Fluorine-19 Nuclear Magnetic Resonance Characterization of Ternary Complexes of Folate Derivatives, 5-Fluorodeoxyuridylate and *Lactobacillus casei* Thymidylate Synthetase†

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ABSTRACT: Numerous biochemical techniques have been employed to characterize the covalent inhibitory ternary complex of thymidylate synthetase consisting of enzyme, 5-fluorodeoxyuridylate, and 5,10-methylenetetrahydrofolate. ^{19}F NMR studies of this covalent ternary complex reveal a single, broad resonance centered at 12.7 ppm to higher shielding from the free nucleotide, while the 5-fluorodeoxyuridylate–enzyme binary complex exhibits two resonances to higher shielding of free nucleotide, one at 1.4 ppm representing noncovalently bound ligand and the other at 34.5 ppm indicative of covalently bound 5,6-dihydro-5-fluorodeoxyuridylate. In order to follow the transformation of the latter binary complex to a ternary complex, we have employed ^{19}F NMR to profile changes in the environment of the nucleotide which result from the interaction of folates with the coenzyme binding site. At low molar excesses of folates (5-fold), the effects observed in the ^{19}F NMR spectrum fall into three major classes. (1) 5-Methyltetrahydrofolate exhibited a weak interaction with the binary complex. (2) Methotrexate and aminopterin, antifolate drugs, were observed to increase the exchange rate among the species detected in the ^{19}F NMR spectrum of the binary complex. (3) Folate, dihydrofolate, and a series of tetrahydrofolate derivatives were found to shift the equilibrium of the binary complex toward the covalent 5,6-dihydro-5-fluorodeoxyuridylate–enzyme complex. With the latter folates the chemical shifts for the covalent species of these ternary complexes were found in the range of 35–40 ppm to higher shielding and are interpreted to reflect subtle differences in the strength and steric nature of the interaction of the folate ligand with the binary complex. These data illustrate that the latter folates promote the conversion of the enzyme-bound nucleotide to a species which would be poised to form the second covalent bond of the ternary complex, namely the linkage of the methylene group of the coenzyme with carbon 5 of the nucleotide.

The interaction of the substrate analogue FdUMP[1] with thymidylate synthetase (EC 2.1.1.45) has been exploited to investigate the mechanism of the reductive methylation of dUMP catalyzed by this enzyme by using CH_2-H_4folate as the source of the methyl group (Friedkin, 1973; Danenberg, 1977; Pogolotti & Santi, 1977). Substitution by fluorine of the proton on carbon 5 of dUMP produces the pseudosubstrate FdUMP which can only proceed through the enzymatic reaction to the formation of an enzyme–FdUMP–CH_2-H_4folate inhibitory ternary complex.[2] Covalent bonds to the pyridimine ring of FdUMP, one from the methylene group of CH_2-H_4-folate and the other from the catalytic cysteine of the enzyme, have been shown to be present in this intact ternary complex (Langenbach et al., 1972; Danenberg et al., 1974) and the isolated active-site peptide (Sommer & Santi, 1974; Danenberg & Heidelberger, 1975; Pogolotti et al., 1976). The latter inhibitory ternary complex is believed to be directly analogous to the catalytic complex involving dUMP and has been thoroughly characterized by a variety of biochemical and spectroscopic techniques. Extensive ^{19}F NMR studies performed in these laboratories (Byrd, 1977; Byrd et al., 1977, 1978) on the native and denatured forms of this ternary complex in the intact enzyme resulted in the determination of the relative stereochemistry of the enzyme and folate ligands on the saturated pyrimidine ring of FdUMP.

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[1] Abbrevations used: FdUMP, 5-fluorodeoxyuridylate; dUMP, deoxyuridylate; CH_2-H_4folate, (±)-5,10-methylene-5,6,7,8-tetrahydrofolate; 10-CH_3-H_4folate, (±)-10-methyl-5,6,7,8-tetrahydrofolate; H_4homofolate, (±)-5,6,7,8-tetrahydrohomofolate; 5-CH_3-H_4folate, (±)-5-methyl-5,6,7,8-tetrahydrofolate; 5-CHO-H_4folate, (±)-5-formyl-5,6,7,8-tetrahydrofolate; methotrexate, 2,4-diamino-10-methylfolate; aminopterin, 2,4-diaminofolate; H_2folate, 7,8-dihydrofolate; H_4folate, (±)-5,6,7,8-tetrahydrofolate; 10-CH_3-folate, 10-methylfolate; 5,10-CH^+-H_4folate, (±)-5,10-methenyl-5,6,7,8-tetrahydrofolate; 10-CHO-H_4folate, (±)-10-formyl-5,6,7,8-tetrahydrofolate; $NaDodSO_4$, sodium dodecyl sulfate; FdU, 5-fluorodeoxyuridine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

[2] In the thymidylate synthetase literature the terms "inhibitory complex" and "ternary complex" have been used exclusively to describe the unique association of thymidylate synthetase, FdUMP, and CH_2-H_4folate in which the cofactor is attached covalently to C-5 of FdUMP through a methylene bridge and the enzyme is linked to C-6 of FdUMP through a thioether bond. Since ternary complexes and inhibitory ternary complexes of this enzyme exist whose compositions differ from that described above, we find the latter terminology to be inappropriate. As suggested by the editors, we propose the adoption of a more general nomenclature in which a binary complex refers to the binding of a single type of ligand (nucleotide or folate) and a ternary complex indicates the interaction of both types of ligands (nucleotide and folate) with the enzyme. In order to distinguish between the noncovalent and covalent linkages of ligands, we use the following notation. For binary complexes, enzyme···ligand (nucleotide or folate) symbolizes noncovalent binding and enzyme–ligand (nucleotide or folate) represents covalent linkage. For ternary complexes, the binding of two types of ligands is represented by enzyme–nucleotide–folate which indicates the presence of two covalent linkages as in the classical thymidylate synthetase–FdUMP–5,10-methylenetetrahydrofolate complex, whereas the term enzyme···nucleotide···folate reflects a noncovalent interaction of both ligands. It will be recognized that most of the ternary complexes studied in this paper are of the following form, enzyme–FdUMP···folate, in which the FdUMP is attached covalently to the enzyme and the folate ligands are noncovalently associated with the system.

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In recent papers we began a study of the steps preceding formation of the enzyme–FdUMP–CH_2-H_4folate complex by examining the association of FdUMP with thymidylate synthetase in the absence of CH_2-H_4folate by ^{19}F NMR (Lewis et al., 1978, 1980). Precedent existed in the literature for the formation of stable enzyme–nucleotide binary complexes from equilibrium dialysis (Galivan et al., 1976), microcalorimetry (Beaudette et al., 1977, 1980), circular dichroism (Leary et al., 1975; Plese & Dunlap, 1978), and Hummel–Dreyer equilibrium gel filtration (Beaudette et al., 1977; Plese et al., 1979). We have reported that the fluorine NMR spectrum of the binary complex exhibited two resonances for FdUMP bound to the enzyme; one resonance at −1.4 ppm (to higher shielding) from free FdUMP and a second at −34.5 ppm (Lewis et al., 1980). These resonances represent an equilibrium mixture of a noncovalent Michaelis-like complex of the nucleotide inhibitor in the active site of the enzyme and a covalent 5-fluoro-5,6-dihydrodeoxyuridylate–6-enzyme species, respectively. The covalently bound 5,6-dihydro species resulted from protonation of the enolate intermediate which would normally undergo electrophilic attack by the methylene group of CH_2-H_4folate. It is the formation of a comparable enzyme–dUMP binary complex which is responsible for the slow release of tritium from [5-^{3}H]dUMP reported by Pogolotti et al. (1979).

