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_4folate.

References

Becker, A. R., Richardson, D. J., & Bruice, T. C. (1977) *J. Am. Chem. Soc. 99*, 5058.

Blair, J. A., & Saunders, K. J. (1970) *Anal. Biochem. 34*, 376.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, p 81, American Elsevier, New York.

Cram, D. J. (1965) in *Fundamentals of Carbanion Chemistry*, Chapter 1, pp 1–20, Academic Press, New York.

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

Danenberg, P. V., Langenbach, R. J., & Heidelberger, C. (1974) *Biochemistry 13*, 926.

Friedkin, M. (1973) *Adv. Enzymol. 38*, 235.

Galivan, J. H., Maley, G. F., & Maley, F. (1976) *Biochemistry 15*, 356.

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

Garrett, C., Wataya, Y., & Santi, D. V. (1979) *Biochemistry 18* (following paper in this issue).

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

James, T. L., Pogolotti, A. L., Ivanetich, K. M., Wataya, Y., Lam, S. S. M., & Santi, D. V. (1976) *Biochem. Biophys. Res. Commun. 72*, 404.

Lomax, M. I. S., & Greenberg, G. R. (1967) *J. Biol. Chem. 242*, 1302.

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

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

Roberts, D. (1966) *Biochemistry 5*, 3546.

Santi, D. V., & Sakai, T. T. (1972) *Biochem. Biophys. Res. Commun. 46*, 1320.

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

Segel, I. H. (1975) *Enzyme Kinetics*, pp 860–864, Wiley, New York.

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

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

Weill, C. L. (1974) Ph.D. Dissertation, University of California, Santa Barbara, CA.

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

Charles Garrett, Yusuke Wataya, and Daniel V. Santi*

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 α-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_4folate. 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-

† From the Department of Biochemistry and Biophysics and the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143. *Received November 17, 1978.* This work was supported by U.S. Public Health Service Grant CA 14394 from the National Cancer Institute and by U.S. Public Health Service Training Grant GM-02197 (C.G.). D.V.S. is the recipient of a National Institutes of Health Career Development Award. Preliminary communications of portions of this work have appeared [Wataya, Y., & Santi, D. V. (1975) *Biochem. Biophys. Res. Commun. 67*, 818; Wataya, Y., & Santi, D. V. (1977) *J. Am. Chem. Soc. 99*, 4534].

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

action is accompanied by a large inverse α-secondary tritium isotope effect at C-6 of the heterocycle which, together with extensively studied chemical counterparts, provides conclusive evidence that this enzyme-catalyzed reaction also proceeds via nucleophilic attack at the 6 position of the heterocycle and transient formation of 5,6-dihydropyrimidines.

Experimental Procedures

Materials. BrdUMP, IdUMP, folate, H_2folate, 5-CH_3-H_4folate, 5-CHO-H_4folate, and aminopterin were purchased from Sigma Chemical Co. dUMP, dTMP, BrdUrd, and CF_3dUrd were obtained from P-L Biochemicals and FdUMP was obtained from Terra-Marine Bioresearch. Pteroic acid, homofolate, methotrexate, dichloromethotrexate, and 10-deazafolate were gifts of the Drug Research and Development Division of the National Cancer Institute. N^{10}-Methylfolate was a gift from Lederle Laboratories and N^{10}-methyl-5,8-deazafolate from Parke, Davis and Co. [6-^{3}H]BrdUrd and [2-^{14}C]BrdUrd were purchased from New England Nuclear. *dl*-L-H_4Folate was prepared from folate by the procedure of Hatefi et al. (1960), and CH_2-H_4folate was formed by the addition of a 15- to 30-fold excess of H_2CO. Other materials were of reagent grade or better quality from commercial sources.

Chromatographic Techniques. Chromatography on Aminex A-27 was as described by Garrett et al. (1977), except that equipment capable of operating at 2000–3000 psi was used. The buffer systems used with Aminex A-27 are as follows: system A was 0.75 M ammonium acetate, pH 4.4, containing 8% 1-propanol; system B contained 0.5 mol of ammonium bicarbonate and sufficient NH_4OH to give a final pH of 9.0 in a total volume of 1 L; system C contained 0.35 mol of ammonium bicarbonate, NH_4OH to give pH 9.0, and 80 mL of 1-propanol in a total volume of 1 L. Separations were performed at ambient temperature except that, when one was using system B, a temperature of 85 °C was maintained with a water-jacketed column. Chromatography on Lichrosorb C_{18} (4.6 × 250 mm column, 10-μm particle size) was performed at ambient temperature by using an eluting buffer (pH 7.0) containing 5 mM tetrabutylammonium hydrogen sulfate, 5 mM potassium phosphate, and 1% acetonitrile. Two-dimensional paper chromatography of nucleotides was performed on Whatman 3MM paper by using isopropyl alcohol–28% NH_4OH–H_2O (7:1:2) in the first direction and isobutyric acid–0.5 N NH_4OH (5:3) in the second; two-dimensional thin-layer chromatography of nucleosides was performed on 20 × 20 cm cellulose plates (Merck, 0.10 mm) by using 1-butanol–H_2O (86:14) in the first direction and 1-butanol–acetic acid–H_2O (2:1:1) in the second.

