91111-94-3; **70**, 91111-95-4; (6*S*)-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, 71165-90-7; (6*R*)-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, 88243-65-6; (carbethoxymethylene)triphenylphosphorane, 1099-45-2; phenyldiazonium chloride, 100-34-5; ethyl 10-[(dimethylamino)methylene]-4-oxo-4,6,7,8,9,10-hexahydroazepino[1,2-*a*]pyrimidine-3-carboxylate, 87932-12-5; 4*H*-pyrido[1,2-*a*]pyrimidin-4-one, 23443-10-9.

Interaction of N^4-Hydroxy-2′-deoxycytidylic Acid with Thymidylate Synthetase

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The interaction of dTMP synthetase with N^4-hydroxy-2′-deoxycytidylate (N^4-HOdCMP) has been investigated. With use of standard assay conditions, N^4-HOdCMP is a competitive inhibitor with an apparent K_i of 8.0 μM. Incubation of N^4-HOdCMP with dTMP synthetase in the presence of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) resulted in a rapid time-dependent inactivation of the enzyme which was not first order and the formation of complexes which could be isolated on nitrocellulose filter membranes. With use of radioactive ligands, the isolable native complex was shown to possess 2 mol of N^4-HOdCMP and 2 mol of CH_2-H_4folate/mol of dimeric enzyme; the apparent dissociation constant of N^4-HOdCMP was 1.0 μM. Ultraviolet difference spectroscopy of the ternary complex showed a loss of the pyrimidine chromophore which did not reappear upon denaturation with $NaDodSO_4$. The rate of dissociation of N^4-HOdCMP from the ternary complex was biphasic in which one-half of the initially bound ligand dissociated with $t_{1/2} \simeq 2.3$ min and the remainder with $t_{1/2} \simeq 13$ min. When the N^4-HOdCMP–CH_2-H_4folate–enzyme complex was denatured, one-half of the CH_2-H_4folate dissociated whereas all of the N^4-HOdCMP remained bound to the enzyme. Taken together, our results indicate that N^4-HOdCMP forms a covalent bond with dTMP synthetase and reveal an unusual asymmetry in the two subunits of the N^4-HOdCMP–CH_2-H_4folate–enzyme complex. It appears that one subunit is covalently bound to N^4-HOdCMP, which, in turn, is covalently linked to CH_2-H_4folate whereas the other subunit is covalently bound to N^4-HOdCMP but CH_2-H_4folate is bound by noncovalent interactions.

dTMP synthetase (EC 2.1.1.45) catalyzes the reductive methylation of dUMP to dTMP with concomitant conversion of CH_2-H_4folate to H_2folate. Because this enzyme is essential for de novo synthesis of dTMP, much effort has been invested in the development and studies of inhibitors (see ref 3–5). Thus far, the most successful inhibitors have been 5-substituted dUMPs, which, in general, bind reasonably well to the enzyme. further, some 5-substituted dUMPs act as potent mechanism-based inhibitors; such inhibitors reversibly bind to the enzyme and undergo events in a manner analogous to the normal catalytic reaction, ultimately leading to stable covalent complexes involving covalent bond formation between the catalytic thiol of the enzyme and the 6-position of the heterocycle. Although other analogues of dUMP have been examined, modifications at positions other than the 5-carbon generally lead to substantial decreases in affinity for the enzyme.[5] One interesting exception to this is N^4-HOdCMP, which reversibly binds to dTMP synthetase about as well as the substrate, dUMP.[6,7] In addition, it has been reported that incubation of N^4-HOdCMP and CH_2-H_4folate with the enzyme from chick embryo results in time-dependent inactivation of the enzyme and a resultant change from competitive to noncompetitive inhibition kinetics.[7] In this report, we describe studies of the interaction of N^4-HOdCMP with dTMP synthetase from *Lactobacillus casei* which demonstrate that it is a mechanism-based inhibitor of the enzyme and reveal an unusual asymmetry in binding of the analogue to the subunits of the dimeric enzyme.

