Brockerhoff, H. (1974) *Lipids 9*, 645.
Buckley, J. T., Halasa, N., & MacIntyre, S. (1982) *J. Biol. Chem. 257*, 3320.
Demel, R. A., & deKruyff, B. (1976) *Biochim. Biophys. Acta 457*, 109.
Dennis, E. A. (1973a) *Arch. Biochem. Biophys. 158*, 485.
Dennis, E. A. (1973b) *J. Lipid Res. 14*, 152.
Glomset, J. A., & Norum, J. R. (1973) *Adv. Lipid Res. 11*, 1.
Huang, C. H. (1976) *Nature (London) 259*, 242.
Huang, C. H. (1977a) *Lipids 12*, 348.
Huang, C. H. (1977b) *Chem. Phys. Lipids 19*, 150.
Jain, M. K. (1975) *Curr. Top. Membr. Transp. 6*, 1.
Kitabatake, K., Piran, U., Kamio, Y., Doi, Y., & Nishida, T. (1979) *Biochim. Biophys. Acta 573*, 145.
MacIntyre, S., & Buckley, J. T. (1978) *J. Bacteriol. 135*, 402.
MacIntyre, S., Trust, T. J., & Buckley, J. T. (1979) *J. Bacteriol. 139*, 132.
Norby, G., & Norum, K. R. (1975) *Scand. J. Lab. Clin. Invest. 35*, 677.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Pugh, E. L., & Kates, M. (1975) *Biochim. Biophys. Acta 380*, 442.
Roberts, M. F., Otnaess, A.-B., Kensil, C. A., & Dennis, E. A. (1978) *J. Biol. Chem. 253*, 1252.
Waite, M., & Sisson, P. (1974) *J. Biol. Chem. 249*, 6401.

Secondary α-Hydrogen Isotope Effects on the Interaction of 5-Fluoro-2′-deoxyuridylate and 5,10-Methylenetetrahydrofolate with Thymidylate Synthetase†

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ABSTRACT: Secondary α-hydrogen isotope effects have been used to study the covalent interactions of FdUMP and (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2-H_4folate) with dTMP synthetase. Dissociation of 6-tritiated FdUMP from the FdUMP–CH_2-H_4folate–dTMP synthetase ternary complex proceeds with a large α-hydrogen secondary kinetic isotope effect (k_H/k_T = 1.23). In contrast there is no isotope effect upon initial formation of the complex, but isotopic equilibration slowly occurs with $k = 5.5 \times 10^{-2}$ h^{-1}. The equilibrated complex is enriched in tritium, and the equilibrium isotope effect determined by two independent experimental methods was K_H/K_T = 1.24. This is in excellent agreement with values calculated from simple models and provides the first experimental verification of a fractionation factor for -CR_2CHTSR. Together with other data, these results lead us to conclude that the covalent bond between the cysteine residue of the enzyme and the 6 position of FdUMP is formed after the rate-determining step in the formation of the complex and, by microscopic reversibility, is cleaved before the slow step in dissociation of the complex. Dissociation of [^{3}H]-CH_2-H_4folate from the ternary complex occurs with a normal secondary kinetic isotope effect of k_H/k_T = 1.03 which is some 8% higher than the calculated equilibrium isotope effect between bound and free cofactor. We explain this by proposing that the normal isotope effect which accompanies formation of the putative 5-iminium ion of the cofactor from the complex during dissociation is not completely canceled by the inverse isotope effect which accompanies its subsequent conversion to CH_2-H_4folate in the rate-determining step of the reaction. The rate-determining step may also involve a conformational change of the enzyme, driven by reversible interactions of the cofactor and the protein, which may play a role in the interconversion of CH_2-H_4folate and the 5-iminium ion in the normal enzymic reaction as well as in formation of the FdUMP–CH_2-H_4folate–dTMP synthetase complex.

Thymidylate (dTMP) synthetase (EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate[1] to dTMP and H_2-folate. Much of what is currently known of the mechanism of this enzyme has been derived from studies of its interaction with the mechanism-based inhibitor FdUMP (cf. Pogolotti & Santi, 1977; Danenberg, 1977). This inhibitor participates in two or more steps of the normal enzymic reaction and results in the formation of a stable complex in which the 6 position of FdUMP is covalently bound to the nucleophilic catalyst of the enzyme and the 5 position is linked to the one-carbon unit of the cofactor. Since the ternary complex is structurally analogous to a steady-state intermediate of the normal enzymic reaction, studies of the mechanism of its formation provide an accessible model for understanding features of the partial catalytic reaction it mimics. In this paper we describe secondary α-hydrogen isotope effects at C-6 of FdUMP and the one-carbon unit of CH_2-H_4folate which occur during formation/dissociation of the complex. The results of these studies

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[1] Abbreviations: CH_2-H_4folate, (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydrofolate; CH_2-H_4PteGlu$_5$, (6*R*)-L-5,10-methylene-5,6,7,8-tetrahydropteroylpentaglutamate; H_2folate, 7,8-dihydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; Tris, tris(hydroxymethyl)aminomethane; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; KIE, kinetic isotope effect; FF, fractionation factor; HPLC, high-performance liquid chromatography. All other abbreviations are as suggested by IUPAC.

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are used in an attempt to ascertain where covalent bond changes occur with respect to the rate-limiting step(s) of the interaction.

