which occur upon formation of the reversible ternary complexes is yet unknown, we suggest that they result from perturbations of the p-aminobenzoylglutamate moiety of the cofactor analogs which result from their environment within the ternary complex. This perturbation is believed to be a manifestation of a conformational change which is related to the aforementioned synergism in binding of the two ligands. The difference spectrum of the E·FdUMP·CH_2FAH_4 complex (Figure 4) is similar to those observed for the cofactor analogs, suggesting that similar changes in environment occur with the natural cofactor, CH_2FAH_4. There is one striking difference in that there is a loss of differential absorbance at 269 nm in the complex formed with CH_2FAH_4 (18, 19) which we have not observed in complexes formed with cofactor analogs; the reason for this will become apparent in the ensuing discussion.

The complex formed with thymidylate synthetase, FdUMP, and the natural cofactor CH_2FAH_4, has been extensively investigated. This complex is extremely tight and may readily be isolated by a variety of techniques (18, 19, 21, 23, 24). Using radioactive FdUMP and CH_2FAH_4, the enzyme has been titrated and shown to possess two FdUMP and two cofactor binding sites per mole (19). The stoichiometry of binding has been verified in a number of laboratories by a variety of methods (11, 25, 26). This is in accord with the earlier finding

that thymidylate synthetase from L. casei has two apparently identical subunits of MW 35,000 each (9, 27).

Studies of the rate of association of FdUMP with the $E{\cdot}CH_2FAH_4$ complex (k_{on}) and its dissociation (k_{off}) have

$$E{\cdot}CH_2FAH_4 + [^3H]FdUMP \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}CH_2FAH_4{\cdot}[^3H]FdUMP$$

allowed us to calculate the dissociation constant of the complex to be ca. 10^{-13} M. This provides an explanation for the discrepancies in K_d values reported for FdUMP in the literature; namely, previous experiments were using concentrations of enzyme higher than the K_d, and were in concentration ranges where FdUMP was behaving as a stoichiometric inhibitor. The kinetically determined K_d is approximately 10^8-fold lower than that for the binary complex; in effect, the presence of the cofactor increases the affinity of the enzyme for FdUMP by over 10 kcal/mol in binding energy. Clearly, a most pertinent feature of the interaction of FdUMP and thymidylate synthetase involves changes which occur within the bound ternary complex.

Several lines of evidence demonstrate rather conclusively that a covalent bond is formed between FdUMP and thymidylate synthetase within the complex. (a) The $E{\cdot}[^3H]FdUMP{\cdot}CH_2FAH_4$ complex may be treated with a number of protein denaturants (urea, guanidine hydrochloride, etc.) without apparent loss of protein-bound radioactivity. With few exceptions, such treatment is sufficient to disrupt noncovalent interactions between low molecular weight ligands and their protein receptors. (b) Upon formation of the complex, there is a decrease of absorbance at 269 nm which corresponds to stoichiometric loss of the pyrimidine chromophore of FdUMP. This result strongly suggests that the 5,6-double bond of the pyrimidine is saturated in the bound complex. (c) The rate of dissociation of [6-^{3}H]FdUMP from the complex shows a secondary tritium isotope effect (k_H/k_T) of 1.23. This would correspond to $k_H/k_D = 1.15$ and clearly demonstrates that the 6-carbon of the heterocycle undergoes sp^3 to sp^2 rehybridization during the process as required if the 5,6-double bond of FdUMP is saturated in the complex. (d) Proteolytic digestion of the complex yields a peptide which is covalently bound to both FdUMP and CH_2FAH_4. The ultraviolet and fluorescence spectra of this peptide are characteristic of 5-alkyltetrahydrofolates and, as with the native complex, there is no evidence of ultraviolet absorption of the FdUMP chromophore.

From these lines of evidence, together with information gathered from model chemical counterparts, the structure of the enzyme·FdUMP·CH_2FAH_4 complex is currently believed to be as depicted in Figure 5. Here, a nucleophile of the enzyme has added to the 6-position of FdUMP, and the 5-position of the pyrimidine is coupled to the 5-position of FAH_4 via the methylene

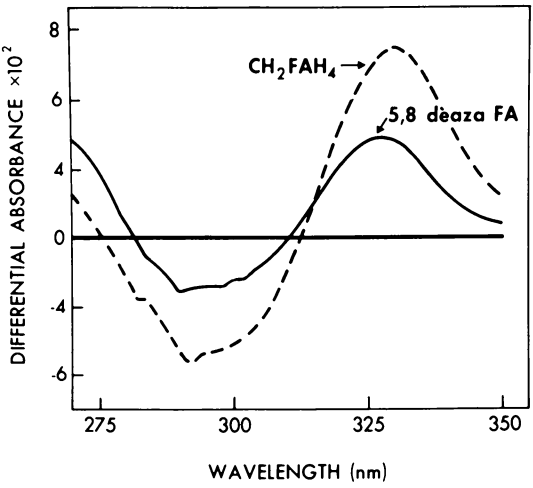

Figure 4. Dashed line: Ultraviolet difference spectra of FdUMP, CH_2FAH_4 and thymidylate synthetase vs. CH_2FAH_4 and thymidylate synthetase. Solid line: FdUMP, 5,8-deazofolate and thymidylate synthetase vs. enzyme and 5,8-deazafolate.

