Mechanism of Interaction of Thymidylate Synthetase with 5-Fluorodeoxyuridylate†

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ABSTRACT: A study of the properties of the complex containing 5-fluoro-2'-deoxyuridylate (FdUMP), 5,10-methylenetetrahydrofolate, and thymidylate synthetase is described. In the presence of the cofactor, isolable complexes contain two tightly bound molecules of FdUMP per enzyme molecule of 70,000 daltons. A number of folate analogs also stimulate binding of FdUMP, albeit to a lesser degree than the cofactor. Kinetic data indicate the rate constant for association of FdUMP with the enzyme-methylenetetrahydrofolate complex to be 2×10^7 M^{-1} min^{-1} at 24". The unimolecular dissociation rates of FdUMP from the complex are highly temperature dependent and show $\Delta F^{\ddagger} = 21.5$ kcal/mol, $AH^* = 28.4$ kcal/mol, and $AS^{\ddagger} = 0.023$ eu; there is no indication that homotropic interactions, if existent, are manifested in the rate of dissociation of FdUMP. From kinetic data, an assocation constant for the interaction of FdUMP with the enzyme-cofactor complex is calculated to be ca. 2×10^{10} M^{-1} at 24". Within the enzyme–cofactor–FdUMP complex, a nucleophilic group of the enzyme forms a covalent bond by a Michael-type addition to the 6 position of the nucleotide. This is demonstrated by (i) the stability of the complex toward guanidine hydrochloride, (ii) the loss of absorbance of the pyrimidine chromophore at 269 nm, (iii) isolation of peptide fragments associated with FdUMP after pronase digestion, and (iv) a large secondary tritium isotope effect on the rate of dissociation of [6-^{3}H]FdUMP from the complex. Ultraviolet difference spectra also demonstrate that CH_2FAH_4 is converted to a new chemical species concomitant with or following binding of FdUMP. In conjunction with other studies, it is proposed that the interaction of FdUMP with thymidylate synthetase parallels a portion of the normal enzymic reaction. The analog behaves as a quasisubstrate which undergoes and/or stimulates conversions up to the stage of one-carbon transfer from the cofactor and, in effect, results in a buildup of a complex which resembles a steady-state intermediate of the normal enzymic reaction.

Thymidylate synthetase catalyzes the conversion of dUMP to TMP,[1] with concomitant transfer and reduction of the one carbon unit of CH_2FAH_4. In this process the hydrogen at C-6 of FAH_4 is directly transferred to the methyl group of

$$dUMP + 5,10\text{-}CH_2FAH_4 \longrightarrow TMP + FAH_2$$

TMP (Pastore and Friedkin, 1962) and 5-hydroxymethyl dUMP is not formed as an intermediate (Santi and Sakai, 1971; Flaks and Cohen, 1959).

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 (see Santi and Brewer, 1973; Santi and Pogolotti, 1974) have suggested 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. With the recent availability of a source of homogeneous, stable enzyme (Crusberg et al., 1970; Dunlap et al., 1971a), we undertook studies which might provide direct support for proposals based on nonenzymic models.

The first, and perhaps most important, point to be verified was whether a nucleophilic group of the enzyme played the catalytic role of initiating addition-elimination reactions across the 5,6-double bond of dUMP. To answer this question we sought a quasisubstrate of the reaction; that is, an analog of dUMP which after formation of a reversible complex would undergo nucleophilic addition at the 6 position to form a covalent bond sufficiently stable for characterization. It has been known for some time (Cohen et al., 1958; Heidelberger et al., 1960) that FdUMP is an extremely potent inhibitor of thymidylate synthetase but the nature of inhibition has been the topic of considerable controversy (see Blakley, 1969). Since the 6 position of 5-fluorouracil is quite susceptible toward nucleophilic attack (Fox et al., 1966; Otter et al., 1969; Reist et al., 1964), we suspected that FdUMP might be the quasisubstrate we were seeking. In this report we describe data which confirm this supposition.

Materials and Methods

Folate, FAH_2, and pronase were obtained from Sigma Chemical Co. Pteroic acid, homofolate, methotrexate, dichloromethotrexate, and 5-fluorodeoxyuridine were gifts from the Drug Research and Development division of the National Cancer Institute. N^{10}-Methylfolate was supplied by Lederle Laboratories. 5,8-Deazafolate and 5,8-deaza-N^{10}-methylfolate were gifts from Parke Davis & Company. [3',5',9-^{3}H]Folate (27 Ci/mmol) was purchased from Amersham/Searle. Carrot phosphotransferase was a geneous gift from Dr. Alex Nussbaum of Hoffmann-La Roche and FAH_2 reductase (ca. 50 % pure) from rat liver (Baker and Vermeulen, 1970) was a gift from Dr. Larry Kirk. [6-^{3}H]Fluorodeoxyuridine (1.37 $\times 10^4$ dpm/pmol) was obtained from New England Nuclear and [2-^{14}C]fluorodeoxyuridine (34.4 dpm/pmol) was obtained from Schwarz/Mann; the stated specific

[1] Abbreviations used are: FdUMP, 5-fluoro-2'-deoxyuridylic acid; FdUR, 5-fluoro-2'-deoxyuridine; FAH_4, dl-L-tetrahydrofolic acid; CH_2FAH_4, 5,10-methylenetetrahydrofolic acid; $7,8\text{-}FAH_2$, 7,8-dihydrofolic acid; TMP, thymidylic acid.

activities were verified by spectrophotometric measurement using ϵ_{268} 8.3 $\times$ 10³ (pH 7.23). The corresponding nucleotides were prepared from carrot phosphotransferase as described by Harvey *et al.* (1970). The purity of the preparations was routinely monitored by high voltage electrophoresis (5% acetic acid, 0.5% pyridine) and paper chromatography (CH₃CN–0.1 M NH₄Cl, 6:4). Unlabeled FdUMP was prepared by treatment of the 3 '-*O*-acetyl nucleoside with phosphorus oxychloride in triethyl phosphate (Yoshikawa *et al.,* 1967), followed by blocking group removal with NH₄OH; complete details will be published elsewhere. Sources of other materials used are described in the accompanying paper (Santi *et al.,* 1974).

Unless otherwise noted the formation and nitrocellulose filter assays of enzyme. CH₂FAH₄ . FdUMP complexes were performed as described in the accompanying paper (Santi *et al.,* 1974). Complexes are freed from unbound ligands by Sephadex G-25 filtration at 4" on a 1 .O $\times$ 25 cm column using 60 mM *N*-methylmorpholine-HCl buffer (pH 7.4), 30 mM MgCl₂, 1.2 mM EDTA, and 90 mM 2-mercaptoethanol. Spectrophotometric assays of enzyme activity were performed as previously described (Santi and Sakai, 1971). Specific activity is defined as units (μmol of TMP formed/min) per mg of protein.

Filters containing bound radioactivity were dissolved in Bray's mixture (Bray, 1960) for counting and aqueous solutions were counted in a fluid containing 0.3% Omnifluor in xylene-Triton X-l 14 (3 : 1) in a Nuclear Chicago Isocap 300 liquid scintillation counter. Counting efficiencies were determined by the dual channel ratio method except for experiments with dual labels and low radioactivity where the external standards ratio method was used. Isotopes were generally counted to an accuracy of $\pm$ 2 % except for stoichiometry and double label experiments where sufficient counts were obtained to ensure $\pm 0.5\%$ accuracy. Dpm calculations were aided by a PDP-10 tape-fed computer using a program provided by R. Anderson of this laboratory.

Thymidylate synthetase was obtained from an Amethopterin-resistant strain of *Lactobacillus casei* (Crusberg *et al.,* 1970) grown at the New England Enzyme Center and provided to us as a crude cell-free suspension. Most of the studies described here used a purified preparation obtained by the method of Leary and Kisliuk (1971). To process large amounts of protein a sequence was developed by V. A. Peña of this laboratory which consists of modifications of reported methods. The salient features are described in the original papers cited and only the modifications pertinent to the preparation of large amounts of pure enzyme are reported here.

Step 1. Hydroxylapatite chromatography was performed essentially as described by Leary and Kisliuk (1971) except the support contained 10% (w/w) cellulose and the flow rate was 2 ml/min. At least 10 g of protein could be chromatographed on a 4.5 $\times$ 65 cm column without affecting resolution. Fractions having specific activities over 1.0 were pooled, and dialyzed against 0.08 M potassium phosphate (pH 6.8)–5 mM β-mercaptoethanol. Lower specific activity fractions were pooled and treated similarly.

