# Thymidylate Synthetase Catalyzed Exchange of Tritium from [5-$^3$H]-2′-Deoxyuridylate for Protons of Water†

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ABSTRACT: Thymidylate synthetase catalyzes an exchange of tritium of [5-$^3$H]dUMP for protons of water in the absence of $CH_2$-$H_4$folate. The turnover number for this reaction is some 45 000-fold lower than that of dTMP formation and $K_m$ is $1.2 \times 10^{-5}$ M, similar to the dissociation constant of the enzyme–dUMP complex determined by equilibrium dialysis. The presence of 1 mM folate has no effect on $V_{max}$ but results in a decrease in the $K_m$ of dUMP to a value close to that in the normal enzymic reaction. The exchange reaction provides definitive evidence that the enzymic reaction involves attack of a nucleophile of the enzyme on the 6 position of dUMP to provide a 5,6-dihydro-dUMP intermediate which is covalently bound to the enzyme. Stereochemical considerations of the exchange reaction require proposal of a partial reaction which is not completely stereospecific or a complex reaction in which protons of water are handled with complete stereospecificity in a fashion similar to the one carbon unit of the normal enzymic reaction.

Thymidylate synthetase (EC 2.1.1.45) catalyzes the reductive methylation of dUMP to dTMP with concomitant conversion of $CH_2$-$H_4$folate[1] to 7,8-$H_2$folate. The currently accepted mechanism of this reaction involves attack of a nucleophilic group of the enzyme at the 6 position of dUMP as an early event in catalysis. The evidence for the proposed pathway thus far rests solely on studies of model chemical counterparts (Pogolotti & Santi, 1977; Friedkin, 1973) and interactions of the enzyme with inhibitors. Regarding the latter, most notable have been studies which have demonstrated that, in the presence of $CH_2$-$H_4$folate, the 6 position of FdUMP is covalently linked to the putative nucleophilic catalyst of the enzyme to give a stable complex analogous to one of the proposed steady-state intermediates of the normal enzymic reaction. While such studies have resulted in general acceptance of certain aspects of the mechanism, neither nucleophilic catalysis nor 5,6-dihydropyrimidine intermediates have previously been demonstrated in any reactions *catalyzed* by this enzyme.

In this and the accompanying paper (Garrett et al., 1979), we describe two quite different systems which verify that nucleophilic attack at the 6 position of the heterocycle is required for reactions *catalyzed* by thymidylate synthetase. Here, we demonstrate that the enzyme catalyzes the exchange of the H-5 of dUMP for protons of water, a reaction which requires the formation of 5,6-dihydropyrimidine intermediates.

## Materials and Methods

[2-$^{14}$C]dUrd (39 mCi/mmol) and [5-$^3$H]dUrd (16 Ci/mmol) were obtained from Amersham/Searle Corp. and Schwarz/Mann, respectively. $^{14}CH_2O$ (56.8 mCi/mmol) was purchased from New England Nuclear. $CF_3$dUrd and FdUrd were obtained from P-L Biochemicals. Nucleosides were converted to the corresponding 5′-mononucleotides by using *E. coli* thymidine kinase as previously described (Wataya & Santi, 1977) and purified by DEAE-cellulose chromatography (Wataya & Santi, 1977) followed by LC using Aminex A-27 (Garrett et al., 1977). The purity of the nucleotides used was also confirmed by paper and PEI-cellulose chromatography. For routine repurifications before experiments up to 25 μmol of the nucleotide was adsorbed on a small DEAE-cellulose column (0.4 × 1.0 cm), washed with 5–10 mL of 5 mM NaOAc (pH 6.0) or $NH_4HCO_3$ (pH 7.9), and eluted with ca. 2 mL of 100 mM of the same buffer. The position of isotopic labeling of [5-$^3$H]dUMP was verified by complete release of tritium from [2-$^{14}$C,5-$^3$H]dUMP upon thymidylate synthetase catalyzed conversion to dTMP. Folic acid was obtained from Sigma Chemical Corp. and purified by DEAE-cellulose chromatography using a linear $NH_4HCO_3$ gradient (0.05–0.5 M) prior to use; paper chromatography (Whatman No. 1, ascending) using 0.1 M phosphate (pH 7)–0.5% 2-mercaptoethanol (Blair & Saunders, 1970) showed one UV-absorbing spot with $R_f$ 0.37. $H_4$folate was prepared by catalytic reduction of folate (Hatefi et al., 1960).