Experimental Procedures

Materials. dTMP synthetase from methotrexate-resistant *Lactobacillus casei* (Crusberg et al., 1970) was purified by a modification (Wataya & Santi, 1977) of the method of Galivan et al. (1975). The preparation catalyzed the formation of 4 μmol of dTMP min^{-1} mg^{-1} as determined spectrophotometrically (Wahba & Friedkin, 1962) by using conditions previously described (Santi & Sakai, 1971). The concentration of FdUMP binding sites was calculated from the equivalence point determined upon spectrophotometric titration of the enzyme with FdUMP in the presence of CH_2-H_4folate as previously described (Garrett et al., 1979). By use of ϵ_{278} = 1.07×10^5 M^{-1} cm^{-1} (Santi et al., 1974a) and a molecular weight of 70 000 for the enzyme, the preparations used here were calculated to possess 80–90% of the theoretical FdUMP binding sites. Purified H_2folate reductase from *Escherichia coli* RT500 (120 units/mg) was a gift from Dr. David P. Baccanari (Burroughs Wellcome Co.) and was assayed by a reported method (Baccanari et al., 1975). Glucose-6-phosphate (G-6-P) dehydrogenase from *Leuconostoc mesenteroides* (695 NAD units/mg) was purchased from Worthington and was assayed by the method described in their technical bulletin. H_2folate was prepared by the method of Futterman (1957). (6*RS*)-L-H_4folate was prepared by reduction of folate (Hatefi et al., 1960), and (6*R*)-CH_2-H_4Pte-L-Glu_5 was a gift from Professor Jesse Rabinowitz (University of California, Berkeley). FdUrd was obtained from P-L Biochemicals and was converted to FdUMP by using *Escherichia coli* dThd kinase as previously described (Wataya & Santi, 1977); the latter was purified by chromatography on DEAE-cellulose (Wataya & Santi, 1977) and Aminex A-27 (Garrett et al., 1977). Concentrations of FdUMP were determined by using ϵ_{269}^{pH2} = 8950. *N*-Methylmorpholine (NMM) was Pierce Chemical Co. Sequanal grade. All other reagents were of the highest purity commercially available. Doubly distilled, charcoal and Millipore-filtered water was used throughout. The standard NMM buffer contained 50 mM NMM-HCl (pH 7.4), 25 mM $MgCl_2$, 1 mM EDTA, 6.5 mM H_2CO, and 75 mM 2-mercaptoethanol.

[2-^{14}C]Folate (55 mCi/mmol) was obtained from Amersham. [^{3}H]H_2CO (85 mCi/mmol) was obtained from New England Nuclear. [2-^{14}C]FdUrd (52 mCi/mmol) and [6-^{3}H]FdUrd (15 Ci/mmol) were obtained from Moravek Biochemicals. Radioactive FdUMP was prepared and purified as described for the unlabeled nucleotide.

High-Pressure Liquid Chromatography. The purity of nucleotides and folate derivatives used in this work was verified by HPLC analysis shortly before their use. Reverse-phase HPLC was performed at ambient temperature on a Lichrosorb C_{18} column (4.6 × 250 mm). System A contained 5 mM tetra-*n*-butylammonium phosphate (pH 7.5) and 30% (v/v) MeOH; system B contained 0.1 M potassium phosphate (pH 6.0) and 5% (v/v) acetonitrile.

Preparation of Labeled and Unlabeled CH_2-H_4folates. (6*R*)-L-CH_2-H_4folate was prepared by a modification of the method of Pastore & Friedkin (1962). The reaction mixture (4.8 mL) contained 300 mM Tris-HCl (pH 7.2), 21 mM H_2folate, 42 mM H_2CO, 160 mM 2-mercaptoethanol, 61 mM G-6-P, 2.3 mM NADPH, 21 units/mL G-6-P dehydrogenase, and 6.4 units/mL H_2folate reductase; the reaction was initiated with H_2folate reductase. After 30 min at ambient temperture, under argon and protected from light, there was no further increase in A_{340}; the A_{294}/A_{340} had changed from ca. 3 to 18. The reaction was cooled to 0 °C, quenched by adjusting the pH to 9.5 with NaOH or 10 volumes of 0.4 M Et_3N^+·HCO_3^- (pH 9.5), and lyophilized. The residue was redissolved in a minimal volume (ca. 0.5 mL) of 0.4 M Et_3N^+·HCO_3^- (pH 9.5), 20 mM 2-mercaptoethanol, and 1 mM H_2CO, and insolubles were removed by centrifugation. The solution was applied to a Bio-Gel P2 column (1.4 × 73 cm; 200–400 mesh) previously equilibrated with 20 mM Et_3N^+·HCO_3^- (pH 9.5), 20 mM 2-mercaptoethanol, and 1 mM H_2CO. The column was eluted with the same buffer at 15 mL/h, at 4 °C, protected from light, and 2-mL fractions were collected. Fractions 17–23 contained (6*R*)-L-CH_2-H_4folate as assayed with dTMP synthetase; these were pooled, lyophilized, and dissolved in 20 mM Et_3N^+·HCO_3^- (pH 9.5), 20 mM 2-mercaptoethanol, and a 2-fold excess of H_2CO. Typical overall yields were 80–95% of (6*R*)-L-CH_2-H_4folate of ≥95% purity as determined by A_{294}, HPLC (system A, RV = 8.2 mL), and dTMP synthetase assay. Solutions could be stored under argon at −80 °C for at least 1 year without decomposition.

[2-^{14}C]H_2folate was prepared by a modification of previously reported procedures (Futterman, 1957; Coward et al., 1974). A solution (0.45 mL) containing 4 mM [2-^{14}C]folate (21.3 mCi/mmol), 0.2 M 2-mercaptoethanol, 0.2 M potassium phosphate (pH 7.5), and 0.4 M $Na_2S_2O_4$ was incubated for 30 min under argon at ambient temperature, protected from light. To this was added 3.5 mL of a solution containing 0.5 M Et_3N^+·HCO_3^- (pH 7.0) and 50 mM 2-mercaptoethanol. The solution was applied to a DEAE-Sephadex column (9 × 40 mm; 40–120 μm) preequilibrated with the aforementioned buffer at 4 °C. The column was eluted at 4 °C with a 24-mL linear gradient (0.5–1.2 M) of Et_3N^+·HCO_3^- (pH 7.0) containing 50 mM 2-mercaptoethanol; the flow rate was 0.1 mL/min, and the product eluted at ca. 0.9 M salt. Fractions containing the product were combined, lyophilized, and redissolved in 0.5 mL of 0.5 M Tris-HCl (pH 7.2) and 0.25 M 2-mercaptoethanol. The overall recovery of [2-^{14}C]H_2folate was 77%, which was determined to be 97% pure by HPLC (system A, RV = 7.8 mL) and enzymic reduction using H_2-folate reductase.