Step 2. CM-Sephadex chromatography was performed as described by Dunlap *et al.* (1971a) with the exception that the gradient was composed of 0.08 M potassium phosphate (pH 6.8)–5 mM β-mercaptoethanol in the mixing vessel and 0.08 M potassium phosphate (pH 7.7)–700 mM KCl–5 mM β-mercaptoethanol in the reservoir. At least 1.0 g of protein could be applied to a 4.5 $\times$ 71 cm column and a flow rate of l-ml/min could be used without affecting resolution. Fractions

were pooled which had specific activities over 2.6 and dialyzed against 25 mM Tris (pH 7.2)–5 mM β-mercaptoethanol–10 mM MgCl₂. At this stage the enzyme obtained was between 75 and 85% homogeneous as judged by disc gel electrophoresis and specific activity measurements.

Step 3. DEAE-Sephadex A50 column chromatography was performed as described by Dunlap *et a/.* (1971a). At least 0.4 g of protein could be chromatographed on a column 2.5 $\times$ 56 cm without loss in resolution. The enzyme was dialyzed against 50 mM Tris (pH 7.2)–5 mM β-mercaptoethanol followed by dialysis against 50 mM potassium phosphate (pH 6.8)–1 mM dithiothreitol. It was stored as such at O-4" or in 50% glycerol at -20".

The preparation we obtain shows specific activities of 3.7 units/mg using the method of Kalckar (1947) or 6.1 units/mg using ϵ_{278} 1.07 $\times$ 10⁵ (*vida infra*) for protein determinations. It shows one band (>95% by scanning) on disc gel electrophoresis in sodium dodecyl sulfate (Weber and Osborn, 1969). With undenatured enzyme we detected two slower moving bands on polyacrylimide gels (Davis, 1964) which account for *ca.* 10% of the stained protein. However, plots of the logarithm of electrophoretic mobility *us.* gel density indicate that these minor bands result from aggregates of the enzyme (Maurer, 1971). Gels were stained with Coomassie Blue and scanned at 590 nm on a Gilford 2000 spectrophotometer.

Protein concentrations of crude preparations *(viz.* during purification) were measured by the absorption at 260 and 280 nm (Kalckar, 1947). With highly purified preparations measurements made by the methods of Kalckar (1947) and Lowry gave values which were considerably higher than those obtained by the biuret method (Rutter, 1967), causing a high degree of uncertainty in anticipated studies of stoichiometry. To directly determine an extinction coefficient for thymidylate synthetase, the molarity of a solution of our purest enzyme preparation was calculated using the equation [E] = [AA]/n where [AA] is the measured concentration of a particular amino acid and n is the number of molecules of that amino acid per mol of enzyme as determined by amino acid analysis (Dunlap *et al.,* 1971b; R. B. Dunlap, personal communication). Using the method of Edelhoch (1967), spectrophotometric determination of Tyr and Trp of our most highly purified preparation gave ϵ_{278} 1.07 $\times$ 10⁵ (pH 6.8) when the values of 22 Tyr and 12 Trp (R. B. Dunlap, personal communication) per mole of enzyme are used in the calculation. This value is in excellent agreement with that obtained (ϵ_{278} 1.05 $\times$ 10⁵) when protein is measured by the biuret method (R. B. Dunlap, personal communication). Using ϵ_{278} 1 .07 $\times$ 10⁵, 5,5 '-dithiobis(2-nitrobenzoate) titration of thymidylate synthetase freed of dithiothreitol by Sephadex G-25 filtration and denatured with sodium dodecyl sulfate (Habeeb, 1972) indicated the existence of 3.6 sulfhydryl groups per mol of enzyme; this value is in excellent agreement with that obtained by amino acid analysis (Dunlap *et al.,* 1971b) and further substantiates our assignment for the extinction coefficient of the enzyme. From this value, we calculate the catalytic constant of thymidylate synthetase to be 425 (mol of TMP min⁻¹ mol of enzyme-l).

Ultraviolet difference spectra were obtained by adding 20 μl of a 1.84 $\times$ 10⁻⁴ M solution of FdUMP or an equivalent amount of water to two previously balanced cells containing in 1.0 ml: thymidylate synthetase (2.8 nmol), dithiothreitol (6.5 μmol), formaldehyde (7.0 μmol), MgCl₂ (25 μmol), EDTA (1 μmol), and *N*-methylmorpholine-HCl (50 μmol, pH 7.4). The spectrum (255-350 nm) was scanned and after 5 min CH₂FAH₄ was introduced into both cuvettes to give a final

TABLE I: Binding of [³H]CH₂FAH₄ and [³H]Folate to Thymidylate Synthetase.[a]

Components	Total Ligand (dpm/1OO μl)	Bound Ligand (dpm/100 μl)
E + [³H]folate	150,000	*300*
E + [³H]folate + FdUMP	120,000	1,300
E + [³H]CH₂FAH₄	170, 000[b]	*800*
E + [³H]CH₂FAH₄ + FdUMP	140,000"	107,000

[a] The complex was formed by adding 20 μl of the [³H]FAH₄ solution described in Materials and Methods to 800 μl of a solution containing 46 nM thymidylate synthetase, 3.1 μM FdUMP, and the standard buffer components. Aliquots (100 μl) were removed after 1 hr and filtered through nitrocellulose membranes. [b] Approximately 20% of this value represents [³H]folate.

concentration of 7.2 μM and a total volume of 1.045 ml. The spectrum was scanned repeatedly for 100 min.

[3′,5′,9-³H]FAH₄ was prepared and assayed by a modification of the procedure used by Rothenberg (1965). The incubation mixture contained 1.47 μM [3′,5′,9-³H]folate, 28.7 μM TPNH, 67 mM 2-mercaptoethanol, 6.7 mM formaldehyde, 20 mM citrate buffer (pH 5.0), and 3 μg of rat liver folic reductase. After 30 min, 85 % of the folic acid was reduced to the tetrahydro derivative, which was used without further purification.

Results

Stoichiometry of [³H]FdUMP Binding to Thymidylate Synthetase. In the accompanying paper (Santi *et al.,* 1974) it is demonstrated that in the presence of CH₂FAH₄, [³H]FdUMP forms a tight complex with thymidylate synthetase whereas in the absence of the cofactor binding is undetectable in the concentration range used. Similarly, filterable radioactive complexes are not formed between the enzyme and [³H]CH₂FAH₄ unless FdUMP is present (Table I). It is to be noted that binding of [³H]folate, a possible contaminant of our [³H]CH₂FAH₄ preparation, is insignificant compared to cofactor binding in the presence or absence of FdUMP. These results show that formation of the high affinity complex only occurs in the presence of all components, and contains both FdUMP and CH₂FAH₄ bound to the enzyme. It is to be noted that equilibrium measurements do not permit assignment of a dissociation constant since, until saturation of the enzyme occurs, there is no measurable free [³H]FdUMP. We calculate that the dissociation constant must be under 10^{-9} M since, under the conditions used, weaker binding would result in detectable free [³H]FdUMP. In Figure 3 of the accompanying paper, we demonstrated that 12.7 nM enzyme is saturated when 1.70 mol of [³H]FdUMP are bound per mole of thymidylate synthetase. Similarly, titration of 0.14 μM thymidylate synthetase with [³H]FdUMP and 1.1 μM enzyme with [¹⁴C]FdUMP provides values of 1.8 and 2.0 binding sites, respectively, per molecule of enzyme. As an additional test of the stoichiometry of [³H]FdUMP binding, we utilized the method of continuous variation (Chaberek and Martell, 1959). Figure 1 shows that the bound radio-
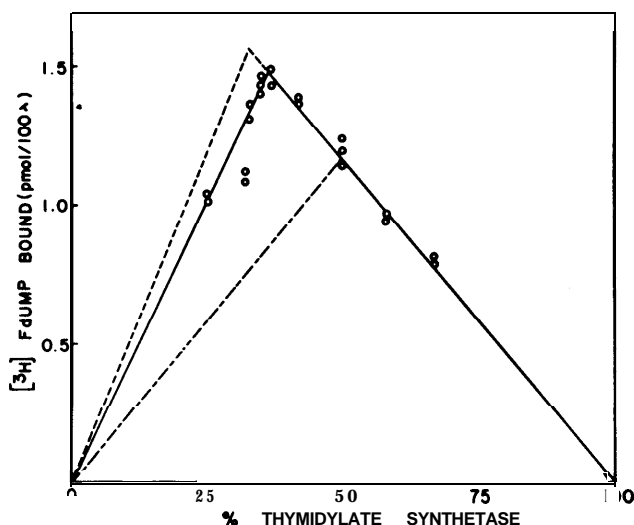

FIGURE 1: Determination of stoichiometry of FdUMP binding to thymidylate synthetase by the method of continuous variation. The total enzyme + FdUMP concentration was 2.35 pmol/100 μl filtration aliquot. The experimentally obtained curve (—) was normalized to 100% filtration efficiency. The theoretical curves calculated for one (- ▪ — –) and two (---) FdUMP binding sites are shown.

activity reaches a maximum when the enzyme–[³H]FdUMP ratio is 1:1.8. For comparison, theoretical curves are presented which were constructed for one and two binding sites with the assumption of stoichiometric binding. Figure 2 shows that the equivalence point for complete inhibition of catalytic activity requires an average of 1.9 mol of FdUMP per mol of enzyme, as determined by the method of Ackermann and Potter (1949).