The present study was undertaken to examine the next step in the reaction mechanism and to determine what further changes in the nucleotide might be imposed upon binding of CH_2-H_4folate, just prior to formation of the covalent bond between the folate and carbon 5 of FdUMP. Folate interaction with the thymidylate synthetase–nucleotide binary complex was first observed by Santi et al. (1974) by isolation on nitrocellulose membranes of enzyme–FdUMP complexes which had been incubated in the presence of large excesses of a variety of folates. To their surprise several folates greatly increased the retention of the nucleotide with the enzyme relative to a native binary complex, but to a much smaller extent than found for the enzyme–FdUMP–CH_2-H_4folate complex. Danenberg et al. (1974) examined the stability of enzyme–FdUMP complexes to Sephadex G-25 column chromatography in both native and heat-denatured states after incubation with one of four tetrahydrofolate derivatives. CH_2-H_4folate, 10-CH_3-H_4folate and H_4homofolate produced comparable binding of FdUMP in the native state, but when denatured only the CH_2-H_4folate-incubated complex retained the full complement of FdUMP. Galivan et al. (1974) reported that thymidylate synthetase isolated from T2 bacteriophage bound 0.5–1.7 mol of FdUMP in the presence of folates when the complexes were isolated on nitrocellulose membranes and that the stoichiometries were equivalent for native and guanidine hydrochloride denatured complexes but were greatly reduced on urea denaturation. Galivan et al. (1975) observed that the circular dichroic spectrum of the *Lactobacillus casei* thymidylate synthetase in the FdUMP binary complex was strikingly altered by the presence of a number of folates, which in the absence of the nucleotide did not alter the circular dichroic spectrum of the enzyme. Equilibrium dialysis experiments of FdUMP–thymidylate synthetase complexes in the presence of folates revealed some striking synergistic effects (Galivan et al., 1976). Monoglutamyl folates exhibited no detectable binding to the native enzyme, but their interaction with the enzyme–nucleotide complexes exhibited dissociation constants of 10^{-5}–10^{-6} M for the folates. Conversely, the dissociation constants of the nucleotides in these ternary complexes were at least 3–10-fold lower than when measured in their binary complexes with the enzyme. These observations suggested that the presence of *both* nucleotide and folate in the active site of the enzyme resulted in significantly greater affinities of the enzyme for *both* ligands. In order to investigate the effects of folates on the interaction of FdUMP with the enzyme, we have observed the changes produced in the ^{19}F NMR spectrum of the binary complex by a variety of folates.

Experimental Procedures

Thymidylate synthetase was isolated in the presence of exogenous thiol from amethopterin-resistant *Lactobacillus casei* according to the procedure of Lyon et al. (1975). Since our previous studies indicated that the greatest extent of binary complex formation was obtained in Tris-HCl buffer with enzyme chromatographed on DEAE-Sephadex, all enzyme employed in these studies was DEAE-thymidylate synthetase (phosphate free) (Byrd, 1977). This enzyme was shown to be homogeneous by disc gel electrophoresis, produced normal enzyme–FdUMP–CH_2-H_4folate ternary complex gels (Aull et al., 1974; Donato et al., 1976), and exhibited specific activities in excess of 2.5 units/mg of protein when assayed spectrophotometrically by the procedure of Wahba & Friedkin (1961) as modified by Dunlap et al. (1971).

5-CH_3-H_4folate, aminopterin, (6*RS*)-5-CHO-H_4folate (Sigma Chemical Co.), and folic acid (Calbiochem) were used without further purification. Methotrexate was subjected to DEAE-cellulose column chromatography to remove a number of contaminants, especially 10-CH_3-folate (Gallelli & Yokoyama, 1967; Chatterji et al., 1978). 10-CH_3-folate, as obtained above or from the alkaline hydrolysis of methotrexate, was purified by DEAE-cellulose chromatography and reduced catalytically to yield 10-CH_3-H_4folate (Gupta & Huennekens, 1967). H_2folate (Sigma) was purified by DEAE-cellulose column chromatography (Zakrewski & Sansone, 1971). The racemic mixture (±) of H_4folate was obtained by catalytic hydrogenation of folic acid in glacial acetic acid as described by Hatefi et al. (1960). The fluffy lyophilized product was stored under nitrogen in sealed vials and was used without further purification. 5,10-CH^+-H_4folate was produced by acidifying of an aqueous 2-mercaptoethanol solution of 5-CHO-H_4folate, monitoring the changing ultraviolet spectrum, and collecting and drying the white powder (Rabinowitz, 1963). Neutralization of an aqueous suspension of 5,10-CH^+-H_4folate in 0.1 M Tris-HCl, pH 7.4, yielded 10-CHO-H_4folate over the course of ~6 h as indicated by the ultraviolet spectrum (Rabinowitz, 1963). The purity of all folates employed in these studies was judged by the agreement of their ultraviolet spectra, in 0.1 N HCl, pH 7.0 buffer, and 0.1 N NaOH, with those reported in the literature [Blakley (1969) and references cited above].

Enzyme samples were prepared for NMR spectroscopy by concentrating a solution containing ~35 mg of the purified DEAE-thymidylate synthetase in an Amicon ultrafiltration cell to a volume of ~4 mL and a final enzyme concentration of 90–100 μM. Activation was accomplished by dialysis in 2 L of 0.1 M Tris-HCl at pH 7.4 containing 1 mM EDTA and 50 mM 2-mercaptoethanol, followed by two dialyses in 150 mL of the same buffer prepared with 30% deuterium oxide (Bio-Rad Laboratories) to provide a lock signal for the NMR spectrometer. No correction was made for the deuterium isotope effect on the glass electrode, so all pH values are those actually measured. Enzyme concentration was measured spectrophotometrically at 278 nm by using the extinction coefficient of 1.05×10^5 reported by Lyon et al. (1975). In these studies of the enzyme–FdUMP···folate ternary com-

plexes, binary complexes were first prepared by mixing a 2.5 molar excess of FdUMP with the activated enzyme and followed by transfer of the solution into an 18-mm flat-bottom NMR tube which was fitted with a nylon vortex plug. The ^{19}F NMR spectrum was then acquired to insure proper conversion of the enzyme into the FdUMP-enzyme binary complex.

Ternary complexes were prepared by conversion of the binary complex through stepwise addition of a concentrated solution of the folate. The folate was dissolved in 1 mL of the final 30% deuterium oxide dialysis buffer and the concentration determined spectrophotometrically. These solutions were typically 10–20 mM so that only 24–40 μL of the solution was required for 1 equiv of folate/enzyme. Stepwise additions of equivalents of folate were made to a final 5–7-fold molar excess over the enzyme, with the ^{19}F NMR spectrum being recorded after each addition. Samples were denatured by addition of a 25% solution of $NaDodSO_4$ to a final concentration of 1.2% (Byrd, 1977).

Fluorine-19 NMR spectra were obtained at 94.1 MHz on the highly modified Varian XL-100-15 NMR spectrometer in the Department of Chemistry at the University of South Carolina utilizing the 18-mm multinuclear probe (Byrd & Ellis, 1977). All data were obtained at 20 ± 1 °C in the Fourier transform mode by using 60° pulse widths, with an acquisition time of 0.4 s, acquiring 5K data points and transforming 8K data points. Free induction decays were weighted by exponential multiplication prior to Fourier transformation, which added 3–6 Hz to the natural line width of the resonances. Spectral windows were 6000 Hz. All spectra were referenced to free FdUMP in the samples, which was observed at 88.9 ppm to higher shielding of 6 mM trifluoroacetic acid in deuterium oxide. Chemical shifts to higher shielding are indicated by negative numbers. Each of the spectra in this study required 18–24 h of data accumulation in order to obtain sufficient definition of signals from noise. Estimates of the percent composition of the individual resonances in each spectrum were obtained by cut and weigh measurements of the areas of the expanded resonances and were usually reproducible to within better than 10% from sample to sample.

The FdUMP used in these experiments was prepared by chemical phosphorylation of FdU (Sigma) and chromatographed on Dowex 1-X10, 200–400 mesh, to purify the 5′-phosphate isomer (Dawson et al., 1977). The FdUMP was chromatographed under slightly basic conditions in D_2O on Chelex (Bio-Rad) to remove metals prior to ^{31}P and ^{19}F analysis. The stock solution of FdUMP was found to be 90% the 5′ isomer of FdUMP, with 5% being the 6-deuterated species of FdUMP and the remainder the 3 isomer of FdUMP and its 6-deuterio species. The deuteration was the result of the small amount of base present in the sample from the Chelex treatment (Lewis et al., 1980). The presence of these small amounts of contaminants was found to have no effect on the quantitation of these spectra.