(6*R*)-L-[^{3}H]CH_2-[2-^{14}C]H_4folate was prepared as previously described for unlabeled cofactor with the following modifications: (a) The reaction was performed in a total volume of 0.8 mL, and the final concentration of [2-^{14}C]H_2folate was 3.8 mM and that of [^{3}H]H_2CO (85 mCi/mmol) was 14.8 mM. (b) Purification was performed on a 1.0 × 27 cm Bio-Gel P2 column at a flow rate of 0.3 mL/min, and H_2CO was omitted from the equilibrating and eluting buffer. The reaction proceeded to at least 97% completion as determined spectrophotometrically, and a 93% recovery of ^{14}C dpm was obtained from the pooled, product-containing fractions of the Bio-Gel column. The cofactor was determined to be at least 95% radiochemically pure by HPLC analysis (system A, RV = 8.2 mL; system B, RV = 28 mL), and the ternary complex formed with FdUMP and dTMP synthetase and isolated by gel filtration had a $^3H/^{14}C$ = 5.00. The final solution of the cofactor contained approximately a 4-fold excess of free [^{3}H]H_2CO because of some coelution in the Bio-Gel chromatography. The pooled fractions were lyophilized, redissolved in 0.80 mL of 20 mM Et_3N^+·HCO_3^- (pH 9.5) and 20 mM 2-mercaptoethanol, and stored under argon at −80 °C.

Formation and Isolation of Ternary Complexes. FdUMP–CH_2-H_4folate–dTMP synthetase complexes were formed under conditions specified under Results. Filter assays of the complex were performed by using Bac-T-flex nitro-

cellulose filters (0.45 μm; 2.4 cm; Schleicher & Schuell) as previously described (Santi et al., 1974b) except that filters were presoaked and washed in 75 mM potassium phosphate (pH 7.4); filtration efficiencies were typically ca. 90%. Separations of the complex from free ligands by Sephadex G-25 filtration were performed at 4 °C by using 75 mM potassium phosphate (pH 7.4) and 10 mM 2-mercaptoethanol for equilibration and elution. In all chromatographic separations of double-labeled isotopes, the entire radioactive peak was pooled prior to counting to avoid isotopic separation.

Determination of Secondary α-Hydrogen Isotope Effects. Double-labeled [^{3}H,^{14}C]FdUMP–CH_2-H_4folate–enzyme complexes were separated from free radioactive ligands by adsorption to nitrocellulose filters or by gel filtration as described elsewhere. The ^{3}H and ^{14}C radioactivities were obtained by counting samples in 10 mL of aqueous counting scintillant (Amersham) 3–10 times and collecting a minimum of 2×10^5 total counts of ^{14}C, and dpm values for each isotope were calculated with appropriate corrections for ^{14}C spillover by the external standard ratio method. Standard errors (SE) for determination of ^{3}H/^{14}C ratios and ^{14}C dpm were maximally 0.25% and 0.5%, respectively. Isotope effects accompanying the dissociation of the FdUMP–CH_2-H_4folate–enzyme complex were calculated from the isotopic ratios remaining in the reactant complex as described by Melander (1960). These also were calculated as the ratio of the first-order rate constants for dissociation of ^{3}H and ^{1}H (^{14}C) as previously described (Santi et al., 1974a). Both methods gave essentially identical results. For equilibrium isotope effects, the complex was separated from unbound ligands after equilibrium was reached, and the equilibrium isotope effect was calculated as the ratio of ^{3}H/^{14}C of the complex to ^{3}H/^{14}C of the unbound ligands. All statistical estimates are presented as the mean ± SE.

Miscellaneous. UV spectra were obtained on a Cary 118 spectrophotometer, and radioactivity was monitored on an Isocap 300 liquid scintillation counter. dpm calculations were performed by the external standard ratio method and were aided by a tape-fed Hewlett-Packard 11255 A computer. HPLC was performed by using a Hewlett-Packard 1084B instrument.

Results

Stability of the FdUMP–CH_2-H_4folate–dTMP Synthetase Complex. In the presence of excess ligands, formation of the ternary complex is extremely rapid (Santi et al., 1974a; Danenberg & Danenberg, 1978) and is complete within the time period of any assays performed here. Many of the experiments described here are performed over prolonged periods, and their validity requires that the ternary complex and its kinetic properties remain stable for the duration of the experiment. For verification of this important point, the ternary complex was formed in the standard NMM buffer with 0.17 mM (6*R*)-L-CH_2-H_4folate, 7.5 μM [6-^{3}H]FdUMP (253 mCi/mmol), and 1.5 μM dTMP synthetase and kept at 25 °C under N_2 protected from light. Nitrocellulose filtration of 20-μL aliquots of this solution showed that the bound radioactivity remained constant for over 100 h (Figure 1). At t = 0.5, 60, and 85 h, a 100-fold excess of unlabeled FdUMP was added to 200-μL aliquots of this solution, and the rate of dissociation of [^{3}H]FdUMP from labeled complex was measured over ca. 1.5 half-lives; as shown in Figure 1, the first-order rate constants for dissociation of [6-H^3]FdUMP throughout this period are unchanged ($k = 2.0 \times 10^{-3}$ min^{-1}) and demonstrate that the kinetic properties of the complex are unaltered during this prolonged period of incubation.

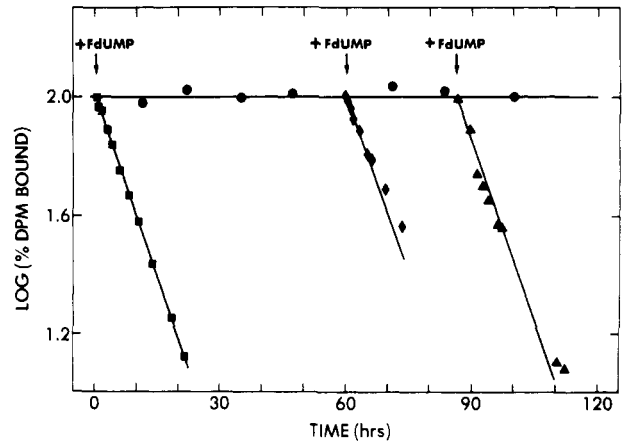


FIGURE 1: Stability of the [^{3}H]FdUMP–CH_2-folate–dTMP synthetase complex. The filled circles (●) represent the unperturbed complex kept at 25 °C. As indicated, a 100-fold excess of unlabeled FdUMP was added, and the rates of dissociation of [6-^{3}H]FdUMP from the complex were determined after 0.5 (■), 60 (♦), and 85 (▲) h of incubation. The solid lines were obtained by least-squares fits of the data.