CysdUrd and CysdUMP. CysdUrd was prepared by incubating a solution (0.5 mL) containing 5 mg (16 μmol) of BrdUrd and 0.25 M cysteine at pH 9.0 for 2.5 h at 37 °C (Wataya et al., 1973). The product was purified on a 9 × 250 mm Aminex A-27 column by using system B at 55 °C. CysdUMP was prepared from BrdUMP (5 mg, 12 μmol) in an identical manner; the product eluted between dUMP and BrdUMP on Aminex A-27 (system B) and was well separated from small amounts of contaminating dUrd and CysdUrd. Its identity was confirmed by the following criteria: (a) it had UV spectra identical with that of CysdUrd in both acidic and alkaline solution; and (b) upon treatment with *E. coli* alkaline phosphatase, it was converted to a compound indistinguishable from CysdUrd by chromatography on Aminex A-27 systems B and C and on Lichrosorb C_{18}.

Thymidylate Synthetase. 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 by using ϵ_{278} 1.07 × 10^5 M^{-1} cm^{-1} (Santi et al., 1974). FdUMP binding sites were titrated spectrophotometrically as follows. One of two previously balanced cuvettes contained in 1.0 mL: ca. 5 μM thymidylate synthetase, 50 mM *N*-methylmorpholine hydrochloride (pH 7.4), 6.5 mM dithiothreitol, 25 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetate. Enzyme was omitted in the reference cuvette, and the UV absorbance of the enzyme at 278 nm was recorded. CH_2-H_4folate was then added to both cuvettes to give a final concentration of 0.20 mM, the base line was recorded, and successive additions (10 μL) of a 0.12 mM solution of FdUMP were made to each cuvette until no further increase of UV absorbance at 330 nm was observed. The concentration of titratable enzyme binding sites was then calculated from the equivalence point or by using $\Delta\epsilon_{330}$ 17 700 M^{-1} cm^{-1} for formation of the enzyme–CH_2-H_4folate–FdUMP complex (Santi et al., 1976). The molar amount of titratable enzyme was calculated by assuming two binding sites per 70 000-dalton dimer and corresponded to 85% of the amount of enzyme calculated from the A_{278}.

Standard Assay Conditions. Unless otherwise noted, dehalogenation reactions were performed at 30 °C in a buffer containing 50 mM *N*-methylmorpholine hydrochloride (pH 7.4), 25 mM $MgCl_2$, 1 mM ethylenediaminetetraacetate, and 75 mM β-mercaptoethanol. Kinetic assays were performed by monitoring the decrease in absorbance at 285 nm for BrdUMP ($\Delta\epsilon$ 5320) and at 290 nm for IdUMP ($\Delta\epsilon$ 6520); reference cuvettes lacked enzyme.

Calculation of Secondary Tritium Isotope Effects. Unless otherwise specified, reactants and products were separated by high-performance liquid chromatography as described; care was taken to collect each radioactive peak in its entirety to avoid isotope separation. The radioactivity in each peak was counted three to four times and a minimum of 2 × 10^5 ^{14}C counts collected; standard errors (SE) for determination of ^{3}H/^{14}C ratios and ^{14}C dpm's were ca. 0.25% and 0.5%, respectively. In the case of determinations on CysdUMP, fewer total counts were collected; SE's of isotopic ratios and ^{14}C dpm's were 0.5–2.0%. Isotopic effects were calculated from the isotopic ratios of either product or reactant as described by Melander (1960). All statistical estimates are presented as mean ± SE.

Miscellaneous. [6-^{3}H,2-^{14}C]BrdUMP was prepared from [6-^{3}H,2-^{14}C]BrdUrd by phosphorylation with thymidine kinase and purification on diethylaminoethylcellulose (Wataya & Santi, 1977). The product was further purified on Aminex A-27 (system A) to remove contaminating AMP. Radiochemical purity was greater than 99.9% as judged by chromatography on Aminex-27 using systems A or B. CF_3dUMP was prepared similarly, except that final purification was performed by ascending paper chromatography (Whatman no. 1 paper; 1-butanol–acetic acid–H_2O, 2:1:1; R_f 0.47).

Measurements of free thiol concentration were made by the method of Ellman (1959) using ϵ_{412} 14 100 for the thiophenolate product (Collier, 1973). *E. coli* alkaline phosphatase treatment of CysdUMP was performed by adding 0.9 unit of an enzyme suspension (Worthington BAPF, 32.3 U/mg) to

[1] Abbreviations other than those recommended by IUPAC–IUB are as follows: XdUrd and XdUMP (where X = Br, Cl, F, I), 5-halogenated 2′-deoxyuridines or deoxyuridylates; CysdUrd, *S*-[5-(2′-deoxyuridyl)]-cysteine; CysdUMP, *S*-[5-(5′-monophosphoryl-2′-deoxyuridyl)]cysteine.

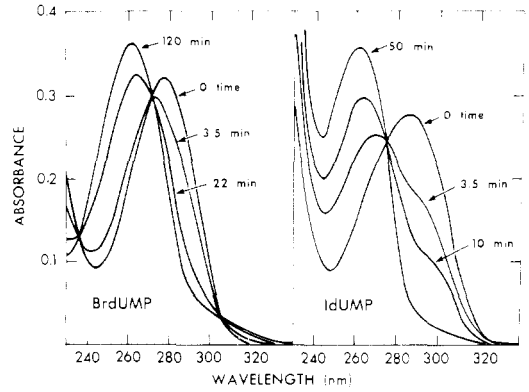


FIGURE 1: Ultraviolet spectral changes of (a) 40 μM BrdUMP and (b) 38 μM IdUMP when reacted with 0.6 μM thymidylate synthetase in the presence of 6.5 mM dithiothreitol. Other components of the reaction mixture are as described under Experimental Procedures.

