It was found that 0.073 M saline afforded optimal extraction of hepatic and splenic iron, superior to isotonic saline or other molarities between 0.014 to 0.145. The recoveries of exogenous iron in the 2–5-μg range were 82–104% for tissues and 84–114% for excreta (12 determinations for each tissue and excreta). The precision of atomic absorption determination indicated a 2% coefficient of variation for urine and 10% for liver homogenates. In 36 separate bioassays with DFB at a dose of 250 mg/kg, the mean decrease ± SE in liver iron was 23.8 ± 4.7% and the increase in urinary iron was 270 ± 14%.

The following compounds failed to exhibit iron chelating activity when tested in the above screen: 2,2′-dihydroxy-5,5′-dimethylbiphenyl (**7**),[22,23] 2,6-bis(2-hydroxy-5-methylphenyl)-4-methylphenol (**8**),[22,23] 2-[(*o*-hydroxybenzyl)amino]phenol (**9**),[23] 2-[[(*o*-hydroxyphenyl)imino]methyl]phenol (**10**),[20] 2-(*o*-hydroxyanilino)-Δ^2-penten-4-one (**11**),[24] 2-[[(*o*-carboxyphenyl)imino]methyl]pyridine (**12**),[25] 2-[[(*o*-carboxyphenyl)amino]methyl]pyridine (**13**), *N*,*N*′-bis(2,3-dihydroxybenzoyl)-1,4-diaminobutane (**14**), *N*,*N*′-bis[4-(2,3-dihydroxybenzamido)butyl]-2,3-dihydroxyterephthalic acid diamide (**15**), (*o*-aminobenzal)acethydrazide (**16**), (*o*-aminobenzal)benzhydrazide (**17**), 2-[[(*o*-hydroxyphenyl)imino]methyl]-8-acetoxyquinoline (**18**), 2-[[(*o*-hydroxyphenyl)imino]methyl]pyridine (**19**), 2-[[(*o*-hydroxyphenyl)amino]methyl]pyridine (**20**), 2-[(*o*-hydroxybenzyl)imino]pyridine (**21**), 2-[(*o*-hydroxybenzyl)amino]pyridine (**22**), 2-(8-quinolylamino)-Δ^2-penten-4-one (**23**),[26] 8-[(α-pyridylmethylene)amino]quinoline (**24**).[27]

Acknowledgment. We are grateful to Dr. D. G. Badman (NIADDK) and Dr. W. F. Anderson (NHLBI, NIH) for their encouragement and assistance to the program, which was supported by the NIADDK, NIH, under Contract No. NO1-AM-0-2207. The chelator **3** was kindly provided by W. R. Grace and Co.

Registry No. **1**, 10328-28-6; **1**·2Li, 102212-18-0; **1**·2HCl, 102212-19-1; **1**·2Na, 102212-20-4; **2**, 21972-77-0; **3**, 63651-93-4; **4**, 102212-27-1; **5**, 35998-29-9; **5** (dimethyl ester), 85120-52-1; **5** (diethyl ester), 102212-10-2; **5** (diisopropyl ester), 102212-11-3; **5** (dibenzyl ester), 102212-12-4; **5** (dipentyl ester), 98318-32-2; **5** (dimethyl ester diacetate), 102212-13-5; **5** (di-*tert*-butyl ester), 102212-14-6; **5** (di-*tert*-butyl ester diacetate), 102212-17-9; **6**, 1170-02-1; **6** (dimethyl ester), 90044-13-6; **6** (diethyl ester), 98318-25-3; **9**, 36282-74-3; **13**, 5691-02-1; **13** (imino precursor), 78604-78-1; **14**, 71636-73-2; **14** (bis dimethoxy precursor), 73630-96-3; **15**, 102212-21-5; **16**, 102212-22-6; **17**, 102212-23-7; **18**, 102212-25-9; **19**, 3860-58-0; **20**, 102212-26-0; **21**, 1823-47-8; **22**, 70301-52-9; $PhCH_2O_2CCl$, 501-53-1; $HO_2CCH_2NH(CH_2)_2NHCH_2CO_2H$, 5657-17-0; $HO_2CCH_2N(Z)(CH_2)_2N(Z)CH_2CO_2H$, 102212-15-7; *t*-$BuO_2CCH_2NH(CH_2)_2NHCH_2CO_2Bu$-*t*, 102212-16-8; *o*-$AcOC_6H_4CH_2Br$, 704-65-4; *o*-$MeOC_6H_4NH(CH_2)_2NHC_6H_4OMe$-*o*, 37460-52-9; MeO_2CCH_2Br, 96-32-2; *o*-$HOC_6H_4CH{=}NC_6H_4OH$-*o*, 1761-56-4; $AcNHNH_2$, 1068-57-1; $NH_2(CH_2)_4NH_2$, 110-60-1; *o*-$NH_2C_6H_4OH$, 95-55-6; $PhCONHNH_2$, 613-94-5; Fe, 7439-89-6; 2,3-dimethoxybenzoic acid, 1521-38-6; 2,3-dimethoxyterephthalic acid, 7168-95-8; *N*-(2,3,dimethoxybenzoyl)-1,4-diaminobutane, 102212-24-8; 8-acetoxyquinoline-2-carboxaldehyde, 36456-52-7; 2-pyridinecarboxaldehyde, 1121-60-4; 2-aminopyridine, 504-29-0; salicylaldehyde, 90-02-8.

