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Reaction of 5-Ethynyl-2′-deoxyuridylate with Thiols and Thymidylate Synthetase†

Philip J. Barr,‡ Morris J. Robins, and Daniel V. Santi*

ABSTRACT: Thymidylate synthetase has been found to catalyze addition reactions at the normally inert acetylene moiety of 5-ethynyl-dUMP (EdUMP). A chemical counterpart for this reaction has also been found in which 5-ethynyl-dUrd (EdUrd) and EdUMP react with 2-mercaptoethanol to give 5-[1-[(2-hydroxyethyl)thio]vinyl]-dUrd (HETVdUrd) and -dUMP (HETVdUMP). If one uses 6-tritiated EdUrd, the model reaction proceeds with a large α-secondary inverse isotope effect (k_T/k_H = 1.23), indicating that nucleophilic attack of thiolate at the 6 position of the heterocycle is an early event in the reaction. Kinetic studies are in accord with the proposal that the next step of this reaction involves rate-determining general acid catalyzed protonation of the acetylene moiety and rearrangement to an allene conjugated with the 4-carbonyl group of the heterocycle. Subsequent reaction of thiol with the reactive Michael center of the allene and β-elimination across the 5,6 double bond account for the formation of the product, HETVdUrd. In the presence of CH_2-H_4folate, EdUMP causes a time-dependent inactivation of dTMP synthetase [Barr, P. J., Nolan, P. A., Santi, D. V., & Robins, M. J. (1981) *J. Med. Chem. 24*, 1385–1388]. However, in the absence of the cofactor, dTMP synthetase catalyzes the conversion of EdUMP and 2-mercaptoethanol to HETVdUMP. As in the model chemical counterpart, a large inverse α-secondary hydrogen isotope effect (k_T/k_H = 1.22–1.23) is observed with 6-tritiated EdUMP, which indicates that nucleophilic attack of the catalytic thiol group of the enzyme at the 6 carbon of EdUMP is an early step in this reaction. In analogy with model reactions, it is proposed that subsequent steps involve the formation of a reactive, enzyme-bound allene intermediate that undergoes reaction with a thiol group at the Michael center and β-elimination across the 5,6 double bond of the heterocycle to provide HETVdUMP and catalytically competent enzyme. Interestingly, certain nucleophilic buffers such as *N*-methylmorpholine and morpholine can effectively compete with 2-mercaptoethanol for reaction at the Michael center of the enzyme-bound allene intermediate. Such reactions are not observed in model chemical counterparts and may result from binding of these buffers to the site occupied by the cofactor in the normal enzymic reaction.

Thymidylate synthetase (EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate. It is known that an early event in catalysis involves covalent bond formation between a thiol group of the enzyme—Cys-198 in the *Lactobacillus casei* enzyme (Bellisario et al., 1979)—and the 6 position of dUMP to give transient 5,6-dihydropyrimidine intermediates [cf. Pogolotti & Santi (1977)]. A similar reaction occurs with a number of 5-substituted analogues of dUMP, giving rise to either mechanism-based inhibitors (Danenberg, 1977; Santi, 1980) or alternate substrates (Pogolotti et al., 1979; Garrett et al., 1979). Detailed studies of the interactions of such inhibitors and substrates have provided much information regarding the mechanism of dTMP synthetase. With mechanism-based inhibitors, the covalent bond that is formed may be stable, or it may activate a latent reactive chemical group at the 5 position that subsequently forms a covalent bond with the enzyme. Position-5 substituents of alternate substrates are activated in a similar manner but react with nucleophiles of the media, and the modified analogue is released to regenerate the catalytically active enzyme. Interestingly, the presence of the cofactor, CH_2-H_4folate, may alter the course of many such interactions, either by serving as a chemical partner in the reaction(s) or by indirectly modifying the binding or reactivity of the nucleotide analogue.

Interest in the interaction of EdUMP[1] with dTMP synthetase has arisen from observations that EdUrd potently

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[1] Abbreviations: EdUrd, 5-ethynyl-2′-deoxyuridine; EdUMP, 5-ethynyl-2′-deoxyuridylate; HETVdUrd, 5-[1-[(2-hydroxyethyl)thio]-vinyl]-2′-deoxyuridine; HETVdUMP, 5-[1-[(2-hydroxyethyl)thio]-vinyl]-2′-deoxyuridylate; NMMVdUMP, 5-[1-(4-methylmorpholinium-4-yl)vinyl]-2′-deoxyuridylate; AcdUMP, 5-acetyl-2′-deoxyuridylate; CH_2-H_4folate, (±)-L-5,10-methylenetetrahydrofolic acid; TEAB, triethylammonium bicarbonate; NMM, *N*-methylmorpholine; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonate; EDTA, ethylenediaminetetraacetic acid; RV, retention volume.

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inhibits the growth of cells in culture in a manner consistent with the inhibition of this enzyme (De Clercq et al., 1981; Kalman & Yalowich, 1979). Recently, this and another laboratory reported that in the presence of CH_2-H_4folate, EdUMP causes a time-dependent inactivation of dTMP synthetase, in which the inactive enzyme was bound to both ligands (Barr et al., 1981; Danenberg et al., 1981). In accord with the mechanism of other acetylenic suicide inactivators of enzymes and what is known of the mechanism of dTMP synthetase, some workers have suggested that nucleophilic attack at the 6 position of EdUMP could generate a reactive conjugated allene at the 5 position that could subsequently form a covalent bond with an enzyme nucleophile (Barr et al., 1981; Kalman & Yalowich, 1979; Rando, 1977). Other workers (Danenberg et al., 1981) suggested that such covalent conversions did not occur but rather that the inhibition resulted from slow formation of tight, noncovalent complexes.

In previous reports (Barr et al., 1981; Danenberg et al., 1981), time-dependent inhibition of the enzyme by EdUMP was not observed in the absence of CH_2-H_4folate, and it was presumed that the analogue was inert. In the present paper, we describe experiments that demonstrate that EdUMP is an excellent alternate substrate for dTMP synthetase in the presence of thiols. We also describe a chemical counterpart for this enzymic reaction and details of its mechanism. The results confirm the previous proposal that nucleophilic attack at the 6 position of the heterocycle leads to the generation of a reactive allene at the 5 position and further suggest the necessity for general acid–base catalysis in the alkyne–allene rearrangement.