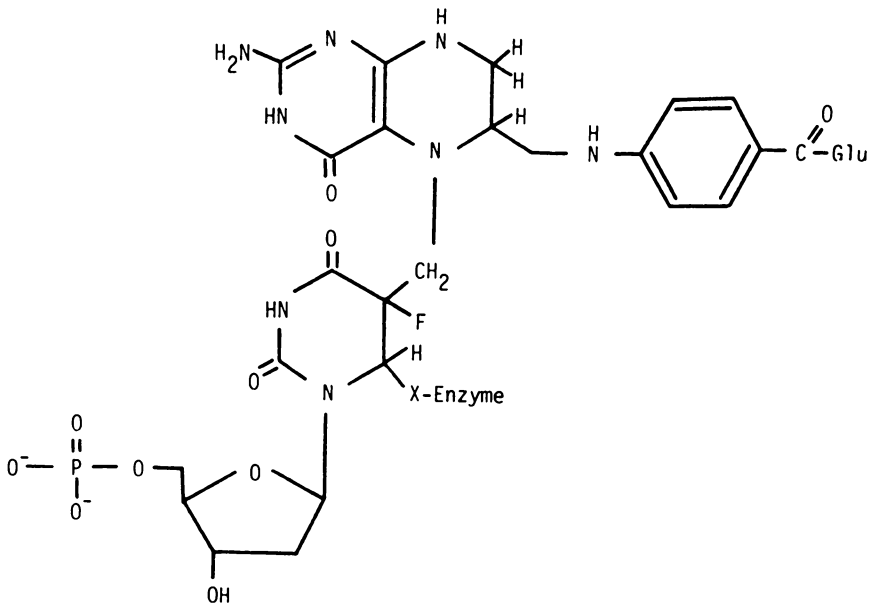

Figure 5. Structure of the FdUMP · CH_2 · FAH_4 thymidylate synthetase ternary complex where X represents a nucleophile of one of the enzyme amino acids

group of the cofactor. A similar structure was proposed from evidence obtained independently in another laboratory (20).

Referring to Figure 5, it is noted that the assigned structure for the E•FdUMP•CH_2FAH_4 complex is analogous to one of the proposed steady state intermediates of the normal enzymic reaction (viz II, Figure 2). They differ in that II possesses a proton at the 5-position of the nucleotide which is abstracted in a subsequent step, whereas the E•FdUMP•CH_2FAH_4 complex (Figure 5) possesses a stable fluorine at the corresponding position. Thus, it appears that FdUMP behaves as a "quasi-substrate" for this reaction. That is, it enters into the catalytic reaction as depicted for the substrate dUMP in Figure 2 up to the point where an intermediate is formed which can proceed no further; in effect, a complex is trapped which resembles a steady state intermediate (viz II, Figure 2) of the normal catalytic reaction.

We have recently obtained the fluorine-19 nmr spectrum of an FdUMP•CH_2FAH_4•peptide obtained upon proteolytic digestion of the FdUMP•CH_2FAH_4•thymidylate synthetase complex. As shown in Figure 6, the 94 MHz ^{19}F spectrum consists of a doublet of triplets located 87.2 ppm upfield of the external reference, trifluoroacetic acid. Our current interpretation of this spectrum is as follows: The doublet is caused by splitting of the ^{19}F resonance by the proton at the 6-position of the uracil ring (H_A) with a coupling constant J_{AF} of 32.5 Hz. Each component of the doublet is split further into a triplet (intensity ratio (1:2:1) caused by coupling of the fluorine with the adjacent methylene protons (H_B) of the cofactor with the magnitude of the coupling constant H_{BF} being 19.2 Hz. The innermost lines of the triplets overlap so the ^{19}F resonance appears to be a quintet with intensity ratio 1:2:2:2:1.

It has been well established that the trigonal geometry of the carbonyl atoms in uracil derivatives saturated across the 5,6-double bond results in a half-chair conformation with substituents on carbon atoms 5 and 6 staggered (28-30) as commonly found in cyclohexane.

The ^{19}F spectrum, in conjunction with previously reported ultraviolet and fluorescence spectral data (31), of the FdUMP•CH_2FAH_4•peptide yields definitive evidence for its structure. The doublet of triplets implies that the fluorine-bonded carbon is flanked by CH and CH_2 groups (i.e. $CHCFCH_2$). The CH, of course, occurs at the 6-position of the nucleotide which is attached to the nucleophile of the enzyme. The most logical assignment for the CH_2 is the bridging group between the nucleotide and cofactor as depicted for the native complex in Figure 5. Based on the stability of the FdUMP•CH_2FAH_4•peptide in the absence of antioxidants, we have surmised that the CH_2 group bridges the nucleotide to the 5-and not to the 10-nitrogen of the cofactor.