Kinetic Isotope Effect upon Dissociation of the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4PteGlu$_{1-5}$–dTMP Synthetase Complex. To a solution (3.4 mL) of the ternary complex was added unlabeled FdUMP (1.1 mL) to give a final solution (4.5 mL) containing, in the standard NMM buffer, 0.45 μM dTMP synthetase, 51 μM (6*R*)-L-CH_2-H_4folate, 2.3 μM [2-^{14}C,6-^{3}H]FdUMP (49.7 mCi of ^{14}C/mmol; ^{3}H/^{14}C = 4.549), and 340 μM FdUMP. The mixture was kept at 25.0 °C under nitrogen, protected from light. Triplicate aliquots were removed at intervals up to ca. 35 h, and the ternary complex was isolated by adsorption on nitrocellulose filters; the aliquots filtered were progressively larger (20–500 μL) with time to obtain sufficient and constant dpm in the isolated complex as the reaction proceeded. The triplicate samples were counted, showing standard errors for ^{3}H/^{14}C within 0.8% of the mean. From these, the dissociation of the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4folate–dTMP synthetase complex was shown to be first order with k_H = 0.12 h^{-1}. Figure 2A shows the data from which k_H/k_T was calculated to be 1.229 ± 0.009 (n = 12). Similar experiments using variable amounts of unlabeled FdUMP (10–100-fold excess over labeled FdUMP) demonstrated that both the rate constant for dissociation and the kinetic isotope effect were independent of the concentration of the free ligand. The isotope effect we obtain here is in excellent agreement with that previously reported (Santi et al., 1974a).

The kinetic isotope effect upon dissociation of complexes made by using (6*R*)-CH_2-H_4Pte-L-Glu$_5$ was determined identically as described above, except that aliquots were removed from the final mixture up to ca. 140 h. Dissociation of the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4PteGlu$_5$–dTMP synthetase complex was shown to be first order with $k_H = 2.02 \times 10^{-2}$ h^{-1}. Figure 2B shows the data from which the kinetic isotope effect was calculated to be 1.211 ± 0.027 (n = 12).

Equilibrium Isotope Effect for the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4folate–dTMP Synthetase Complex. Method a. The ternary complex (300 μL) was prepared in the standard NMM buffer with 51 μM (6*R*)-L-CH_2-H_4folate, 1.5 μM dTMP synthetase, and 0.34 mM [2-^{14}C,6-^{3}H]FdUMP (49.7 mCi ^{14}C/mmol; ^{3}H/^{14}C = 3.912) and kept under N_2 at 25 °C protected from light; controls omitted CH_2-H_4folate. Aliquots (20 μL) were withdrawn, and the radioactivity in the complex was determined by nitrocellulose filtration. The binding of FdUMP was rapid, and the amount bound was unchanged as

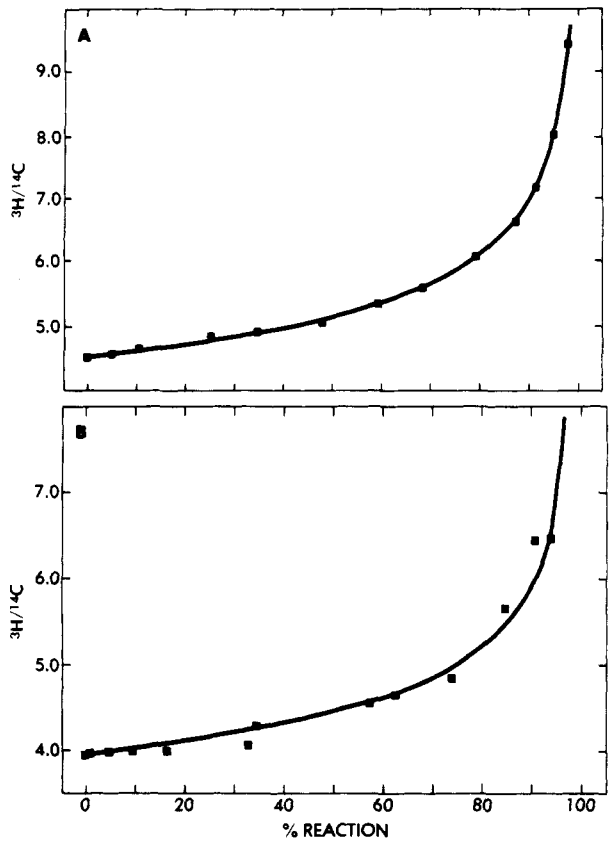


FIGURE 2: Secondary α-hydrogen isotope effect upon dissociation of (A) the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4folate–dTMP synthetase complex and (B) the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4PteGlu$_5$–dTMP synthetase complex. Data points represent the average of triplicate determinations of the $^3H/^{14}C$ ratio in the bound complex at specified times. The solid lines are best fits to the equation $k_T/k_H = 1 + \log (R_S/R_0)/\log (1 - x)$ where R_0 is the $^3H/^{14}C$ of the complex at $t = 0$ and R_S at the time of analysis and x is the fraction of complex dissociated (Melander, 1960).