Materials and Methods

Thymidylate synthetase was obtained from a methotrexate-resistant strain of *Lactobacillus casei* (Crusberg et al., 1970) and purified as previously described (Wataya & Santi, 1977). Protein concentration was determined with $\epsilon_{278} = 1.07 \times 10^5$ M^{-1} cm^{-1} (Santi et al., 1974), and binding sites were determined by EdUMP titration (Garrett et al., 1979). [6-^{3}H]IdUrd and [2-^{14}C]dUrd were obtained from Moravek Biochemicals. H_4folate was obtained from Sigma. *Escherichia coli* thymidine kinase was prepared by the reported procedure (Wataya & Santi, 1977). *E. coli* alkaline phosphatase (30 units/mg) was obtained from Worthington Biochemicals. The synthesis of unlabeled EdUrd has been described previously (Robins & Barr, 1981). EdUMP was synthesized by enzymatic phosphorylation of EdUrd with *E. coli* thymidine kinase as described (Barr et al., 1981). 5-Acetyl-dUMP was prepared by phosphorylation of 5-acetyl-dUrd (Barr et al., 1980) with carrot phosphotransferase and *p*-nitrophenyl phosphate as described (Harvey et al., 1970). Other materials were analytical grade or better quality from commercial sources.

Chromatographic Techniques. High-performance liquid chromatography (HPLC) on Lichrosorb C_{18} (4.6 × 250 mm column, 10-μm particle size) was performed at a 2 mL/min flow rate and 30 °C. The mobile-phase systems used with Lichrosorb C_{18} are as follows: system A was 0.25 M ammonium acetate (pH 6); system B was a 50-mL linear gradient of 10–24% methanol in 5 mM tetra-*n*-butylammonium sulfate and 5 mM potassium phosphate (pH 7). HPLC on Ultrasphere ODS (4.6 × 250 mm column, 5-μm particle size) was performed at 1 mL/min and 30 °C in methanol–water mixtures as follows: system C was 12% methanol; system D was 20% methanol; system E was 50% methanol for 20 mL followed by a 20-mL gradient to 75% methanol; system F was 5% methanol; system G was 10% methanol; system H was 8% methanol.

Kinetic Assays. Initial velocity measurements of dTMP synthetase catalyzed dTMP formation were performed spectrophotometrically at 25 °C as previously described (Wataya et al., 1980). Kinetics of dTMP synthetase catalyzed reactions of EdUMP were performed at 25 °C by monitoring the decrease in absorbance at 295 nm ($\Delta\epsilon$ = 6900) in solutions containing specified amounts of EdUMP, 25 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 50 mM *N*-methylmorpholine or specified buffer (pH 7.4), and limiting enzyme. Chemical conversions of EdUrd or EdUMP were monitored at 25 ± 0.1 °C by the decrease in absorbance at 295 nm in solutions containing specified amounts of *N*-methylmorpholine and 2-mercaptoethanol at an ionic strength of 0.5 M (KCl).

[2-^{14}C]EdUrd and [6-^{3}H]EdUrd. The procedures described below are analogous to reported methods (Robins et al., 1982; Robins & Barr, 1981) that have been modified to permit small-scale preparation of radiolabeled materials. 3′,5′-Di-*O*-acetyl-[6-^{3}H]IdUrd was prepared as follows: A dried sample of 0.02 mg of [6-^{3}H]IdUrd (1 mCi, 16 Ci/mmol) in a Siliclad container was treated with 1 mL of Ac_2O containing 2 mg of 4-(dimethylamino)pyridine for 18 h at 25 °C, evaporated to dryness, and coevaporated with three 2-mL portions of EtOH. [2-^{14}C]IdUrd was not commercially available, and 3′,5′-di-*O*-acetyl-[2-^{14}C]IdUrd was prepared as follows: 9.7 mg of [2-^{14}C]dUrd (2.5 mCi, 59 mCi/mmol) was acetylated and processed as described above. To the remaining residue was added 2 mL of CH_2Cl_2 containing 55 mg of ICl (0.34 mmol), and the solution was refluxed for 2 h. After the solution cooled, 2 mL of CH_2Cl_2 and 2 mL of water were added, and the stirred mixture was *carefully* titrated with 5% $NaHSO_3/H_2O$ until the dark organic phase just became colorless. The organic phase was separated, washed with two 2-mL portions of water, and evaporated to dryness. The remainder of the preparation of both [2-^{14}C]EdUrd and [6-^{3}H]EdUrd was identical.

To the residue of radioactive 3′,5′-di-*O*-acetyl-IdUrd was added a mixture containing 1 mL of Et_3N, 1 mL of (trimethylsilyl)acetylene, 1 mg of $(PPh_3)_2PdCl_2$, and 2 mg of CuI (Robins & Barr, 1981). The mixture was heated at 50 °C for 2 h, evaporated to dryness, dissolved in 2 mL of CH_2Cl_2, and washed successively with two 4-mL portions of 5% EDTA in water and 4 mL of water. After evaporation of solvent, the residue was dissolved in 0.25 mL of MeOH and subjected to HPLC in system E. In this system, most of the radioactivity (48% for ^{14}C; 70% for ^{3}H) comigrated as a single peak with an authentic sample of 3′,5′-di-*O*-acetyl-5-[(trimethylsilyl)-ethynyl]-dUrd (RV = 39 mL), the remainder eluting as an early peak with RV = 6 mL. The desired product contained in 4 mL was collected and evaporated to dryness. The residue was treated with 0.1 mL of 0.1 M NaOEt/EtOH for 3 h at 25 °C and neutralized with 0.1 M HOAc. Final purification of radiolabeled EdUrd was by HPLC in system F, which showed a single radioactive peak; further verification of purity was provided by cochromatography with authentic unlabeled EdUrd with HPLC system G and characteristic reactions described elsewhere. The yield of radiolabeled EdUrd determined by the radioactivity eluting from each of the two HPLC purification steps used was quite good (ca. 50–70% overall). However, the total [2-^{14}C]EdUrd and [6-^{3}H]EdUrd recovered was only 5 and 3%, respectively. It was found that most of the remaining radioactivity was adsorbed to the vessels used in the preparations.

[2-^{14}C]EdUMP and [6-^{3}H,2-^{14}C]EdUMP. [2-^{14}C]EdUMP and [6-^{3}H,2-^{14}C]EdUMP were prepared by phosphorylation of [2-^{14}C]EdUrd and appropriate mixtures of [6-^{3}H]EdUrd

and [2-^{14}C]EdUrd with *E. coli* thymidine kinase as described previously (Wataya & Santi, 1977; Barr et al., 1981), except that final purification was performed by HPLC in system A (RV = 16 mL). Fractions of 2 mL were collected and the appropriate fractions lyophilized to dryness and colyophilized with water to remove volatile salts. Yields were determined by scintillation counting to be greater than 85%.