Demonstration That Active Enzyme and FdUMP Are Released from the Complex. To demonstrate that FdUMP was released unmodified from the E · CH₂FAH₄ · FdUMP complex, the complex was formed using limiting [³H]FdUMP and isolated by Sephadex G-25 filtration. The isolated complex (35.5 nM) was divided into two portions; CH₂FAH₄ (130 μM final concentration) was added to one which was stored under argon as a control, while the other was not protected and permitted to dissociate, presumably due to CH₂FAH₄ oxidation prior to or concomitant with dissociation (Table
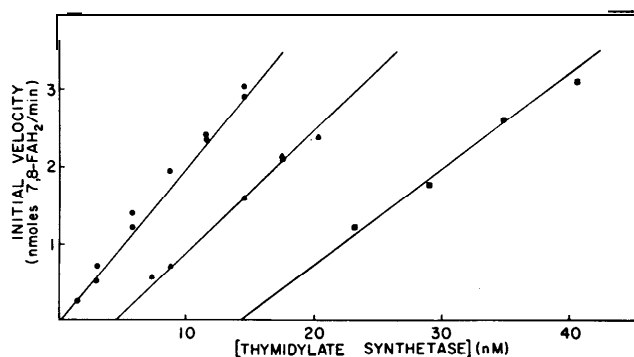

FIGURE 2: Titration of FdUMP by thymidylate synthetase. The indicated amount of the enzyme was incubated for 75 min at room temperature in a 1.2 ml solution containing 8.75 nM FdUMP (▲), or 26.3 nM FdUMP (■) and the standard components of the spectrophotometric assay (0.13 mM CH₂FAH₄) except dUMP; the control (●) contained no FdUMP. The reaction was initiated by the addition of 20 μl of a solution containing 197 nmol of dUMP and 130 nmol of CH₂FAH₄ and initial velocity measured by the increase in absorbance at 340 nm (Wahba and Friedkin, 1961).

TABLE II : Demonstration that FdUMP Is Not Modified upon Dissociation from the E· CH₂FAH₄· FdUMP Complex.'

Time (hr)	% Protein Bound [³H]FdUMP Complex [b] (−CH₂FAH₄)	Control [c] (+CH₂FAH₄) (%)
9	76	98
24	31	90
53	8	94
53 hr, then add E and CH₂FAH₄[d]	96	

[a] The complex (0.85 ml) was formed as described in Materials and Methods using 114 nM [³H]FdUMP and 340 nM enzyme and isolated by filtration through Sephadex G-25. The isolated complex contained 35.5 nM FdUMP binding sites. [b] The complex was allowed to stand at room temperature and aliquots (50 μl) were removed at specified times and filtered through nitrocellulose membranes. [c] The control was prepared by the addition of CH₂FAH₄ to a portion of the complex and assayed in an identical manner as described above. [d] Enzyme (0.23 μM) and CH₂FAH₄ (130 μM) were added and after 1 hr, 50-μl aliquots were filtered through nitrocellulose filters.

II). After 53 hr, there was a loss of 92% of the filter-bound radioactivity from the complex not containing excess CH₂-FAH₄ while the control was essentially unchanged. Paper chromatography of the dissociated complex showed 91% of the radioactivity to move as free FdUMP, 3% as the nucleoside, and 6% as the protein-bound complex which remained at the origin. Additional evidence that the dissociated radioactivity was in fact [³H]FdUMP was also
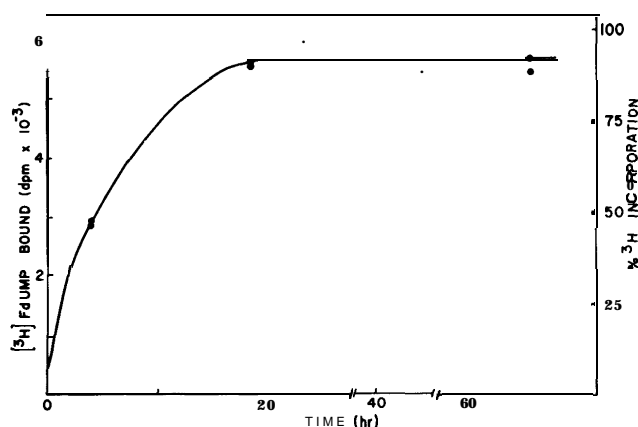

FIGURE 3: Incorporation of [³H]FdUMP into the E–CH₂FAH₄–FdUMP complex. The complex (0.3 ml) was formed as described in Materials and Methods using 1.56 X 10⁻⁷ M enzyme, 1.5 X 10⁻⁶ M [³H]FdUMP (51.1 dpm/pmol), and 0.13 mM CH₂FAH₄ and filtered through Sephadex G-25. The three leading fractions of the macromolecular peak were combined and treated with 5,10-CH₂-FAH₄ (0.13 mM for 30 min); the mixture (4.4 ml) contained 7.7 pmol (394 dpm) of bound[³H]FdUMP/ml. After the addition of [³H]FdUMP (1.37 x 10⁴ dpm/pmol; 10.6 nM) 100-μl aliquots (0.77 pmol of complex) were filtered through nitrocellulose filters. At equilibrium the specific activity of [³H]FdUMP was 7.95 x 10³ dpm/pmol; the data shown are corrected for filtration efficiency (88%).
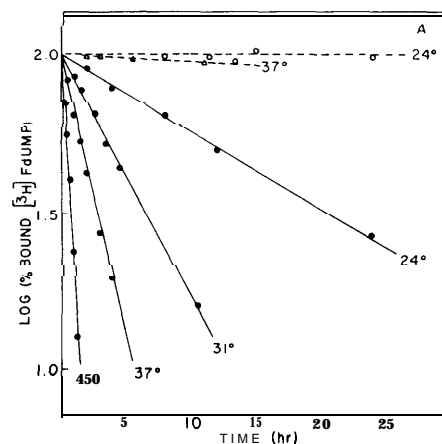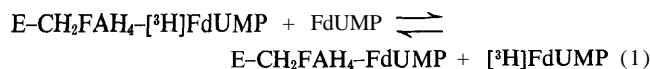
FIGURE 4: Dissociation of [³H]FdUMP from the E·CH₂FAH₄·[³H]FdUMP complex at various temperatures. The complex was formed from cu. 1 x 10⁻⁸ M enzyme, 6 X 10⁻⁸ M [³H]FdUMP, and 3 X 10⁻⁴ M CH₂FAH₄ as described in Materials and Methods. Unlabeled FdUMP (1.2 x 10⁻⁵ M) was added and aliquots were filtered through nitrocellulose membranes to determine protein-bound [³H]FdUMP. Dashed lines represent loss of bound radioactivity when no labeled FdUMP is added. Temperature control was ±0.2° of that specified.

obtained by demonstrating that replenishment of enzyme and CH₂FAH₄ resulted in 96% recovery of protein-bound radioactivity (Table II).

To demonstrate that association-dissociation of the E–CH₂FAH₄–FdUMP complex was not accompanied by modification of the enzyme, we performed an experiment to show that [³H]FdUMP could be incorporated into the performed complex. The initial complex was formed with a saturating level of [³H]FdUMP of low specific activity and freed from unbound ligands by gel filtration. The complex in the void volume was quantitated, treated with CH₂FAH₄, and permitted to equilibrate with [³H]FdUMP of high specific activity. As shown in Figure 3, [³H]FdUMP could be exchanged into at least 92% of the preformed complex; moreover, over the time period examined numerous association–dissociation events occur (*vide infra)* and it may be concluded that such events do not modify the enzyme.