0.03–0.75 μmol of the nucleotide in 0.3 mL of 0.1 M NH_4CO_3 buffer, pH 9.0, and incubating overnight at 37 °C. Spectrophotometric determinations were made by using a Cary 118 recording spectrophotometer. Radioactive isotopes were measured by counting in a fluid containing 0.4% Omnifluor in xylene–Triton X-114 (3:1) or in Aquasol-2 (New England Nuclear) by using a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. Counting efficiencies were determined by the external standards ratio method and dpm calculations were aided by a tape-fed Hewlett-Packard computer.

Results

Reaction of BrdUMP and IdUMP with Thymidylate Synthetase and Identification of dUMP as the Major Product. Figure 1 shows the ultraviolet spectral changes which occur upon treatment of BrdUMP or IdUMP with thymidylate synthetase; in these reactions, dithiothreitol was used in place of β-mercaptoethanol to minimize end absorption due to atmospheric oxidation of the thiol and side product formation (see below). In both cases there is a time-dependent change of the absorption maximum of the halogenated substrates to 262 nm. No spectral changes were observed when CldUMP or FdUMP were treated similarly, and inhibition of the enzyme (0.8 μM) by pretreatment with CF_3dUMP (3.7 μM) for 30 min at room temperature decreased the rate of reaction of IdUMP by at least 95%.

The major product of the reaction was ascertained to be dUMP by the following criteria. (i) The products of the reaction of BrdUMP and thymidylate synthetase were analyzed by two-dimensional paper chromatography and by chromatography on Aminex A-27 (systems A, B, and C). In all cases, the major UV-absorbing product had a mobility identical with that of dUMP. Elution of the major spot obtained from the paper chromatography yielded a product which had an ultraviolet spectrum identical with that of dUMP in acidic and alkaline pH regions. (ii) After completion (2 h) of the reactions depicted in Figure 1, excess CH_2-H_4folate was added to the reaction mixture to give a final concentration of 0.18 mM and the increase in absorbance at 340 nm (characteristic of the formation of dTMP and H_2folate from dUMP and CH_2-H_4folate) was monitored. From $\Delta\epsilon_{340}$ 6400 (Wahba & Friedkin, 1962), the products obtained from BrdUMP and IdUMP were ascertained to be 89 and 100% dUMP, respectively. The dehalogenation of IdUMP is accompanied by a corresponding increase in absorbance at 230–260 nm (Figure 1) which is attributable to end absorption of I^-; Br^- does not absorb in this region, and the conversion

Table I: Kinetic Parameters for the Thymidylate Synthetase Catalyzed Dehalogenation of BrdUMP and IdUMP[a]

substrate	K_m (μM)	k_{cat} (min^{-1})[b]
BrdUMP	5.7	5.9
IdUMP	1.4	2.1
dUMP (dTMP formation)[c]	3.0	384

[a] The assay mixture contained 0.21 μM enzyme and other components as described under Experimental Procedures. Kinetic parameters were estimated by nonlinear curve-fitting techniques. [b] Values are per 70 000-dalton dimer. [c] The reaction mixture contained 0.2 mM CH_2-H_4folate.

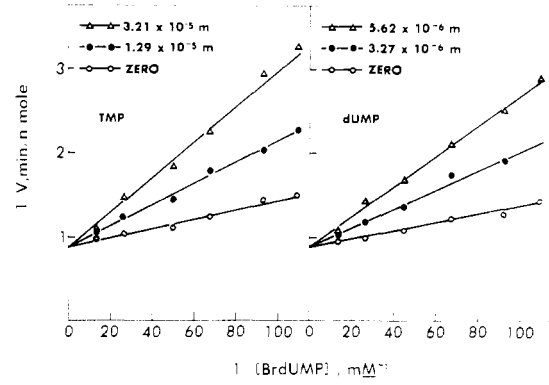


FIGURE 2: Double-reciprocal plots of the inhibition of enzymatic debromination by dUMP and dTMP. Reaction mixtures were as described under Experimental Procedures; enzyme concentration was 0.25 μM.

of BrdUMP to dUMP shows a well-defined isosbestic point at 236 nm. Using ϵ_{240} 330 for I^-, ϵ_{240} 4290 for dUMP, and ϵ_{240} 330 for oxidized thiol (see Discussion), it can be calculated that I^- and dUMP are formed in stoichiometrically equivalent amounts during the deiodination reaction.

In an attempt to measure the reversibility of the reaction, 50 μM BrdUMP, 1.2 μM enzyme, and [6-^{3}H]dUMP (1.1 μCi/mL, 21 Ci/mmol) were incubated with other components of the standard reaction mixture. After approximately 50% completion of dehalogenation of BrdUMP, a 100-μL aliquot was separated on Aminex A-27, system C, and fractions were counted for tritium content. We detected no formation of BrdUMP from dUMP ($\leq$0.4%) by this method even when 100 mM NaBr was included in the reaction mixture.

Kinetics of Dehalogenation. Under the standard conditions described under Experimental Procedures, the initial reaction velocity is linearly proportional to enzyme concentration up to 6 μM enzyme with 75 μM BrdUMP as the substrate, and up to at least 8 μM using 75 μM IdUMP. Kinetics parameters of the thymidylate synthetase catalyzed dehalogenation are shown in Table I; values for dTMP synthesis from dUMP and CH_2-H_4folate are also given for comparison. Both dTMP and dUMP are competitive inhibitors of the dehalogenation of BrdUMP (Figure 2), with K_i values of 8.7 and 2.3 μM, respectively; dTMP and dUMP also inhibit the deiodination of IdUMP.