Supplementary Material Available: Figure 2 shows the structure of the chelators **7–24** (1 page). Ordering information is given on any current masthead page.

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Spin Probes as Mechanistic Inhibitors and Active Site Probes of Thymidylate Synthetase

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C-4- and C-5-substituted analogues of dUMP were examined as inhibitors of thymidylate synthetase and as topographical probes of its active site by electron spin resonance (ESR). The C-5-substituted spin-labeled analogues pDUAP (**2**) and a pDUTT (**3**) as well as the unlabeled AAdUMP (**1**) were competitive inhibitors with K_i's of 9.2, 89, and 7.9 μM, respectively. The C-4-spin-labeled pls^{4}dU (**4**) displayed no inhibition activity. Scatchard plots as determined by ESR gave similar association constants for **2** (K_{assoc} = 1.9 × 10^5 M^{-1}) and for **3** (K_{assoc} = 2.4 × 10^5 M^{-1}). Both of these values are similar to the K_{assoc} of FdUMP indicating that the bulky substituent in position 5 does not interfere with the formation of the binary complex. The enzyme–C-5-spin-labeled nucleotide complexes indicate the presence of similarly immobilized spin labels by ESR, whereas no binding and immobilization were noticed with the C-4-spin-labeled nucleotide. A model for the active-site geometry of the enzyme was derived which suggests that the C-5 substituents point toward the opening of the binding cavity whose depth is at least 12 Å. Also, the approximate 10-fold increased inhibitory activity of **2** as compared to that of **3** may be attributed to the significant electron withdrawing properties of the C-5 substituent in **2**. Finally, the set of probes used for the binding and inhibition of thymidylate synthetase gives direct experimental evidence that an electron-withdrawing C-5 substituent primarily affects the formation of the ternary complex and will not substantially influence the stability of the binary complex.

Thymidylate synthetase, the key enzyme in the sole de novo pathway for thymidylate synthesis, is recognized as a clinically effective target enzyme for the control of neoplastic cell proliferation.[1,2] The active form of the drug, 5-fluorouracil, in the chemotherapeutic control of certain forms of cancer at the enzymatic level has been shown to be 5-fluoro-2′-deoxyuridylic acid (FdUMP), which acts as a powerful inhibitor of the enzyme. The enzyme catalyzes the conversion of dUMP and dTMP in the presence of the cofactor 5,10-methylene tetrahydrofolate (Scheme I).