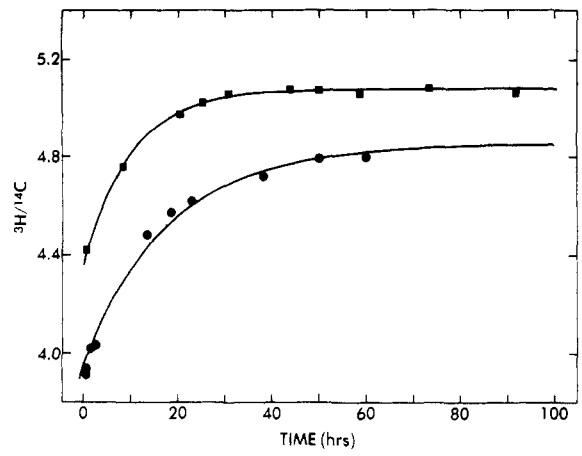


FIGURE 3: Secondary α-hydrogen isotope effect upon formation of the [2-^{14}C,6-^{3}H]FdUMP–CH_2-H_4folate–dTMP synthetase complex and its approach to isotopic equilibrium. Complexes were formed with 1.5 μM dTMP synthetase and 7.5 (■) or 340 μM (●) [2-^{14}C,6-^{3}H]FdUMP. Each data point is the average of the $^3H/^{14}C$ in the triplicate determinations, and solid lines depict the theoretical isotopic ratios fit to the equation for first-order isotopic approach to equilibrium (Jencks, 1969). Values for $^3H/^{14}C$ at equilibrium and the first-order rate constants, k, were obtained by computer-aided best fits of the data to the equation $kt = \ln [(^3H/^{14}C_0 - {}^3H/^{14}C_{eq})/(^3H/^{14}C_t - {}^3H/^{14}C_{eq})]$. The equilibrium isotope effect was calculated from data by using 340 μM [2-^{14}C,6-^{3}H]FdUMP (●) as described in the text.

demonstrated by the constancy of ^{14}C dpm bound to the filters. At $t = 0$, the $^3H/^{14}C$ of the complex was identical with that of the FdUMP used, but it gradually increased until isotopic equilibrium was reached (Figure 3). The change of the $^3H/^{14}C$ in the complex was first order with $k = 5.5 \times 10^{-2}$ h^{-1}, and the flux of isotope exchange, R, was 1.6×10^{-7} M h^{-1} (Jencks, 1969). Because of the large molar excess of labeled FdUMP over enzyme sites (>100-fold), the $^3H/^{14}C$ of the free FdUMP does not significantly change with time, and the equilibrium isotope effect may be calculated as the ratio of the $^3H/^{14}C$ of the complex at equilibrium (4.794 ± 0.015) to the $^3H/^{14}C$ of FdUMP present in the initially formed complex (3.874 ± 0.014); $K_H/K_T = 1.237 \pm 0.008$ ($n = 10$).

Method b. Three separate solutions (800 μL) of the complex were prepared with 0.17 mM (6*R*)-L-CH_2-H_4folate, 1.5 μM dTMP synthetase, and 7.5 μM [2-^{14}C,6-^{3}H]FdUMP (49.7 mCi ^{14}C/mmol; $^3H/^{14}C = 4.419$) and were kept under nitrogen at 25.0 °C, protected from light. As in method a, aliquots were filtered through nitrocellulose to ensure isotopic equilibrium was achieved (Figure 3). At 60–65 h, three 200-μL aliquots of each solution were passed through a Sephadex G-25 column (1.0 × 29 cm), equilibrated, and eluted with 75 mM potassium phosphate–10 mM 2-mercaptoethanol (pH 7.4) at 4 °C, and ca. 0.8-mL fractions were collected. The fractions containing bound and free [2-^{14}C,6-^{3}H]FdUMP were individually pooled, and triplicate aliquots of each were counted. Samples of the free [2-^{14}C,6-^{3}H]FdUMP also were subjected to HPLC analysis (system A) and were demonstrated not to have undergone appreciable decomposition during the period of incubation and analysis; >96% of the radioactive material was FdUMP (RV = 8.0 mL). The equilibrium isotope effect was calculated as the ratio of $^3H/^{14}C$ in the bound fraction (5.320 ± 0.010) to that in the free (4.291 ± 0.007). The equilibrium isotope effect calculated from these data gave $K_H/K_T = 1.240 \pm 0.004$ ($n = 9$).

Kinetic Isotope Effect upon Dissociation of the FdUMP–[^{3}H]CH_2-[2-^{14}C]H_4folate–dTMP Synthetase Complex. The complex was formed in a solution (2.5 mL) containing, in the standard NMM buffer, 19 μM dTMP synthetase, 2.1 mM FdUMP, and 94 μM (6*R*)-L-[^{3}H]CH_2-[2-^{14}C]H_4folate (1.1×10^7 ^{14}C dpm). After 30 min at 4 °C, under argon and protected from light, the complex was isolated by gel filtration through a Sephadex G-25 column (1.8 × 57 cm), equilibrated, and eluted with NMM buffer minus formaldehyde at 4 °C (gel filtration was necessary to remove free [^{3}H]H_2CO which interfered with the nitrocellulose filter assay). Under identical conditions, <1.5% of the total ^{14}C dpm recovered was eluted in the macromolecular peak when [2-^{14}C]H_2folate was used in place of the double-labeled cofactor. Triplicate solutions (7.8 mL each) were prepared which contained 1.2 μM isolated complex (3×10^6 ^{14}C dpm; $^3H/^{14}C = 5.00$), 48 μM FdUMP, and 340 μM (6*R*)-L-CH_2-H_4folate in the standard NMM buffer and were incubated at 25.0 °C, under argon and protected from light; controls omitted the cofactor. Duplicate aliquots (30–520 μL), containing ca. 3000 bound ^{14}C dpm, were removed at intervals up to ca. 35 h, and the ternary complex was isolated on nitrocellulose filters; controls lacking FdUMP showed negligible adsorption of radioactivity (0.4%) providing free [^{3}H]H_2CO was removed as described above. The duplicate aliquots of each of the three experiments (total of six determinations per time point) were counted, each showing standard errors of $^3H/^{14}C$ within 0.68% of the mean. The dissociation of the radioactive ligands was first-order for at least six half-lives with $k_H = 0.14$ h^{-1}. The $^3H/^{14}C$ ratio of the complex remaining did not change appreciably during the period when over 85% of the labeled cofactor had dissociated (Figure 4), and k_H/k_T was calculated

Table I: Secondary α-Tritium Isotope Effects for the Reactions of FdUMP-dTMP Synthetase-Cofactor Complexes

ternary complex[a]	reaction	isotope effect
[2-^{14}C,6-^{3}H]FdUMP-CH_2-H_4folate-Enz	dissociation	k_H/k_T = 1.229 ± 0.009 (n = 12)
[2-^{14}C,6-^{3}H]FdUMP-CH_2-H_4PteGlu$_5$-Enz	dissociation	k_H/k_T = 1.211 ± 0.027 (n = 12)
[2-^{14}C,6-^{3}H]FdUMP-CH_2-H_4folate-Enz	isotope exchange	K_H/K_T = 1.240 ± 0.004 (n = 9)[b] K_H/K_T = 1.237 ± 0.008 (n = 10)[c]
FdUMP-[^{3}H]CH_2-[2-^{14}C]H_4folate-Enz	dissociation	k_H/k_T = 1.033 ± 0.004 (n = 16)

[a] Cofactors used to make complexes have 6R and L stereochemistry. [b] Determined by Sephadex G-25 filtration. [c] Determined by adsorption of ternary complexes to nitrocellulose filters.