The pH optimum of the debromination reaction is ca. 7.4, with one-half maximal rates at pH 6.6 and 8.0. The reaction rate was affected by the solvent buffer concentration, increasing more than threefold between 25 and 200 mM *N*-methylmorpholine. Figure 3 shows the rate dependence of debromination on the concentration of dithiothreitol, β-mercaptoethanol, and cysteine. Glutathione also supported the debromination reaction; over the concentration range 0.5–10 mM, it was nearly as effective as cysteine. In the absence of added thiols the reaction proceeded extremely

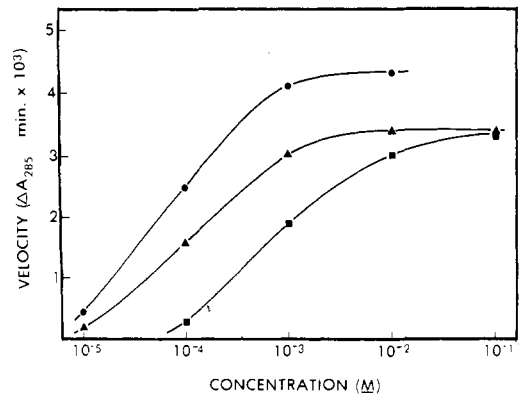


FIGURE 3: Effect of thiols on the thymidylate synthetase catalyzed debromination of BrdUMP. The standard reaction mixture contained 50 μM BrdUMP, 0.29 μM enzyme, and thiol reagents as indicated: (●) dithiothreitol; (▲) β-mercaptoethanol; (■) cysteine. Incubations were at 25 °C.

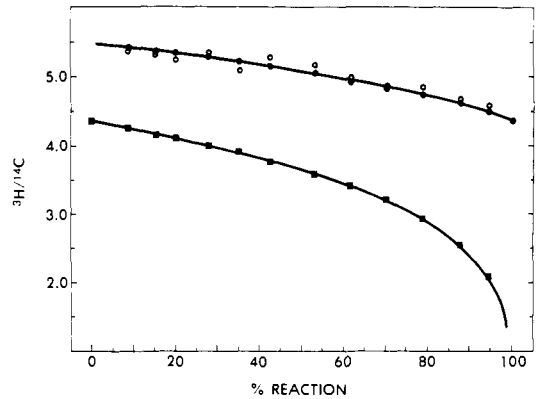


FIGURE 4: Inverse secondary isotope effect in the thymidylate synthetase catalyzed debromination of [6-^{3}H,2-^{14}C]BrdUMP. The standard reaction mixture (3.5 mL) contained 50 μM BrdUMP (5.21 Ci/mol of ^{14}C, $^3H/^{14}C$ = 4.360), 10 mM cysteine, and 1.1 μM enzyme. Aliquots (0.15 mL) were withdrawn, dUMP and BrdUMP were added as chromatographic markers (ca. 1 mM each), and the aliquots were kept on ice until separation of the reactant (BrdUMP) and products (dUMP and CysdUMP) on Aminex A-27 system B. Data points represent the $^3H/^{14}C$ ratios of dUMP (●), CysdUMP (○), and BrdUMP (■); also depicted are the theoretically expected changes in isotopic ratios of product (upper line) and reactant (lower line) for a reaction with k_T/k_H = 1.258 (Melander, 1960).

slowly; however, at high enzyme concentrations (5 μM), some reaction was noted, perhaps due to participation of enzyme thiol groups as reductants in the reaction.

Formation of Minor Nucleotide Products. During the course of the enzymatic dehalogenation of BrdUMP, but not IdUMP, a distinct increase in UV absorbance at 310–320 nm occurs (Figure 1). This observation, together with the lower yield of dUMP as compared with that obtained during the dehalogenation of IdUMP, suggested the formation of minor product in the debromination reaction. When [2-^{14}C]BrdUMP (50 μM) was allowed to react with enzyme (1.1 μM) until all spectral changes had stopped and the reaction mixture was separated on Aminex A-27, a second radioactive peak corresponding to a product other than dUMP was found. Both the amount of this product and its chromatographic properties were determined by the thiol reagent present in the reaction. After separation on Aminex A-27 with the indicated buffer systems, the following amounts of minor product are formed, expressed as a percentage of total product: with 75 mM and 0.2 mM β-mercaptoethanol (system C), 30% and 32%, respectively; with 10 mM cysteine (system B), 9%; with 6.5 mM dithiothretiol (system C), 7%. In each case the product elutes between dUMP and BrdUMP, a position characteristic of 5-substituted deoxyuridylates (Garrett et al., 1977). With β-mercaptoethanol and cysteine, the indicated chromatographic system allows full separation of the second product from both dUMP and BrdUMP, and it was seen that the minor product is formed in constant proportion to dUMP throughout the course of the reaction; thus it is not an intermediate in the conversion of BrdUMP to dUMP. No indication of side product formation was found with IdUMP as the substrate.

Identification of CysdUMP as the Minor Reaction Product Formed in the Presence of Cysteine. [2-^{14}C]BrdUMP (50 μM, 23 Ci/mol) was reacted with thymidylate synthetase (1.1 μM) in the presence of 10 mM cysteine; after 25 min at 30 °C, the reaction was 97% complete. (In a parallel control reaction lacking enzyme ca. 1% dehalogenation was observed.) CysdUMP was added to a portion of the reaction mixture as a chromatographic marker, and the mixture was separated on Aminex A-27 by using system B. In addition to the radioactive peaks corresponding to dUMP and BrdUMP, a third peak corresponding to the minor product was found intermediate between them and at the exact position of CysdUMP. The product was isolated from the remaining reaction solution and converted to the nucleoside with *E. coli* alkaline phosphatase; after this treatment it was indistinguishable from CysdUrd by chromatographic analysis on Aminex A-27 systems B and C, on Lichrosorb C_{18}, and on the four paper chromatographic systems used for the identification of CysdUrd by Wataya et al. (1973).