5-[1-[(2-Hydroxyethyl)thio]vinyl]-2'-deoxyuridine (HETVdUrd) (**5a**). A solution of EdUrd (5 mg, 20 μmol) in 0.2 M 2-mercaptoethanol and 0.1 M *N*-methylmorpholine (pH 7.4) was kept at 30 °C for 2 h. The product was purified by HPLC in system C (RV = 36 mL) and represented over 90% of the total UV-absorbing material eluted. The product was obtained as a colorless powder after lyophilization. The following data confirmed the product to be HETVdUrd. The product at 0.065 mM gave no reaction with 0.3 mM DTNB in 80 mM Na_2HPO_4 (pH 8.0), confirming the absence of free thiol, and treatment with 0.1 M HCl (18 h, 25 °C) gave a quantitative yield of 5-acetyl-2'-deoxyuridine (AcdUrd), as confirmed by HPLC in system D (RV = 6.1 mL) and UV comparison with an authentic sample (Barr et al., 1980): NMR (D_2O) δ 8.05 (s, 1 H, H-6), 6.22 (t, 1 H, H-1'), 5.56, 5.48 (2 s, 2 H, C=CH_2), 4.40 (m, 1 H, H-3'), 3.96 (m, 1 H, H-4'), 3.65 (m, 4 H, H-5' and $-CH_2O-$), 2.80 (t, 2 H, $-CH_2S-$), 2.32 (m, 2 H, H-2') (relative to external Me_4Si); high-resolution mass spectrum, *m/e* 330.08699 (M^+) (calcd for $C_{13}H_{18}N_2O_6S$, 330.08857); UV_{max} (H_2O, pH 6) 254 nm (ε 12000).

5-[1-[(2-Hydroxyethyl)thio]vinyl]-dUMP (HETVdUMP) (**5b**). A solution (400 μL) containing EdUMP (1.7 μmol) and 0.1 M 2-mercaptoethanol in 50 mM NMM–HCl (pH 7.4) was kept at 30 °C under N_2 for 2.5 h. The product was purified by HPLC in system A in 5% methanol (RV = 18.7 mL) and lyophilized to dryness to give 0.91 μmol (53% yield) of a white powder. NMR and UV spectra were essentially identical with those of HETVdUrd; treatment of 0.56 μmol with 2 units of *E. coli* alkaline phosphatase (0.5 M TEAB, pH 8.5) for 2 h at 37 °C gave HETVdUrd as the sole product.

5-[1-(4-Methylmorpholinium-4-yl)vinyl]-dUMP (**6b**). A solution (20 mL) containing 0.2 M NMM–HCl (pH 7.4), 25 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 0.2 mM EdUMP, and 1.1 μM thymidylate synthetase was incubated at 25 °C for 90 min. The solution was deproteinized by addition of 2 volumes of methanol, centrifuged, and then diluted to 200 mL with water. This solution was applied to a DEAE-cellulose column (1.5 × 30 cm) previously equilibrated with 5 mM triethylammonium bicarbonate (pH 7.4). After the sample was loaded, the column was eluted with a 160-mL linear gradient of 5–150 mM triethylammonium bicarbonate (pH 7.4) at a flow rate of 160 mL/h. Fractions containing the first peak eluted were pooled and evaporated to dryness, redissolved in 0.2 mL of water, and further purified by HPLC in system A. Repeated lyophilization of aqueous solutions of the product to remove salts gave a colorless powder that was identified as NMMVdUMP by the following criteria: 360-MHz NMR (D_2O) δ 8.25 (s, 1 H, H-6), 6.33 (t, 1 H, H-1'), 6.21 (d, 1 H, vinylic H, J = 4 Hz), 5.87 (d, 1 H, vinylic H, J = 4 Hz), 4.58 (m, 1 H, H-3'), 4.24 (m, 1 H, H-4'), 4.10, 4.05 (m, 4 H, $-CH_2-O$ and H-5'), 3.95, 3.69 (m, 4 H, $-CH_2N^+$), 3.51 (s, 3 H, CH_3N^+), 2.43 (m, 2 H, H-2') (relative to HDO arbitrarily assigned to 4.8); positive ion fast-atom bombardment mass spectrum, *m/e* 434 (M^+) (calcd for $C_{16}H_{25}N_3O_9P$, 434); UV_{max} (H_2O, pH 6) 267 nm.

Calculations of Secondary Tritium Isotope Effects. Reactants and products were separated by HPLC as described; care was taken to collect each radioactive peak in its entirety to avoid isotope separation. The radioactivity in each peak was counted several times, and a minimum of 2 × 10^5 ^{14}C counts were collected; standard errors (SE) for determination of ^{3}H/^{14}C ratios and ^{14}C dpms were ca. 0.25% and 0.5%, respectively. Isotope effects were calculated from the isotope ratios of reactant and/or product as described by Melander (1960). All statistical estimates are presented as mean ± SE.

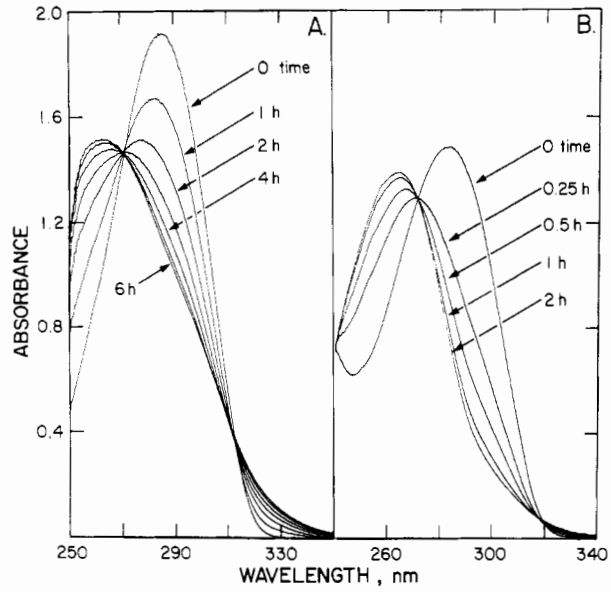


FIGURE 1: Ultraviolet spectral changes of (A) 0.17 mM EdUrd when reacted with 75 mM 2-mercaptoethanol and (B) 0.13 mM EdUMP when reacted with 1 μM dTMP synthetase and 25 mM 2-mercaptoethanol. Other components are described in the text.