Thymidylate synthetase was obtained from an amethopterin-resistant strain of *Lactobacillus casei* (Crusberg et al., 1970). Partially purified enzyme was purchased from the New England Center and purified by a modification (Wataya & Santi, 1977) of the method of Gallivan et al. (1976). By using assay conditions previously described (Santi & Sakai, 1972), this enzyme showed a specific activity of 5.3 μmol min$^{-1}$ mg$^{-1}$ at 25 °C using $\epsilon_{278}$ $1.07 \times 10^5$ M$^{-1}$ cm$^{-1}$ for protein determination. The concentration of active thymidylate synthetase referred to in experiments was determined by spectrophotometric titration as described in the accompanying paper (Garrett et al., 1979). All UV spectra were obtained on a Cary Model 118 recording spectrophotometer. Isotopes were counted in a Nuclear Chicago Isocap 300 spectrometer by using 25% (v/v) Triton X-114 in xylene containing 0.4% Omnifluor. Sufficient counts (ca. $10^6$) were collected to ensure no greater than a 0.25% standard error. Corrections for $^{14}$C spillover and dpm calculations were performed by the external standard ratio method.

*Tritium Release from [5-$^3$H]dUMP.* Incubations were carried out at room temperature (ca. 22 °C) and contained 50 mM *N*-methylmorpholine hydrochloride (pH 7.40), 26 mM $MgCl_2$, 1 mM EDTA, 80 mM 2-mercaptoethanol, and the

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[1] Abbreviations used: dUMP, 2′-deoxyuridylic acid; dTMP, 2′-deoxythymidylic acid; $CH_2$-$H_4$folate, 5,10-methylenetetrahydrofolic acid; $H_2$folate, 7,8-dihydrofolic acid; FdUrd, 5-fluoro-2′-deoxyuridine; $CF_3$dUrd, 5-trifluoromethyl-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylic acid; $CF_3$dUMP, 5-trifluoromethyl-2′-deoxyuridylic acid; HmdUMP, 5-hydroxymethyl-2′-deoxyuridylic acid; LC, high-pressure liquid chromatography.

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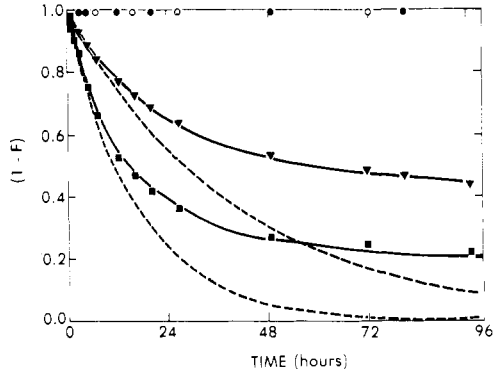


FIGURE 1: Plot of $(1 - F)$ vs. incubation time where $F$ = the fraction of isotopic exchange of the 5-$^3$H of dUMP catalyzed by thymidylate synthetase. All reactions were performed at 22 °C and contained 0.37 μM enzyme and 5.7 μM [2-$^{14}$C,5-$^3$H]dUMP; (■) 1 mM folate; (▲) no additions; (●) 1 mM folate and 50 μM FdUMP; (○) minus enzyme. Points were experimental with dashed (- - -) and solid (—) lines calculated from eq 1 and 2, respectivley, presented in the text.

indicated amounts of thymidylate synthetase, [2-$^{14}$C,5-$^3$H]-dUMP, folate derivative and formaldehyde. At specified intervals aliquots (20–50 μL) were placed in glass scintillation vials which contained 100 μL of ethanol. After gentle drying under a lamp, three additional portions of ethanol (75 μL) were applied and gently dried to ensure complete removal of $^3H_2O$ released from the nucleotide. The efficacy of this procedure was demonstrated by complete volatilization of radioactivity when $^3H_2O$ (ca. $10^5$ dpm) was used in control experiments. Scintillation fluid containing 5% water was added, and, after standing for 24 h, samples were counted. The fraction of tritium remaining in dUMP at time $t$ was evaluated by the equation ($^3$H dpm/$^{14}$C dpm)$_t$($^{14}$C dpm/$^3$H dpm)$_0$ (Santi & Brewer, 1973).