The generally accepted mechanism of action of the enzyme involves the initial formation of a reversible binary

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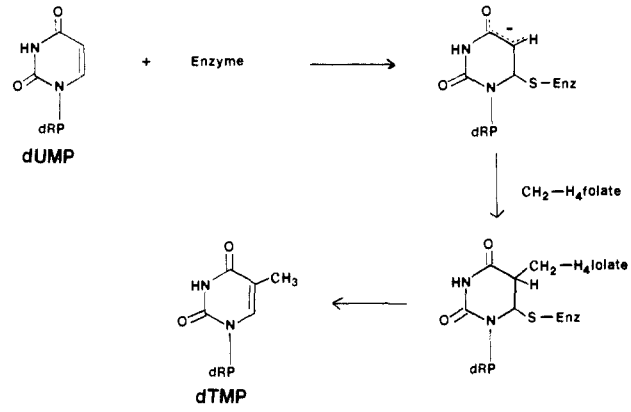
Scheme I



complex between the enzyme and the substrate[3-5] (Scheme I). Subsequent reaction with the cofactor results in the formation of the enzyme–substrate–cofactor ternary complex as a transient intermediate in the enzymatic reaction. Mechanism studies with the purified enzyme from *Lactobacillus casei* support the view that this ternary complex is formed by the interaction of the thiol group of an active-site cysteine with carbon 6 and the cofactor with carbon 5 of the pyrimidine ring.[3-7] Mechanism-based inhibitors undergo a similar sequence of reactions resulting in the formation of analogues binary and ternary complexes.

Most of the binding studies with the enzyme have been performed on the ternary complex formed,[8-10] although the enzymatic reaction has been shown to proceed through an ordered binding sequence of nucleotide binding to the enzyme first followed by the cofactor.[11] More recently, Lewis et al.[12,13] reported observing the formation of the binary complex between the enzyme and FdUMP using light spectroscopic methods and ^{19}F NMR.

Since it has been established that large substituents in the 5-position of the pyrimidine ring do not interfere with the active site binding[14] and that electron-withdrawing substituents at this position enhance binding,[1,15,16] spin-labeled analogues of dUMP substituted at C-5, pDUAP (**2**) and pDUTT (**3**), as well as the C-4 analogue pls^{4}dU (**4**) were examined as in vitro inhibitors of thymidylate synthetase and as probes to monitor the binding of the

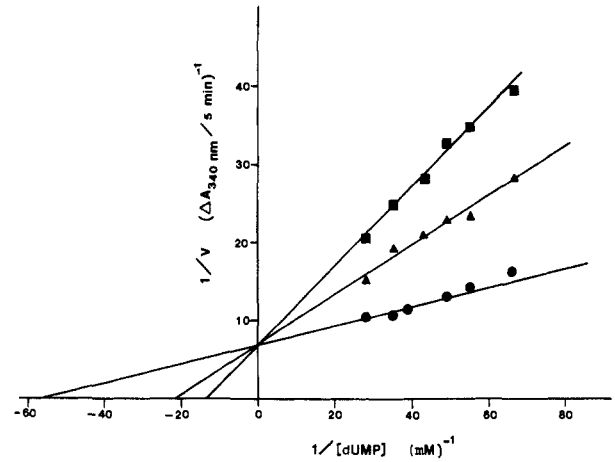
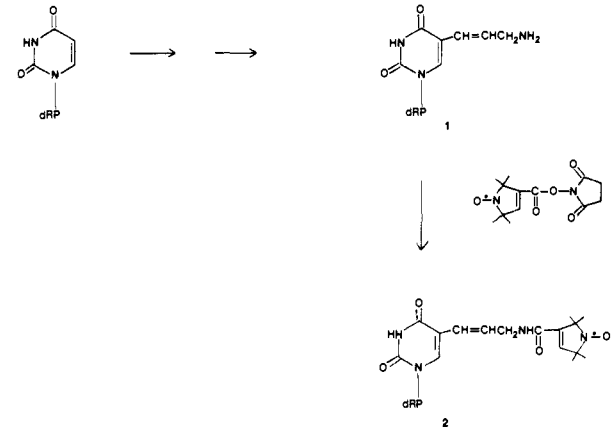


Figure 1. Double-reciprocal plot of the inhibition of thymidylate synthetase in the presence of AA-dUMP (**1**), with dUMP as the variable substrate: (●) no inhibitor, (▲) 1.2×10^{-5} M inhibitor, (■) 2.5×10^{-5} M inhibitor. The reaction mixtures contained 100 mM β-mercaptoethanol, 15 mM HCHO, 25 mM Mg^{2+}, 35 mM Tris buffer, pH 7.4, 0.7 mM EDTA, and 0.6 unit of enzyme.