Miscellaneous. Spectrophotometric measurements were made with either a Cary 118 spectrophotometer equipped with repetitive scan and automatic cuvette changing accessories or a Hewlett-Packard 8450A spectrophotometer. Radioisotopes were measured by counting in a fluid containing 0.4% Omnifluor in xylene–Triton X-114 (3:1) on a Nuclear Chicago Isocap 300 liquid scintillation counter. Counting efficiencies were determined by the external standard ratio method, and dpm calculations were aided by a tape-fed Hewlett-Packard computer. The pK_a of EdUrd was determined spectrophotometrically at 0.5 M ionic strength (KCl) and 25 °C. The change in absorbance at 301 nm as a function of pH was used to calculate a pK_a value of 8.53 ± 0.02.

Results

Reactivity of EdUrd with 2-Mercaptoethanol. Figure 1A shows the ultraviolet spectral changes that occur upon treatment of EdUrd with 75 mM 2-mercaptoethanol in 50 mM NMM (pH 7.4).[2] Using [2-^{14}C]EdUrd, HPLC analysis (system H) showed that only a single product was formed, which was subsequently identified as HETVdUrd by NMR and mass spectral analysis (see Materials and Methods). The conversion of EdUrd to HETVdUrd proceeded to completion as determined by UV, HPLC, and isolation of the product in near quantitative yield. Further, when HETVdUrd was kept in D_2O containing 75 mM 2-mercaptoethanol and 50 mM NMM (pD 7.4), there was no detectable loss of the vinylic proton signals after as long as 18 h. Since the interconversion of HETVdUrd to EdUrd in D_2O would be accompanied by

[2] Danenberg et al. (1981) reported that no change in the UV spectrum occurred when EdUMP was incubated with dTMP synthetase for as long as 24 h; however, substantially less enzyme was used than in the present study.

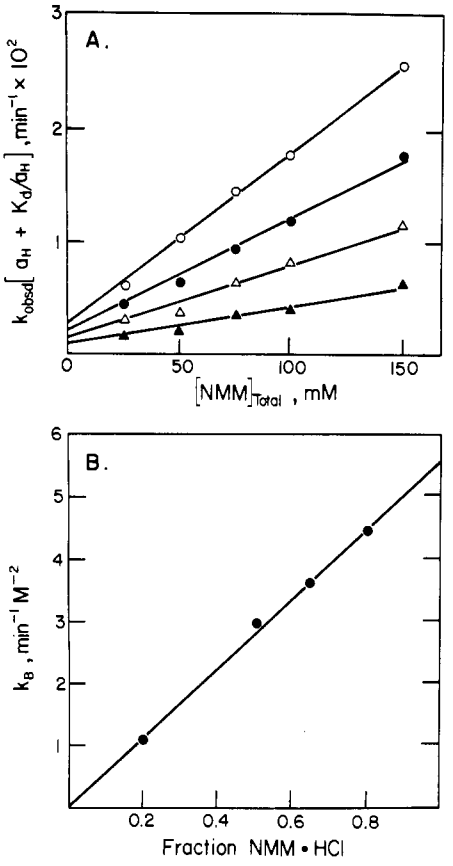


FIGURE 2: Dependence of rate of conversion of EdUrd to HETVdUrd on buffer concentration and pH. All solutions contained 0.1 mM EdUrd, 75 mM 2-mercaptoethanol, and specified concentrations of NMM. (A) Plots of k_{obsd} times the mole fraction of EdUrd vs. total buffer at pH 6.78 (▲), 7.13 (△), 7.4 (●), and 8.02 (○). (B) Dependence of the apparent rate constant k_B (eq 1) on the mole fraction of protonated NMM. The data are obtained from replots of slope/$[RS^-]$ in panel A and use a pK_a value of 7.4 for NMM–HCl.

exchange of the vinylic protons for deuterium of solvent, this experiment shows that under the conditions used the formation of EdUrd from HETVdUrd is insignificant. UV changes similar to those shown in Figure 1 also occurred when EdUrd was treated with 10–50 mM DTT in NMM (pH 7.4); although the product was not further characterized, we presume it is analogous to that which is formed with 2-mercaptoethanol.

In 50 mM NMM–HCl (pH 7.4), the reaction was first order with respect to 2-mercaptoethanol over the range of 10–75 mM, giving an apparent second-order rate constant of 0.12 ± 0.02 (SE) M^{-1} min^{-1}, which is calculated for the reaction of total mercaptoethanol and total EdUrd in solution; likewise, in 50 mM NMM–HCl (pH 7.4), EdUMP and 2-mercaptoethanol gave rise to HETVdUMP with k = 0.05 M^{-1} min^{-1}. Shortly after the outset of these studies, we found that the reaction of EdUrd with mercaptoethanol was also influenced by the concentration of NMM buffer. The kinetic data are described by eq 1, which transforms to eq 2 when the

$$v = k_0[RS^-][EdUrd] + k_B[NMM]_T[RS^-][EdUrd] \quad (1)$$

$$k_{obsd} = k_0[RS^-][a_H/(a_H + K_d)] + k_B[NMM]_T[RS^-][a_H/(a_H + K_d)] \quad (2)$$

reaction is pseudo first order in EdUrd.

Here, K_d is the dissociation constant for the 3-NH of EdUrd (pK_a = 8.53), a_H the hydrogen ion concentration as measured by the glass electrode, $a_H/(a_H + K_d)$ the fraction of the neutral form of EdUrd, $[NMM]_T$ the total concentration of NMM present, and $[RS^-]$ the concentration of thiolate anion present

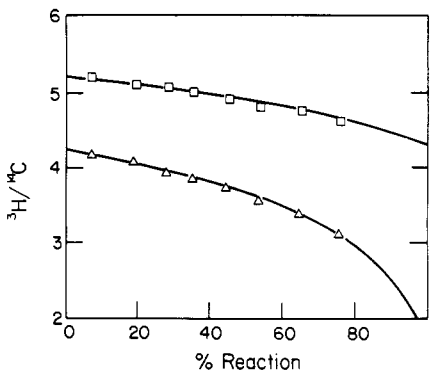


FIGURE 3: Secondary α-tritium isotope effect in the conversion of [2-^{14}C,6-^{3}H]EdUrd (△) to HETVdUrd (□) in 75 mM 2-mercaptoethanol and 50 mM NMM–HCl (pH 7.4). The points are experimental, and the lines are theoretical for k_T/k_H = 1.22.