*Product Analysis.* Aliquots (50–100 μL) of reaction mixtures were quenched with 1–1.5 volumes of EtOH along with 10 μL of a solution containing 0.1 M each of dUrd, dUMP, and dTMP (>$10^3$-fold excess). Separation was achieved by LC on Aminex A-27 as described by Garrett et al. (1977). The UV-absorbing peaks were collected and radioactivity was determined. Nucleotides were quantitated by the $^{14}$C content and the extent of exchange of [2-$^{14}$C,5-$^3$H]dUMP was determined by its $^3$H/$^{14}$C ratio.

## Results

Previous assays for tritium release from the 5 position of dUMP monitored the amount of tritium released into water (Lomax & Greenberg, 1967; Roberts, 1966) or remaining in the nucleotide (Crusberg et al., 1970). Because such assays require quantitative recovery of either nucleotide or water and are subject to manipulative errors, the precision is somewhat poor especially at low levels of tritium release. In the present work, we used a modified procedure of Crusberg et al. (1970) utilizing [2-$^{14}$C,5-$^3$H]dUMP; aliquots were removed from reaction mixtures, $^3H_2O$ was completely removed by repeated evaporations, and the $^3$H/$^{14}$C ratio of the nonvolatile nucleotide was used as a measure of tritium release. This modified version is not subject to manipulative errors and the precision of the assay is limited only by the accuracy of counting the dual isotopes. As an example, control points shown in Figure 1 (and others not shown), representing individual aliquots, were processed as described under Materials and Methods and showed the same $^3$H/$^{14}$C ratio; the $^3$H/$^{14}$C of these was 4.80 ± 0.03 (mean ± SD; $n$ = 9). As will be described later, the limit of accuracy we have chosen as acceptable for the work described here is ±0.5% total exchange; however, even this

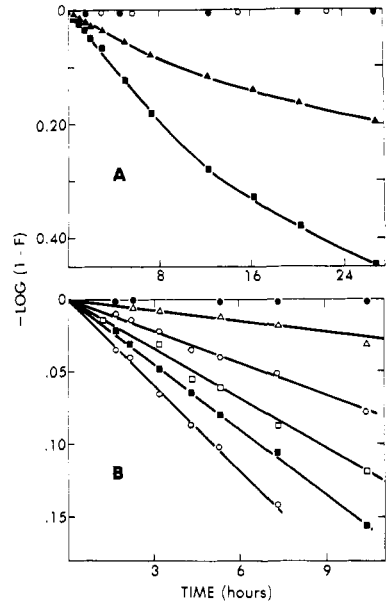


FIGURE 2: (A) Semilog replot of the data presented in Figure 1 where $F$ = the fraction of isotopic exchange of [2-$^{14}$C,5-$^3$H]dUMP catalyzed by thymidylate synthetase at various times. Reactions were performed as described in Figure 1 except that additional time points are included; (■) 1 mM folate; (▲) no additions; (●) 1 mM folate and 50 μM FdUMP; (○) minus enzyme. (B) Semilog plot of initial velocity of 5-$^3$H exchange with varying [2-$^{14}$C,5-$^3$H]dUMP concentrations catalyzed by thymidylate synthetase in the presence of 1 mM folate. Reactions were performed at 22 °C by using 0.37 μM enzyme. Substrate concentrations were (○) 4.1 μM; (■) 6.1 μM; (□) 8.1 μM; (◇) 14. μM; and (△) 40.7 μM. Control points (●) contained 4.1 μM substrate and 50 μM FdUMP.

could be increased by repeated determinations and/or more precise determination of the dual isotopes.