Scheme II



nucleotides to the enzyme by means of electron spin resonance (ESR) spectroscopy. In addition, a precursor of **2**, AA-dUMP (**1**), was also examined as an inhibitor. The results of the inhibition and binding studies are correlated to the structure of these analogues and to the mechanism of action of the enzyme. On the basis of ESR binding studies, the mode of binding of nucleotides to the enzyme to form the binary complex is proposed. The spin-labeled analogues and ESR spectroscopy are shown to present a potential means of probing the size and topography of the active site and for use in further mechanistic studies.

Results

Chemistry. Compound **1** was synthesized on the basis of the procedure employed by Langer et al.[17] for the synthesis of the corresponding triphosphate (Scheme II). Purification of this monophosphate differs from that of other monophosphates in that at the pH of the solvent used for elution of the nucleotides from the DEAE-Sephadex column (NH_4HCO_3), the amine group is positively charged, and hence it elutes well before other monophosphates typically do and the nucleotide exhibits a blue fluorescence during paper chromatography. Conversion of **1** to the spin-labeled form of **2** required that **1** has Na^+ as its cation rather than NH_4^+ since the latter interferes with the spin-labeling reaction. A Na^+-Dowex column was

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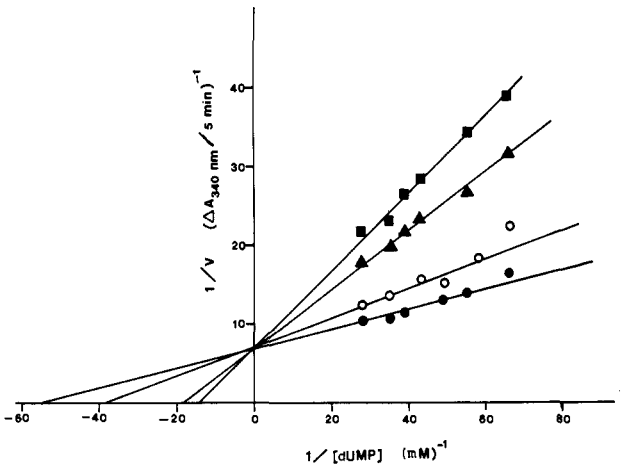
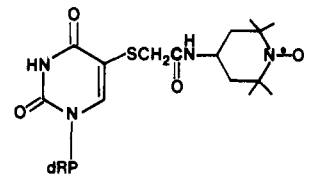
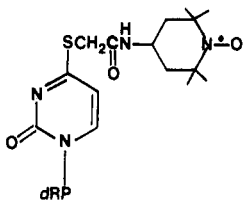


Figure 2. Double-reciprocal plot of the inhibition of thymidylate synthetase in the presence of the C-5-substituted spin-labeled analogues, with dUMP as the variable substrate: (●) no inhibitor, (○) 3.1×10^{-5} M pDUTT (3), (▲) 1.8×10^{-5} M pDUAP (2), (■) 2.6×10^{-5} M (2). The reaction mixtures contained 100 mM β-mercaptoethanol, 15 mM HCHO, 25 mM Mg^{2+}, 35 mM Tris buffer, pH 7.4, 0.7 mM EDTA, and 0.6 unit of enzyme.

employed for this conversion. Compound 3 was synthesized from deoxyuridine by a combination of published procedures.[19-22]



3



4

Inhibition Studies. Three spin-labeled analogues of dUMP were examined as possible inhibitors of thymidylate synthetase activity in vitro. The enzymatic activity was monitored by the spectrophotometric assay of Wahba and Friedkin.[23] The K_m value for the substrate dUMP toward this enzyme preparation was 1.9×10^{-5} M at 25 °C. In the presence of the cofactor, analogues 1 and 2 were good competitive inhibitors of the enzyme. By use of double-reciprocal plots (Figures 1 and 2), the inhibition constants (K_i) were calculated to be 7.9 and 9.2 μM, respectively, for compounds 1 and 2.