It is possible to assign the stereochemistry of the

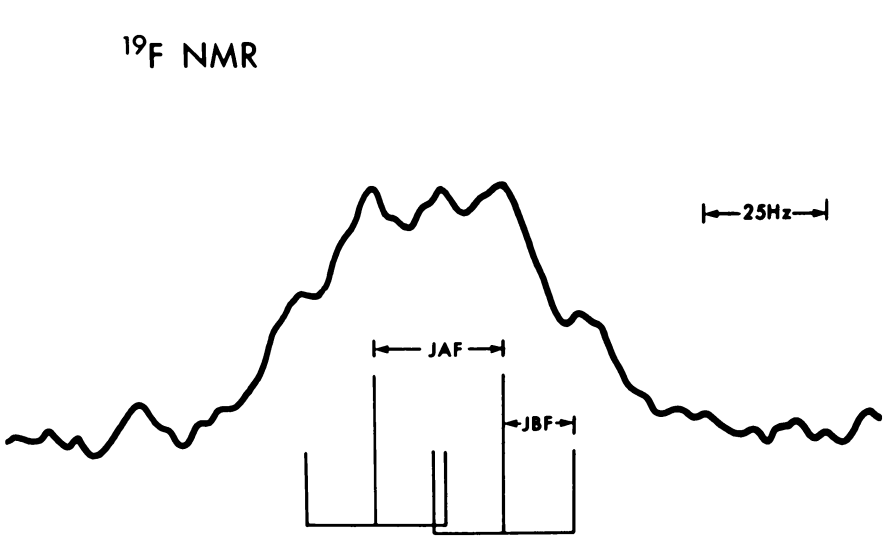

Figure 6. 94 MHz Fluorine-19 nmr spectrum of the FdUMP · CH_2FAH_4 · peptide

substituents which have added across the 5, 6-double bond of FdUMP to give the ternary complex by comparing the observed coupling constants with those from extensively studied models. As shown in Figure 7, the 5-fluoro and 6-hydrogen of the FdUMP·CH_2FAH_4·peptide are proposed to be in a trans pseudo-axial conformation. The enzyme nucleophile and cofactor are therefore trans pseudoequatorial.

The addition of a nucleophile of thymidylate synthetase to the 6-position of dUMP is a primary event in the enzyme-catalyzed reaction (Figure 8). The resultant carbanion (1) reacts with CH_2FAH_4 to produce an intermediate with a structure analogous to that of the ternary complex formed with FdUMP and the cofactor (2, 3). Abstraction of the 5-hydrogen followed by a series of steps involving reduction of the one carbon unit and elimination of the nucleophile results in the observed products of the reaction. These steps have been previously depicted in detail in Figure 2. With the logical assumption that the normal enzyme-catalyzed reaction occurs in a manner similar to the formation of the thymidylate synthetase·FdUMP·CH_2FAH_4 complex, several details concerning the mechanism of the normal enzymic reaction may be inferred.

First, the overall stereochemical pathway of the enzyme-catalyzed reaction may be deduced. The addition of the nucleophile and cofactor across the 5, 6-double bond must occur in a trans fashion. Consequently, the subsequent elimination of the 5-hydrogen and the enzymic nucleophile must occur as a cis-elimination .

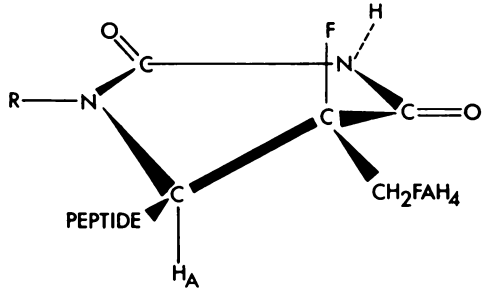

Figure 7. Stereochemical projection of the FdUMP · CH_2FAH_4 · peptide as determined by its ^{19}F nmr spectrum; R = 5-phospho-2′deoxyribosyl