Figure 1 shows that, in the presence of thymidylate synthetase, there is a slow release of tritium from [2-$^{14}$C,-5-$^3$H]dUMP into water. The apparent rate of release is increased when folate is present, but if enzyme is omitted or specific inhibitors of thymidylate synthetase such as FdUMP or $CF_3$dUMP are present, tritium release is not observed. In the experiments shown, a 6.7% release of tritium from the 5 position of dUMP would correspond to a single turnover of the enzyme; although the reaction is slow, it is clear that multiple turnovers occur and tritium release is indeed catalyzed by thymidylate synthetase. When reaction mixtures that had undergone 13 and 15% tritium release in the absence and presence of 1 mM folate, respectively, were analyzed by LC, $^{14}$C was quantitatively recovered in the peak corresponding to dUMP and the $^3$H/$^{14}$C ratio was identical with that obtained in the tritium release assay. This verifies that tritium release from [2-$^{14}$C,5-$^3$H]dUMP occurs by isotope exchange for protons of water and is not an artifact resulting from displacement by another electrophile (e.g., dTMP formation).

Equation 1 describes the expected first-order exchange of tritium for protons of water (Segel, 1975), where $F$ is the fraction of isotopic exchange of [2-$^{14}$C,5-$^3$H]dUMP and $k$ is

$$1 - F = e^{-kt} \quad (1)$$

the apparent first-order rate constant for exchange. Over the first 5–8 h of the experiment shown in Figure 1, tritium release follows linearity and fits eq 1 (dashed line) with rate constants of 0.025 and 0.059 h$^{-1}$ in the absence and presence of folate, respectively. This is clearly illustrated in the semilog replot of these data (Figure 2A). The deviation from eq 1 observed after this time (Figure 1, dashed lines) can best be explained by comcomitant first-order loss of enzyme activity during

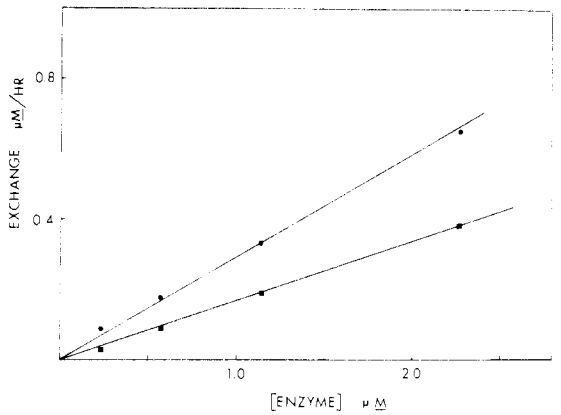


FIGURE 3: Initial velocity of 5-$^3$H exchange vs. enzyme concentration in the presence and absence of folate. Reactions were performed at 22 °C and contained 50 μM [2-$^{14}$C,5-$^3$H]dUMP. The enzyme concentration was varied from 0.23 μM to 2.3 μM; (●) 1 mM folate; (■) minus folate. Velocities are expressed in terms of μM exchange/h.

Table I: Catalytic Indices for Thymidylate Synthetase Catalyzed 5-H Exchange of dUMP and dTMP Formation

| addition | $K_m$ (μM) | catalytic constant (min$^{-1}$) |
|---|---|---|
| dUMP[a] | 16 | 0.010 |
| dUMP + folate[a] | 1.2 | 0.012 |
| dUMP + $CH_2$-$H_4$folate[b] | 3.0 | 486 |

[a] Kinetic parameters of the exchange reaction in the presence and absence of folate were determined at 22 °C as described in the legend to Figure 2B. [b] Formation of dTMP was monitored spectrophotometrically (Wahba & Friedkin, 1961) at 25 °C by using conditions described by Santi & Sakai (1972); differences in constants at 22 and 25 °C were within experimental error.