Analogue 3 was a very weak inhibitor of the enzymatic activity with a K_i value of 89 μM (Figure 2). The K_i/K_m ratio of 5 indicated that this analogue had a much lower affinity for the enzyme in the presence of the cofactor than the substrate, and hence, 3 competed only weakly for the active site of the enzyme in the presence of the substrate.

The C-4-substituted analogue 4 exerted no effect on the activity of the enzyme. Since the spin-labeled substituents are identical in both 3 and 4 except for the position of the substitution, and spin label itself was not inherently responsible for the lack of inhibitory activity of this derivative. However, this substitution at C-4 did result in the loss of the N-3 proton, which is believed to be essential for the binding of dUMP and its analogues to the enzyme.[15,16] An unfavorable geometry of the molecule due to the

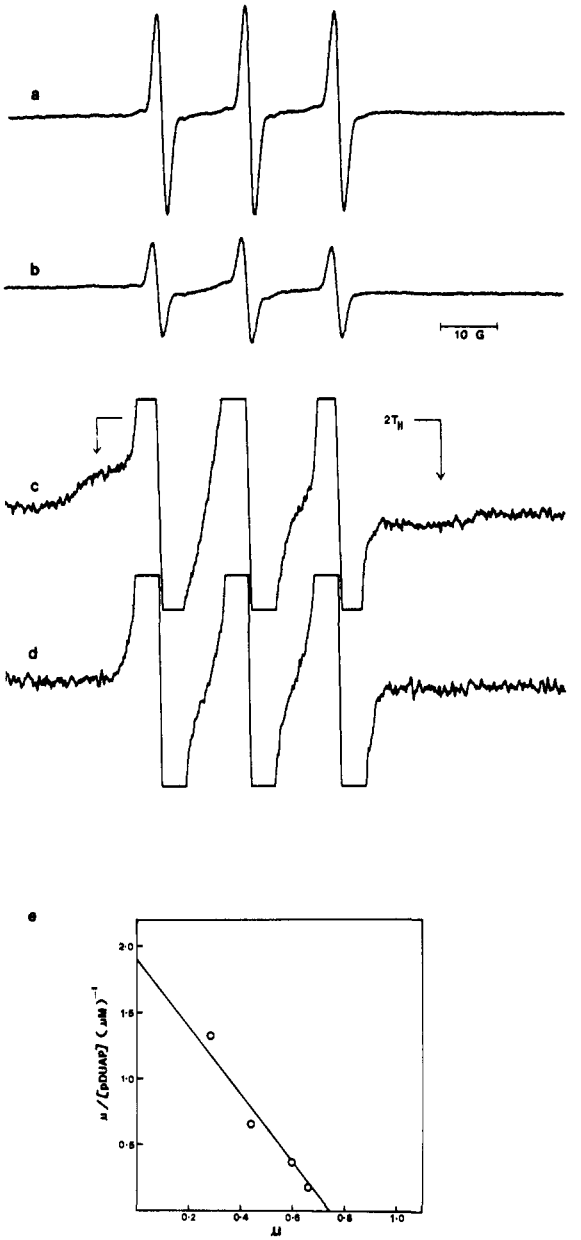


Figure 3. ESR titration of pDUAP (2) with thymidylate synthetase in 50 mM Tris buffer, pH 7.4, 15 mM DTT, and 2 mM EDTA: (a) free ESR spectrum of 2 (6.6 μM), (b) 6.6 μM 2 plus 9.9 μM enzyme, (c) high-gain spectrum of b, measured at 10 times the receiver gain and 2 times the modulation amplitude of the normal three-line spectrum. The immobilized nitroxide peaks are indicated by arrows and the distance between these two hyperfine extrema was 60 ± 0.5 G: (d) high-gain spectrum of 6.6 μM 2 plus 9.9 μM enzyme plus 90 μM FdUMP, (e) Scatchard plot of the binding of 2 (3.7–37 μM) to the enzyme (5.6 μM). Typical instrumental parameters were 13.3-mW microwave power, 9.42-GHz frequency, 1.0–5.0-G modulation amplitude, 3360-G applied field, and 100-G scan range.