Results

A variety of changes was observed in the ^{19}F NMR spectrum of the binary complex in the presence of low molar excesses of the added folate derivatives. An experiment with 10-CH_3-folate is shown in Figure 1 in order to illustrate these changes. After the native binary complex spectrum was acquired (Figure 1A), 10-CH_3-folate was added in 1-, 2-, 5-, and 7-fold molar excess over the enzyme. Note that the addition of the first equivalent (Figure 1B) resulted in increases in the peak height and area of the resonance at −34.5 ppm for co-

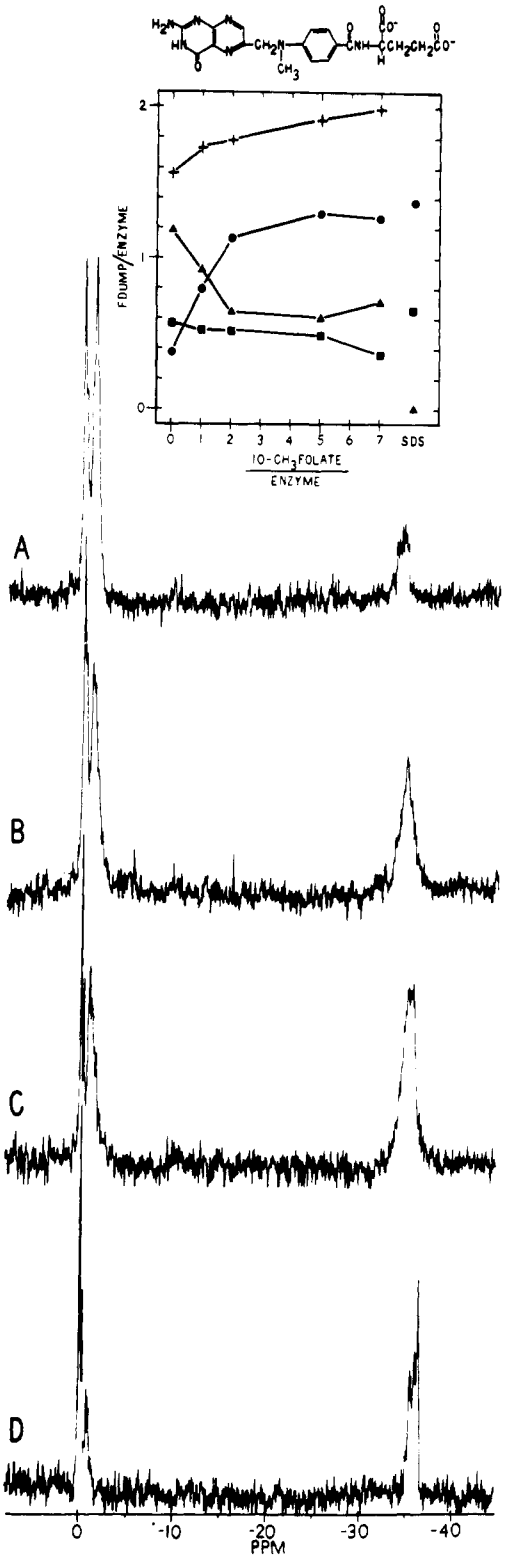


FIGURE 1: Fluorine-19 NMR spectra of the thymidylate synthetase–FdUMP binary complex titrated with 10-CH_3-folate in 0.1 M Tris-HCl, pH 7.4, at 20 °C. (A) Native binary complex. (B) The binary complex spectrum after the addition of 10-CH_3-folate to a concentration equivalent to that of the enzyme. (C) The binary complex spectrum after the addition of a 7-fold excess of 10-CH_3-folate. (D) The same sample as in (C), but after denaturation by addition of $NaDodSO_4$ to 1.2%. The inset plot represents the changes during the titration of the amount of free FdUMP (■) and FdUMP bound to the enzyme noncovalently (▲) and covalently as the 5,6-dihydro derivative indicated by the resonance near −35 ppm (●). The total nucleotide bound to the enzyme during the titration is indicated by (+). The FdUMP free in solution or remaining associated with the enzyme in the presence of 1.2% of $NaDodSO_4$ is indicated in the column above SDS.

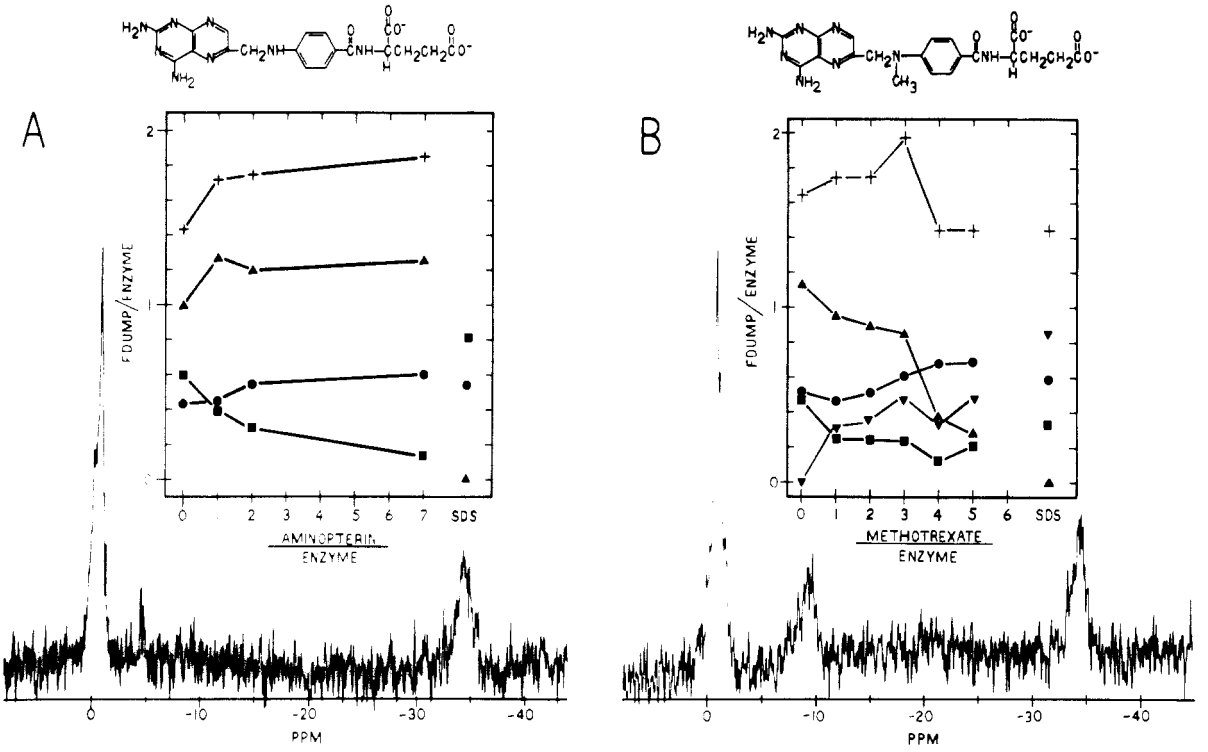


FIGURE 2: Behavior of the fluorine-19 NMR spectrum of the binary complex upon addition of folate antimetabolites. Addition of aminopterin (A) produced increased line widths of all resonances, as indicated by the spectrum in the presence of the 7-fold excess. Methotrexate (B), as well as broadening the resonances of the binary complex, produced a new covalent resonance which appeared at −9.5 ppm (▼). All other symbols are as defined in Figure 1.

valently bound nucleotide, while the resonance at −1.4 ppm due to noncovalently bound nucleotide decreased. Further additions of 10-CH_3-folic acid produced spectra comparable to that for the 7-fold excess (Figure 1C). The chemical shift of the resonance for covalently bound FdUMP increased during the titration to −35.5 ppm with a slight increase in line width (120–145 Hz). Denaturation by addition of $NaDodSO_4$ produced a large well-resolved doublet and singlet characteristic of the 5,6-dihydro derivative of FdUMP covalently bound to the enzyme, with a chemical shift of −36.0 ppm (Lewis et al., 1980). In this case no covalently bound FdUMP was released from the enzyme upon denaturation. A few of these ternary complexes (containing H_2folate and 5-CHO-H_4folate) did exhibit depleted amounts of covalently bound FdUMP in the denatured state, and we cannot offer a complete explanation for this phenomenon. However, we have observed that the resonance for the covalently bound nucleotide disappears within a few days after denaturation. This is apparently due to a slow dissociation of the covalently bound nucleotide from the denatured complex.

To more clearly illustrate the flux in the pools of FdUMP observed in the fluorine NMR spectra of these ternary complexes, we constructed plots of FdUMP/enzyme vs. folate/enzyme as shown in the inset of Figure 1. This plot presents the changes observed in the amount of FdUMP free in solution and noncovalently bound or covalently bound to the enzyme as the folate was added. Formation of the enzyme–FdUMP···10-CH_3-folate ternary complex resulted in a slight overall increase in the amount of nucleotide bound to the enzyme. However, the equilibrium of nucleotide binding was displaced in favor of the covalent complex by a factor of 3 over the binary complex. Since only slight differences were visible in the spectra with a 2-, 5-, and 7-fold excess of 10-CH_3-folate, it appeared that stoichiometric binding of one 10-CH_3-folate molecule per active site produced the observed spectral changes. The symbols on the plot above SDS indicate the amounts of FdUMP which remained bound to the enzyme and free in solution under the denaturing conditions. For all subsequent ternary complexes investigated herein, an endpoint ^{19}F NMR spectrum will be accompanied by an inset plot of the titration.