Stoichiometry of Thiol Utilization. A reaction mixture, containing 100 μM BrdUMP, 1.2 μM enzyme, and an initial concentration of 200 μM β-mercaptoethanol, was incubated at room temperature (21 °C) in an N_2-purged chamber. Progress of the reaction was monitored spectrophotometrically and aliquots were removed for determination of thiol concentration; over the time period of the experiment (1.5–2.0 h), control reactions lacking enzyme or BrdUMP showed no significant changes in thiol concentration.

The results of three such experiments demonstrate that the disappearance of thiol groups is proportional to the amount of BrdUMP dehalogenated up to at least 50% completion of the reaction and that under these conditions 1.8 ± 0.1 (n = 12) equiv of thiol are oxidized per mol of BrdUMP debrominated. By analysis of Aminex A-27 (system C) of an identical reaction mixture in which the substrate was radioactively labeled, it was determined that the product distribution was 68% dUMP and 32% another nucleotide, presumably 5-β-hydroxyethylthio-dUMP. Assuming that 1 equiv of thiol is used in the formation of each mol of side product (see Discussion), it may be calculated that 2.1 mol of thiol is consumed per mol of dUMP formed. Similar experiments were attempted by using cysteine in place of β-mercaptoethanol, but rapid oxidation of cysteine in the control reaction prevented accurate determination of the stoichiometry of its consumption.

Inverse Secondary Isotope Effect upon Enzymatic Debromination of BrdUMP. As shown in Figure 4, when [6-^{3}H,2-^{14}C]BrdUMP is allowed to react with thymidylate synthetase in the presence of 10 mM cysteine, the $^3H/^{14}C$ ratios of the reactant (BrdUMP) and the products (dUMP and CysdUMP) change in a manner indicating a more rapid dehalogenation of the 6-tritiated compound. Dehalogenation in a parallel reaction lacking enzyme was negligible. The inverse secondary tritium isotope effects (k_T/k_H) are 1.253 ± 0.003 and 1.31 ± 0.03 calculated from the $^3H/^{14}C$ ratios of dUMP and CysdUMP, respectively (n = 12). If, for each

Table II: Inverse Secondary Isotope Effect in the Thymidylate Synthetase Catalyzed Debromination of [6-^{3}H]BrdUMP

thiol additive	isotope effect[a]
10 mM cysteine	1.258 ± 0.002 (n = 24)[b]
6.5 mM dithiothreitol	1.243 ± 0.002 (n = 5)[c]
0.01 mM dithiothreitol	1.229 ± 0.002 (n = 5)[c]
75 mM β-mercaptoethanol	1.212 ± 0.012 (n = 20)[b]

[a] Isotope effects were determined as described in the text. In the case of dithiothreitol and β-mercaptoethanol, separations of product and reactant were performed by using Aminex A-27 system C. [b] Average of all determinations as calculated from products and reactant. [c] Calculated from dUMP at ≤30% reaction.

determination during the course of the reaction, we combine the isotopic content of each product, the mean isotope effect for product formation computed in this way is 1.256; this agrees well with the value of 1.260 ± 0.003 (n = 12) calculated from the decreasing isotopic ratio of the substrate BrdUMP. These and other data collected in similar experiments in which the nature or concentration of thiol reagent was changed are summarized in Table II.

As pointed out by Kirsch (1977), small deterministic errors may far outweigh the statistically determined standard errors in any single experiment, and we do not consider the relatively small differences in kinetic isotopic effects observed in separate experiments reported here to be significant.

Inverse Secondary Isotope Effect upon Cysteine Catalyzed Debromination of BrdUrd. [6-^{3}H,2-^{14}C]BrdUrd (10 mM) was allowed to react with 0.25 M cysteine (pH 7.3) at 37 °C. The extent of debromination was monitored spectrophotometrically, and aliquots were removed at intervals. The reactant (BrdUrd) and product (dUrd and CysdUrd) were separated by two-dimensional thin-layer chromatography, and the isotopic ratio of each was determined after elution with water. Values of k_T/k_H calculated for formation of dUrd and CysdUrd are 1.187 ± 0.006 and 1.156 ± 0.007, respectively; k_T/k_H from dehalogenation of BrdUrd is 1.174 ± 0.005 (n = 9). Upon completion of the reaction, CysdUrd constituted 8% of the product.

Effect of Folate Analogues on Dehalogenation. The effect of a variety of folate analogues on the rate of dehalogenation is shown in Table III. Most of the compounds tested have either no effect or are inhibitory to the reaction. MTX and aminopterin are notable exceptions, showing marked stimulatory effect on the reaction rate. Folic acid was observed to demonstrate intersecting hyperbolic noncompetitive inhibition (Segel, 1975) of the debromination reaction (data not shown).

Discussion

Treatment of BrdUMP and IdUMP with catalytic amounts of thymidylate synthetase in the presence of thiol reagents results in their facile conversion to dUMP, the natural substrate of the enzyme. By monitoring the loss of free thiol groups from the reaction mixture and, in the case of deiodination of IdUMP, the formation of halide anion, we have determined the stoichiometry of the reaction to be as follows:

$$\text{BrdUMP(IdUMP)} + 2\text{RSH} \rightarrow \text{dUMP} + (\text{RS})_2 + \text{HBr(HI)}$$

Neither CldUMP nor FdUMP serves as substrate for the reaction, and we could detect no reversal of the reaction.