prolonged incubation periods. Equation 2 describes simultaneous 5-tritium exchange and enzyme inactivation, where $k_E$ is the apparent first-order rate constant for loss of enzyme

$$1 - F = \exp\left[-\frac{k}{k_E}(1 - e^{k_E t})\right] \quad (2)$$

activity. The value of $k_E$ includes inactivation of free and bound forms of the enzyme and would likely vary depending upon concentrations of ligands present in solution. By using the aforementioned values for $k$, eq 2 provides an excellent fit of all of the data in Figure 1 (solid line) and gives $k_E$ values of 0.036 and 0.029 h$^{-1}$ in the presence and absence of folate, respectively. The important point is that for initial periods of the reaction the exchange reaction fits eq 1 within acceptable error limits. For all initial velocity experiments described in this paper, five or more measurements of the tritium content of [2-$^{14}$C,5-$^3$H]dUMP were obtained over the initial period where *all* data points fit eq 1 as shown in Figure 2B; this usually encompassed a period not exceeding 8 h and where the tritium release did not exceed 15%.

Figure 3 shows that the initial velocity of 5-tritium exchange in the presence and absence of folate from [2-$^{14}$C,5-$^3$H]dUMP is a linear function of enzyme concentration from 0.23 to 2.3 μM. Kinetic parameters of the exchange reaction using 0.37 μM enzyme, as well as dTMP formation, are given in Table I. In the exchange reaction, folate decreases $K_m$ for dUMP but has no effect on $V_{max}$. The $K_m$ for dUMP in the normal enzymic reaction (viz. dUMP to dTMP) is similar to that obtained in the folate stimulated exchange of the 5-tritium, but $V_{max}$ is some 45 000-fold greater.

Addition of 10 mM $CH_2O$ to reaction mixtures containing 1 mM folate had no effect on the rate of tritium release from

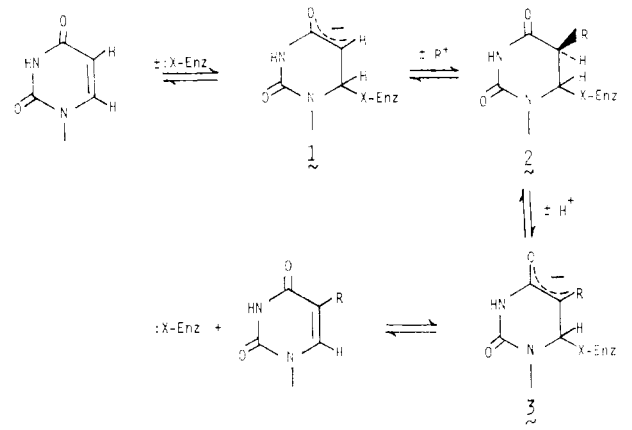


FIGURE 4: General mechanism for the thymidylate synthetase catalyzed 5-$^3$H exchange of [2-$^{14}$C,5-$^3$H]dUMP by an electrophile ($R^+$).

[2-$^{14}$C,5-$^3$H]dUMP. In order to demonstrate that under these conditions the tritium release was a result of exchange for protons of water rather than displacement by formaldehyde, the reaction was performed by using $^{14}CH_2O$ and [6-$^3$H]-dUMP, and the isolated nucleotide was analyzed for $^{14}$C content. A total volume of 0.27 mL contained 1 mM folate, 2.2 mM $^{14}CH_2O$ (7.3 × 10$^7$ dpm; 56.8 mCi/mmol), 6 μM [6-$^3$H]dUMP (2 × 10$^7$ dpm; 6 Ci/mmol), 0.37 μM enzyme, and other components of the reaction mixture. A parallel experiment was performed which differed only in that unlabeled $CH_2O$ and [2-$^{14}$C,5-$^3$H]dUMP were used; this was monitored for tritium release. When exchange of the control had proceeded to 55%, the former reaction mixture was applied to a DEAE-cellulose column (0.4 × 1.5 cm) equilibrated with 5 mM $NH_4OAc$ (pH 6.0). The column was washed with 25 mL of the equilibration buffer to remove over 95% of the $^{14}CH_2O$. The nucleotide was then eluted with 2 mL of 100 mM $NH_4OAc$ buffer (75% yield), lyophilized, and purified by LC (Garrett et al., 1977). Fractions corresponding to dUMP, HmdUMP, and dTMP markers were collected, concentrated, and counted. Tritium was only found in the peak corresponding to dUMP and the dUMP peak contained $^3H/^{14}C$ 8600. If tritium release (55%) occurred from displacement by $^{14}CH_2O$, the $^3H/^{14}C$ ratio would have been 166; thus, the rate of enzyme catalyzed reaction of dUMP with $^{14}CH_2O$, if it occurs at all, must be less than 2% of the overall exchange.