at any given pH. The terms k_0 and k_B represent the apparent rate constants for the spontaneous and buffer-catalyzed reactions, respectively. Figure 2A shows a plot of $k_{obsd}[(a_H + K_d)/a_H]$ vs. total NMM in the presence of 75 mM 2-mercaptoethanol; as depicted in eq 1 and 2, there is a spontaneous reaction between EdUrd and 2-mercaptoethanol anion as well as a buffer-catalyzed reaction. The increase in the rate of the nonbuffer-catalyzed reaction (ordinate intercept) with increasing pH is in accord with the first term of eq 1 and 2 where the reactive species of mercaptoethanol is depicted as the thiolate anion. If a pK_a of 9.5 is used for the thiol group of mercaptoethanol, k_0 is calculated from the data to be 2.55 ± 0.55 M^{-1} min^{-1}. The buffer-catalyzed reaction is reflected in the slopes of the lines of Figure 2, and a replot of slopes/$[RS^-]$ (i.e., k_B) vs. the mole fraction of buffer present in the protonated form is presented in Figure 2B. As shown, the reaction of RS^- with EdUrd is formally general acid catalyzed, with an apparent catalytic rate constant of k = 5.57 ± 0.16 min^{-1} M^{-2}. Alternatively, the data can be interpreted as the kinetically equivalent general base catalyzed reaction of RSH with EdUrd. Mechanistic interpretations of these data are provided under Discussion.

[6-^{3}H,2-^{14}C]EdUrd (1.0 mM) was allowed to react with 75 mM 2-mercaptoethanol in 50 mM NMM buffer (pH 7.4) under anaerobic conditions (N_2). At appropriate intervals aliquots (100 μL) were removed for analysis. The reactant (EdUrd) and product (HETVdUrd) were separated by HPLC in system H. As shown in Figure 3, the $^3H/^{14}C$ ratios of the reactant and the product change in a manner indicating a more rapid reaction of the 6-tritiated compound. The inverse secondary tritium isotope effect (k_T/k_H) is 1.223 ± 0.003 as calculated from the $^3H/^{14}C$ ratio of the product (n = 8) and 1.225 ± 0.009 (n = 8) as calculated from the decreasing isotopic ratio of the reactant.

Reactions of EdUMP with dTMP Synthetase. Figure 1B shows the UV spectral changes that occur upon treatment of EdUMP with dTMP synthetase, 25 mM 2-mercaptoethanol, and 50 mM NMM–HCl (pH 7.4); as in the nonenzymic reaction, there is a shift of the maximum to lower wavelength (λ = 266 nm) with a single isosbestic point at 272 nm. The initial velocity of the reaction is linear with respect to enzyme concentration up to at least 1.0 μM and is dependent on thiol concentration, with at least 15 mM mercaptoethanol required for maximal activity.

In subsequent studies, 2-mercaptoethanol was used at 25 mM, buffer concentration was kept at 50 mM, and sufficient enzyme was used to minimize the chemical conversion of EdUMP to HETVdUMP (k_{obsd} = 1.15 × 10^{-3} min^{-1}). By a

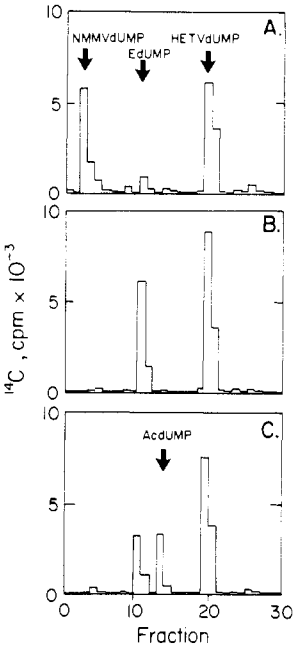


FIGURE 4: HPLC of partially complete reactions of [2-^{14}C]EdUMP with dTMP synthetase and 2-mercaptoethanol in different buffers: (A) NMM–HCl, (B) Tris-HCl, and (C) morpholine–HCl. Elutions of authentic standards are designated by arrows. Reaction conditions are described under Results.

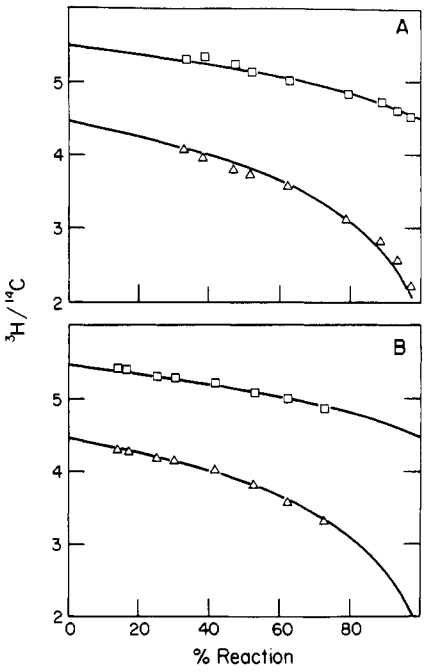


FIGURE 5: Secondary α-tritium isotope effects in the reaction of [6-^{3}H,2-^{14}C]EdUMP with dTMP synthetase and 25 mM 2-mercaptoethanol and (A) 50 mM NMM–HCl (pH 7.4) or (B) 50 mM Tes (pH 7.4). Points are experimental for the reactant (△) and product(s) (□), and the lines are the theoretical best fits for the isotope effects described in the text.

monitoring of the decrease in absorbance at 295 nm, kinetic parameters of the dTMP synthetase catalyzed conversion of EdUMP to product(s) were determined to be K_m = 3.2 μM and k_{cat} = 18 min^{-1}. For the time period used for initial velocity determinations, we calculate that less than 0.23% of the EdUMP would have undergone chemical reaction. dUMP was found to be a competitive inhibitor of the reaction with K_i = 2.6 ± 0.6 μM, in good accord with the dissociation constant of the enzyme–dUMP complex determined by other methods (Wataya et al., 1977; Galivan et al., 1976). The product HETVdUMP inhibited the reaction (K_i = 6.2 μM), and as previously reported (Barr et al., 1981), in the presence of CH_2-H_4folate, EdUMP inhibited the normal enzyme-catalyzed conversion of dUMP to dTMP with K_i = 0.1 μM.

In order to identify the reaction products, 0.1 mM [2-^{14}C]EdUMP (2.3 mCi/mmol) was treated with 25 mM mercaptoethanol, 50 mM NMM, and dTMP synthetase for 1 h and the reaction mixture analyzed by HPLC (system B). As shown in Figure 4A, three radioactive products were obtained: 6% of the radioactivity eluted as the reactant EdUMP (RV = 21 mL), and 50% was the expected product HETVdUMP (RV = 39 mL); the identity of the latter was confirmed by comparison of UV and HPLC properties to an authentic standard. In addition, 44% of the radioactivity eluted early (RV = 4.8 mL) and was determined by NMR and mass spectral analysis to be the unusual nucleotide NMMVdUMP. This finding prompted us to investigate similar reactions with different buffers.