The optimal pH for the reaction under the standard conditions described here using N-methylmorpholine buffer is pH 7.4. Since the rate of reaction increased at higher buffer concentrations, the pH optimum might vary depending on the buffer used. The rate of the reaction is also dependent upon the concentration of thiol reagents in the reaction mixture. With dithiothreitol or β-mercaptoethanol, approximately 0.01 M concentrations are sufficient for maximal activity; with cysteine, a tenfold higher concentration is required.[2]

Table III: Effect of Various Folate Analogues on the Rate of the Thymidylate Synthetase Catalyzed Dehalogenation of IdUMP and BrdUMP[a]

analogue	relative rate IdUMP	relative rate BrdUMP
none (control)	1.00	1.00
H_4folate	1.00	0.57
N^5-CHO-H_4folate	0.67	0.39
N^5-CH_3-H_4folate	0.90	0.89
folic acid	0.93	0.54
N^{10}-CH_3-folic acid	0.58	0.31
pteroic acid	0.89	0.93
homofolic acid	0.84	0.56
aminopterin	1.55	3.21
methotrexate	2.08	3.25
dichloromethotrexate	0.95	0.97
10-deazafolic acid	0.86	0.50
5,8-deaza-N^{10}-CH_3-folic acid	0.19	0.05

[a] The standard reaction mixture contained 40 μM substrate and 0.17 μM enzyme. All analogues were used at a concentration of 21 μM. Rates (initial velocities) were determined as ΔA_{290} per unit time.

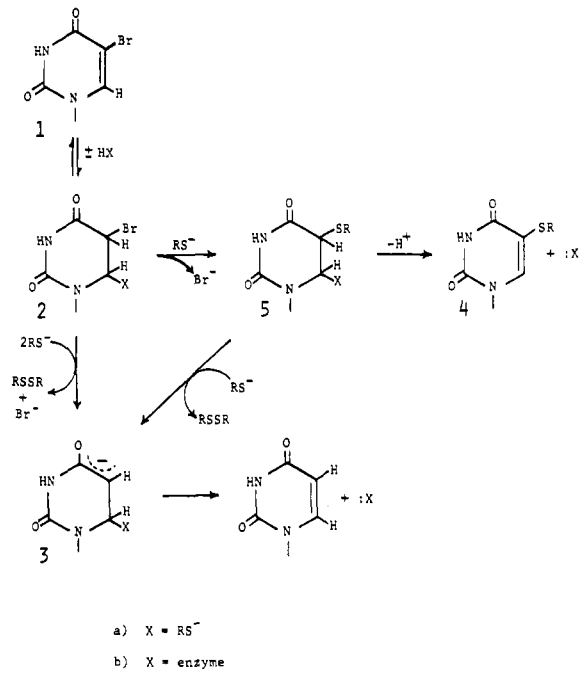
Unlike the normal enzymatic reaction, dehalogenation does not require the presence of CH_2-H_4folate.[3] Folate was observed to demonstrate intersecting hyperbolic noncompetitive inhibition kinetics (Segel, 1975), and, although the complexity of the system did not permit an exact evaluation of kinetic constants, it is clear that within the enzyme–folate–BrdUMP complex dehalogenation occurs, albeit at a slower rate than in the enzyme–BrdUMP complex. In contrast, methotrexate and aminopterin enhance the rate of dehalogenation of BrdUMP, demonstrating that the complex formed with these analogues is more reactive than the binary enzyme–BrdUMP complex. Whether these effects result from subtle catalytic differences when the folate analogues are bound to the same site of the enzyme as the cofactor or from different binding characteristics is currently unknown.

The K_m values of BrdUMP (5.7 μM) and IdUMP (1.4 μM) are similar to that of dUMP (3.0 μM) in the normal enzymic reaction, but V_{max} for dehalogenation is some 100-fold slower than it is for dTMP synthesis. Both BrdUMP and IdUMP are competitive inhibitors with respect to dUMP in the normal enzymic reaction, having K_i values of 1.4 and 1.6 μM, respectively (Wataya et al., 1977). Likewise, dUMP (K_i = 2.3

[2] The glutathione concentration in mammalian tissues is 0.5–10 mM (Jocelyn, 1972). This level of glutathione is sufficient to support the dehalogenation reaction described here, and it appears possible that an important pathway for biological catabolism of 5-bromo- and 5-iodouracil derivatives may involve their dehalogenation at the deoxyribonucleotide level by thymidylate synthetase. With CH_2-H_4folate, the overall reaction involves conversion of these halogenated nucleotides to dTMP and represents a route by which they may be utilized as precursors of DNA. This fact warrants consideration in experiments involving in vivo incorporation of 5-bromouracil into DNA.

[3] A standard assay for thymidylate synthetase activity involves monitoring the increase in UV absorbance at 340 nm which is characteristic of the formation of dTMP and H_2folate from dUMP and CH_2-H_4folate (Wahba & Friedkin, 1962). The accuracy of this assay is dependent upon the purity of the highly unstable cofactor and for this reason it often suffers from day-to-day variations using the same enzyme sample. Since the dehalogenation reaction has no requirement for folate cofactors, it may serve as a convenient assay for the enzyme in situations where the greater sensitivity of the conventional method is not necessary.

Scheme I



a) X = RS^-

b) X = enzyme

μM) and dTMP (K_i = 8.7 μM) are competitive inhibitors of dehalogenation of BrdUMP. These data, as well as similarities in the catalytic mechanisms of the reactions discussed below, support the view that the same site of the enzyme is involved in the binding of nucleotide substrates and in catalysis of both dehalogenation and dTMP formation.