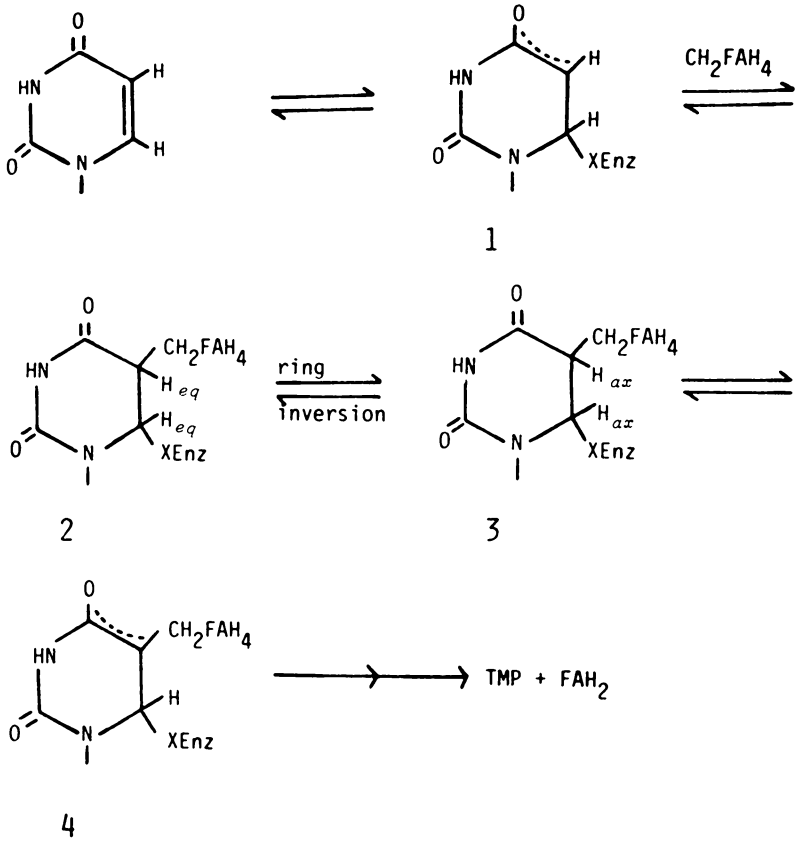

Figure 8

Second, the stereochemistry concerning the transient intermediates shown in Figure 8 may be inferred. The mechanistic details discussed below follow from the principle that a group reacting with the π-system of the uracil heterocycle approaches approximately perpendicular to the plane of the ring; by microscopic reversibility, a similar orientation is required when a group departs to reform the π-system. Thus, the initial attack of the nucleophile of the enzyme at the electrophilic 6-carbon of dUMP should be perpendicular to the plane of the heterocycle. The resultant carbanion (1) will be delocalized throughout the carbonyl groups and will be high in sp^2 character. The approach of CH_2FAH_4 to the 5-position would be perpendicular to the plane of the ring and, based on the ^{19}F data presented above, trans to the nucleophile attached to the 6-position. As a result, as shown in structure 2, the cofactor would exist in a pseudoaxial position, and the 5-hydrogen would be pseudoequatorial. For the subsequent elimination reaction, the proton from the 5-position must be in the pseudoaxial position (3) prior to its abstraction to form the carbanion (4). This mechanistic interpretation necessitates a previously unrecognized conformational change, which occurs after addition of the cofactor but before abstraction of the 5-proton; and results in ring inversion about the 5-and 6-positions of the nucleotide intermediates (i. e. 2→3). The results of ^{19}F nmr studies described here are preliminary; a detailed nmr study of the interaction of FdUMP with thymidylate synthetase will be published elsewhere.

5-Trifluoromethyl-2'-deoxyuridylate (CF_3dUMP). CF_3dUMP is a potent inhibitor of thymidylate synthetase. Reyes and Heidelberger have reported that upon preincubation CF_3dUMP causes irreversible inhibition of thymidylate synthetase from Ehrlich Acsites cells (32). Based on the observation that trifluoromethyluracil (CF_3U) acylates amines in aqueous media to give uracil-5-carboxamides (33), it was suggested that the irreversible inactivation of thymidylate synthetase might result from a similar acylation of an amino group at or near the active site of the enzyme as shown in Figure 9 (32).

A question that arose is why the trifluoromethyl group at the 5-position of uracil derivatives should be at all susceptible to these reactions. The carbon-fluorine bond is quite strong (34) and an outstanding characteristic of most trifluoromethyl groups is their unusual resistance toward chemical degradation. As relevant examples, it is noted that benzotrifluorides, derivatives of 6-trifluoromethyluracils (35), derivatives of 2-trifluoromethyl-4-oxopyrimidines (36) and 5-trifluoromethyl-6-azauracil (37) are quite stable toward hydrolytic reactions. In contrast, CF_3U is rapidly converted into 5-carboxyuracil (CU) (33) in basic media and, although somewhat slower, nucleosides of CF_3U are converted into the corresponding

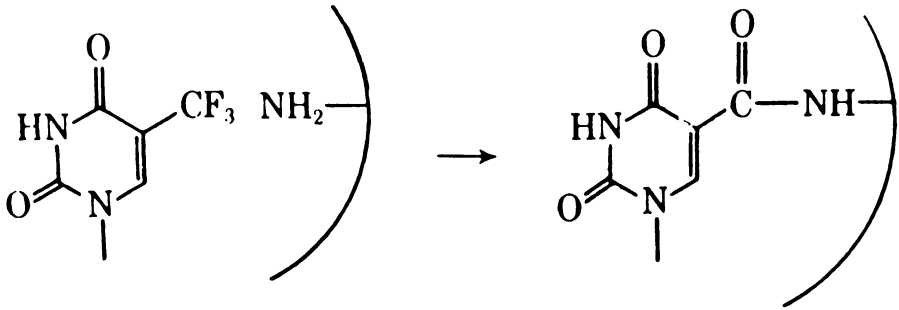

Figure 9

nucleosides of CU (38-40). In vivo, the metabolism of CF_3U and CF_3dUR provides CU and not the normal products expected from pyrimidines (41).