Two folate antimetabolites, aminopterin and methotrexate, were found to alter the ^{19}F NMR spectrum of the binary complex by increasing the exchange rate of the nucleotide in the binary complex. Increased line widths for the resonances of free (30–50 Hz) and noncovalently bound FdUMP (55–90 Hz) were observed, although the line width of the resonance for the covalently bound nucleotide was unaltered in the ternary complexes containing aminopterin and methotrexate (Figure 2). This indicated an increased rate of exchange of FdUMP in solution with that noncovalently bound to the enzyme. Methotrexate, in contrast (Figure 2B), appeared to increase the rate of exchange between the two bound species as well as to give rise to a new resonance in the ^{19}F NMR spectrum for bound FdUMP. The nucleotide bound to the enzyme appeared to be nearly equally distributed among the resonances at −1.4, −9.4, and −34.5 ppm. Denaturation of this ternary complex exhibited two bound resonances: the doublet and singlet at −36.4 ppm and an unresolved multiplet at −11.4 ppm. Note that although methotrexate is a 10-CH_3 derivative of folic acid, there is no increase in the resonance due to covalently bound FdUMP at −34.5 ppm.

The N^5-substituted tetrahydrofolate derivatives were found to exert quite different effects on the ^{19}F NMR spectrum of the binary complex. 5-CH_3-H_4folate (Figure 3A) appeared to interact feebly with the complex since no change was observed until a 6-fold excess was present in the sample. Under these conditions a small resonance appeared at −38.8 ppm, which was the only indication that 5-CH_3-H_4folate was interacting at all with the binary complex. This was not unexpected since this folate was reported to be a very weak noncompetitive inhibitor of the enzyme (Danenberg et al.,

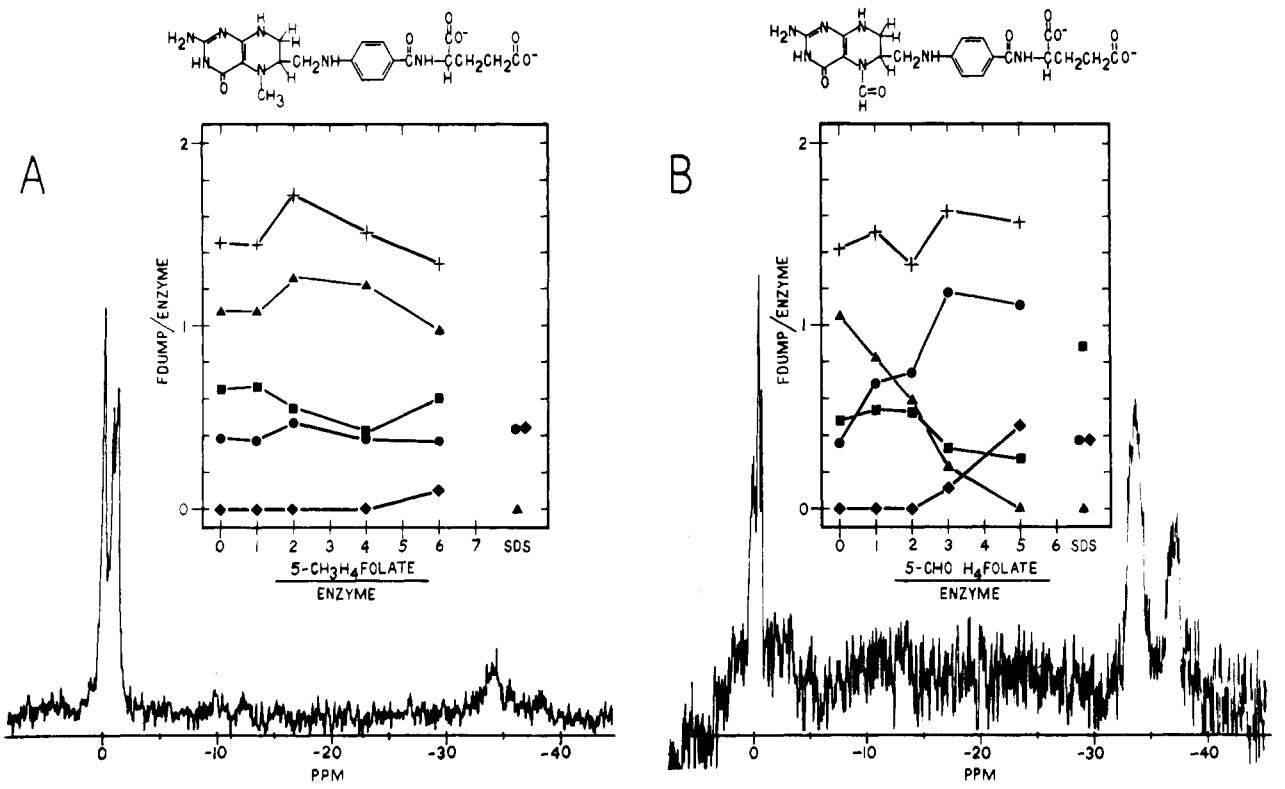


FIGURE 3: Changes in fluorine-19 NMR spectrum of binary complex produced by N^5-substituted H_4folates. 5-CH_3-H_4folate (A) had only slight effects on the binary complex until a 6-fold excess was present in the illustrated spectrum. 5-CHO-H_4folate (B), in contrast, produced significant alterations in the fluorine spectrum of the binary complex, including two distinct chemical shifts for the covalently bound 5,6-dihydro derivative of FdUMP (♦). Other symbols are defined in Figure 1.

1974). In marked contrast, (6*RS*)-5-CHO-H_4folate, which is employed to rescue patients from high-dose methotrexate chemotherapy, strikingly altered the fluorine NMR spectrum of the binary complex (Figure 3B). Initial additions of this folate increased the resonance at −34.0 ppm corresponding to covalently bound FdUMP. Subsequent additions continued this trend and generated a second resonance for covalently bound nucleotide at −37.7 ppm. The presence of two resonances corresponding to covalently bound nucleotides in the ternary complexes containing (6*RS*)-5-CHO-H_4folate may result from the interaction of the two carbon 6 diastereomers with two different active sites containing covalently bound FdUMP. Since the resonance at −34.0 ppm was observed to increase prior to the appearance of the resonance at −37.7 ppm, it may be concluded that one diastereomer has a greater affinity for complex formation than the other. The presence of two species is supported by 300-MHz ^{1}H NMR spectra of (6*RS*)-5-CHO-H_4folate reported recently by Poe & Benkovic (1980). Feeney et al. (1980) have recently employed saturation transfer experiments to show that 5-CHO-H_4folate is an unequal mixture of two slowly interconverting conformations. Increases in covalently bound FdUMP in the two highly shielded resonances resulted from depletion of nucleotide that was originally noncovalently bound. Although only a slight *overall* increase in FdUMP binding was observed, the presence of (6*RS*)-5-CHO-H_4folate resulted in the complete covalent binding of the nucleotide to the enzyme.

To evaluate the effect which the oxidation state of the pyrazine ring of the folate has on the formation of enzyme–FdUMP···folate ternary complexes, we added folate, H_2folate, and H_4folate individually to the binary complex. Folate (Figure 4A) produced a significant increase in the resonance at −36.5 ppm for covalently bound nucleotide, the result of covalent binding of FdUMP which had been free in solution or noncovalently associated with the enzyme. H_2folate (Figure 4B) shifted the equilibrium of the binary complex in favor of the covalent species, which resulted in a tripling of the amount of covalently bound FdUMP indicated by the resonance at −37.4 ppm. A new resonance was observed at +2.0 ppm in the enzyme–FdUMP···H_2folate ternary complex but disappeared upon denaturation. Addition of the racemic mixture of (±)-H_4folate to the binary complex was done with a large excess of dimedone (Sigma) present to scavenge any trace of formaldehyde which might be associated with the H_4folate and support enzyme–FdUMP–CH_2-H_4folate complex formation. Dimedone itself was found to have no effect on the binary complex spectrum. Additions of H_4folate produced a dramatic shift in the binary complex equilibrium which was reflected in the large amount of the 5,6-dihydro-FdUMP covalently bound to the enzyme, as indicated by the resonance at −39.2 ppm.

To further illustrate the effect of reduction of the pyrazine ring of the folate on the interaction of the folate with the binary complex, we examined the addition of 10-CH_3-H_4folate (Figure 5). This folate has been shown by Danenberg et al. (1974) to be a potent competitive inhibitor of thymidylate synthetase. In contrast to the observations of Figure 1, 10-CH_3-H_4folate produced a much greater shift in the binary complex equilibrium favoring the covalent binding of FdUMP, as indicated by a resonance at −38.3 ppm. This was accomplished by complete depletion of the free ligand and nucleotide that was noncovalently bound to the enzyme. Also observed in this spectrum are three very small resonances at −4.5, −9.6, and −11.8 ppm. The first of these resonances appeared to be a very minor contaminant in the FdUMP due to its narrow line width and its invariant chemical shift upon denaturation of the sample. The latter two resonances with 80-Hz line widths may be covalently bound species, since upon denaturation they move to −2.0 ppm. The presence of the latter species were minor results of the addition of 10-CH_3-H_4folate.