## Discussion

Thymidylate synthetase from *E. coli* has been reported to catalyze an exchange of the H-5 of dUMP for protons of water (Lomax & Greenberg, 1967). The reaction was reported to require the cofactor $CH_2$-$H_4$folate and under optimal conditions proceeded at 5–10% of the rate of dTMP synthesis. Two general mechanisms can logically be envisioned for this exchange reaction (Figure 4). The first involves condensation of dUMP and the cofactor along the normal pathway to provide an intermediate in which the H-5 has been displaced by the cofactor **3** (R = –$CH_2$-$H_4$folate), followed by reversal of these steps. This mechanism requires that the slow step of the normal enzymic reaction occur after formation of the condensed intermediate and that the proton released from the 5 position of dUMP exchange with solvent protons at a rate competitive with reversal to reactants. The second pathway would involve direct exchange of the H-5 of dUMP for protons of water without displacement by $CH_2$-$H_4$folate; here, the requirement for the cofactor might be one of providing or

inducing an environment necessary for catalysis of the exchange reaction. From a chemical standpoint, direct abstraction of the H-5 of dUMP is untenable since its p$K_a$ may be estimated to exceed 50 (Cram, 1965). Numerous chemical studies (for a review, see Pogolotti & Santi, 1977) have substantiated that H-5 exchange of 1-substituted uracils must proceed via a mechanism analogous to what Becker et al. (1977) have termed "nucleophilic addition–elimination catalysis". That is, a nucleophile attacks the 6 position of the heterocycle to provide a reactive enolate analogous to **1** which upon protonation provides the 5,6-dihydropyrimidine intermediate **2** (R = H); reversal of these steps results in exchange of the H-5 for protons of water. If thymidylate synthetase catalyzes a direct exchange of the H-5 of dUMP, it would be logical to conclude that the enolate (or enol) and 5,6-dihydro-dUMP intermediates are indeed formed. While **1** is believed to be the reactive species which condenses with the cofactor in the formation of dTMP, the dihydro-dUMP intermediate **2** (R = H) lies off the reaction pathway and, as will be discussed later, poses interesting questions regarding the stereochemistry of proton transfer at the 5 position of dUMP.

In principle, the simplest approach to ascertain whether the latter mechanism is operative would be to demonstrate tritium release from [5-$^3$H]dUMP in the absence of cofactor, $CH_2$-$H_4$folate. However, the assumption that tritium release from [5-$^3$H]dUMP is synonymous with exchange can be misleading since such assays do not distinguish between replacement by protons and other electrophiles. This is especially pertinent when $H_4$folate analogues are present since they yield unpredictable byproducts upon degradation which could cause displacement of tritium from [5-$^3$H]dUMP (Blakely, 1969). This was first encountered by Lomax & Greenberg (1967) who found it necessary to resort to the tedious technique of monitoring tritium incorporation into dUMP from $^3H_2O$ to demonstrate that the *E. coli* enzyme catalyzed exchange of the H-5 of dUMP for protons of water in the presence of $H_4$folate. However, since their preparation of $H_4$folate contained traces of formaldehyde, these experiments did not authenticate an uncoupling of the H-5 exchange from the normal enzymatic reaction. Using the same enzyme source, Friedkin (1973) did not observe tritium labelization in the presence of $H_4$homofolate. In early experiments performed in this laboratory (Weill, 1974), we observed that *Lactobacillus casei* thymidylate synthetase catalyzed tritium release from [2-$^{14}$C,5-$^3$H]dUMP in the presence of excess 5-$CH_3$-$H_4$folate which had been extensively purified. Unexpectedly, product analysis demonstrated that the tritium release was a consequence of dTMP formation which must have resulted from contamination of our 5-$CH_3$-$H_4$folate preparation by less than 1% $CH_2$-$H_4$folate. It has also been reported (Danenberg et al., 1974) that in the presence of 10-$CH_3$-$H_4$folate thymidylate synthetase catalyzes release of tritium from [5-$^3$H]dUMP. Unfortunately, product analysis was not performed to ensure that tritium release resulted from exchange with protons of water rather than displacement by some other electrophile.