A number of other compounds have been reported to have reactive C-F groups (see reference 42). The reactivity of C-F bonds in most cases has been attributed to hyperconjugative effects (43, 44), hydrogen bonding effects (44, 45), and direct displacement (S_N2) reactions (46). Model reactions involving the hydrolysis of compounds possessing C-F bonds were examined in this labotatory in an attempt to understand the underlying features which resulted in the reactivity of some of these molecules (42). From such studies we proposed that C-F bond labilization usually involves one of several general mechanisms. (a) As depicted in Figure 10a, proton removal is at an atom α to the carbon bearing the fluorine atom with the resultant negative charge, either in a stepwise or concerted manner, aiding in the formation of an intermediate (fluoro) alkene. Depending on the stability of the alkene, it may or may not react with solvent. (b) The proton may be situated on an atom such that the negative charge resulting from the ionization of the proton can exert its influence through an extended π-system (Figure 10b). (c) When the compound is an allylic fluoride incapable of undergoing either of the mechanisms described above, it may undergo nucleophilic (Michael-type) attack at the β-carbon with assistance by the developing carbanion to give an intermediate similar to those previously described (Figure 10c). In any of the above, trifluoromethyl groups give carboxylic acids or derivatives, difluoromethyl groups give aldehydes or ketones, and fluoromethyl groups give alcohols or alkenes.

Most of the C-F bond cleavages thus far reported can be explained then in terms of the aforementioned mechanisms; the ability of the compounds to form olefinic intermediates of the type described appears necessary for such reactions to occur. The mechanism(s) by which the olefinic intermediates are transformed to products is believed to involve alternate addition of

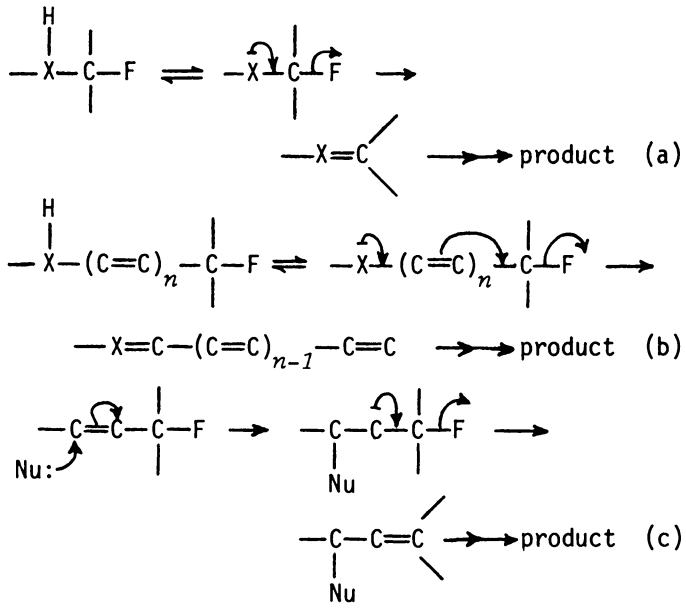

Figure 10

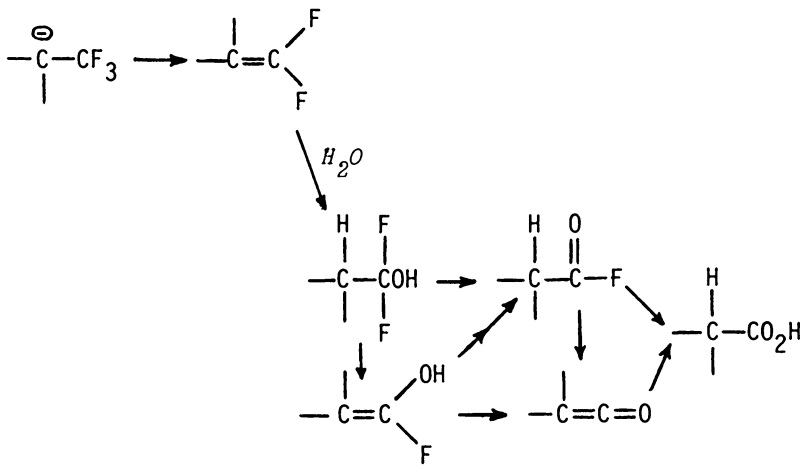

Figure 11

nucleophile (or solvent) to the intermediate, and elimination of fluoride ion. A possible mechanism for hydrolysis of the CF_3 group is depicted in Figure 11 and, as shown, may involve the intermediacy of acyl fluorides and ketenes in the transformation of a trifluoromethyl group to a carboxylate function, although these intermediates have, as yet, not been detected.

The above provided insight into the possible mechanisms by which CF_3dUMP might act as an acylating agent. To obtain direct supporting evidence, the mechanisms of hydrolytic reactions of 5-trifluoromethyluracil and its N-alkylated derivatives were examined in detail (47). The results of these studies are summarized below, and depicted in Figures 12-14.