Results

By use of the conventional spectrophotometric assay for *L. casei* dTMP synthetase and initiation of the reaction with enzyme, N^4-HOdCMP was a competitive inhibitor with respect to dUMP with an apparent K_i of 8.0 μM. When N^4-HOdCMP was incubated with the enzyme in the absence of CH_2-H_4folate, there was no loss of activity for as long as 60 min. As previously reported for the enzyme from chick embryo,[7] when the cofactor was included in the incubation buffer, there was a time-dependent loss of activity. For example, with use of 15 nM dTMP synthetase, 0.15 mM CH_2-H_4folate, and 80 μM N^4-HOdCMP, 50% loss of activity occurred at about 20 s. However, experimental difficulties did not permit a kinetic evaluation of the time-dependent inactivation. First, there were considerable uncertainties in determinations of remaining enzyme activities by initial velocity measurements; when excess dUMP was added to aliquots of the incubation mixture, the initial velocity increased with time ultimately approaching that of the control. As described below, this phenomenon is attributable to dissociation of the enzyme–inhibitor complex during the period of assay. Second, the loss of activity did not appear to be a first-order process and classical analysis of time-dependent inhibition was not possible.

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(1) Present address: Department of Dermatology, Veterans Administration Hospital, San Francisco, CA 94121.
(2) Abbreviations used are: N^4-HOdCMP, N^4-hydroxy-2′-deoxycytidylic acid; 5-CH_3-N^4-HOdCMP, 5-methyl-N^4-hydroxy-2′-deoxycytidylic acid; FdUMP, 5-fluoro-2′-deoxyuridylic acid, CH_2-H_4folate, (6*RS*)-L-5,10-methylenetetrahydrofolic acid; $(n\text{-}Bu)_4N^+HSO_4^-$, tetrabutylammonium hydrogen sulfate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate. All other abbreviations are as suggested by IUPAC.
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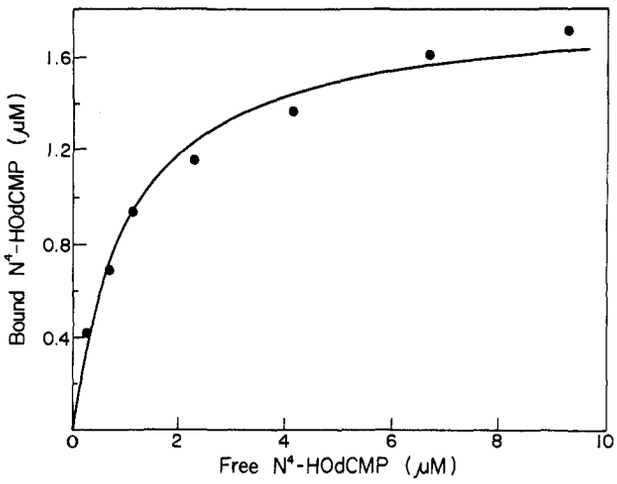

Figure 1. Titration of 0.82 μM dTMP synthetase in the presence of 0.15 mM CH_2-H_4folate with varying [2-^{14}C]N^4-HOdCMP. The concentration of labeled ligand was varied from 0.67 to 11 μM with nitrocellulose binding assays performed as described in the Experimental Section.