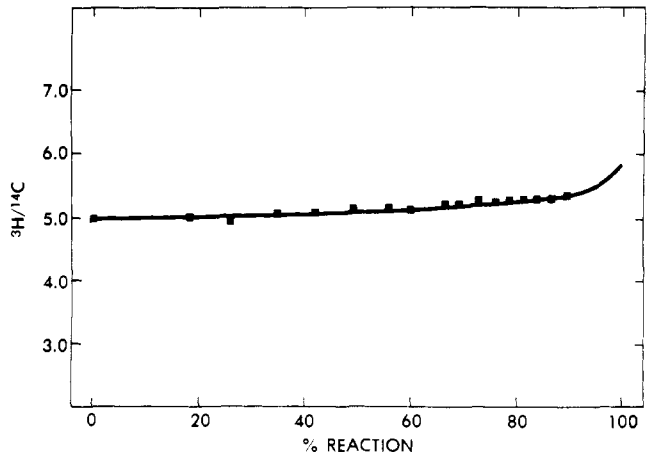


FIGURE 4: Secondary α-hydrogen isotope effect in the dissociation of the FdUMP-[^{3}H]CH_2-[2-^{14}C]H_4folate complex. Data points are the average of duplicates of three experiments, and the solid line is the best fit to the equation given in the legend to Figure 2.

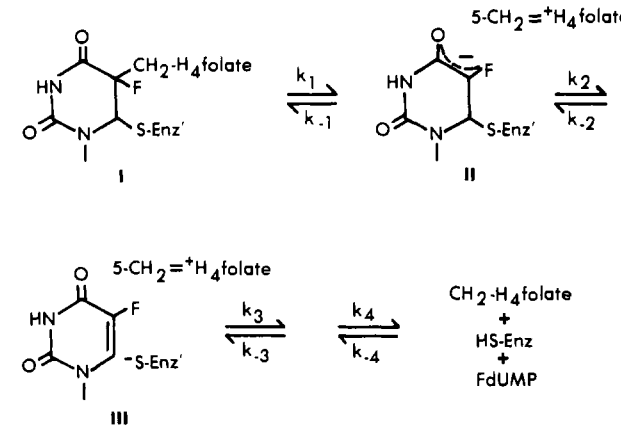
to be 1.033 ± 0.004 (n = 16). In the absence of added unlabeled cofactor no dissociation of the radioactive complex was observed after as long as 35 h.

Discussion

Scheme I depicts what is currently believed to be a minimal mechanism for dissociation/association of the covalent FdUMP-CH_2-H_4folate-dTMP synthetase complex (cf. Pogolotti & Santi, 1977). The first step in dissociation of the complex (I), k_1, involves cleavage of the linkage between the 5 position of the bound FdUMP and the cofactor to form the covalently bound enolate II (or equivalent enol) and the 5-iminimum ion form of the cofactor, CH_2=$^{+}H_4$folate; this step involves sp^3 to sp^2 rehybridization of the one-carbon unit of the bound cofactor. While the sp^2 hybridized iminium ion intermediate has not been directly demonstrated in this pathway, its existence is strongly supported by chemical rationale and model studies and is widely accepted to be an intermediate in the dTMP synthetase reaction (cf. Kallen & Jencks, 1966; Benkovic & Bullard, 1973; Pogolotti & Santi, 1977; Benkovic, 1978). The second step in dissociation, k_2, involves β elimination of the enzyme thiol of II to provide noncovalently bound FdUMP; it is here where sp^3 to sp^2 rehybridization of the 6 carbon of FdUMP occurs. The step k_3/k_{-3} represents a conformational change of the enzyme, and perhaps of the cofactor (to be discussed later), which we will suggest occurs upon formation of noncovalent, reversible, ternary complexes (III).

In this study, secondary α-hydrogen isotope effects have been used in an attempt to clarify the relationships of covalent bond changes at the 6 position of FdUMP and at the one-carbon unit of CH_2-H_4folate with rate-limiting processes in the dissociation and formation of the FdUMP-CH_2-H_4-folate-dTMP synthetase complex. A summary of the isotope effects determined here is provided in Table I. The use and

Scheme I



interpretation of secondary α-hydrogen isotope effects have been extensively reviewed (Melander, 1960; Collins & Bowman, 1970; Kirsch, 1977), and only a brief summary relevant to interpretation of this study is provided here. Isotopic substitution of a nonreactive, nonexchangeable hydrogen attached to a carbon atom which undergoes a covalent bond change may alter the equilibrium and rate of a reaction. The maximal α-hydrogen secondary isotope effect of a reaction is manifested at equilibrium and can be determined experimentally or estimated by the use of fractionation factors derived from simple models of the two relevant states (Hartshorn & Shiner, 1972; Buddenbaum & Shiner, 1977; Cleland, 1980). Comparisons of the magnitude of secondary α-hydrogen kinetic isotope effects (KIE) with that of the equilibrium isotope effect can provide information regarding where covalent bond changes at the isotopically substituted carbon atom occur with respect to the rate-determining step of a reaction. In general, maximal KIE's are observed when the pertinent covalent bond change occurs before the rate-determining step, or in a very late transition state of the rate-determining step. Such KIE's are attenuated when the bond change occurs earlier in the rate-determining step and are not observed when the bond change is post rate determining.