All reactions appear to proceed by formation of a highly reactive intermediate having an exocyclic difluoromethylene group at the 5-position which subsequently reacts with water or hydroxide ion in a series of rapid steps to give corresponding 5-carboxyuracils. For those derivatives which possess an ionizable proton at the 1-position, the predominant mechanism involves ionization to the conjugate base and assistance by the 1-anion in the expulsion of fluoride ion (Figure 12). When ionization at the 1-position is precluded by the presence of an alkyl substituent (Figure 13), acylation reactions proceed by rate determining attack of hydroxide ion at the 6-position of the neutral or negatively charged (3-anion) heterocycle to provide the reactive intermediate. In order to obtain suitable intramolecular models, and to verify the primary site of reaction of 1-substituted derivatives, a series of 1-(ω-aminoalkyl)trifluoromethyluracils were prepared and their hydrolyses examined (Figure 14). Neighboring group participation was apparent where attack of the amino group on the 6-position of the heterocycle results in the formation of five-, six-, and seven-membered rings; in the case of 1-(3-aminopropyl)-5-trifluoromethyluracil, apparent first-order constants were more than 10^4 times greater than simple 1-alkyl derivatives not possessing a neighboring nucleophile.

With regard to the enzymic reaction, the salient feature of these studies is that the trifluoromethyl group of CF_3dUMP derivatives would only behave as an acylating agent when a secondary driving force is furnished by reactions which occur at other parts of the heterocycle. That is, it is necessary that a nucleophile is added to the 6-position of the heterocycle; in this manner, the normally inert trifluoromethyl group would be converted into a highly reactive exocyclic difluoromethylene intermediate which might then acylate a nucleophilic group of the enzyme.

In accord with proposals for the involvement of nucleophilic catalysis in the enzymic reaction (viz I, Figure 2), these studies led us to propose a related minimal mechanism for the reported irreversible inactivation of thymidylate synthetase by CF_3dUMP. In the pathway depicted in Figure 15, it was suggested that juxtaposed within the active site, a nucleophilic group of the enzyme (:Z) adds to the 6-position of CF_3dUMP, promoting the expulsion of fluoride ion and the formation of a reactive exocyclic difluoromethylene intermediate similar to those encountered in our model studies. The reactive intermediate would then be trapped by a nucleophilic group of