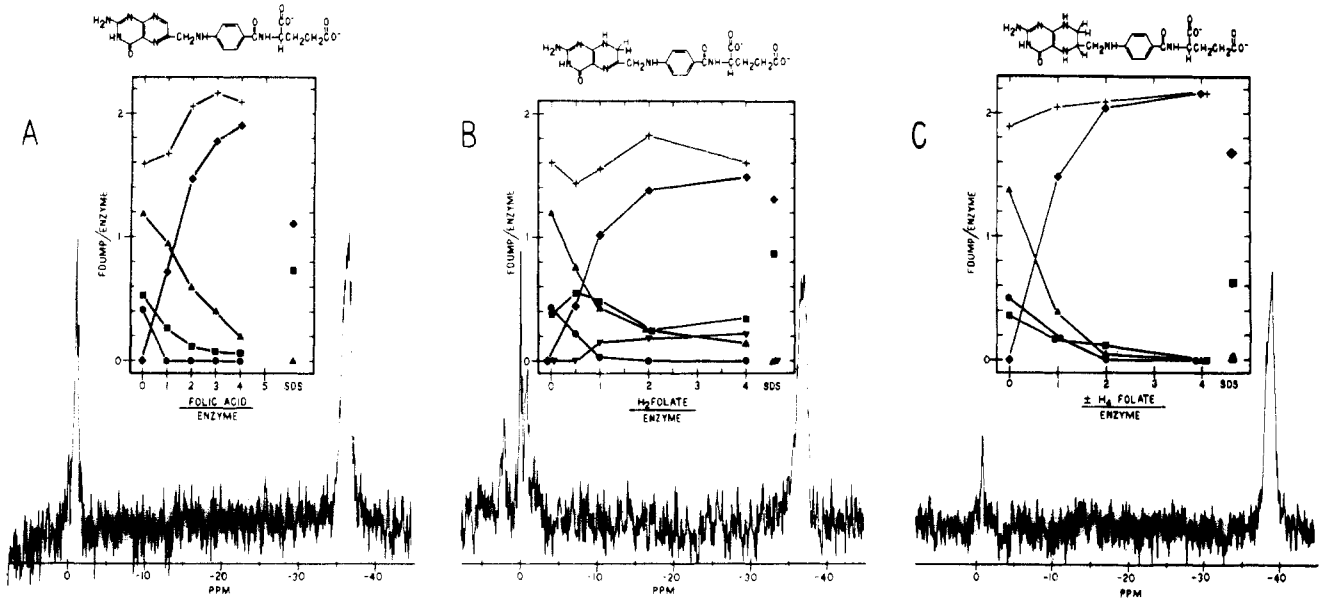


FIGURE 4: Effect of the oxidation state of folate on the fluorine-19 NMR spectrum of the binary complex. Folate (A), H_2folate (B), and (±)-H_4folate (C) were added to the binary complex stepwise to a 4-fold excess illustrated in this figure. In each case, a new chemical shift for the covalently bound 5,6-dihydro species of FdUMP (♦) was observed between −36 to −39 ppm from free FdUMP. Dimedone was added to the H_4folate sample to prevent free formaldehyde from combining with H_4folate and supporting enzyme–FdUMP–CH_2-H_4folate complex formation. In the enzyme–FdUMP···H_2folate complex, a resonance due to apparently noncovalently bound FdUMP appeared at +2.0 ppm (▼). Other symbols are identified in Figure 1.

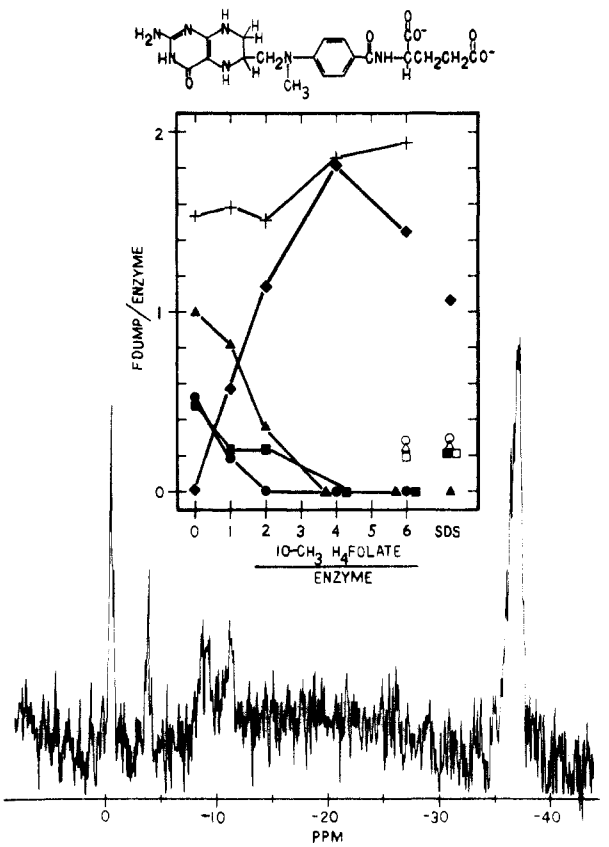


FIGURE 5: Fluorine-19 NMR spectral changes observed during titration of the binary complex with 10-CH_3-H_4folate. The chemical shift of the covalently bound 5,6-dihydro derivative of FdUMP was −38.3 ppm (♦). In the spectrum at the 6-fold excess of the folate, small resonances at −4.5, −9.6, and −11.8 ppm (indicated by the open symbols) were observed and are probably caused by decomposition products of the excess 10-CH_3-H_4folate present in the sample. All other symbols are defined in Figure 1.

To approximate the presence of CH_2-H_4folate binding to the binary complex prior to formation of the covalent bond to FdUMP, we titrated the complex with 5,10-CH^+-H_4folate. NMR (Khalifa et al., 1979; Poe et al., 1979a,b) and crystallographic (Fontecilla-Camps et al., 1979a,b) studies have shown comparable orientations of the proton on carbon 6 and the bridging carbon unit between N^5 and N^{10} and the presence of equivalent conformations of the tetrahydropyrazine rings in the methenyl and methylene H_4folates. The double bond and positive charge on N^5 of this folate might allow it to mimic the N^5 iminium cation, which has been proposed as the catalytic species of CH_2-H_4folate in the enzymatic reaction (Benkovic & Bullard, 1973; Benkovic, 1980). Since 5,10-CH^+-H_4folate is unstable in neutral aqueous solution and hydrolyzes to 10-CHO-H_4folate (Rabinowitz, 1963), two control experiments were also performed. The hydrolysis was monitored spectrophotometrically and found to be complete in 4 h, which was more than sufficient time for the 5,10-CH^+-H_4folate to interact with the binary complex. Second, 10-CHO-H_4folate was prepared and added directly to a binary complex so its effects on the ^{19}F NMR spectrum could be identified. These controls, along with the data for 5-CHO-H_4folate (Figure 3B), ensured that the effects observed in the ^{19}F NMR spectra of the enzyme–FdUMP···5,10-CH^+-H_4folate ternary complex were due to 5,10-CH^+-H_4folate, although it is not possible to know the identity explicitly of the bound folate in this ternary complex. As shown in Figure 6A additions of 5,10-CH^+-H_4folate produced a dramatic shift in the nucleotide binding equilibrium of the binary complex which greatly favored the covalent binding of FdUMP as indicated by the large resonance at −39.2 ppm (see Figure 6A). This enhanced covalent binding of nucleotide depleted the pools of FdUMP which were free in solution and noncovalently associated with the enzyme. In contrast, addition of 10-CHO-H_4folate produced a more complex spectrum for the enzyme–FdUMP··· 10-CHO-H_4folate ternary complex (Figure 6B). Through the addition of a 3-fold excess of this folate, a large resonance was observed at −39.6 ppm with smaller resonances at −34.5 and −37.2 ppm, indicative of the dramatic shift in the binary complex equilibrium typical of the interaction of the H_4folates. Further addition of 10-CHO-H_4folate generated a large resonance at −12.8 ppm typical of the enzyme–FdUMP–CH_2-