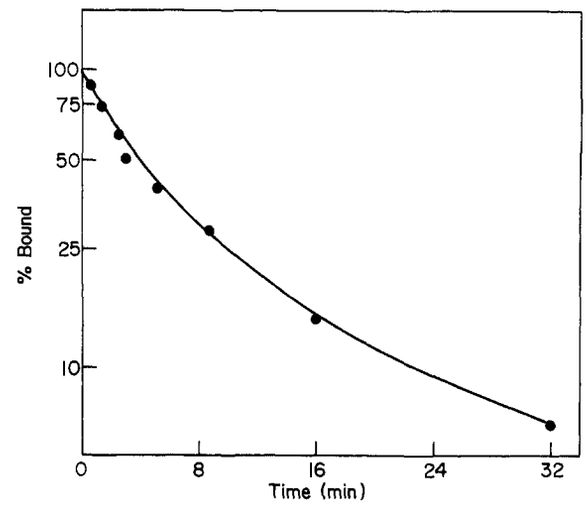

Figure 2. Dissociation of [2-^{14}C]N^4-HOdCMP from the N^4-HOdCMP–CH_2-H_4folate–dTMP synthetase complex. The complex was formed as described in the Results section with duplicate 50-μL filter assays performed at the indicated times. The points are experimental values, and the line is the best fit of the data to eq 1, where k_1 = 0.295 min^{-1} and k_2 = 0.054 min^{-1}.

When dTMP synthetase was incubated with CH_2-H_4-folate and [2-^{14}C]N^4-HOdCMP, radioactive complexes could be isolated on nitrocellulose filters; as in time-dependent inhibition of the enzyme, radioactive complexes could not be isolated if the cofactor was omitted. Although not extensively investigated, formation of isolable complexes was rapid; with use of 1.0 μM enzyme, 0.15 mM CH_2-H_4folate, and 24 μM [2-^{14}C]N^4-HOdCMP, complex formation was about 50% maximal at 30 s. In experiments described here, incubations were generally allowed to proceed for at least 10 min, which was more than sufficient for maximal formation of the complex. Figure 1 shows an experiment in which solutions containing dTMP synthetase (0.82 μM) and CH_2-H_4folate (0.15 mM) were incubated at 25 °C for 10 min with varying amounts of [2-^{14}C]N^4-HOdCMP (0.67–11 μM) and the complex assayed by adsorption to nitrocellulose. Scatchard analysis of this data (not shown) indicated that 1.90 mol of nucleotide was bound/mol of enzyme and an average apparent K_d of 1.0 μM for binding of N^4-HOdCMP to the two subunits of the enzyme.

The rate of dissociation of N^4-HOdCMP from the ternary complex was determined as follows. The complex was formed by incubating a solution (0.8 mL) containing 3.8 μM enzyme, 5.0 μM [2-^{14}C]N^4-HOdCMP, and 0.15 mM CH_2-H_4folate for 10 min at 25 °C. After filter binding assays of duplicate 50-μL aliquots, a 100-fold excess of unlabeled N^4-HOdCMP was added, and at intervals aliquots were analyzed by the nitrocellulose binding assay. As shown in Figure 2, the dissociation is biphasic. Independent dissociation of a ligand bound to two sites of a protein is described by eq 1; here, k_1 and k_2 are first-order

$$f_B = e^{-k_1 t} + e^{-k_2 t} \quad (1)$$

rate constants for dissociation from each site, and f_B is the fraction of bound ligand at a given time. Using nonlinear least-squares analysis, the best fit of the data in Figure 2 to eq 1 indicated that one-half the radioactivity dissociated with $t_{1/2}$ = 2.3 min and the other with $t_{1/2}$ = 13.0 min. When the complex was incubated for 3.5 h prior to addition of unlabeled N^4-HOdCMP, similar $t_{1/2}$ values of 2.5 and 14 min were obtained.

Although unlikely, it was considered possible that dTMP synthetase might catalyze modification of N^4-HOdCMP. When N^4-HOdCMP was substituted for dUMP in the normal enzymic reaction, there was no absorbance change at 340 nm for as long as 30 min; this rules out formation of H_2folate which would occur if 5-CH_3-N^4-HOdCMP were formed. In a more definitive experiment, excess enzyme (3.9 μM, 7.8 μM sites), CH_2-H_4folate (0.15 mM), and limiting [2-^{14}C]N^4-HOdCMP (4.5 μM) were incubated at 25 °C in the standard buffer. After 2 h, filter assay of an aliquot indicated that over 90% of the radioactive ligand was bound to the enzyme. A 100-fold excess of unlabeled N^4-HOdCMP was added and the solution kept at 25 °C for 8 h to permit complete dissociation of the radioactive ligand. HPLC analysis revealed that all of the dissociated radioactivity corresponded to N^4-HOdCMP.