Kinetics of Association and Dissociation of [³H]FdUMP. The rate of dissociation of [³H]FdUMP from the complex can conveniently be measured by isotope exchange (eq 1) providing experiments are performed under conditions where the protein is stable. If the isotope dilution is sufficiently large, the

E–CH₂FAH₄–[³H]FdUMP + FdUMP $\rightleftharpoons$
$\qquad$ E–CH₂FAH₄–FdUMP + [³H]FdUMP (1)

probability of recombination of the radioactive ligand with the enzyme will be low and the loss of protein-bound radioactivity measures the rate of dissociation of [³H]FdUMP from the ternary complex. In the experiments described below, the complex was prepared with an excess of [³H]FdUMP under conditions where both binding sites of the enzyme are occupied. The cofactor was also present at a saturating concentration which was kept constant throughout the series of experiments. Reactions were initiated by adding unlabeled FdUMP and protein-bound radioactivity was determined by nitrocellulose filtration of aliquots at various time intervals. Figure 4 shows typical first-order plots of the loss of protein-bound radioactivity from the E–CH₂FAH₄–[³H]FdUMP complex in the presence of excess unlabeled FdUMP and

CH_2FAH_4. The first-order rate constants for dissociation at a given temperature are identical over a 400-fold range (3-1200 μM; $k_{obsd} = 6 \times 10^{-4}$ min^{-1} at 21°) of unlabeled FdUMP and, under conditions of constant isotope dilution, the rates are not a function of the concentration of the bound complex initially present. These experiments demonstrate that free FdUMP does not influence the dissociation rate of the E–CH_2FAH_4–[^{3}H]FdUMP complex and the dissociation is truly a first-order process. The rates of dissociation are highly temperature dependent and, from an Arrhenius plot, we calculate activation parameters of E_a = 29.0 kcal/mol, AF* = 21.5 kcal/mol, AH‡ = 28.4kcal/mol, and $\Delta S^{\ddagger} = 0.023$ eu.

For the measurements described above, we have assumed that the E–CH_2FAH_4–FdUMP complex is stable over the time period of the experiments; should protein denaturation occur prior to release of [^{3}H]FdUMP, complexes might result which have different dissociation rates than the native form. Since a modified protein would not be expected to rebind [^{3}H]FdUMP, and since numerous turnovers occur over the time periods examined, such occurrences should be manifested as a time-dependent loss of protein-bound radioactivity in the *absence* of unlabeled FdUMP. At temperatures up to 37°, the loss of protein-bound radioactivity is not significant over the time scale examined (Figure 4); in fact, we observed no detectable loss of filterable radioactivity at 4" and 24° for 14 and 4 days, respectively. At 45°, the loss of radioactivity proceeds at *ca.* 8% of the rate of that measured in the presence of excess unlabeled FdUMP; regardless, the first-order rate of release of nucleotide correlates well with the temperature dependence of rates obtained under nondenaturing conditions. At higher temperatures (>60°), over 50% of the bound radioactivity is lost within 10 min. That the apparent instability of the complex is due to protein denaturation was shown by adding aliquots of the heated reaction mixtures to cold solutions containing CH_2FAH_4 and [^{3}H]-FdUMP, and allowing time (3 hr) for reequilibration. Under these conditions, any native protein remaining would re-form the ternary complex and the loss of filterable counts would not be apparent; in fact, such experiments did not result in increases in filterable radioactivity.

As previously described, when the complex is formed with [^{3}H]FdUMP $\geq$ enzyme both binding sites are occupied and the isolated complex contains 2 mol of [^{3}H]FdUMP per mol of enzyme. Barring the occurrence of unusually large positive homotropic interactions, the complex formed with enzyme $\gg$ [^{3}H]FdUMP should be largely populated with species containing 1 mol of [^{3}H]FdUMP per mol of enzyme. When dissociation rates are measured in the presence of unlabeled FdUMP, both sites will be occupied by nucleotide, but the observed rates of dissociation would measure the release of [^{3}H]FdUMP from one site or both sites, depending on the ratio of enzyme to [^{3}H]FdUMP used in formation of the complex. Complexes were formed with varying excess sites (1–100-fold) over [^{3}H]FdUMP (23 nM), and the release of protein bound radioactivity was monitored during equilibration with unlabeled FdUMP as described in the legend to Figure 4. The calculated rate constants from these experiments were invariable and identical with those obtained in experiments where excess [^{3}H]FdUMP was used in formation of the complex.

Rates of association of [^{3}H]FdUMP and E·CH_2FAH_4 were measured by the initial velocity (v_i) of appearance of nitrocellulose filterable radioactivity when [^{3}H]FdUMP was combined with a mixture containing enzyme (6 nM) and a saturating level of CH_2FAH_4 (123 μM). Second-order rate constants
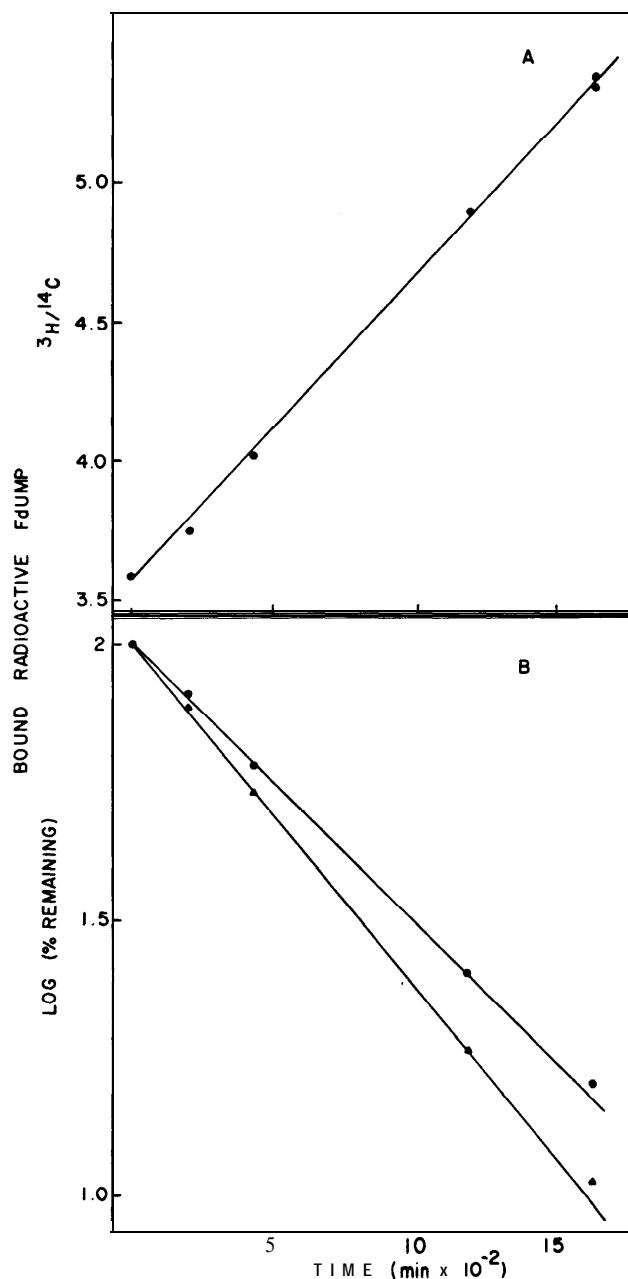

FIGURE 5: Secondary tritium isotope effect on dissociation rate. The complex was formed as described in Materials and Methods using 200 μM CH_2FAH_4, 1.18 μM thymidylate synthetase, 1.64 μM [2-^{14}C]-FdUMP, and 16 nM [6-^{3}H]FdUMP. The reaction was initiated by the addition of 10 μl of FdUMP (50 mM) to 800 μl of this solution. Aliquots (100 μl) were removed at intervals and filtered through nitrocellulose membranes and both isotopes counted to an accuracy of $\pm 0.5\%$; ^{14}C spillover into the ^{3}H channel was determined by the external standard ratio method. (A) Variance of the ^{3}H : ^{14}C ratio with time. (B) First-order plots for dissociation of [^{3}H]FdUMP (●) and [^{14}C]FdUMP (▲) from the complex.

were evaluated from the data using the equation $k_{assoc} = v_i/[FdUMP_i][Enz_i]$. Rate constants were invariable over a 57-fold concentration range of [^{3}H]FdUMP (2-l 15 nM) and gave a nominal value of 2×10^7 M^{-1} min^{-1} for the apparent association of [^{3}H]FdUMP and E·CH_2FAH_4.