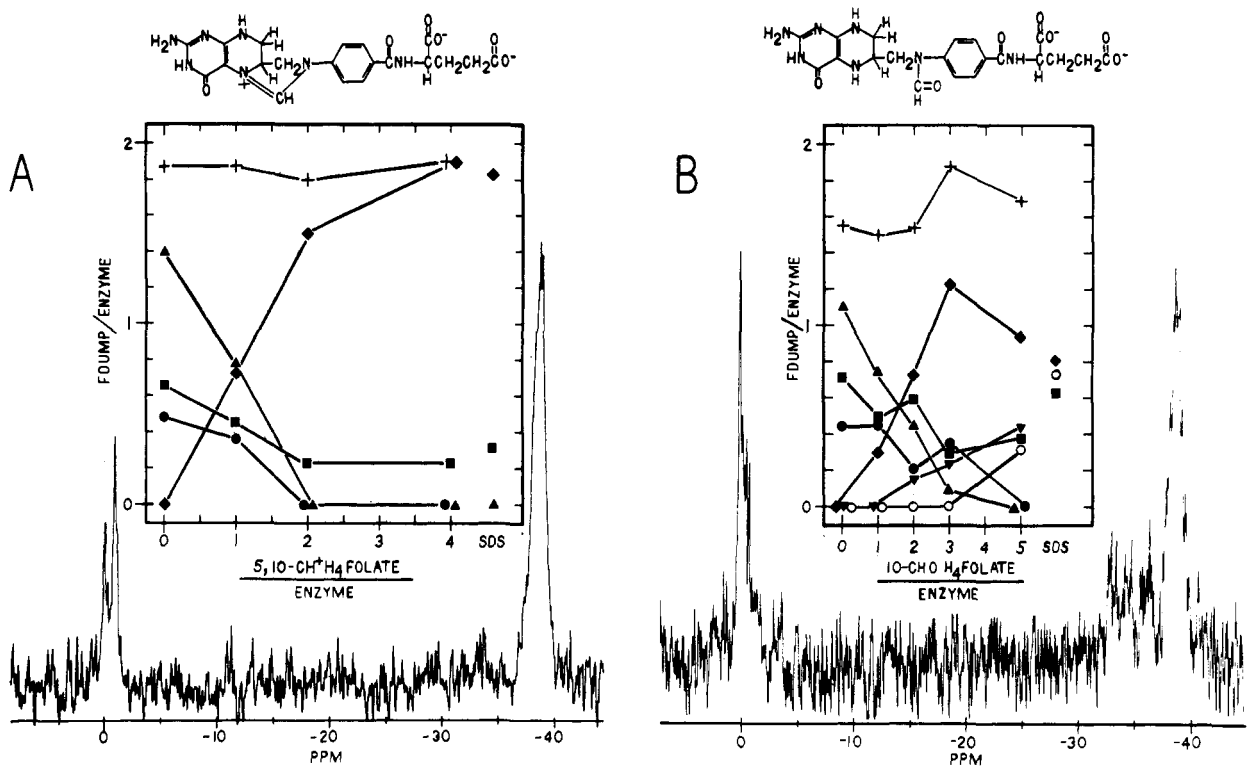


FIGURE 6: Fluorine-19 NMR spectral behavior of the binary complex in the presence of 5,10-CH^+-H_4folate (A) and 10-CHO-H_4folate (B). Addition of the methenyl derivative produced a new chemical shift for the covalently bound 5,6-dihydro-FdUMP of −39.2 ppm (♦), with a coordinate decrease of the other resonances. The less stable 10-formyl derivative exhibited more complex behavior with three distinct chemical shifts of the 5,6-dihydro-FdUMP at −34.5 (●), −37.2 (▼), and −39.6 ppm (♦). Above the 3-fold excess of 10-CHO-H_4folate concentration, the spectrum began to exhibit a resonance at −12.8 ppm due to inhibitory ternary complex formation (○), due to decomposition of the folate. All other symbols are defined in Figure 1.

H_4folate ternary complex. This was verified by the addition of $NaDodSO_4$ to the sample which resulted in the shift of the latter resonance to −2.0 ppm, an effect which is indicative of the ternary complex formed with CH_2-H_4folate. Apparently, decomposition of the 10-CHO-H_4folate released some formaldehyde, which in combination with free H_4folate, produced enough CH_2-H_4folate in the sample to support enzyme–FdUMP–CH_2-H_4folate complex formation.

It should be noted that in a number of the spectra presented in this paper, a sharp resonance is often clearly observed at −0.8 ppm due to the 3′ isomer of FdUMP (Lewis et al., 1980). The narrow line width of this resonance (~20 Hz) indicated that this material was not interacting with the enzyme. In these spectra there was also a large variation in the noise level in the base line of the spectra. This resulted from the large changes in the dynamic range of each spectrum due to the accumulation of the fluorine signal in very broad resonances (120–150 Hz).

Discussion

The folate derivatives employed in this ^{19}F NMR study were required to be (1) substrates, products, or analogues of reactive species in the enzymatic reaction, (2) stable forms of the N^5 or N^{10} iminium cations, (3) chemotherapeutic agents which might exhibit a secondary reactivity with thymidylate synthetase, (4) species which might chemically react with the enzyme–FdUMP complex, mimicking the normal reaction sequence, or (5) compounds which might provide insight into the linkage of the methylene group to N^5 or N^{10} in enzyme–FdUMP–CH_2-H_4folate complex. The resulting fluorine NMR spectra presented in this paper confirmed the formation of ternary complexes by the interaction of folate derivatives with the thymidylate synthetase–FdUMP binary complex. In all but one complex, the spectra indicated that the folates were interacting noncovalently with the FdUMP in the binary complex. The apparently covalent interaction of methotrexate will be discussed below. As shown in the titration plots, interaction of the folates with the binary complex occurred at essentially stoichiometric concentrations. Such behavior was not unexpected, since the tight binding of folates to enzyme–nucleotide complexes had been reported by several investigators in the literature. Table I summarizes the observations from the NMR experiments, and further details are described below.

Changes in Binary Complex Equilibrium. The thymidylate synthetase–FdUMP binary complex was previously shown to be an equilibrium mixture of unbound and noncovalently and covalently bound nucleotide in slow exchange (Lewis et al., 1980). The 2,4-diaminofolates, aminopterin and methotrexate, produced pronounced line broadening in the ^{19}F NMR spectrum of the binary complex. This effect indicated an increase in the rate of exchange of the nucleotide among the unbound and two bound species. The increased exchange rate produced a slight shift of the binary complex equilibrium toward covalent binding of FdUMP.

The predominant effect observed in Table I was a dramatic shift in the binary complex equilibrium toward covalent binding of FdUMP to the enzyme. The ^{19}F NMR spectra showed increases of 3–4-fold in the resonances observed within the range −34 to −40 ppm. The increased extent of covalent binding of FdUMP was equivalent to that obtained when a binary complex sample was converted to the enzyme–FdUMP–CH_2-H_4folate complex by addition of CH_2-H_4folate. The accumulation of the covalently bound species apparently resulted from the folate derivatives binding to the binary complex in a manner which effectively decreased the rate of dissociation of FdUMP from the active site. The increased residence time of FdUMP within the active site would stabilize the sulfide bond to carbon 6 of the nucleotide. This may be

Table 1: Changes in Binding of FdUMP and Chemical Shift of Covalently Bound 5,6-Dihydro-FdUMP in Enzyme-FdUMP···Folate Complexes

folate	FdUMP in ternary complex[a]/FdUMP in binary complex: free	noncovalent	covalent	chemical shift of covalently bound 5,6-dihydro-FdUMP (ppm)	effect on equilibrium
5-CH_3-H_4folate	0.92	0.87	1.20	−34.5 and −38.8	weak interaction
methotrexate[b]	0.44	0.24	1.32	−34.5	increased exchange rate
aminopterin	0.21	1.25	1.50	−35.0	increased exchange rate
H_2folate[c]	0.99	0.37	2.93	−37.4	shifted toward covalent complex
10-CH_3-folate	0.64	0.58	3.36	−35.5	shifted toward covalent complex
10-CH_3-H_4folate	0.0	0.0	3.41	−38.3	shifted toward covalent complex
5,10-CH^+-H_4folate	0.34	0.0	4.00	−39.2	shifted toward covalent complex
10-CHO-H_4folate	0.48	0.08	4.04	−34.5, −37.2, and −39.6	shifted toward covalent complex
H_4folate	0.0	0.0	4.32	−39.2	shifted toward covalent complex
5-CHO-H_4folate	0.58	0.26	4.43	−34.0 and −37.7	shifted toward covalent complex
folate	0.13	0.17	4.47	−36.5	shifted toward covalent complex

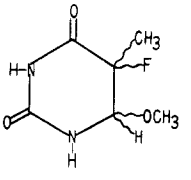
[a] The ratio, FdUMP in ternary complex/FdUMP in binary complex, was obtained as follows: the cut and weigh procedure was used to determine the percentages of FdUMP in the free, noncovalent, and covalent species for the binary and ternary complexes. These numbers were used to compute the ratios shown. [b] Methotrexate-containing ternary complexes also exhibited a resonance at −9.4 ppm in the native state, which upon denaturation shifted to −11.4 ppm indicative of a covalent attachment of the FdUMP to the enzyme and a possible covalent linkage of the folate to carbon 5 of the nucleotide. See text for further details. [c] Ternary complexes containing H_2folate exhibited a resonance at +2.0 ppm in the native state, which was lost upon denaturation by $NaDodSO_4$. This appeared to be nonspecific noncovalent association.

represent one of the most important aspects of the binding of CH_2-H_4folate, prior to any of the chemical events which subsequently occur.