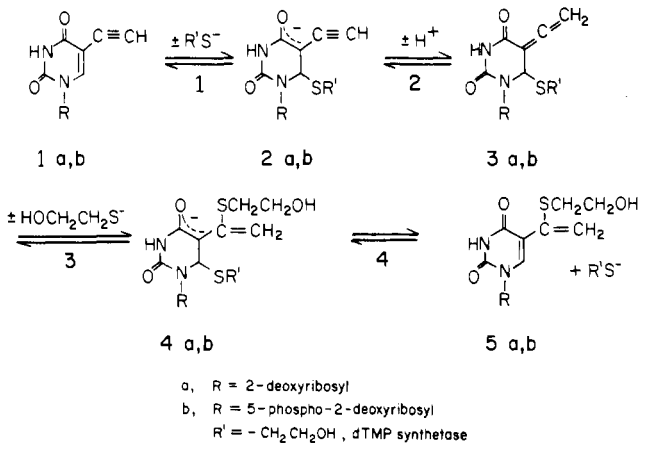
When the reaction was performed with [2-^{14}C]EdUMP and 25 mM 2-mercaptoethanol in 50 mM Tris-HCl buffer (pK_a = 8.1) at pH 7.4, after 1-h incubation at 25 °C, the only product observed was HETVdUMP (62%) along with starting material (38%) (Figure 4B). Similarly, with 50 mM Tes (pK_a = 7.5) and 50 mM triethanolamine (pK_a = 7.77) buffers at pH 7.4 the only product observed was HETVdUMP (64% and 68%, respectively), along with starting material (36% and 32%, respectively). However, an identical reaction in 50 mM morpholine (pK_a = 8.36) gave HETVdUMP (58%), starting EdUMP (22%), and a further product (20%) identified by cochromatography on HPLC (system B) and UV analysis as AcdUMP (Figure 4C).

As shown in Figure 5A, when [2-^{14}C,6-^{3}H]EdUMP is allowed to react with 9 μM dTMP synthetase in 25 mM 2-mercaptoethanol–50 mM NMM–HCl (pH 7.4), the $^3H/^{14}C$ ratios of the reactant and products change in a manner indicating a more rapid reaction of the 6-tritiated compound. A parallel reaction omitting enzyme indicated that chemical conversion of EdUMP was insignificant over the period of reaction (ca. 7% under conditions where enzymic conversion proceeded to over 97%). The inverse secondary tritium isotope effects are calculated to be 1.230 ± 0.007 (n = 9) for reaction of EdUMP and 1.23 ± 0.01 (n = 9) for formation of products. Likewise, when the reaction was performed in 50 mM Tes buffer (pH 7.4), the observed isotope effects were k_T/k_H = 1.218 ± 0.003 (n = 8) for the single product (HETVdUMP), and k_T/k_H = 1.220 ± 0.007 (n = 8) for the reactant EdUMP (Figure 5B).

Discussion

Considerations of the properties of dTMP synthetase and the mechanisms of acetylenic suicide inactivators have led a number of workers to suggest that dTMP synthetase might catalyze the conversion of EdUMP to a reactive allene analogue covalently bound to the active site of the enzyme (Kalman & Yalowich, 1979; Rando, 1977). First, the acetylenic moiety is small and electron withdrawing—features that enhance both reversible binding to the enzyme and nucleophilic attack at the 6 position of the heterocycle (Wataya et al., 1977). Second, addition of a nucleophile at C-6 would generate a β-acetylenic carbonyl moiety with a carbanion situated at the α position; upon protonation of the γ carbon of the acetylene moiety such intermediates can rearrange to provide allenes that may capture nucleophiles at their reactive β carbon. Indeed, we and other workers have suggested that this may be the mechanism responsible for the time-dependent

Scheme I



inactivation observed when dTMP synthetase is incubated with EdUMP and CH_2-H_4folate (Barr et al., 1981; Kalman & Yalowich, 1979). Nevertheless, other than analogy, there was no direct evidence for the conversion of EdUMP to a reactive allene, and its mechanism of interaction with dTMP synthetase was speculative. In the ensuing discussion we describe a reaction in which dTMP synthetase catalyzes the conversion of EdUMP and 2-mercaptoethanol to HETVdUMP. We also show that the reaction occurs chemically, albeit at a slower rate, and will argue that the mechanistic details of both reactions are, in general, as described above.

Model Chemical Reactions. Under conditions used for most assays of dTMP synthetase in this laboratory [75 mM 2-mercaptoethanol, 50 mM NMM–HCl (pH 7.4)], EdUrd is converted to HETVdUrd in a first-order reaction with $k_{obsd} = 6.2 \times 10^{-3}$ min^{-1}. Likewise, EdUMP is converted to HETVdUMP at a rate that is about 2-fold slower. The conversion of EdUrd to HETVdUrd appears to be quantitative, and no reversal of the reaction was detected after as long as 18 h.

It is well-known that alkynes are quite inert toward nucleophiles in neutral media, and the possibility of direct addition of 2-mercaptoethanol to the acetylene moiety of EdUrd was considered highly unlikely at the outset. However, upon protonation β-alkynyl carbonyl compounds that possess α carbanions can rearrange to give α,β-unsaturated allenes that are powerful Michael acceptors; such reactions have provided the rationale for the design of a popular class of mechanism-bound inhibitors of enzymes that generate carbanions in their catalytic sequence (Morisaki & Bloch, 1972; Abeles & Maycock, 1976; Rando, 1977). It has been well established that the 6 position of the uracil heterocycle is susceptible to attack by nucleophiles, in particular thiolate, which results in generation of a stabilized carbanion at the 5 position [cf. Pogolotti & Santi (1977)]. Taken together, these data permit the proposal of a reasonable mechanism for the chemical conversions of EdUrd to HETVdUrd. As shown in Scheme I, thiolate attack at the 6 position of EdUrd (**1a**) would generate the carbanion **2a**, which upon protonation/rearrangement would provide the reactive allene **3a**; thiol attack at the electrophilic β carbon of **3a** followed by β-elimination across the 5,6 carbon–carbon bond would provide HETVdUrd (**5a**). Excepting for addition–elimination across the 5,6 double bond, the conversions depicted in Scheme I are analogous to what has previously been described for a large number of β,γ-alkynyl carbonyl compounds [cf. Abeles & Maycock (1976) and Rando (1977)].