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presence of the substituent at C-4 could also play a role in the absence of inhibition observed with the analogue.

ESR Binding Studies. The spin-labeled analogues were used as probes to monitor the binary complex formation between the enzyme and nucleotide by means of ESR spectroscopy. Purified, DTT-activated enzyme formed such a bound complex with **2** as evidenced by the decrease in the narrow line, free spectral amplitude upon binding (Figure 3b), which was also accompanied by a line shape change. In order to observe the bound species, a "high-gain" spectrum was recorded at 10-fold the receiver gain. The high-gain spectrum of **2** and thymidylate synthetase (Figure 3c) showed the presence of the strongly immobilized nitroxide spectral peaks at both high and low fields (indicated by arrows). The separation between these two hyperfine extrema, $2T_{11} = 60 \pm 0.5$ G, is related to the tumbling rate of the spin-label ring moiety.[24] The specificity of binding of **2** to the active site of the enzyme was established by the addition of a large excess of FdUMP, which resulted in the disappearance of the bound spectral component (Figure 3d) and a simultaneous reversal to the free-label spectrum. It has been established by other investigators that FdUMP binds to the same site on the enzyme as the normal substrate, dUMP.[1,2] Hence, it can be concluded that the competitive inhibitor **2** also binds to the active site of the enzyme.

A Scatchard[25] plot of the binding of **2** to thymidylate synthetase is presented in Figure 3e. The amount of bound and free species was calculated from the center field (h_0) peak heights after correcting for some loss of ESR signal that was not due to binding. This correction factor was determined by carrying out the titrations in the presence of a very large molar excess of the competitor, FdUMP. The h_0 peak height observed was correlated directly to the amount of the free analogue in the reaction mixture with h_0 measured in the absence of the enzyme corresponding to the total free state of the spin analogue. An association constant of 1.9×10^5 M^{-1} for the binding of **2** to the enzyme was obtained with a binding site stoichiometry of 0.75, indicating the availability of one binding site for the binary complex formation with **2**.

The weak inhibitor **3** was also found to bind to the enzyme in a manner similar to that observed with compound **2**. Decrease in the free spectral component accompanied by line shape change was observed in the presence of the enzyme (Figure 4b). The high-gain spectrum of **3** and the enzyme (Figure 4c) showed the strongly immobilized spectral peaks with a $2T_{11}$ value of 61 ± 0.5 G, indicating a slightly stronger binding interaction than that observed with the enzyme and compound **2**, attributable to the slightly shorter length and different dihedral angles of the substituent at C-5. Addition of a large excess of the competitor, FdUMP, resulted in the abolishment of the bound component demonstrating the specificity of binding (Figure 4d). A Scatchard plot of the binding of **3** to the enzyme, presented in Figure 4e, gave an association constant for this binding of 2.4×10^5 M^{-1} for a single binding site.

The noninhibitor, compound **4**, did not bind to the enzyme under conditions similar to those employed in the other binding experiments (results not shown). This established the binding of nucleotide analogues to the enzyme to be discriminatory, with the noninhibitors prohibited from forming the binary complex, the first step in the enzymatic mechanism.