Changes in Chemical Shift of Covalently Bound 5,6-Dihydro-FdUMP in Enzyme–FdUMP···Folate Complexes. Denaturation of these complexes in all cases yielded the doublet and singlet pattern at −36.0 ppm, characteristic of the 5,6-dihydro derivative of FdUMP covalently attached to the enzyme (Lewis et al., 1980), although in the native complexes the resonance was observed between −34 and −40 ppm depending upon the added folate (see Table I). It might be proposed that folate binding to the binary complex might select sterically for a particular stereoisomer of the 5,6-dihydro species, which might have a different characteristic fluorine chemical shift. However, in the bisulfite adducts of FdUMP a chemical shift difference of only 1.4 ppm was observed for the two magnetically nonequivalent species (representing a total of four stereoisomers) (Lewis et al., 1980). If the chemical shift for the enzyme-bound 5,6-dihydro derivative of FdUMP under denaturing conditions, −36.0 ppm, is assumed to represent the chemical shift of this species free from the effects of the enzyme and exchange processes, an interpretation of the chemical shift behavior can be formulated. In the native binary complex, the fluorine nucleus exhibited a chemical shift that was deshielded 2 ppm from −36.0 ppm due to the presence of the enzyme and the chemical exchange process. In contrast, some of the folates were found to increase the shielding of the fluorine nucleus by ~4 ppm to −40 ppm. The tight binding of the folates may not only halt the exchange process but may orient the folates in close proximity to the fluorine nucleus on the nucleotide. The resultant alteration in the microenvironment of the fluorine nucleus would be reflected in the chemical shift of the resonance corresponding to covalently bound FdUMP. If the aromatic rings of the folate or amino acid side chains of the enzyme were oriented such that their π clouds interacted with the fluorine nucleus, a ring current shift of 4–5 ppm might result. The actual resultant effect of changes in the microenvironment and ring currents on the fluorine nucleus would be dependent upon the strength and steric nature of the interaction of the folate with the binary complex. Perhaps the most drastic environmental change was indicated by our observation of the insensitivity of the fluorine resonance of the inhibitory ternary complex to changes in solution pH, over a range where the free ligand and the denatured complex exhibited a 2.5-ppm change in chemical shift.[3] This may reflect the dramatic contraction in the structure of the enzyme observed by gel filtration and sedimentation studies when the inhibitory ternary complex was formed (Lockshin & Danenberg, 1980b).

New Resonances. Ternary complexes containing FdUMP and H_2folate or methotrexate exhibited new resonances, which were characteristic of these complexes. In the complex containing H_2folate shown in Figure 4B, a sharp resonance was observed at +2.0 ppm, with a line width comparable to that of noncovalently bound nucleotide. Denaturation by addition of $NaDodSO_4$ resulted in the loss of this resonance, further supporting a noncovalent interaction with the enzyme. It is unclear what mechanism of binding in the presence of H_2folate might produce a resonance which is deshielded with respect to free FdUMP.

The enzyme–FdUMP···methotrexate complex exhibited a broad resonance at −9.4 ppm which contained ~20% of the FdUMP in the sample (see Figure 2B). Denaturation afforded little decrease in the line width of this resonance, nor did it serve to improve the resolution of the structure of the resonance, which moved to −11.4 ppm. The covalent nature of this resonance was surprising since the range of chemical shift in which it was observed was defined by Byrd et al. (1977) as that expected for the fluorine nucleus of FdUMP which also had a heavy atom substituent on carbon 5 of the nucleotide. In fact, their model compound, 5-fluoro-5-methyl-6-methoxy-5,6-dihydrouracil (see structure **1**), was reported to have



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a chemical shift of −9.5 ppm from FdUMP (Byrd et al., 1977). In order for a 5-fluoro-5-methyl-5,6-dihydro–6-enzyme derivative of dUMP to be formed from the enzyme–FdUMP binary complex, we hypothesize that the 10-methyl group of methotrexate might be transferred to the carbanion of the covalently bound 5,6-dihydro derivative. This reaction would

[3] C. A. Lewis, Jr., unpublished results.

liberate aminopterin as a byproduct, leaving the enzyme covalently blocked by the 5-fluoro-5-methyl-5,6-dihydro derivative. Further experiments are being undertaken to confirm or disprove this hypothetical course of events.

Structural Requirements for Folate Binding. The most striking structural feature which appeared to prohibit the strong interaction of folates with the enzyme–FdUMP binary complex was found to be the orientation of the substitutent on N^5 of the tetrahydropyrazine ring. 5-CH_3-H_4folate exhibited the weakest interaction with the binary complex, even if the presence of the racemic mixure was taken into account, while 5-CHO-H_4folate and 5,10-CHO-CH^+-H_4folate exhibited ^{19}F NMR spectral changes indicative of their very strong interaction. Crystallographic (Bieri & Viscontini, 1977b) and NMR (Poe et al., 1979a,b) investigations of 5-CH_3-H_4folate have shown that the 5-CH_3 group is a pseudoaxial substituent in both carbon 6 stereoisomers of this folate. Similar studies of the 5-formyl- (Bieri & Viscontini, 1977a,b), 5,10-methenyl- (Fontechilla-Camps et al., 1979a,b; Khalifa et al., 1979), and 5,10-methylenetetrahydrofolates (Poe et al., 1979a,b) have found that the N^5 substituent was equatorial and nearly in the same plane described by the tetrahydropyrazine ring. Therefore, the axial orientation of the methyl group of 5-CH_3-H_4folate would appear to prohibit the productive association of this folate with the enzyme–FdUMP complex. This may be the ultimate explanation for the weak noncompetitive inhibition by 5-CH_3-H_4folate of the enzymatic reaction (Danenberg et al., 1974).

The replacement of the 4-keto group of folic acid by an amino group, as observed in aminopterin and methotrexate, produced increased rates of exchange in the ternary complex spectra. The substitution of the hydrogen bond accepting keto group by a hydrogen bond donating species apparently destabilized the binding of these two folates to the enzyme–nucleotide complex. Apparently, the presence of the 4-amino group and its effect on the exchange rate overshadows the effect of the 10-methyl group (compare the spectra of the methotrexate and 10-CH_3-folate complexes, Figures 1 and 2B).

Strikingly, the level of oxidation of the pyrazine ring of unsubstituted folate had very little effect on the strength of interaction of the folates with the binary complex. The increased amounts of the covalent component of the binary complex were comparable for folate, H_2folate, and H_4folate as seen in Figure 4 and Table I. This may result from similarities in the nearly planar structure of the pterin rings of these three derivatives, of which only the H_4folate has been shown to have carbons 6 and 7 in a slightly half-chair conformation (Bieri, 1977; Weber & Viscontini, 1977; Poe & Hoogsteen, 1978; Furrer et al., 1978), a feature in common with the normal cofactor CH_2-H_4folate. In contrast, comparison of the data for 10-CH_3-folate and 10-CH_3-H_4folate (Figures 1 and 5) illustrated that in substituted folate derivatives the oxidation state of the pyrazine ring becomes the dominant factor in the effect observed on the binary complex NMR spectrum.