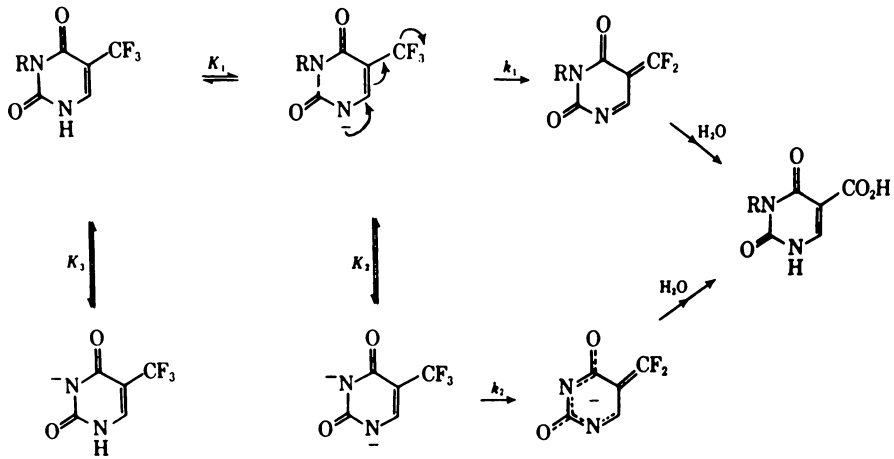

Figure 12

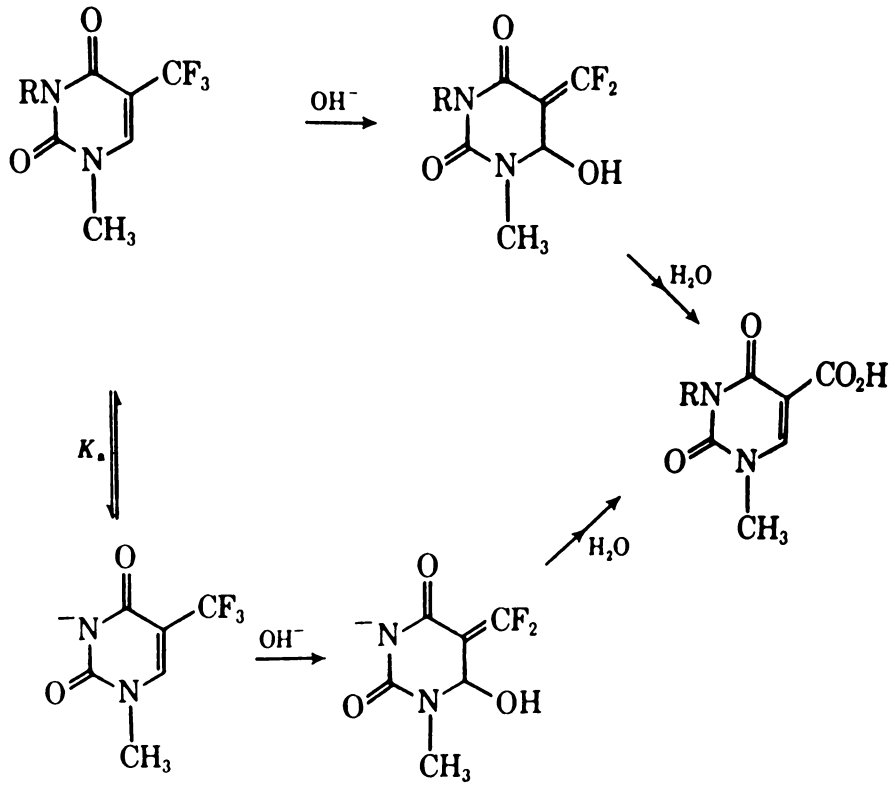

Figure 13

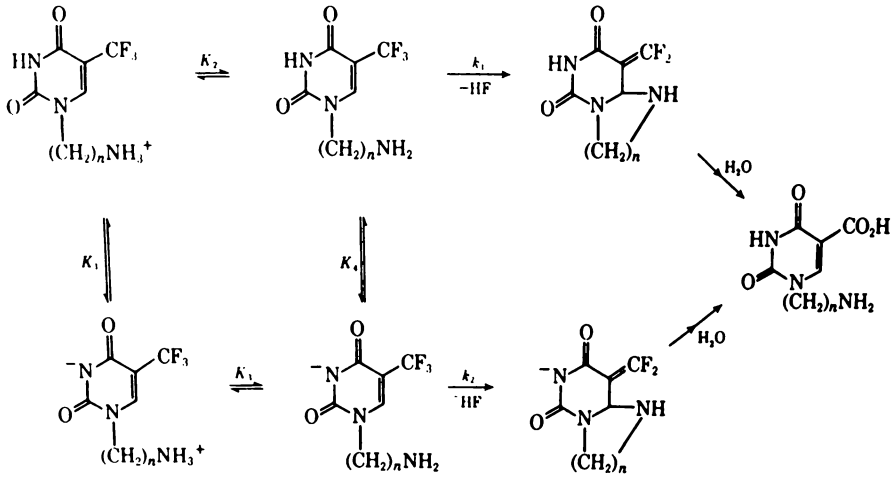

Figure 14

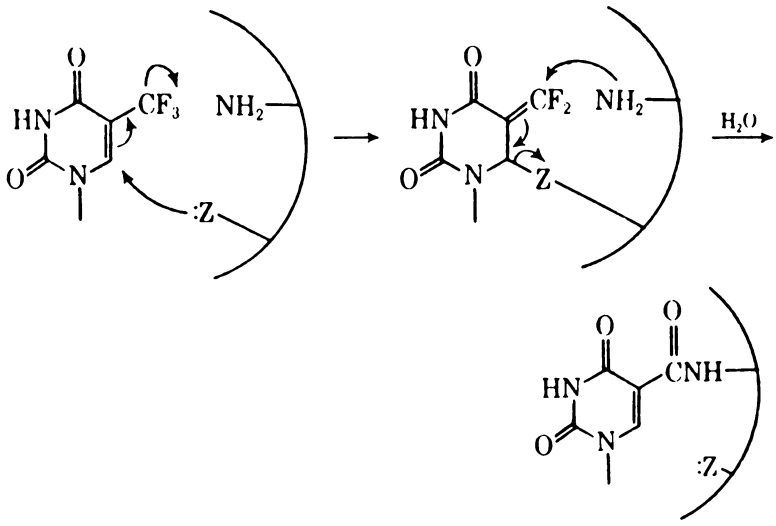

Figure 15

the enzyme to give, after a number of steps, the acylated enzyme.

Subsequent to completion of these model studies, the interaction of CF_3dUMP with thymidylate synthetase was reinvestigated in another laboratory using the enzyme from L. casei (21). These workers observed that CF_3dUMP, CH_2FAH_4 and thymidylate synthetase formed a tight ternary complex which was isolatable by disc gel electrophoresis under non-denaturing conditions. However, unlike the FdUMP·CH_2FAH_4·enzyme complex, no change in the difference spectra was observed when CF_3dUMP was used. Furthermore, gel electrophoresis in the presence of a protein denaturant resulted in apparent destruction of the complex. After denaturation of the complex, the nucleotide product was observed to migrate identically with authentic CF_3dUMP on DEAE-cellulose paper. From these results, it was concluded that C-F bonds of the nucleotide were not cleaved by the enzyme and a nucleophile of the enzyme did not add to the 6-position of CF_3dUMP.

Recent results obtained in this laboratory are not in accord with these findings. Although the mechanism of reaction of CF_3dUMP with thymidylate synthetase has not been ascertained at this time, it appears certain that C-F bond cleavage is catalyzed by the enzyme, probably via nucleophilic catalysis. Our preliminary results which lead to this conclusion are summarized below.

Contrary to the previous report (21), we observe that subtraction of the ultraviolet spectrum of enzyme and CH_2FAH_4 from that of the enzyme, CH_2FAH_4 and CF_3dUMP produces a difference spectra (Figure 16a) which is very similar to that observed with the FdUMP·CH_2FAH_4·enzyme ternary complex (Figure 4). As with the thymidylate synthetase·FdUMP·CH_2FAH_4 complex, there is the characteristic increase of absorbance at 330 nm and a decrease at 261 nm; the latter is in accord with saturation of the 5, 6-double bond of the nucleotide. Upon addition of sodium dodecyl sulfate, the only differential absorbance is that characteristic of a nucleotide with $\lambda_{max}^{\approx}$ 267 nm; although this is upfield from the maximum of CF_3dUMP, we have not yet ascertained whether an alteration of structure is involved or whether the shift is artifactual.