The N^4-HOdCMP–CH_2-H_4folate–enzyme complex could also be detected by UV difference spectroscopy. Sample and reference cuvettes contained 6.5 μM enzyme (13 μM sites) and 27 μM CH_2-H_4folate in the standard buffer except 5 mM DTT was used instead of 2-mercaptoethanol; 25 μL of 364 μM N^4-HOdCMP (final concentration = 8.9 μM) was added to the sample cuvette and 25 μL of water was added to the reference cuvette. Using K_d = 1.0 μM, we calculate that, under these conditions, about 80% of the N^4-HOdCMP should be bound to the enzyme. As shown in Figure 3, there is a maximum at 330 nm and a minimum at 282 nm, which are characteristic of such complexes and are believed to result from pertubations of chromophores of the cofactor and enzyme.[8-10] In addition, as with the FdUMP–CH_2-H_4folate–enzyme complex there is a large decrease in absorbance at 272 nm, the λ_{max} of N^4-HOdCMP, suggesting that the 5,6-double bond may be saturated in the complex. When $NaDodSO_4$ was added to both cuvettes, giving a final concentration of 2%, there was a complete loss of the differential absorbance at 330 and 282 nm. Importantly, if the chromophore of N^4-HOdCMP was intact, the difference spectrum of the denatured complex should have shown a maximum at 272 nm with OD = 0.053. Instead, the residual absorbance at 272 nm was 0.012, corresponding to 23% of the total N^4-HOdCMP present. Since we have estimated that only

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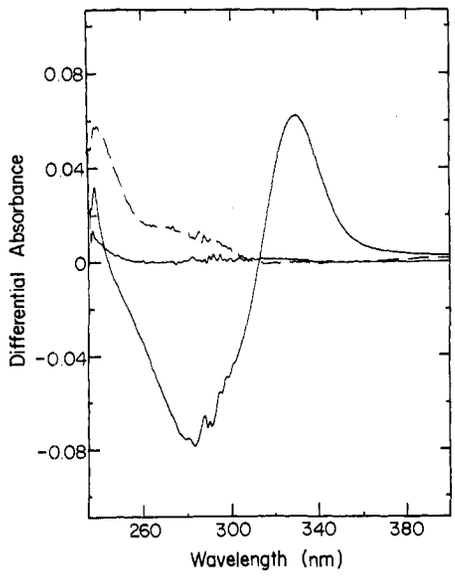

Figure 3. Ultraviolet difference spectra of the dTMP synthetase–N^4-HOdCMP–CH_2-H_4folate ternary complex vs. enzyme and CH_2-H_4folate. The solid line (–) represents the difference spectrum obtained from a solution containing 6.5 μM enzyme (13 μM sites), 27 μM CH_2-H_4folate, and 8.9 μM N^4-HOdCMP. The dashed line (--) represents the difference spectrum of the same solution after addition of $NaDodSO_4$ as described in the Results section.

80% of the total N^4-HOdCMP was bound to the enzyme prior to denaturation, the residual absorbance is attributed to the free nucleotide.

As previously described, filter binding assays indicated that about 2 mol of N^4-HOdCMP are bound/mol of dimeric dTMP synthetase. The following experiments were performed to determine the stoichiometry of CH_2-H_4folate binding and the stability of the complex toward denaturation. A double-labeled complex was formed by incubation of 50 μM [2-^{14}C]N^4-HOdCMP (9.36 mCi/mmol), 0.16 mM (6*R*)-L-CH_2-[6-^{3}H]H_4folate (30 mCi/mmol), and 5 μM dTMP synthetase for 2 h at 25 °C. Triplicate filter assays demonstrated that 2.05 ± 0.07 nmol of [2-^{14}C]N^4-HOdCMP and 1.95 ± 0.09 nmol of (6*R*)-L-CH_2-[6-^{3}H]H_4-folate were bound/nmol of dimeric enzyme; this corresponds to a ratio of bound nucleotide to bound cofactor of 1.03. $NaDodSO_4$ was added to the double-labeled complex to give a final concentration of 2% and the solution was incubated for 2 h at 37 °C. The macromolecular radioactivity isolated by Sephadex G-25 chromatography corresponded to 1.98 nmol of N^4-HOdCMP and 1.07 nmol of (6*R*)-L-CH_2-[6-^{3}H]H_4folate/nmol of enzyme. The molar ratio of bound nucleotide to bound cofactor corresponds to 1.85. A similar ratio of 1.80 was obtained after Sephadex G-25 chromatography of the complex which had been treated with 2% $NaDodSO_4$ for 24 h at 37 °C.