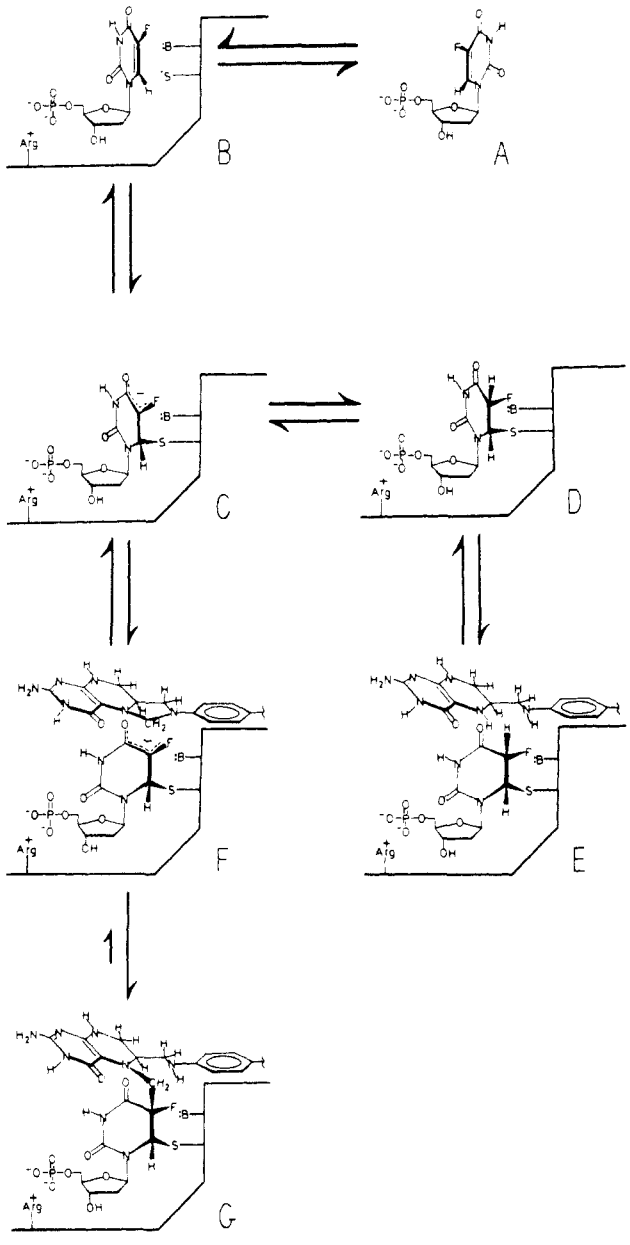
Although the structures of the folate derivatives discussed above are known in either the crystalline state or in solution, it is not possible to predict what alterations in their structure may occur upon binding to the enzyme to form the enzyme–FdUMP···folate complexes. It is apparent from the spectra presented in this paper that a combination of subtle changes in the conformation of the nucleotide, the enzyme, and the folate are probably occurring (see the preceding section on the chemical shift of the resonance due to covalently bound FdUMP).

Formation of the Binary and Ternary Complexes. Our fluorine NMR studies of the binary complex with FdUMP (Lewis et al., 1980), enzyme–FdUMP–CH_2-H_4folate (Byrd, 1977; Byrd et al., 1977, 1978), and enzyme–FdUMP···folate complexes, presented here, have provided the molecular details of the structural changes occurring in FdUMP upon binding to the enzyme and subsequent reaction with CH_2-H_4folate. By direct analogy, this information also may provide an accurate description of the changes occurring in the substrate dUMP as it proceeds through the first half of the enzymatic reaction. These studies provide a detailed description of the events occurring in the enzymatic reaction up to ternary complex formation, with regard to the nucleotide (see Scheme I). FdUMP in aqueous solution and many other uracil nucleotides exist in the anti conformation as shown in (A) of Scheme I, with the 2-keto group of the pyrimidine pointed away from the 5′-phosphate group (Emerson et al., 1967). Association of the nucleotide with the enzyme appears to be accompanied by a rotation about the glycosidic bond toward the syn conformation (Leary et al., 1975) to form a Michaelis-like complex [(B) of Scheme I]. An essential arginine residue within the active site (Cipollo & Dunlap, 1978, 1979) provides an ionic interaction for the 5′-phosphate group to stabilize the nucleotide. This complex gives rise to the resonance observed at −1.4 ppm in the ^{19}F NMR spectrum of the binary complex assigned to noncovalently bound FdUMP. The thiolate anion of the catalytic cysteine attacks carbon 6 of the pyrimidine ring generating the reactive anionic intermediate [(C) of Scheme I]. Although this intermediate is probably unobservable by fluorine NMR spectroscopy due to its short lifetime, its presence has been demonstrated by the resonance at −34.5 ppm assigned to the covalently bound 5,6-dihydro derivative of FdUMP [(D) of Scheme I], which resulted from protonation of the intermediate in (C). The pyrimidine ring of species C and D are probably further rotated toward the syn conformer since 6-substituted uridine derivatives have been found to prefer that orientation (George et al., 1978).

The covalently bound 5,6-dihydro species of FdUMP proved to be a valuable probe for approximating the behavior of the anionic intermediate in the presence of a nonreactive bound folate, as well as for measuring the strength of interaction of the folate. The spectra presented in this paper indicated that folate binding produced as much as a 4-fold increase in stability for covalently bound FdUMP. Both the nucleotide and the environment of the active site appeared to have been signficiantly altered. Circular dichroic spectra of the enzyme–FdUMP···folate complexes show greater negative ellipticities in the nucleotide region, suggesting a continued rotation about the glycosidic bond toward the syn conformation.[4] Further features of these circular dichroic spectra indicate changes in the enzyme due to binding of the folate. The fluorine chemical shift of the covalently bound FdUMP in these ternary complexes was more shielded than the resonance for the binary complex reflecting possible indirect electronic interactions with the folate or aromatic residues of the enzyme, changes in solvation within the active site, or a conformational change in the enzyme similar to that observed for enzyme–FdUMP–CH_2-H_4folate complex formation (Lockshin & Danenberg, 1980b). Complex E illustrates the proposed structure of the enzyme–FdUMP···folate ternary complexes observed in the ^{19}F NMR spectra. By analogy, complex F corresponds to the binding of CH_2-H_4folate to the complex containing the anionic intermediate. This complex probably has only a fleeting existence before the enzyme–

[4] W. E. Hopper, Jr., unpublished results.

Scheme I



FdUMP–CH_2-H_4folate complex is formed [(G) of Scheme I].

Consequences of Binary and Ternary Complexes. Until relatively recently, FdUMP was considered to be a potent inhibitor of thymidylate synthetase only in conjunction with CH_2-H_4folate to form an inhibitory ternary complex. The significance of the FdUMP binary and enzyme–FdUMP··· folate complexes reported in the literature was clouded by uncertainty as to the chemical nature of their binding to the enzyme and their structure. Our ^{19}F NMR studies revealed both noncovalently and covalently bound species of FdUMP in the binary complex and demonstrated that a variety of folate derivatives served to increase the stability of the covalent species. Although careful reexamination of the physical characteristics of these complexes is only now being undertaken, several important aspects are already apparent.

Recent studies by Lockshin & Danenberg (1980a) have considered the extent of formation of the thymidylate synthetase–FdUMP–CH_2-H_4folate complex within the cell. Interestingly, these researchers concluded that the intracellular concentrations of CH_2-H_4folate, dUMP, and inorganic phosphate would determine how much FdUMP actually inhibited the enzyme as a component of the enzyme–FdUMP–CH_2-H_4folate complex. Further, Lockshin & Danenberg (1980a) suggested that the enzyme might be complexed with either FdUMP or dUMP along with the intracellular folates, based on the literature evidence described earlier, such that even less FdUMP might find its way into the enzyme–FdUMP–CH_2-H_4folate complex. Our fluorine NMR studies provide strong support for this contention since strong, essentially stoichiometric enzyme–FdUMP···folate complexes were observed (see Table I). This provides a new mechanism for the inhibition of thymidylate synthetase by a wide variety of enzyme–FdUMP···folate complexes, which although not as strong as the classical ternary complex, $K_D^{FdUMP} = 10^{-12}$ M (Santi et al., 1974; Danenberg et al., 1974; Murinson et al., 1979; Santi, 1980; Lockshin & Danenberg, 1980a), may be strong enough, $K_D^{FdUMP} = 10^{-6}$–10^{-7} M (Galivan et al., 1976), to effectively suppress thymidylate synthetase activity.

Second, a mechanism for the interaction of folate antimetabolites as potential thymidylate synthetase inhibitors has been revealed. The interaction of methotrexate or aminopterin with the enzyme–FdUMP binary complex may serve two functions. A decrease in the concentration of available folate antimetabolite may somewhat alter the effectiveness of the drug against dihydrofolate reductase while also slightly suppressing thymidylate synthetase activity.

Finally, the use of citrovorum factor (5-CHO-H_4folate) rescue in methotrexate chemotherapy might be viewed with new insight since this folate was found to form a very strong ternary complex. Moran et al. (1979) have investigated the role of thymidylate synthetase in methotrexate cytotoxicity and found that FdU, probably active as FdUMP within the cell, and 5-CHO-H_4folate acted to moderate or eliminate the cell-killing power of methotrexate by minimizing the rate of reduced folate consumption by thymidylate synthetase. Such an inhibition might likely result from the formation of the enzyme–FdUMP···5-CHO-H_4folate complex.

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