For the sp^3 to sp^2 conversion of the 6-CH[T]SR of I to FdUMP, a maximal (equilibrium) isotope effect of 1.21–1.23 is calculated from fractionation factors, with tritium enrichment in the FdUMP complex (I).[2] This is in excellent

[2] The fractionation factor relative to acetylene (FF) for CH_3CHDSR has been estimated by Dr. V. J. Shiner, Jr., from fractionation factors calculated by Hartshorn & Shiner (1972) and by T. E. Newmann (personal communication) at Indiana University using the method of Hartshorn & Shiner (1972). When calculated FF's for CH_3SH (1.329), CH_3CH_3 (1.361), and CH_4 (1.246) were used, the FF for CH_3CHDSR was estimated as 1.329 × 1.361/1.246 = 1.452. Alternatively, when FF's for CH_3SH (1.329), CH_3Cl (1.405), and EtCl (1.518) are used, the FF for CH_3CHDSR is 1.329 × 1.518/1.405 = 1.436. When the FF for CH_2=CHD (1.257 relative to acetylene) is used, the deuterium isotope effect for the interconversion of CH_2=CHD and CH_3CHDSR is calculated to be 1.436 to 1.452/1.257 = 1.142 to 1.155 with deuterium concentrating in the thiol. Raised to the 1.442 power, the corresponding tritium isotope effect is 1.21–1.23.

agreement with the experimentally determined measurement made by comparison of the $^3H/^{14}C$ ratio of bound and free [2-^{14}C,6-^{3}H]FdUMPs which were separated by gel filtration or nitrocellulose adsorption of the complex after equilibrium was reached ($K_H/K_T = 1.24$). By use of reported fractionation factors (Cleland, 1980), the overall conversion of the C_5-C-H_2[T]-N^5 of I to the N^5-CH_2[T]-N^{10} of CH_2-H_4folate is calculated to proceed with an equilibrium α-tritium isotope effect of $K_H/K_T = 0.95$, with isotopic enrichment in CH_2-H_4folate. Unfortunately, difficulties associated with the instability of the cofactor and interference of excess [^{3}H]H_2CO in our assays did not permit experimental verification of this value. Although no simple model is available to estimate a fractionation factor for CH_2[T]=$^+H_4$folate, it would certainly be larger than for CH_2[T]=CH_2, and equilibrium α-tritium isotope effects greater than 13% would be expected in conversions between CH_2=$^+H_4$folate and I or CH_2-H_4folate (Cleland, 1980).

When [2-^{14}C,6-^{3}H]FdUMP was added to dTMP synthetase and CH_2-H_4folate, the complex which initially formed had the same $^3H/^{14}C$ ratio as the reactant, demonstrating that there is no KIE in the initial formation of the complex. However, tritium enrichment of the complex occurred commensurate with isotopic equilibration; at equilibrium, comparison of the bound $^3H/^{14}C$ to that of the initially formed complex gave an isotope effect which was identical with the equilibrium isotope effect determined by gel filtration ($K_H/K_T = 1.24$). Dissociation of [2-^{14}C,6-^{3}H]FdUMP from I proceeded with a KIE which was essentially identical with equilibrium isotope effect. The combined findings that there is a maximal KIE during dissociation of the [6-^{3}H]FdUMP complex and no KIE during its formation demonstrate that, in the direction of dissociation, cleavage of the covalent bond between the enzyme thiol and FdUMP (k_2) must either be (a) pre rate determining or (b) rate determining with a very late transition state in which sp^2 rehybridization of C-6 is essentially complete. We favor the former for the following reasons: First, the probability is low that the transition state would be so late that the KIE would be identical with the equilibrium isotope effect. Second, dissociation of [6-^{3}H]FdUMP from the complex formed with CH_2-H_4PteGlu$_5$ proceeds some 6-fold slower than that from the complex formed with the monoglutamate but still shows a maximal KIE ($k_H/k_T = 1.21$). To accommodate these data in a mechanism involving k_2 as the rate-determining step, one must propose that the additional glutamate moieties cause slower covalent bond cleavage (i.e., raise $\Delta G^{\ddagger}$) but do not significantly alter the position of the transition state on the reaction coordinate. While this is possible, it is more likely that the difference in rates of dissociation of FdUMP from complexes formed with CH_2-H_4PteGlu$_5$ vs. CH_2-H_4folate reflects a rate-determining step(s) subsequent to k_2. This is in agreement with a number of studies (Galivan et al., 1976; Galivan et al., 1977; Dolnick & Cheng, 1978; Kisliuk & Gaumont, 1979) which have demonstrated that pteroylpolyglutamates bind much tighter to dTMP synthetase than do pteroylmonoglutamates. Last, a number of noncovalent FdUMP–dTMP synthetase complexes formed with cofactor analogues dissociate as slow or slower than the covalent FdUMP–CH_2-H_4folate–dTMP synthetase complex. For example, [^{3}H]FdUMP dissociates from complexes formed with 5,8-deaza-10-methylfolate and 5,8-deaza-10-propargylfolate with $k = 1.14 \times 10^{-3}$ min^{-1} and 0.8×10^{-4} min^{-1}, respectively (unpublished results), compared to 1.3×10^{-3} min^{-1} for dissociation of the FdUMP–CH_2-H_4folate–dTMP synthetase complex. Likewise, [^{3}H]FdUMP dissociates from the complex formed with PteGlu$_3$ some 1.5-fold slower than from the FdUMP–CH_2-H_4folate–dTMP synthetase complex (Danenberg & Locksin, 1982). Considered together, the above strongly argues that cleavage of the covalent bond linking the enzyme to C-6 of FdUMP (k_2/k_{-2}) is pre rate determining in dissociation and post rate determining in formation of the covalent complex.

The dissociation of [^{3}H]CH_2-H_4folate from I proceeds with a small normal KIE ($k_H/k_T = 1.03$). From the KIE studies of [6-^{3}H]FdUMP, we have concluded that conversion of I to the sp^2 hybridized iminium ion in II occurs before the rate-determining step. We may thus deduce that subsequent conversion of the iminium ion to 5,10-CH_2-H_4folate must also occur before or at the rate-determining step, since a substantial inverse kinetic isotope effect would be necessary to cancel the large, normal KIE expected upon preequilibrium formation of the iminium ion from I. The calculation that conversion of the one carbon unit of I to that of CH_2-H_4folate would proceed with a maximal inverse isotope effect of $k_H/k_T \simeq 0.95$ (Cleland, 1980) places additional relevance on the fact that we observe a small, normal isotope effect of $k_H/k_T = 1.03$. This indicates that the inverse KIE expected upon conversion of the iminium ion to CH_2-H_4folate does not completely cancel the normal isotope effect which accompanies formation of II (i.e., the actual KIE observed is 8% and normal). This would have the important implication that conversion of the iminium ion to CH_2-H_4folate occurs during the rate-determining step of the reaction. Unfortunately, experimental determination of the equilibrium (i.e., maximal) isotope effect between I and CH_2-H_4folate has not been experimentally feasible, and the validity of this interpretation rests on the reliability of the value calculated from fractionation factors.