Discussion

The structure, mechanism, and inhibition of *L. casei* dTMP synthetase have been extensively studied over the past decade (see ref 3–5). Salient features of the enzyme which are relevant to the present study are as follows. First, the native enzyme consists of two identical subunits, each of which possesses binding sites for both the nucleotide substrate and cofactor, or related analogues. Second, the enzyme possesses a catalytic thiol which adds to the 6-position of dUMP in the normal enzymatic reaction and forms stable covalent adducts with certain analogues of dUMP. Third, there have been reports which suggest that there may be asymmetry in the binding of

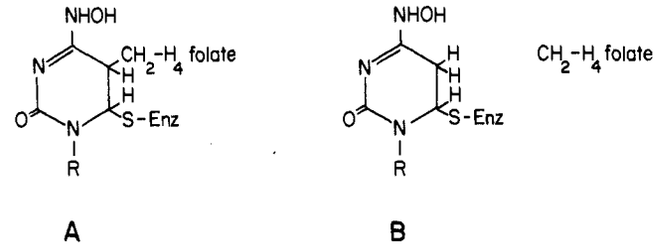

Figure 4. Proposed structures for the complexes formed between the subunits of dTMP synthetase and N^4-HOdCMP in the presence of CH_2-H_4folate; R = 5-phospho-2'-deoxyribosyl.

some ligands to the subunits of the enzyme.[11-14]

As reported for the dTMP synthetase from chick embryo,[7] we have shown that N^4-HOdCMP is a competitive inhibitor with respect to dUMP of the *L. casei* enzyme (K_i = 8.0 μM) and, in the presence of CH_2-H_4folate, results in a rapid time-dependent inactivation of the enzyme. As previously noted,[15] the K_i value of time-dependent inhibitors obtained by double-reciprocal plots may not reflect the true dissociation constant, but we do not know whether this is true in the present case. It has been reported that, by equilibrium dialysis, 1 mol of CH_2-H_4folate is bound/mol of enzyme in the presence of N^4-HOdCMP.[13,14] However, with use of radioactive ligands, complexes possessing 2 mol of CH_2-H_4folate and 2 mol of N^4-HOdCMP/mol of dimeric enzyme have been isolated on nitrocellulose membranes. The apparent K_d of N^4-HOdCMP in the isolable complex was determined to be 1.0 μM, some eightfold lower than the K_i value obtained by kinetic experiments. In the absence of CH_2-H_4folate, binary N^4-HOdCMP–enzyme complexes are not isolable by this method. It appears that after formation of rapidly reversible ternary complexes in which N^4-HOdCMP behaves as a competitive inhibitor, conversions occur which lead to a rapid time-dependent inhibition and formation of complexes isolable on nitrocellulose membranes. Interestingly, the rate of dissociation of N^4-HOdCMP from the isolable complex is biphasic; approximately one-half of the bound radioactivity dissociates with $t_{1/2} \simeq 2.3$ min and the remainder with $t_{1/2} \simeq 13$ min.

Two lines of evidence demonstrate that N^4-HOdCMP is covalently bound to dTMP synthetase. First, in the UV difference spectrum of the complex vs. enzyme and CH_2-H_4folate, there is a loss of absorbance of the pyrimidine chromophore which does not reappear upon denaturation with $NaDodSO_4$. Second, when the complex formed with [2-^{14}C]N^4-HOdCMP is denatured, the radioactivity remains bound to the protein. From what is known of the mechanism of dTMP synthetase and its interaction with inhibitors such as FdUMP, it is reasonable to conclude that a covalent bond is formed between the catalytic thiol of the enzyme and the 6-position of N^4-HOdCMP.