Secondary Isotope Effect upon Dissociation of [6-^{3}H]FdUMP. Reactions in which a carbon atom undergoes sp^3 to sp^2 rehybridization at or before the rate-limiting step may show secondary isotope effects (for a review, see Shiner (1971) and Sunko and Borčić (1971)). Since it was believed that the 6

TABLE III : Facilitation of [^{3}H]FdUMP Binding to Thymidylate Synthetase by Various Folate Analogs.[a]

Effector	[^{3}H]FdUMP Bound (dpm/100 μl)	Relative [b] Efficacy
None	150	
CH_2FAH_4	10,500	1.0
FAH_2	6,500	0.61
FA	3,750	0.35
N^{10}-CH_3FA	5,200	0.49
Pteroic acid	230	0.008
Homo-FA	3,300	0.30
Methotrexate	2,300	0.21
Dichloromethotrexate	800	0.06
5,8-Deaza-FA	6,300	0.59
5,8-Deaza-N^{10}-CH_3FA[c]	7,900	0.75

[a] The assay mixture containing 0.5×10^{-8} M enzyme, 3.6×10^{-8} M [^{3}H]FdUMP, and 1.6×10^{-4} M of the specified effector was allowed to stand at room temperature for 2 hr; 100-μl aliquots (4.93×10^4 total dpm) were filtered through nitrocellulose membranes and protein bound radioactivity determined as in Materials and Methods. [b] Calculated as fraction of [^{3}H]FdUMP bound as compared to that obtained with 5,10-CH_2FAH_4. [c] Concentration was 1.0×10^{-4} M.

carbon of FdUMP was covalently attached to a nucleophilic group of the enzyme in the bound complex (*i.e.,* sp^3 hybridized) a secondary isotope effect at C-6 was anticipated upon dissociation to free FdUMP. To test for this, a complex formed with a mixture of [2-^{14}C]- and [6-^{3}H]FdUMP was allowed to dissociate in the presence of a 380-fold excess of unlabeled FdUMP. Aliquots were filtered through nitrocellulose membranes and the protein-bound ^{3}H and ^{14}C determined as the dissociation proceeded. As shown in Figure 5A, the ^{3}H : ^{14}C ratio of bound FdUMP increases with time. Since secondary carbon isotope effects are extremely small (Sunko and Borčić, 1971), this effect must be attributed to a slower rate of dissociation of the 6-^{3}H nucleotide than the 6-^{1}H ligand. First-order plots of the loss of the individual isotopes (Figure 5B) show $k_H/k_T = 1.23$ which from the relationship log $k_H/k_T = 1.44$ log k_H/k_D (Swain et *al.,* 1958) corresponds to $k_H/k_D = 1.15$. This strongly suggests that a sp^3 to sp^2 hybridization change at the 6 position occurs upon dissociation. Previously reported kinetic values are not corrected for the observed secondary isotope effect upon dissociation and would correspond to 81% of the rate of dissociation of unlabeled FdUMP.

Complex Formation in the Presence of Folate Analogs. Table III shows that a number of analogs of CH_2FAH_4 will serve as effectors for binding of [^{3}H]FdUMP, *albeit* not as effectively as CH_2FAH_4. It is to be noted that optimal conditions were not established for the analogs listed and a complete study will be forthcoming.

Treatment of Complex with Guanidine Hydrochloride and Pronase. Treatment of the preformed E–CH_2FAH_4–[^{3}H]-FdUMP complex with 6 M guanidine hydrochloride does not result in rapid dissociation of the radioactive ligand (Figure 6); in fact, dissociation in the presence of the denaturant occurs at a similar or lower rate than observed in its absence. In this experiment, an excess of unlabeled FdUMP was added with the denaturant to preclude reassociation of the radio-
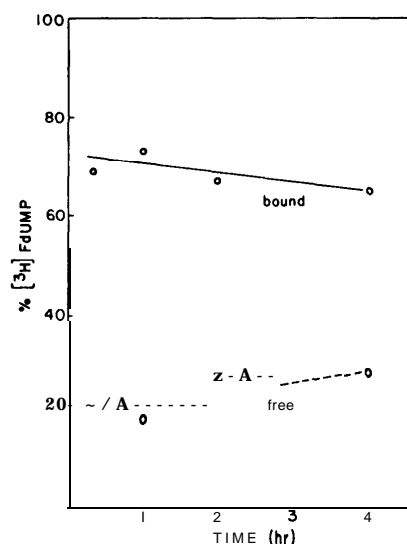

FIGURE 6: The effect of 5 M guanidine hydrochloride on the rate of dissociation of the E·CH_2FAH_4·[^{3}H]FdUMP complex. The complex was formed as described in Materials and Methods and isolated by Sephadex G-25 filtration. To 2 ml (0.14 nmol of complex) of the filtrate was added 2.85 ml of a solution containing 8.5 M guanidine hydrochloride and 11 nmol of unlabeled FdUMP. At intervals 100 μl of aliquots were filtered through Sephadex G-25 and the radioactivity in the effluent was determined. Protein-bound radioactivity was calculated as the per cent of total radioactivity recovered in the void volume; in all cases radioactivity in the volume was filterable on nitrocellulose whereas included radioactivity was not.

active ligand and the loss of radioactivity represents the effect of the denaturant on the rate of dissociation. In contrast, when thymidylate synthetase was incubated with [^{3}H]FdUMP and CH_2FAH_4 in the presence of 5 M guanidine hydrochloride, protein-bound radioactivity could not be detected by Sephadex G-25 or nitrocellulose filtration. Although it is conceivable that the bound complex is inherently more stable toward this denaturant than the native protein, we are unaware of examples in which ligand protection is manifested to the degree exhibited here. In concert with other data presented, the stability of the bound complex toward guanidine hydrochloride is best in accord with covalent bond formation between the enzyme and [^{3}H]FdUMP.

Treatment of the gel filtered E–CH_2FAH_4–[^{3}H]FdUMP complex with pronase resulted in a loss ($t_{1/2} = 27$ min) of nitrocellulose filterable counts. This result indicates that either the radioactive ligand had dissociated, or that it was bound to peptide fragments sufficiently small to preclude their adsorption to the membranes. That the latter was the case could be shown by chromatographic and electrophoretic analysis of the digestion products. After a 3-hr incubation with pronase, the mixture was concentrated by lyophilization and applied, along with unlabeled FdUMP and FdUR markers, to chromatography paper. Paper chromatography (Figure 7A) showed most (77%) of the radioactivity to move with R_F 0-0.16; 18 and 5% moved with FdUMP and FdUR, respectively. On high voltage electrophoresis (pH 3.5), the radioactivity moved 0-7 cm toward the cathode (Figure 7B), clearly separated from FdUMP which moved 11 cm toward the anode. It is to be noted that FdUR also moves slightly toward the cathode in this system and is not separable from the bulk of radioactivity; however, from paper chromatography (*vide supra*) it was shown that the amount of nucleoside present did not exceed 5%. Likewise, since only 2% of the radioactivity moved with FdUMP on electrophoresis,
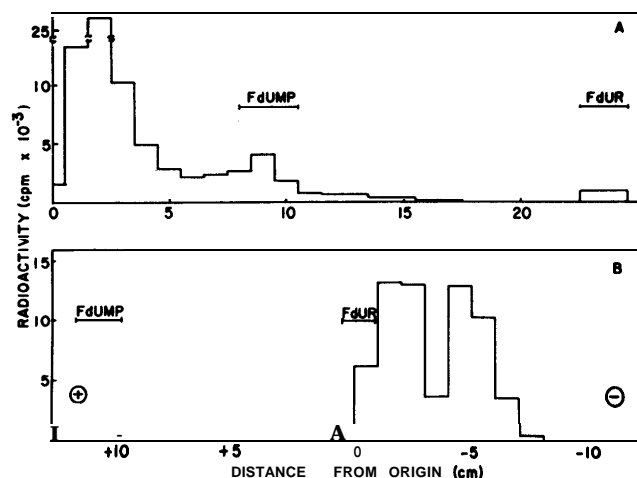
FIGURE 7: Paper chromatography and high voltage electrophoresis of pronase digests of the $E \cdot CH_2FAH_4 \cdot [^3H]FdUMP$ complex. The enzyme–$[^3H]FdUMP$ complex (2.0 ml) was formed as described in Materials and Methods using 2×10^{-6} M FdUMP, 10^{-6} M enzyme, and 0.12 mM CH_2FAH_4. Filtration through Sephadex G-25 using the buffer described in Materials and Methods gave 4 ml of complex containing 2.7×10^6 dpm bound $[^3H]FdUMP$; 1 ml of the isolated complex was diluted with an equal volume of the eluent buffer and treated with 140 μl of pronase (10 mg/ml) in 5 mM $CaCl_2$–0.1 M sodium borate (pH 7.4). The reaction was monitored by filtration of 100-μl aliquots through nitrocellulose membranes. After 3 hr the mixture was lyophilized and applied to chromatography paper along with unlabeled FdUMP and FdUR markers: (A) paper chromatography (Whatman No. 1; ascending) using EtOH–0.5 N NH_4OAc (7 : 3) for development; (B) high voltage (2.5 kV, 180 mA) electrophoresis (Whatman No. 3) using 0.5% pyridine–0.5% AcOH (pH 3.5).

most of the radioactivity which moves with FdUMP (18 %) on the paper chromatogram is probably not the nucleotide. In a control experiment, the pronase preparation used was shown to possess significant phosphatase activity and under identical conditions used for the above experiment, FdUMP was almost completely converted to FdUR. Since FdUR is only a minor product of pronase digestion of the complex, this provides further evidence that the loss of filterable radioactivity upon proteolysis is not due to dissociation of $[^3H]$-FdUMP. From these experiments, we can only conclude that pronase treatment of the $E–CH_2FAH_4–[^3H]FdUMP$ complex results in the formation of small peptide fragments which remain bound to the radioactive ligand. Experiments aimed at the purification and identification of these fragments are in progress.