During the enzymic debromination of BrdUMP in the presence of cysteine, a minor side product is formed which we identified to be CysdUMP. This is directly analogous to the chemical cysteine-promoted dehalogenation of BrUrd in which CysdUrd is formed as a side product (Wataya et al., 1973). Enzymic debromination of BrdUMP in the presence of other thiols also produces side products with chromatographic properties characteristic of 5-substituted dUMPs, and it is reasonable to conclude that these are also corresponding 5-alkylthio-5′-deoxyuridylates. For reasons unclear, no such side products are formed during the dehalogenation of IdUMP; this too is in accord with chemical studies in which the cysteine-promoted dehalogenation of IdUrd is not accompanied by significant formation of CysdUrd (H. Hayatsu, personal communication).

Numerous chemical studies on the thiol mediated dehalogenation of 1-substituted 5-bromo(iodo)uracils have resulted in the proposal of the mechanisms depicted in Scheme I (for a review, see Sander, 1977). The initial and perhaps rate-determining step almost certainly involves attack of thiolate anion at the 6 position of the heterocycle (**1a**); subsequent protonation of C-5 yields the 5-bromo-6-dihydrouracil **2a**. Two general pathways have been proposed to account for subsequent steps leading to the dehalogenated product. The first, E2 Hal, involves abstraction of bromonium (Br^+) ion from **2a** to provide intermediate **3a** and a sulfenyl halide. The latter would rapidly react with thiol to yield the halide ion and disulfide, and a β elimination of **3a** would yield products. The second mechanism (S_N2) involves nucleophilic displacement of Br^- from **2a** by thiolate to give the intermediate **5a**. Further reaction with RS^- would yield the oxidized thiol $(RS)_2$ and intermediate **3a**, which is common with the E2 Hal mechanism and would yield the dehalogenated pyrimidine upon β elimination. As previously mentioned, the cysteine-induced dehalogenation of BrdUrd is accompanied by formation of CysdUrd, the mechanism of which has been proposed to involve conversion of **5a** to **4a**. While the mechanism proposed for the thiol mediated dehalogenation is in accord with kinetic data, it has largely rested upon analogy with the extensively studied bisulfite mediated dehalogenation where direct evidence of 5,6-dihydropyrimidine intermediates has been firmly established. In the case of the thiol-mediated reaction, the proposed 5,6-dihydropyrimidine intermediates are unstable and their existence has not heretofore been directly demonstrated.

The observation of a deuterium isotope effect of greater than 10%, equivalent to a tritium effect of 15% (Swain et al., 1958), is generally accepted to provide conclusive evidence for carbon rehybridization at or before the rate-limiting step in a reaction. Thus, the magnitude of the inverse secondary isotope effect reported here for the cysteine-promoted nonenzymatic dehalogenation of 6-tritiated BrdUrd (k_T/k_H = 1.174) demonstrates the occurrence of sp^2 to sp^3 rehybridization at the 6-carbon of the pyrimidine heterocycle at or before the rate-determining step of the reaction and confirms the existence of 5,6-dihydropyrimidine intermediates early in this reaction pathway. Likewise, the substantial secondary isotope effect in the thymidylate synthetase catalyzed conversion of BrdUMP to dUMP (k_T/k_H = 1.212–1.258) convincingly demonstrates that an early catalytic event in the enzymic reaction also involves nucleophilic attack at the 6 position of the pyrimidine heterocycle and consequent formation of a 5,6-dihydropyrimidine intermediate. We therefore conclude that the thymidylate synthetase catalyzed dehalogenation reaction proceeds as described in Scheme Ib. Since the 5-alkylthiol-dUMP **4b** is formed during debromination of BrdUMP, it would also appear that intermediate **5b** is formed and that the S_N2 mechanism plays a significant role. As is the case with the nonenzymatic reaction, the relative contribution of the E2 Hal mechanism (**2b** → **3b**) is not easily determined.

In summary, studies of model chemical counterparts and interaction with mechanism-based inhibitors have resulted in general acceptance that the mechanism of thymidylate synthetase involves nucleophilic attack at the 6 position of dUMP to form 5,6-dihydropyrimidine intermediates in which normally inert moieties of the pyrimidine heterocycle are activated. However, until this time no direct evidence has been reported which demonstrates that reactions *catalyzed* by this enzyme proceed in this manner. In this and the accompanying paper, we demonstrate that thymidylate synthetase catalyzes the exchange of the H-5 of dUMP for protons of water and the dehalogenation of BrdUMP and IdUMP. From chemical precedent and analysis of α-secondary isotope effects, conclusive evidence has been obtained that these enzyme-catalyzed reactions proceed via nucleophilic attack at the 6 position of the heterocycle to form transient 5,6-dihydropyrimidine intermediates. These results are in complete accord with and verify important aspects of the mechanism originally proposed for this enzyme (Santi & Brewer, 1968; Pogolotti & Santi, 1974, 1977).

References

Collier, H. B. (1973) *Anal. Biochem. 56*, 310.