In the present work, we demonstrate that *L. casei* thymidylate synthetase catalyzes an exchange of the H-5 of dUMP for protons of water in the *absence* of $CH_2$-$H_4$folate. Tritium release from the 5 position of dUMP was monitored as the decrease in the $^3H/^{14}C$ ratio of [2-$^{14}$C,5-$^3$H]dUMP. The increased sensitivity of this assay permitted assessment of initial velocities under conditions where exchange had proceeded 5–10% and obeyed the expected first-order kinetics (Figure 2B). In addition, reaction mixtures were analyzed by an LC system which readily separates 5-substituted 2′-deoxyuridylates. By demonstrating that dUMP was the sole nucleotide present and that it had the same $^3H/^{14}C$ ratio as observed in the tritium release assay, we verified that loss of tritium from [2-$^{14}$C,5-$^3$H]dUMP was a result of exchange for protons of water and not displacement by some other electrophile. We performed experiments utilizing $^{14}CH_2O$ in an attempt to trap the reactive intermediate **1**. When sufficient time had elapsed for 55% tritium release from the 5 position of dUMP, the reaction mixture was analyzed by LC. No radioactivity was associated with the 5-HmdUMP marker applied and it is concluded that tritium release from [5-$^3$H]dUMP in the presence of formaldehyde is also due to exchange for protons of water.

Although the exchange reaction is slow, it does not occur when the enzyme is omitted or treated with specific nucleotide inhibitors. Folate stimulated the rate of tritium release which, as before, was demonstrated to be a result of exchange for protons of water by product isolation and isotopic analysis. The presence of folate does not affect $V_{max}$ of the exchange reaction, but $K_m$ is decreased by about tenfold. Thus, it appears that folate increases the affinity of the enzyme for dUMP but has no effect on catalytic events leading to the exchange. This is in accord with other studies indicating a synergism in binding of ligands to this enzyme. The $K_m$ for H-5 exchange of dUMP (16 $\mu$M) in the absence of folate is quite similar to the dissociation constant for the binary enzyme–dUMP complex as determined by equilibrium dialysis (Galivan et al., 1976), while $K_m$ for the exchange reaction in the presence of folate is similar to the $K_m$ of dUMP for dTMP formation (3 $\mu$M) and dissociation constants of dUMP from complexes formed in the presence of other folate analogues (Galivan et al., 1976).

While numerous factors can be proposed to account for the large differences in the rates of H-5 exchange and dTMP formation, the most reasonable involve the fact that **2** (R = H) is a requisite intermediate in the exchange reaction but is *not* on the reaction pathway to dTMP synthesis.

Stereochemical aspects of the enzyme-catalyzed H-5 exchange of dUMP must be considered since, a priori, the reaction appears to require nonstereospecific protonation of the enolate **1**. This follows from the fact that, if **1** were protonated stereospecifically on one face of the pyrimidine, the same proton would be removed upon reversal to dUMP and H-5 exchange would not be observed. If both faces of **1** were accessible to solvent, albeit to different degrees, the observed rate of exchange would reflect but a fraction of the rate of interconversion of dUMP and the dihydro-dUMP intermediate **2**. Indeed, this could account for the large differences in $V_{max}$ observed for H-5 exchange and dTMP formation.