In Figure 8 is shown the difference spectrum obtained when FdUMP is added to the sample cuvette of two cuvettes containing equal amounts of thymidylate synthetase. The spectrum obtained is that of FdUMP and shows no change for at least 5 min. Also shown is the scan obtained when equal amounts of CH_2FAH_4 are then added to the sample and reference cuvettes. There is a complete loss of absorbance at 269 nm, the absorbance maximum of FdUMP. In addition, there are also marked spectral changes which can only be attributed to the cofactor, CH_2FAH_4; notably, there is a loss of absorbance in the region below 294 nm, and an appearance of a differential peak at 330 nm.[2] Although the concentration of the 330-nm absorbing species cannot be measured, minimal values of the
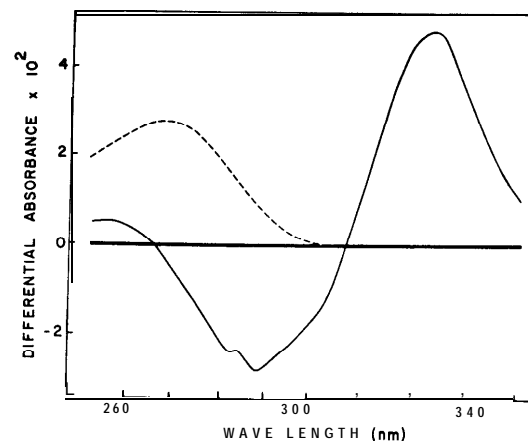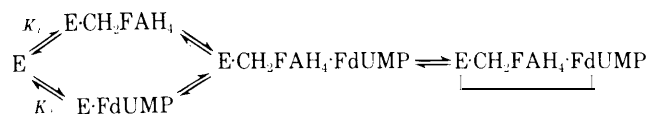

FIGURE 8: Difference spectra showing the loss of FdUMP and CH_2-FAH_4 absorbance, and appearance of a new peak at 330 nm. Reference and sample cuvettes contain equal amounts of enzyme. FdUMP is added to the sample cuvette (---), and then equal amounts of CH_2FAH_4 are added to both cuvettes (—) as described in Materials and Methods.

differential extinction coefficient ($\Delta\epsilon$) may be approximated using the concentrations of the components present. From the concentration of enzyme, a minimum $\Delta\epsilon_{330}$ is calculated to be 1.8×10^4 or 0.9×10^4, respectively, depending on whether 1 or 2 mol of ligand are bound per mol of enzyme. However, if it is assumed that the new species requires binding of at least 1 equiv of FdUMP, or if the concentration of l-L-CH_2FAH_4 is used in the calculations, a minimum value of $\Delta\epsilon_{330} = 1.4 \times 10^4$ is obtained.

Discussion

The results allow considerable insight into the mechanism of interaction of FdUMP with thymidylate synthetase. A scheme which is in accord with current data as we now understand it is given below.

$$E \underset{K_2}{\overset{K_1}{\rightleftarrows}} \begin{matrix} E \cdot CH_2FAH_4 \\ \\ E \cdot FdUMP \end{matrix} \rightleftarrows E \cdot CH_2FAH_4 \cdot FdUMP \rightleftarrows \underline{E \cdot CH_2FAH_4 \cdot FdUMP}$$

As shown, the pathways involve reversible formation of binary complexes which are not of sufficient stability to be isolated by the methods used. We have obtained preliminary data by equilibrium dialysis experiments (D. V. Santi and L. Holland, unpublished observations) which indicate $K_2 \simeq 10^{-5}$ M. In the presence of all components, a tight complex is formed containing enzyme, CH_2FAH_4, and FdUMP which may be isolated by filtration through nitrocellulose membranes or Sephadex G-25. In effect, the presence of either ligand greatly increases the binding of the other. Undoubtedly, the initial central complex which is formed ($E \cdot CH_2FAH_4 \cdot$ FdUMP) is a result of energetically favorable noncovalent interactions. From the data described here, it appears that the covalent complex is responsible for the apparent high affinity of the ligands for binary complexes. For discussion purposes, central complexes containing enzyme, CH_2FAH_4, and FdUMP which are isolable by nitrocellulose or Sephadex G-25 filtration will be collectively referred to as $E–CH_2FAH_4–$FdUMP. Preliminary data (D. V. Santi, C. S. McHenry, and H. Sommer, unpublished results) have provided evidence that the addition and release of ligands may proceed in the random

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[2] While this work was in progress, a similar increase in absorbance at 335 nm was independently observed by Sharma and Kisliuk (1973) and attributed to the formation of 7,8-FAH_2.

fashion depicted. However, since experiments described here were performed with concentrations of CH_2FAH_4 sufficient to complex nearly all free enzyme (i.e., $[CH_2FAH_4] \gg K_1$), and levels of $[^3H]FdUMP$ insufficient to form $E \cdot FdUMP$ complexes (i.e. $[FdUMP] \ll K_2$), the measurements made should largely reflect interactions of FdUMP with the cofactor-bound enzyme or, by microscopic reversibility, dissociation of $E–CH_2FAH_4–FdUMP$ to FdUMP and $E \cdot CH_2FAH_4$.

In the presence of excess cofactor, direct titration of the enzyme with $[^3H]FdUMP$ or the technique of continuous variation demonstrates that two high affinity binding sites exist per mole of enzyme. Since two sites were also found using $[2-^{14}C]FdUMP$ the possibility of a secondary tritium isotope effect causing errors in the assigned stoichiometry need not be considered. Similarly, titration of FdUMP with the enzyme (Ackermann and Potter, 1949) demonstrates that complete inhibition of catalytic activity requires binding of 2 equiv of inhibitor. In this regard it is to be noted that the native enzyme from *L. casei* is composed of two subunits which show identical molecular weights (35,000 daltons) (Dunlap et *al.*, 1971a), N-terminal and penultimate amino acids, and cyanogen bromide fragments (Loeble and Dunlap, 1972).

The finding that the enzyme possesses two binding sites for FdUMP raises the question as to whether they interact. Studies on subunit interactions are in progress, and discussion here is limited to aspects pertinent to the data presented. Of particular concern at this time is the possibility that such interactions might result in inaccuracies in measured rates of dissociation. As previously mentioned, concentrations of CH_2FAH_4 sufficient to saturate free enzyme were used in all experiments so we need only consider the effect that homotropic interactions involving FdUMP might have on our measurements.

When the complex is formed with a large excess of enzyme, only one site should be occupied by radioactive ligand if the sites are equivalent, nonequivalent, or interact by negative homotropic effects. If positive homotropic effects were existent, we calculate that *ca.* half of the complexes formed would have but one site occupied with radioactive ligand under the experimental conditions used (binding sites/$[^3H]FdUMP \simeq 100 : 1$), so long as the difference in affinities for the first and second sites do not exceed two orders of magnitude. Since the loss of protein-bound radioactivity proceeds at identical rates regardless of whether the radioactive complex is formed in the presence of excess $[^3H]FdUMP$ or enzyme, it may be concluded that if nonequivalence of sites or any of the aforementioned homotropic interactions are in effect, they are not detectable in the rate of dissociation of FdUMP from the bound complex. At this time we cannot ascertain whether highly positive homotropic interactions occur in the binding of FdUMP to $E \cdot CH_2FAH_4$ complexes. Classical plotting methods which reveal such behavior (Koshland, 1970) cannot be used because the levels of enzyme required to detect the complex are significantly higher than the dissociation constant of $[^3H]FdUMP$ and, under all measurable conditions of excess enzyme, there is no free nucleotide in solution. Although we tacitly acknowledge this possibility, because of lack of direct evidence we have assumed for present purposes that the enzyme possesses equivalent sites with respect to dissociation rate constants and, for simplicity, refer to the bound complex as if it possesses a single binding site.

$$E \cdot CH_2FAH_4 + [^3H]FdUMP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$
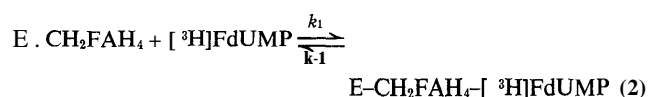
$$E–CH_2FAH_4–[^3H]FdUMP \quad (2)$$

Once formed, $E–CH_2FAH_4–[^3H]FdUMP$ complexes are stable for long periods provided excess cofactor is present and temperatures do not exceed 37". At equilibrium, the free and bound components undergo numerous association-dissociation events without apparent structural change. At higher temperatures, the enzyme is denatured and the ligands dissociate, albeit not necessarily in that order. When the complex is freed of unbound cofactor by gel filtration there is a loss of protein-bound radioactivity with a nominal $t_{1/2}$ of *ca.* 14 hr at 23". This may be attributed to oxidative degradation of the small amount of cofactor present since its replenishment results in rebinding of $[^3H]FdUMP$ to the enzyme.