Evidence in support of the mechanism shown in Scheme I was first obtained by a study of kinetic isotope effects. It has been well established that nucleophilic attack of the thiols at the 6 carbon of the uracil heterocycle may proceed with inverse α-tritium secondary KIE's in the range of 20–24% (Garrett et al., 1979; Wataya et al., 1980; T. W. Bruice and D. V. Santi, unpublished results); these values are in excellent agreement with the maximal (equilibrium) values of 22–24% calculated from fractionation factors of model compounds (T. W. Bruice and D. V. Santi, unpublished results). Likewise, when observed, such isotope effects may be used to confirm the rehybridization at C-6, which is concomitant with nucleophilic attack. Thus, the large inverse isotope effect observed in the conversion of 6-tritiated EdUrd to HETVdUrd ($k_T/k_H = 1.22$) is strong evidence for the sp^2 to sp^3 rehybridization of C-6 that would occur upon addition of thiol. Further, since the isotope effect is within experimental error of the theoretical and observed maximal value, we surmise that the covalent bond change at C-6 occurs either in a preequilibrium step or at the rate-determining step possessing a very late transition state.

The kinetics of the reaction are in accord with the proposed mechanism and, although somewhat incomplete, provide additional mechanistic information. (a) The pH dependence of the reaction indicates that the reactive species are the neutral form of the heterocycle and the thiolate anion of 2-mercaptoethanol. The kinetically equivalent mechanism involving the ionized heterocycle and neutral thiol is unacceptable by chemical rationale; ionization of the NH-3 of uracil heterocycles results in greatly diminished reactivity of the C-6 toward nucleophiles (Santi & Brewer, 1973; Pogolotti & Santi, 1974; Wataya et al., 1980), and neutral thiols are poor nucleophiles. (b) Although the reaction pathway requires 2 equiv of mercaptoethanol, the reaction is first order with respect to thiol concentration up to at least 75 mM. From this, we presume that the rate-determining step precedes the reaction of thiol with the allene intermediate **3a** to give **4a**. (c) The general acid catalysis of the reaction indicates that proton transfer occurs in the rate-determining step and can be explained by several variations of the pathway depicted in Scheme I. The general acid could serve to protonate the oxygen at carbon 4 of the heterocycle in the transition state and thus activate the 6 position toward nucleophilic attack by thiolate. Conversion of EdUrd to **3a** can also be envisioned as a concerted reaction in which the general acid serves to protonate the terminal carbon of the acetylene moiety in the transition state. The kinetically equivalent general base catalyzed reaction of neutral thiol with EdUrd could be envisioned to involve assistance of proton removal from the thiol in the first step of the pathway. Clearly, other mechanisms are feasible, and a more complete study would be required to ascertain which is (are) operative.[3]

Reaction of EdUMP with dTMP Synthetase. Treatment of EdUMP with catalytic amounts of dTMP synthetase in the presence of 2-mercaptoethanol and amine buffers results in the formation of HETVdUMP as a major product. Unlike the normal enzymatic reaction, but similar to the enzyme-catalyzed dehalogenation of BrdUMP and IdUMP, this conversion does not require CH_2-H_4folate; in contrast, in the

[3] Early in this study we observed that when EdUrd was kept in D_2O containing 75 mM 2-mercaptoethanol and 50 mM NMM (pD 7.4), the HETVdUrd formed possessed 1.6–1.8 deuteriums at the vinylic position. This observation lost its mechanistic significance when we subsequently found that the acetylenic proton of EdUrd in D_2O undergoes a buffer-catalyzed exchange for solvent deuterium at a rate significantly faster ($t_{1/2} \simeq 30$ min in 50 mM NMM, pD 7.4) than that of conversion to HETVdUrd ($t_{1/2} \simeq 110$ min). General base catalyzed exchange of acetylenic protons has been previously reported (Halevi & Long, 1961; Kresge & Lin, 1973).

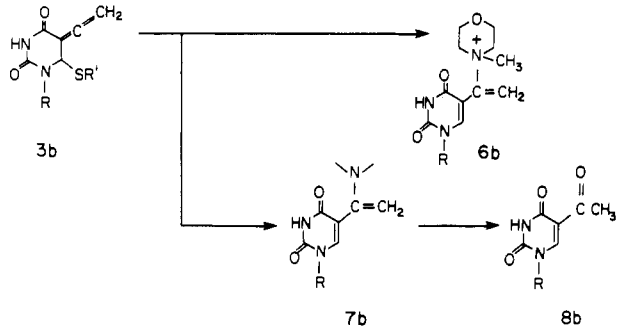
presence of CH_2-H_4folate EdUMP results in inactivation of the enzyme (Barr et al., 1981; Danenberg et al., 1981). The rate of reaction is linear with enzyme concentration and is dependent on thiol concentration, with at least 15 mM 2-mercaptoethanol required for maximal activity. As described below, while HETVdUMP is the major product, some buffers may give rise to secondary products, the formations of which are also enzyme catalyzed. The K_m value of EdUMP (3.2 μM) is similar to that of dUMP in the normal enzymatic reaction, but k_{cat} is some 20-fold lower. The product of the reaction, HETVdUMP, inhibits the initial velocity with K_i = 6.2 μM, and the normal substrate dUMP is a competitive inhibitor with K_i = 2.6 μM; EdUMP is also a competitive inhibitor of the conversion of dUMP to dTMP (Barr et al., 1981). These observations, as well as similarities in the catalytic mechanisms described below, support the view that the same site of the enzyme is involved in the binding of the nucleotide substrates and in catalysis of both the reaction of EdUMP and dTMP formation.

The large inverse secondary isotope effect observed in the dTMP synthetase conversion of 6-tritiated EdUMP (k_T/k_H = 1.22–1.23) provides convincing evidence that an early event in catalysis involves sp^2 to sp^3 rehybridization of the 6 carbon of the heterocycle. Isotope effects of similar magnitude are observed in the addition of 2-mercaptoethanol to C-6 of EdUrd described here and in several examples of addition of the enzyme thiol to the 6 position of 5-substituted analogues of dUMP, which behave as mechanism-based inhibitors or alternative substrates. Thus, we can reasonably conclude that an early event of the dTMP synthetase conversion(s) of EdUMP involves covalent addition of an enzyme thiol to the 6 position of the analogue as depicted in Scheme I. Further, since the magnitude of the isotope effect is, within error, identical with the equilibrium isotope effect, which has been calculated and measured for the addition of thiols to the 6 position of the uracil heterocycle, this step must be either a preequilibrium one or one that is rate determining with a very late transition state. The remaining steps of the dTMP synthetase catalyzed conversion of EdUMP to HETVdUMP may be deduced from what has already been described for the model chemical counterpart and what is known of the chemistry of acetylenic suicide substrates.