An unusual feature of the N^4-HOdCMP–CH_2-H_4folate–enzyme complex is that upon denaturation, one-half of the bound CH_2-H_4folate dissociates from the enzyme whereas all of the N^4-HOdCMP remains covalently bound. We

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interpret this result to indicate that in the native complex one of the subunits is covalently bound to N^4-HOdCMP which, in turn, is covalently linked to CH_2-H_4folate whereas the other is covalently bound to N^4-HOdCMP but CH_2-H_4folate is bound by noncovalent interactions. By analogy to other covalent complexes formed with this enzyme, Figure 4 shows likely structures for these complexes. For one of the subunits, the catalytic thiol of the enzyme would be covalently bound to the 6-position and the CH_2-H_4folate would be covalently linked to the 5-position of the heterocycle to give the structure shown in Figure 4A or, perhaps, a more stable tautomer. This is analogous to the structure of the ternary complexes formed with FdUMP and related analogues.[3,4] The catalytic thiol of the other subunit would also be covalently bound to the 6-position of N^4-HOdCMP to give the structure shown in Figure 4B or perhaps a more stable tautomer; however, in this complex the cofactor would be associated with the enzyme by noncovalent interactions which would be disrupted upon denaturation. Covalent binary complexes analogous to that shown here have been reported for FdUMP[16] and 5-nitro-dUMP.[17]

The asymmetrical nature of the N^4-HOdCMP–CH_2-H_4folate–dTMP synthetase complex supports previous work which suggested that the two subunits of the enzyme may interact in a manner which affects ligand binding.[10-12] The asymmetry also provides an explanation for why time-dependent inactivation of the enzyme by N^4-HOdCMP is not a first-order process and why dissociation of N^4-HOdCMP from the complex shows biphasic kinetics with each rate corresponding to about one-half of the amount of ligand which was initially bound.

Although N^4-HOdCMP is not among the most potent inhibitors of dTMP synthetase, it would be of interest to investigate certain analogues of this nucleotide. For example, certain modifications at the 5-position of dUMP which enhance covalent bond formation with dTMP synthetase might have a similar effect on N^4-HOdCMP. Also, since N^4-HOdCMP represents one of the few 4-substituted dUMP analogues which inhibits dTMP synthetase, further modifications at the 4-position of this analogue could provide more potent inhibitors. In addition to their potential as chemotherapeutic agents, as described here for N^4-HOdCMP, such analogues could also reveal yet undiscovered features of mechanistic aspects of dTMP synthetase.

Experimental Section

Materials. Thymidylate synthetase from a methotrexate resistant strain of *L. casei*[18] was purified as described elsewhere.[19] (6*R*)-L-CH_2-[6-^{3}H]H_4folate (30 mCi/mmol) was prepared enzymatically as previously described.[20] [2-^{14}C]dCMP (29.0 mCi/mmol) was purchased from New England Nuclear. An authentic sample of N^4-HOdCMP was a gift from Dr. F. Maley. Other materials have been previously described[20] or were of the highest grade commercially available.

HPLC separations were performed at 30 °C on a 4.6 × 250 mm LiChrosorb RP-18 column (Altex) using a buffer consisting of 5 mM $(n\text{-Bu})_4N^+HSO_4^-$ and 5 mM KH_2PO_4 (pH 7) as the low concentration eluant and the same buffer, containing 40% MeOH, in the high concentration eluant. A 25-min linear gradient (2 mL/min) going from 0 to 25% of the high concentration buffer gave the following retention volumes: dCMP, 21.5 mL; 5-CH_3dCMP, 25.9 mL; dUMP, 32.0 mL; N^4-HOdCMP, 38.6 mL; and 5-CH_3-N^4-HOdCMP, 46.7 mL.