A reasonable approximation of the rate of association of FdUMP with the $E \cdot CH_2FAH_4$ complex may be made by taking advantage of the nitrocellulose filtration assay and access to $[^3H]FdUMP$ of high specific activity. In these experiments, dilute solutions of the enzyme were first equilibrated with a near-saturating level of cofactor so that the $E \cdot CH_2FAH_4$ complex was the predominating enzyme form in solution. $[^3H]FdUMP$ was added and aliquots were assayed for protein-bound radioactivity at various times. A number of such experiments over a variable range of $[^3H]FdUMP$ concentration gave a second-order rate constant (k_1) of $2 \times 10^7 M^{-1} min^{-1}$ at 24". It is to be noted that this value may have to be refined somewhat because of possible occurrence of a secondary tritium isotope effect in the rate of association. Rates of dissociation of $[6-^3H]FdUMP$ were measured as the loss of protein-bound radioactivity which occurs upon equilibration with excess unlabeled FdUMP. The first-order rate constants (k_{-1}) obtained are independent of the concentrations of the isotope diluent, the enzyme to $[^3H]FdUMP$ ratio, and the concentration of the complex present in solution. Since CH_2FAH_4 was present at levels sufficient to saturate free enzyme, our measurements reflect the rate of dissociation of the complex to $[^3H]FdUMP$ and $E \cdot CH_2FAH_4$. The rates of dissociation so obtained were highly temperature dependent, and showed a negligible entropy of activation and a high enthalpy of activation (28.4 kcal/mol). The activation parameters are consistent with our contention that the FdUMP is covalently attached to the enzyme in the bound complex.

As previously mentioned, the binding assay requires levels of enzyme which are significantly higher than the dissociation constant of the $E–CH_2FAH_4–FdUMP$ complex, thus precluding detection of unbound ligand and assignment of a dissociation constant by equilibrium measurements. However, using the rate constants obtained for the association and dissociation of $[^3H]FdUMP$, a kinetically derived dissociation constant for $[^3H]FdUMP$ (k_1/k_{-1}) at 24" may be calculated to be *ca.* $5 \times 10^{-11} M$, which is minimally two orders of magnitude lower than previously reported values. We ascribe this discrepancy to erroneous interpretations of kinetic inhibition data obtained under conditions where concentrations of enzyme and the inhibitor were sufficiently similar so as to produce observable "stoichiometric" inhibition. Clearly, studies intended to assign the sequence of substrate addition to thymidylate synthetase which utilize classical inhibition profiles of FdUMP (Reyes and Heidelberger, 1965) are erroneous and warrant reinvestigation.

Incubation of FdUMP with thymidylate synthetase in the presence of various CH_2FAH_4 analogs resulted in the binding of FdUMP, although not as tightly as with CH_2FAH_4. This finding would suggest an interaction of the analogs with the CH_2FAH_2 binding site.
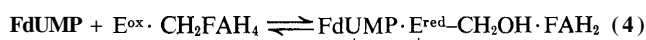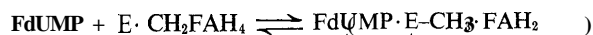
A most pertinent feature of the interaction of FdUMP and thymidylate synthetase involves changes which occur

within the bound complex. Several lines of evidence demonstrate rather conclusively that a covalent bond is formed between FdUMP and thymidylate synthetase. (i) The E–CH₂FAH₄–[³H]FdUMP complex may be treated with 5 M guanidine hydrochloride without apparent increases in the rate of loss of protein-bound radioactivity. With few exceptions, this denaturant is sufficient to disrupt noncovalent interactions between low molecular weight ligands and their protein receptors. (ii) Proteolytic digestion of the complex yields peptide fragments which are strongly associated with the nucleotide; it is difficult to envision a situation not involving covalent bond formation which would yield this result. (iii) 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. (iv) The rate of dissociation of [6-³H]FdUMP from the domplex 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.

From the considerations discussed thus far, it is most reasonable to conclude that a nucleophilic group of the enzyme, probably that one involved in catalysis, adds to the 6 position of FdUMP *via* a Michael-type reaction to give the covalent complex (Figure 9). Elimination would regenerate enzyme and FdUMP and accommodate the observed reversibility of the reaction. In accord with this proposal, it has been well established that the 6 position of the 5-fluorouracil heterocycle is the most susceptible site toward nucleophilic reagents (Reist *et al.,* 1964; Fox *et al.,* 1966; Otter *et al.,* 1969).

In addition to uv spectral changes attributed to saturation of the 5,6-double bond of FdUMP, there are also marked changes that occur upon complex formation which may only be rationalized as a conversion of the cofactor to a different chemical species. Notably, there is a decrease of absorbance at the absorption maxima of the cofactor (294 nm), and an increase of absorbance at higher wavelengths giving a differential absorption maximum for the complex at 330 nm. The possibility that this species is one normally accumulated in the enzymic reaction makes its identification of paramount importance.

At this time, we can envision three chemical species which might explain the anomalous absorption of the enzyme-bound cofactor. First, the difference spectrum shown in Figure 8 is similar to that reported for 7,8-FAH₂ vs. CH₂FAH₄ (Wahba and Friedkin, 1961). The 10-nm differences in the differential maxima and minima could well be a result of perturbations of the environment. The formation of 7,8-FAH₂ could be rationalized if the methylene group of CH₂FAH₄ is transferred to the enzyme with concomitant reduction to provide a methylated enzyme form (eq 3); alternatively, the enzyme could be reduced and the methylene group of the cofactor transferred at the formaldehyde level of oxidation (eq 4). Either of these enzyme forms could serve as the carbon donor and reductant in the catalytic reaction and are in accord with biochemical data reported to date. However, the differential extinction coefficient of 7,8-FAH₂ ($\Delta\epsilon_{max} = 6400$)

$$\text{FdUMP} + \text{E} \cdot \text{CH}_2\text{FAH}_4 \rightleftharpoons \text{FdUMP} \cdot \text{E–CH}_3 \cdot \text{FAH}_2 \quad)$$

$$\text{FdUMP} + \text{E}^{ox} \cdot \text{CH}_2\text{FAH}_4 \rightleftharpoons \text{FdUMP} \cdot \text{E}^{red}\text{–CH}_2\text{OH} \cdot \text{FAH}_2 \quad (4)$$

is lower than our estimations for the 330-nm peak and casts
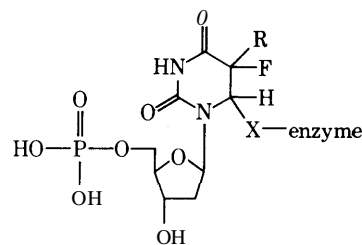
some doubt that 7,8-FAH₂ is the unknown species. It should also be noted that the native protein has been reported to possess no disulfide bonds (Dunlap *et al.,* 1971 b), the functional group of a protein which is most susceptible to reversible reduction, suggesting that reduction of the enzyme (eq 4) is unlikely.

Langenbach *et al.* (1972) have suggested that the enzyme-bound FdUMP may be linked at the 5 position to the cofactor *via* a formaldehyde bridge (Figure 9; R = CH₂FAH₄). This is an interesting speculation which is in accord with the overall mechanisms forwarded by this laboratory (Santi and Brewer, 1973; Santi and McHenry, 1972). However, since 5-alkyl derivatives of FAH₄ show maxima which are shifted to lower wavelengths than CH₂FAH₄ (Gupta and Huennekens, 1967; Blair and Saunders, 1971) this hypothesis would require large electronic perturbations of the cofactor upon binding. Regardless, covalent linkage of the cofactor to FdUMP is not essential for tight binding of FdUMP to the enzyme. We have demonstrated that numerous analogs of FAH₄ which cannot form bridged intermediates stimulate binding of FdUMP. Furthermore, 10-CH₃FAH₄ has been reported (Langenbach *et al.,* 1973) to stimulate the decrease in absorbance at 270 nm we had previously observed with CH₂FAH₄ and associated with saturation of the 5,6-double bond of FdUMP (Santi and McHenry, 1972).