The mechanism of H-5 exchange of dUMP can also be envisioned as a direct counterpart to that proposed for the normal enzymatic reaction (Pogolotti & Santi, 1974). As illustrated in Figure 4, current evidence indicates that addition of the enzyme nucleophile and $CH_2$-$H_4$folate ($R^+$) across the 5,6 double bond occurs by trans addition, and the subsequent elimination of proton and nucleophile proceeds by a cis elimination (James et al., 1976); by microscopic reversibility, the reverse reaction would proceed by cis addition followed by trans elimination. In the case of H-5 exchange of dUMP, **1** could receive a proton ($R^+ = H^+$) from a single face of the pyrimidine trans to the enzyme nucleophile to give the dihydro-dUMP intermediate **2**. The tritium that was originally present at the 5 position of dUMP would be abstracted to provide the carbanion **3**, and subsequent cis elimination would

provide the unaltered enzyme and 5-protio-dUMP. It is to be noted that, in displacement reactions where $R^+$ is not a proton, such as the normal enzymic reaction, **1** and **3** are not equivalent and the nature of the electrophile would dictate the partitioning of intermediates. When $R^+$ is a proton, intermediates **1** and **3** are identical and their protonation to give **2**, albeit stereospecific in any one direction, would occur from both faces of the pyrimidine with equal facility. This mechanism is attractive from the standpoint that it utilizes catalytic features of the enzyme which are analogous to the conversion of dUMP to dTMP, and rate differences between H-5 exchange and the normal enzymic reaction can be accounted for by different accessibility of intermediate **1** toward proton and $CH_2$-$H_4$folate.

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# Thymidylate Synthetase. Catalysis of Dehalogenation of 5-Bromo- and 5-Iodo-2′-deoxyuridylate†

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ABSTRACT: Thymidylate synthetase catalyzes the facile dehalogenation of 5-bromo-2′-deoxyuridylate (BrdUMP) and 5-iodo-2′-deoxyuridylate (IdUMP) to give 2′-deoxyuridylate (dUMP), the natural substrate of the enzyme. The reaction does not require folate cofactors and stoichiometrically consumes 2 equiv of thiol. In addition to dUMP, a minor product is formed during the debromination of BrdUMP which has been identified as a 5-alkylthio derivative formed by displacement of bromide ion by thiolate. The reaction has been found to proceed with a substantial $\alpha$-secondary inverse tritium isotope effect ($k_T/k_H$ = 1.212–1.258) with [2-$^{14}$C,6-$^3$H]-BrdUMP as the substrate. Similarly, an inverse tritium isotope effect of 1.18 was observed in the nonenzymatic chemical counterpart of this reaction, the cysteine-promoted dehalogenation of [2-$^{14}$C,6-$^3$H]-5-bromo-2′-deoxyuridine. Previous evidence for the mechanism of action of this enzyme has rested largely on chemical model studies and on information obtained from its stoichiometric interaction with the inhibitor 5-fluoro-2′-deoxyuridylate. The magnitude of the secondary isotope effect during the enzymatic dehalogenation described here provides direct proof for nucleophilic catalysis and formation of 5,6-dihydroprimidine intermediates in a reaction *catalyzed* by thymidylate synthetase.

In the accompanying paper (Pogolotti et al., 1979), we report that thymidylate synthetase (EC 2.1.1.45) catalyzes the exchange of H-5 of dUMP for protons of water in the absence of the cofactor $CH_2$-$H_4$folate. As this reaction requires addition of a nucleophile to the 6 position of the pyrimidine heterocycle, it represents direct evidence that a reaction *catalyzed* by thymidylate synthetase proceeds via nucleophilic catalysis as originally proposed (Santi & Brewer, 1968; Pogolotti & Santi, 1974). According to this mechanism, initial attack by an enzyme nucleophile at the 6 position of dUMP serves to "activate" the neighboring 5 position for subsequent conversions. Here we describe the facile dehalogenation of BrdUMP[1] and IdUMP by thymidylate synthetase. The re-

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0006-2960/79/0418-2798$01.00/0 