[2-^{14}C]N^4-HOdCMP was prepared by a modification of the procedure of Maley and Maley.[21] A solution consisting of [2-^{14}C]dCMP (0.05 mC, 9.36 mCi/mmol) in 0.25 mL of 2 N NH_2OH (pH 6.5) was kept at 37 °C for 24 h. The reaction was evaporated in vacuo to dryness followed by three 0.20-mL additions of glacial HOAc with evaporation in vacuo after each addition. The crude product was taken up in 2 mL of H_2O and applied to a 0.4 × 7 cm Bio-Rad AG1-X8 (formate) ion-exchange column followed by elution with a 150-mL linear gradient from 0 to 4 M formic acid. The fractions containing radioactivity eluting at about 2 M HCO_2H were pooled, lyophylized to dryness, and dissolved in 0.3 mL of H_2O; on the basis of recovered radioactivity, the yield was 80%. HPLC analysis demonstrated that all radioactivity coeluted with authentic N^4-HOdCMP.

Unlabeled N^4-HOdCMP was prepared by heating a 1-mL solution containing 2 N NH_2OH (pH 6.5) and 11.4 mg (0.035 mmol) of dCMP at 37 °C for 24 h. The reaction was evaporated to dryness in vacuo followed by addition of 5 mL of glacial acetic acid. The resulting suspension was heated to 35 °C for 40 min, filtered free of insolubles, and evaporated to dryness in vacuo. The crude product was dissolved in 5 mL of H_2O and applied to a 1 × 28 cm Bio-Rad AG1-X8 (formate) column followed by elution with a 500-mL linear gradient from 0 to 4 M HCO_2H. The fractions containing product eluting at ca. 2 M HCO_2H were pooled, evaporated to dryness, and dissolved in 3 mL of H_2O. The product had UV spectra as reported for N^4-HOdCMP[21] and HPLC analysis gave one peak (yield 96%) which coeluted with authentic N^4-HOdCMP (RV = 38.6 mL).

Enzyme Assays. Initial velocity measurements of dTMP formation were performed spectrophotometrically at 25 °C.[22] The standard buffer contained 50 mM TES (pH 7.4), 25 mM $MgCl_2$, 75 mM 2-mercaptoethanol, 6.5 mM HCHO, and 1 mM EDTA. For time-dependent inactivation experiments, the enzyme (14 μM) and specified concentrations of N^4-HOdCMP were incubated with 0.20 mM CH_2-H_4folate in a 3-mL cuvette at 25 °C. At various times 0.03 mL of 100 mM dUMP was added (final concentration 1 mM), and initial velocities of dTMP formation were monitored.

Isolation of the [2-^{14}C]N^4-HOdCMP–CH_2-H_4folate–dTMP synthetase complex was performed with nitrocellulose filter membranes (Bac-T-Flex, 2.4-cm filter disks) as described elsewhere.[23] Mixtures contained 2–4 μM dTMP synthetase, 0.28 mM CH_2-H_4folate, and varying amounts of [2-^{14}C]N^4-HOdCMP (29.0 mCi/mmol unless otherwise specified) were incubated at 25 °C in the standard buffer. Aliquots (50–100 μL) were removed, applied to damp filters, and washed five times with 1-mL portions of 75 mM potassium phosphate (pH 7.4). The filters were dissolved in 10 mL of ACS and counted in a liquid scintillation counter; bound radioactivity was corrected for filtration efficiency,[23] which was determined to be 80%. Isolation of the complex by Sephadex G-25 chromatography was performed as described elsewhere.[20]

Instrumentation. Initial velocity measurements and ultraviolet difference spectra were determined with a Hewlett-Packard Model 8450 UV/vis spectrophotometer. Radioisotopes were counted with an Isocap 300 liquid scintillation counter; disintegrations per minute calculations were performed by the external standard ratio method. HPLC analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph equipped with a fixed wavelength (254 nm) UV detector.

Acknowledgment. This study was supported by USPHS Grant CA 14394 from the National Cancer Institute.

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