In the absence of CH_2FAH_4, the incubation of thymidylate synthetase and CF_3dUMP (ratio 1:6) for 20 minutes at 22° results in 89% inactivation of the enzyme. This is in accord with previous reports on the effect of this nucleotide on the enzyme from Ehrlich ascites cells (32). The difference spectra of CF_3dUMP and enzyme vs enzyme is shown in Figure 16b. The maximum of CF_3dUMP at 261 nm decreases, and a transient broad peak appears which has absorbtion up to ca. 340 nm. After 1 hour, the final spectrum exhibits a maxima at 276 nm, resembling 5-acyl derivatives of dUMP. Paper

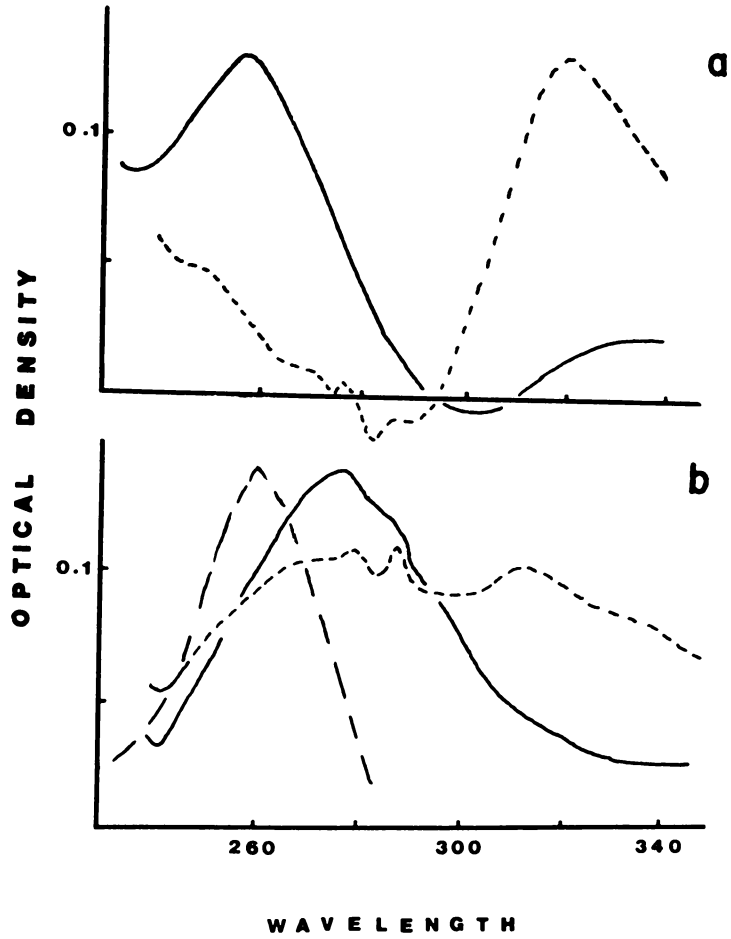

Figure 16. Ultraviolet difference spectra. (a) Dashed line: CF_3dUMP, CH_2FAH_4 and thymidylate synthetase vs. CH_2FAH_4 and thymidylate synthetase. Solid line: after treatment with sodium dodecyl sulfate. (b) Dashed line: CF_3dUMP and thymidylate synthetase vs. thymidylate synthetase after 20 seconds. Solid line: after 1 hr. Broken line: ultraviolet spectrum of CF_3dUMP.

chromatography of this reaction mixture shows a single spot which moves slightly slower than the starting material (CF_3dUMP). Although this product has not yet been identified, it is not 5-carboxy-dUMP. When CF_3dUMP was treated with a limiting amount of thymidylate synthetase (50:1) for 23 hours at 22°, we were able to detect that at least 0.3 equivalents of F^- were released, demonstrating that C-F bonds of the nucleotide were indeed labilized.

Although these results are too preliminary to permit definitive interpretation, certain conclusions may be reached and speculations may be forwarded. It is clear that the interaction of CF_3dUMP and thymidylate synthetase in the absence of cofactor may result in cleavage of C-F bonds of the nucleotide as well as inactivation of the enzyme. From the aforementioned model studies, it is most reasonable to propose that activation of the C-F bond requires addition of a nucleophile of the enzyme to the 6-position of the nucleotide. The mechanism of enzyme inactivation is not known, but it appears to be slower (10%) than the reaction leading to C-F bond cleavage. It is also apparent that the presence of CH_2FAH_4 modulates this reaction in some yet unknown manner; the enzyme·CF_3dUMP·CH_2FAH_4 complex has ultraviolet spectral qualities quite similar to those of the complex formed with FdUMP, indicating that the 5,6-double bond of CF_3dUMP is saturated in the ternary complex. Further experiments are in progress which aim to elucidate the mechanism of interaction of CF_3dUMP with thymidylate synthetase.

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