The fleeting existence of the enzyme-bound allene **3b** does not permit its direct demonstration. However, its presence in the pathway may be inferred by reactions that could only occur through such an intermediate. As previously noted, it is well established that unactivated alkynes are inert toward addition reactions in neutral aqueous media; in contrast, the β carbon of allenes conjugated to carbonyl groups are powerful Michael acceptors. Thus, having established that an enzyme nucleophile does add to the 6 position of EdUMP, the fact that HETVdUMP is isolated as a product in itself provides evidence that an allene, **3b**, is indeed formed. As in Scheme I, the putative allene would react with mercaptoethanol to form **4b**, which upon β-elimination across the 5,6 positions of the heterocycle would provide HETVdUMP and catalytically active dTMP synthetase.

The acetylene to allene conversion depicted in Scheme I has been shown to proceed by general acid catalysis in the model reaction. In view of the low basicity of the terminal acetylenic carbon of EdUMP, together with the low concentration of hydrogen ion under the conditions used, it is possible that enzymic conversion of **2b** to **3b** also enlists the aid of a general acid catalyst. Such catalysis might be provided by the buffer in the medium, but it is more appealing to suggest that some

Scheme II



functional group of the enzyme is involved in this process. While no direct evidence for such a functional group exists, general acid–base catalysis has been proposed to be involved in both the conversions that occur at the 5 position of dUMP (Pogolotti & Santi, 1977) and the one carbon unit of CH_2-H_4folate (Kallen & Jencks, 1966; Benkovic, 1978) in the normal enzymic reaction. It may be envisioned that the proximity of these atoms of the substrates on the active site would place involved catalysts in positions where they could participate in conversion of **2b** to **3b**.

It is interesting that some, but not all, amine buffers used in this study gave rise to products other than HETVdUMP when EdUMP is treated with dTMP synthetase and 25 mM 2-mercaptoethanol (Figure 5). With Tes, Tris, or triethanolamine at 50 mM and pH 7.4, the sole product formed was HETVdUMP. This illustrates that neither the solvent nor these buffers effectively compete with the nucleophilic thiol of 2-mercaptoethanol for the reactive β carbon of the allene intermediate. In contrast, with NMM or morpholine as buffers under similar conditions, NMMVdUMP and 5-acetyl-dUMP were obtained as 44% and 20%, respectively, of the products isolated. The formation of these products is best rationalized by the mechanism depicted in Scheme II. With NMM, the tertiary amine competes with 2-mercaptoethanol for reaction with the allene **3b** to form the stable quaternary enamine, NMMVdUMP (**6b**). Similarly, reaction of **3b** with morpholine would provide a secondary enamine, **7b**, which, unlike **6b**, has an available lone pair of electrons on nitrogen and would readily hydrolyze to form 5-acetyl-dUMP (**8b**). There are two unusual and not completely explicable aspects of these findings. First, neither of these products is formed in the absence of the enzyme; indeed, in the chemical models, HETVdUrd is the only product formed. Compared to the allene formed in the model reaction, the enzyme-bound intermediate may be exposed to a higher effective concentration of NMM or morpholine or perhaps a lower effective concentration of 2-mercaptoethanol. In either case, the enzyme-bound allene must react more effectively with these buffers than that formed in the chemical counterpart. Second, it is curious that of the five buffers examined only NMM and morpholine provided products other than HETVdUMP. There is no obvious correlation of the products formed with the pK_a of the buffers used or the substituents attached to the nucleophilic nitrogen, although the lone pair of electrons on NMM and morpholine are somewhat less sterically hindered than on the unreactive buffers. It is interesting to note that the six-membered ring of both NMM and morpholine bears structural resemblance to the tetrahydropyrazine moiety of the cofactor, CH_2-H_4folate. It is tempting to speculate that if these buffers were bound to the same site of the enzyme as the tetrahydropyrazine ring of the cofactor, their nitrogens would be properly positioned for re-

action with the β carbon of the enzyme-bound allene.

In summary, the work described here shows that dTMP synthetase catalyzes activation of the acetylenic moiety of EdUrd toward addition reactions. The nucleophilic catalyst of the enzyme adds to the 6 position of the heterocycle and permits generation of a reactive allene intermediate; subsequent reaction of the allene with a nucleophile, followed by β-elimination of the enzyme, yields the modified nucleotide and catalytically active enzyme. The enzymic reaction can be mimicked by chemical counterparts in which thiolate anions serve the role of the nucleophilic catalyst of the enzyme. In all respects, the pathway is in complete accord with current combined knowledge of the catalytic mechanism of dTMP synthetase, the chemistry of the uracil heterocycle, and acetylene–allene conversions. It remains unknown why EdUMP is processed as a substrate by dTMP synthetase in the absence of CH_2-H_4folate but causes time-dependent inactivation of the enzyme and formation of ternary EdUMP–CH_2-H_4folate–enzyme complexes in the presence of the cofactor. Indeed, the ability of dTMP synthetase to utilize EdUMP as a substrate is a possible explanation for the transient inhibition of the enzyme, which recovers with time when cells are treated with the corresponding nucleoside, EdUrd (Kalman & Yalowich, 1979). To resolve this issue, it will be necessary to elucidate the structure of the EdUMP–CH_2-H_4folate–dTMP synthetase complex, and such studies are in progress.

Registry No. **1a**, 61135-33-9; **1b**, 79897-02-2; **3a**, 84537-66-6; **3b**, 84537-67-7; **5a**, 84537-68-8; **5b**, 84558-15-6; **6b**, 84537-69-9; **8b**, 84537-70-2; [2-^{14}C]EdUrd, 84537-63-3; [6-^{3}H]EdUrd, 84537-64-4; [2-^{14}C]EdUMP, 84558-07-6; [6-^{3}H,2-^{14}C]EdUMP, 84537-65-5; CH_2-H_4folate, 3432-99-3; NMM, 109-02-4; morpholine, 110-91-8; [6-^{3}H]IdUrd, 14735-03-6; 3′,5′-di-*O*-acetyl-[6-^{3}H]IdUrd, 84537-71-3; [2-^{14}C]dUrd, 17236-65-6; 3′,5′-di-*O*-acetyl-[2-^{14}C]IdUrd, 84537-72-4; 2-mercaptoethanol, 60-24-2; thymidylate synthetase, 9031-61-2.

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