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

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

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

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

Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, Academic Press, New York.
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.
Melander, L. (1960) *Isotope Effects on Reaction Rates*, p 51, Ronald Press, New York.
Pogolotti, A. L., Jr., & Santi, D. V. (1974) *Biochemistry 13*, 456.
Pogolotti, A. L., Jr., & Santi, D. V. (1977) in *Bioorganic Chemistry* (van Tamelen, E. E., Ed.) Vol. 1, p 277, Academic Press, New York.
Pogolotti, A. L., Jr., Weill, S., & Santi, D. V. (1979) *Biochemistry 18* (preceding paper in this issue).
Sander, E. G. (1977) in *Bioorganic Chemistry* (van Tamelen, E. E., Ed.) Vol. 2, p 273, Academic Press, New York.
Santi, D. V., & Brewer, C. F. (1968) *J. Am. Chem. Soc. 90*, 6236.
Santi, D. V., McHenry, C. S., & Sommer, H. (1974) *Biochemistry 13*, 471.
Santi, D. V., Peña, V. A., & Lam, S. S. M. (1976) *Biochim. Biophys. Acta 438*, 324.
Segel, I. H. (1975) *Enzyme Kinetics*, p 166, Wiley, New York.
Swain, C. G., Stivers, E. C., Reuwer, J. J., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885.
Wahba, A. J., & Friedkin, M. (1962) *J. Biol. Chem. 12*, 3794.
Wataya, Y., & Santi, D. V. (1977) *Methods Enzymol. 46*, 307.
Wataya, Y., Negishi, K., & Hayatsu, H. (1973) *Biochemistry 12*, 3992.
Wataya, Y., Santi, D. V., & Hansch, C. (1977) *J. Med. Chem. 20*, 1469.

Isoleucyl Transfer Ribonucleic Acid Synthetase. Steady-State Kinetic Analysis†

John G. Moe‡ and Dennis Piszkiewicz*

ABSTRACT: A steady-state kinetic analysis was conducted of the overall aminoacylation reaction catalyzed by isoleucyl-tRNA synthetase. The patterns of Lineweaver–Burk plots obtained indicated that tRNA adds to the enzyme only after isoleucyl adenylate formation and pyrophosphate release. These kinetic patterns were consistent with the bi-uni-uni-bi Ping Pong mechanism generally accepted for this aminoacyl-tRNA synthetase, but they could also be accommodated by a mechanism in which a second molecule of L-isoleucine added to the enzyme between isoleucyl adenylate formation and aminoacylation of tRNA [Fersht, A.R., & Kaethner, M. M. (1976) *Biochemistry 15*, 818]. The values of the kinetic parameters favor the latter mechanism. The results of this kinetic analysis indicated that the affinity of isoleucyl-tRNA synthetase for Mg·ATP was enhanced upon binding of L-isoleucine and vice versa. It also indicated that the affinity of the enzyme for L-isoleucine is decreased upon binding tRNA and vice versa. The values of dissociation constants calculated for each of the substrates by this study generally compared well with those determined by other authors using a variety of kinetic and equilibrium methods.

The aminoacyl-tRNA synthetases are a group of enzymes which catalyze the esterification of amino acids to their cognate tRNAs in a process of critical importance to protein biosynthesis. The fidelity of translation of genetic information into protein is completely dependent on the specificities of these enzymes. The mechanisms used by these enzymes to ensure faithful esterification of amino acids to the proper tRNAs have therefore been the focus of many years of intensive study [for review articles see Schimmel (1973), Söll & Schimmel (1974), and Kisselev & Favorova (1974)].

The mechanism of the reaction catalyzed by isoleucyl-tRNA synthetase of *Escherichia coli* (EC 6.1.1.5) has been the subject of numerous studies; however, very few of these have attempted to study the overall reaction, as shown in eq 1.

$$\text{Ile} + \text{ATP} + \text{tRNA} \rightarrow \text{Ile-tRNA} + \text{AMP} + \text{PP}_i \quad (1)$$

Steady-state kinetic analysis of the overall reaction has been published in a preliminary, qualitative form (Takeda & Matsuzaki, 1974), but most of our knowledge of the overall reaction mechanism has come from studies of partial reactions. The generally accepted mechanism (Schimmel, 1973; Söll & Schimmel, 1974; Kisselev & Favorova, 1974) holds that isoleucine is first condensed with ATP to form the activated enzyme-bound intermediate, isoleucyl-AMP, followed by transfer of the amino acid to its cognate tRNA, as shown in eq 2. This reaction mechanism, in the terminology of Cleland

$$\begin{gathered}\text{E} + \text{Ile} + \text{ATP} \rightleftharpoons \text{E·Ile-AMP} + \text{PP}_i \\ \text{E·Ile-AMP} + \text{tRNA} \rightleftharpoons \text{Ile-tRNA} + \text{AMP} + \text{E} \quad (2)\end{gathered}$$

(1963a), is bi-uni-uni-bi Ping Pong. On the basis of their studies of partial reactions, Fersht & Kaethner (1976) modified this mechanism. In order to reconcile kinetic constants of various partial reactions, they proposed the addition of a second molecule of isoleucine to the enzyme after the activation step but before the formation of isoleucyl-tRNA, as shown in eq 3a–c.

$$\text{E·tRNA·Ile·ATP} \rightleftharpoons \text{E·tRNA·Ile-AMP} + \text{PP}_i \quad (3a)$$

† From the Department of Biological Chemistry, California College of Medicine, University of California, Irvine, Irvine, California 92717. *Received August 22, 1978.* This investigation was supported by Grant GM 19508 from the National Institutes of Health. This work was done by J.G.M. in partial fulfillment of the requirements for the Ph.D. degree.
‡ Present address: Ph.D. → M.D. Program, University of Miami School of Medicine, Miami, FL 33152.
* Present address: Department of Chemistry, Duquesne University, Pittsburgh, PA 15219.

0006-2960/79/0418-2804$01.00/0