From what has been described, it is reasonable to conclude that the rate-determining step in the dissociation of the FdUMP–CH_2-H_4folate–dTMP synthetase complex occurs after cleavage of the thioether bond linking the enzyme to FdUMP. Under conditions similar to those used here, the release of ligands from the ternary complex has been shown to proceed by an ordered mechanism, with CH_2-H_4folate dissociating before FdUMP (Danenberg & Danenberg, 1978). Thus, it is possible that association/dissociation of a "sticky" cofactor represents the slow step of this reaction. It has been established that formation of the FdUMP–CH_2-H_4folate complex results in a significant conformational change of the enzyme (Lockshin & Danenberg, 1980). Further, spectral studies have indicated an intimate interaction between the pteridine and/or (*p*-aminobenzoyl)glutamate moieties of the cofactor (or related analogues) and chromophores of the enzyme in ternary complexes formed with FdUMP (Santi et al., 1976; Donato et al., 1976; Sharma & kisliuk, 1973; Galivan et al., 1977). Such interactions could provide the driving force for the conformational change which occurs upon ternary complex formation, and together could represent the rate-determining step of the reaction which occurs concomitantly with or shortly after the initial reversible binding of CH_2-H_4folate to the FdUMP–enzyme complex. The tight interaction of the enzyme with the pteridine and (*p*-aminobenzoyl)glutamate residues of CH_2-H_4folate might also strain the labile 5-membered ring of the cofactor and, as a result, induce its conversion to the 5-iminium ion. This hypothesis is attractive because it provides a simple mechanism for interconversion of 5,10-CH_2-H_4folate and the iminium ion and defines a function for the conformational change of the enzyme. Further, it is in accord with the KIE data obtained here with [^{3}H]CH_2-H_4folate which suggests that the rate-deter-

mining step of the reaction may involve the interconversion of CH_2-H_4folate with the iminium ion. Hopefully, rapid kinetic measurements of the formation of the FdUMP–CH_2-H_4folate–dTMP synthetase complex will provide more insight as to the mechanism of this reaction.

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References

Baccanari, D., Phillips, A., Smith, S., Sinski, D., & Burchall, J. (1975) *Biochemistry 14*, 5267.

Benkovic, S. J. (1978) *Acc. Chem. Res. 11*, 314.

Benkovic, S. J., & Bullard, W. P. (1973) *Prog. Bioorg. Chem. 2*, 133.

Buddenbaum, W. E., & Shiner, V. J., Jr. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 1, University Park Press, Baltimore, MD.

Cleland, W. W. (1980) *Methods Enzymol. 64B*, 104.

Collins, C. J., & Bowman, N. S., Eds. (1970) *ACS Monogr. No. 167*, 90–212.

Coward, J. K., Parameswaran, K. N., Cashmore, A. R., & Bertino, J. R. (1974) *Biochemistry 13*, 3899.

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

Danenberg, P. V. (1977) *Biochim. Biophys. Acta 473*, 73.

Danenberg, P. V., & Danenberg, K. D. (1978) *Biochemistry 17*, 4018.

Danenberg, P. V., & Lockshin, A. (1982) *Mol. Cell. Biochem. 43*, 4957.

Dolnick, B. J., & Cheng, Y.-C. (1978) *J. Biol. Chem. 253*, 3563.

Donato, H., Jr., Aull, J. L., Lyon, J. A., Reinsch, J. W., & Dunlap, R. B. (1976) *J. Biol. Chem. 251*, 1303.

Futterman, S. (1957) *J. Biol. Chem. 228*, 1031.

Galivan, J. H., Maley, G. F., & Maley, F. (1975) *Biochemistry 14*, 3338.

Galivan, J. H., Maley, F., & Baugh, C. M. (1976) *Biochem. Biophys. Res. Commun. 71*, 527.

Galivan, J. H., Maley, F., & Baugh, C. M. (1977) *Arch. Biochem. Biophys. 184*, 346.

Garrett, C., Pogolotti, A. L., Jr., & Santi, D. V. (1977) *Anal. Biochem. 79*, 602.

Garrett, C., Wataya, Y., & Santi, D. V. (1979) *Biochemistry 18*, 2798.

Hartshorn, S. R., & Shiner, V. J., Jr. (1972) *J. Am. Chem. Soc. 94*, 9002.

Hatefi, Y., Talbert, P. T., & Osborn, M. J. (1960) *Biochem. Prep. 7*, 89.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology* p 589, McGraw-Hill, New York.

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

Kirsch, J. F. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 100, University Park Press, Baltimore, MD.

Kisliuk, R. L., & Gaumont, Y. (1979) in *Chemistry and Biology of Pteridienes* (Kisliuk, R. L., & Brown, G. M., Eds.) Vol. 4, p 431, Elsevier/North-Holland, New York.

Lockshin, A., & Danenberg, P. V. (1980) *Biochemistry 19*, 4244.

Melander, L. (1960) *Isotope Effects on Reaction Rates*, Ronald Press, New York.

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

Pogolotti, A. L., Jr., & Santi, D. V. (1977) in *Bioorganic Chemistry* (Van Tamelen, E. E., Ed.) Vol. 1, p 277, Academic Press, New York.

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

Santi, D. V., McHenry, C. S., & Sommer, H. (1974a) *Biochemistry 13*, 471.

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

Santi, D. V., Pena, V. A., & Lam, S. S. M. (1976) *Biochim. Biophys. Acta 438*, 324.

Sharma, R. K., & Kisliuk, R. L. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 591.

Wahba, A. J., & Friedkin, M. (1962) *J. Biol. Chem. 237*, 3794.

Wataya, Y., & Santi, D. V. (1977) *Methods Enzymol. 46*, 307.