A third possibility is that binding of FdUMP causes the buildup of an iminium cation, 5-CH₂ = ⁺FAH₄ or 10-CH₂ =
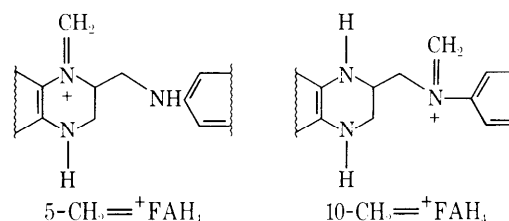
⁺FAH₄. These have recently been considered as the reactive electrophilic species of 5,10-CH₂FAH₄ (Kallen and Jencks, 1966; Benkovic *et al.,* 1969) and one of these species undoubtedly exists on the reactive pathway of the normal enzymic reaction (Santi and Brewer, 1973). Although these are expectedly extremely reactive toward hydrolysis, it is conceivable that they could exist in a protected environment when bound to the enzyme. The formation of an iminium ion from CH₂FAH₄ could arise from a conformational change in the enzyme induced by binding of FdUMP or formation of enzyme–FdUMP covalent bond. Such an intermediate is an attractive possibility since it possesses a methylene group highly activated toward nucleophilic attack by an activated form of deoxyuridylate (Santi and Brewer, 1968, 1973) during the normal thymidylate synthetase catalyzed reaction. Al-

though it is difficult to assign exact spectral characteristics to these species, it is of interest that 5,10-methenyl-FAH$_4$, a folate cofactor which has electronic features similar to the iminium ions, shows a maximum at high wavelength (ca. 355 nm) and an extinction coefficient in the range of that observed for the cofactor in the E–CH$_2$FAH$_4$–FdUMP complex (Rabinowitz, 1960).

Relationship of FdUMP Inhibition of Thymidylate Synthetase to the Catalytic Mechanism. Previous studies from this laboratory have permitted assignment of a minimal mechanism for the thymidylate synthetase reaction (Santi and Brewer, 1968, 1973; Pogolotti and Santi, 1974). One of the primary events has been proposed to involve addition of a nucleophilic group of the enzyme to the 6 position of dUMP. In this manner, the 5 position of dUMP becomes sufficiently nucleophilic to react with the methyl donor. Concomitant with this, changes must occur in the structure of CH$_2$FAH$_4$ to provide a suitably reactive electrophilic species, but the exact nature of these changes or the structure of the ultimate methyl donor are unknown at this time. Once the substrates have been converted to their reactive forms, condensation occurs to give, after several steps, thymidylate and 7,8-FAH$_2$.

From these studies described here, it appears that the reaction of FdUMP closely parallels a part of the sequence of the normal enzymic reaction. First, a covalent bond is formed between a nucleophilic group of the enzyme and the 6 position of the heterocycle, in an identical fashion as proposed for the enzymic reaction; in fact, the ability of FdUMP to behave as a quasisubstrate in this regard provides strong supportive evidence for our proposal of nucleophilic catalysis. Second, upon binding of all components, a structural change in the cofactor occurs to give a product which may be rationalized as one of a number of possible intermediates in the normal enzymic reaction. Our present belief is that FdUMP behaves as a quasisubstrate which undergoes and stimulates enzymic conversions up to the step normally associated with methyl transfer to the heterocycle, and in effect, "traps" a form of the complex which resembles a steady-state intermediate. If this interpretation is correct, delineation of the intricacies of the interaction of FdUMP with thymidylate synthetase should provide information on the mechanism of catalysis which would not be accessible by normal techniques. Studies aimed at this goal are in progress.

Acknowledtment

References

Ackermann, W. W., and Potter, V. R. (1949), *Proc. Soc. Exp. Biol. Med. 72,1.*

Baker, B. R., and Vermeulen, N. M. J. (1970), *J. Med. Chem. 13,* 1143.

Benkovic, S. J., Benkovic, P. A., and Comfort, D. R. (1969), *J. Amer. Chem. Soc. 91,* 5270.

Blair, J. A., and Saunders, K. J. (1971), *Anal. Biochem. 41,332.*

Blakley, R. L. (1969), The Biochemistry of Folic Acid and Related Pteridines, New York, N. Y., American Elsevier.

Bray, G. A. (1960), *Anal. Biochem. I, 279.*

Chaberek, S., and Martell, A. (1959), Organic Sequestering Agents, New York, N. Y., Wiley, pp 508-581.

Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. (1968), *Proc. Nat. Acad. Sci. U. S. 44,* 1004.

Crusberg, T. C., Leary, R., and Kisliuk, R. L. (1970), *J. Biol. Chem. 245,* 5292.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121,404.*

Dunlap, R. B., Harding, N. G. L., and Huennekens, F. M. (1971a), *Biochemistry IO,* 88.

Dunlap, R. B., Harding, N. G. L., and Huennekens, F. M. (1971b), *Ann. N. Y. Acad. Sci. 186,153.*

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Flaks, J. G., and Cohen, S. S. (1959), *J. Biol. Chem. 234,* 2981.

Fox, J. J., Miller, N. C., and Cushley, R. J. (1966), *Tetrahedron Lett.,* 4927-4934.

Gupta, V. S., and Huennekens, F. M. (1967), *Arch. Biochem. Biophys. 120,712.*

Habeeb, A. F. S. A. (1972), *Methods Enzymol. 24,457.*

Harvey, C. L., Clericuzio, E. M., and Nussbaum, A. I. (1970), *Anal. Biochem. 36,413.*

Heidelberger, C., Kaldor, G., Mukherjee, K. L., and Danenberg, P. B. (1960), *Cancer Res. 20,903.*

Kalckar, H. M. (1947), *J. Biol. Chem. 167,461.*

Kallen, R. G., and Jencks, W. P. (1966), *J. Biol. Chem. 241,* 5851.

Koshland, D. E., Jr. (1970), *Enzymes, 3rd Ed. I, 342.*

Langenbach, R. J., Danenberg, P. V., and Heidelberger, C. (1972), *Biochem. Biophys. Res. Commun. 48,* 1565.

Leary, R. P., and Kisliuk, R. L. (1971), *Prep. Biochem. I,* 47.

Loeble, R. B., and Dunlap, R. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 1671.

Maurer, H. R. (1971), Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, New York, N. Y., Walter de Gruyter, pp 1 1-20.

Otter, B. A., Falco, E. A., and Fox, J. J. (1969), *J. Org. Chem. 34,* 1390.

Pastore, E. J., and Friedkin, M. (1962), *J. Biol. Chem. 237,* 3802.

Pogolotti, A. L. and Santi, D. V., (1974), *Biochemistry 13,* 456.

Rabinowitz, J. C. (1960), *Enzymes, 2nd Ed. 2,* 185.

Reist, E. J., Benitez, A., and Goodman, L. (1964), *J. Org. Chem. 29,* 554–558.

Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. I,* 14-30.

Rothenberg, S. P. (1965), *Anal. Biochem. 13,530.*

Rutter, W. J. (1967), *in* Methods in Developmental Biology, Wilt, F. H., and Wessells, N. K., Ed., New York, N. Y., Thomas Y. Crowell, pp 671-683.

Santi, D. V., and Brewer, C. F. (1968), *J. Amer. Chem. Soc. 90,* 6236.

Santi, D. V., and Brewer, C. F. (1973), *Biochemistry 12,* 2416.

Santi, D. V., and McHenry, C. S. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1855.

Santi, D. V., McHenry, C. S., and Perriard, E. R. (1974), *Biochemistry 13,* 467.

Santi, D. V., and Sakai, T. T. (1971), *Biochem. Biophys. Res. Commun. 42,813.*

Sharma, R. K., and Kisliuk, R. L. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 591.

Shiner, V. J., Jr. (1971), *in* Isotope Effects in Chemical Reactions, Collins, C. J., and Bowman, N. S., Ed., New York, N. Y ., Van Nostrand-Reinhold.

Sunko, D. E., and Borčić, S. (1971), *in* Isotope Effects in Chemical Reactions, Collins, C. J., and Bowman, N. S., Ed., New York, N. Y., Van Nostrand-Reinhold.

Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., and Schaad, L. J. (1958), *J. Amer. Chem. Soc. 80*, 5885.

Wahba, A. J., and Friedkin, M. (1961), *J. Biol. Chem. 236,11.*

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Yoshikawa, M., Kato, T., and Takenishi, T. (1967), *Tetrahedron Lett. 50*, 5065.