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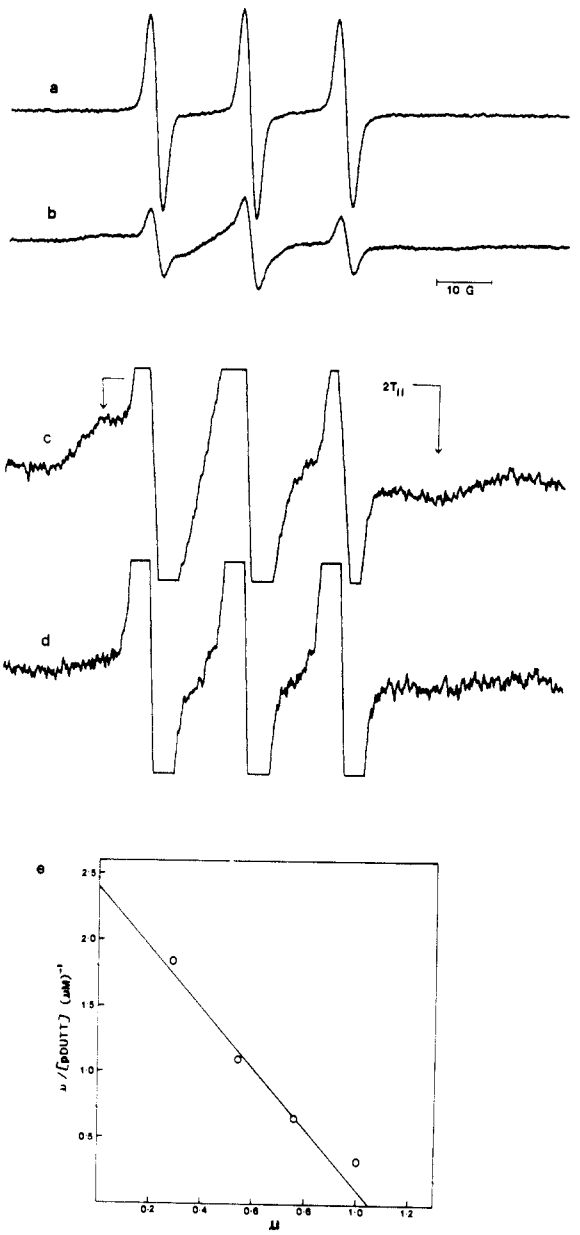

Figure 4. ESR titration of pDUTT (**3**) with thymidylate synthetase in 50 mM Tris buffer, pH 7.4, 15 mM DTT, and 2 mM EDTA: (a) free spectrum of **3** (5.7 μM), (b) 5.7 μM **3** plus 9.9 μM enzyme, (c) high-gain spectrum of b. The hyperfine separation was 61 ± 0.5 G, (d) high-gain spectrum of 5.7 μM **3** plus 9.9 μM enzyme plus 90 μM FdUMP, (e) Scatchard plot of the binding of **3** (3.2–32 μM) to the enzyme (5.6 μM).

Table I. Summary of Inhibition and ESR Binding Studies with Thymidylate Synthetase

compd	inhibition: K_i, M	ESR: K_{assoc}, M^{-1}	ESR: $2T_{\|}$
AA-dUMP (1)	7.9×10^{-6}		
pDUAP (2)	9.2×10^{-6}	1.9×10^5	60 ± 0.5 G
pDUTT (3)	8.9×10^{-5}	2.4×10^5	61 ± 0.5 G
pls^{4}dU (4)			

The results of the inhibition and ESR binding studies are summarized in Table I.

Discussion

The C-5-substituted analogues of dUMP, **1**, **2**, and **3**, competitively inhibited the activity of thymidylate synthetase from *Lactobacillus casei* to varying degrees (Table I), while the C-4-spin-labeled analogue **3** did not

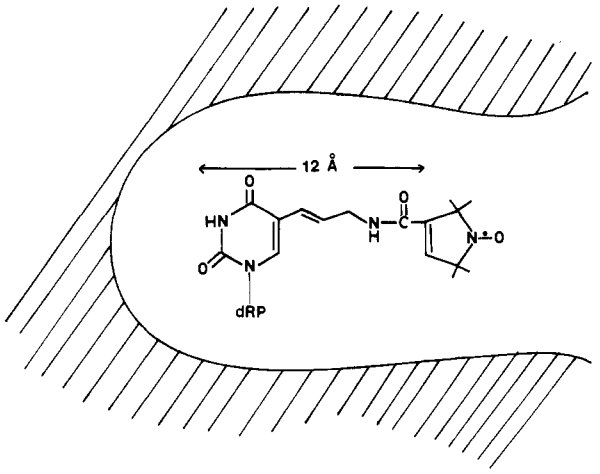

Figure 5. Proposed model of the binding mode of inhibitors to the active site of thymidylate synthetase with pDUAP (2) used as a specific example: dRP = 2′-deoxyribose 5′-monophosphate.

inhibit the enzyme. The analogues containing good electron-withdrawing substituents at C-5, compounds 1 and 2, were about 10 times more active in the inhibition reaction than 3. Mechanism studies indicate that a nucleophilic attack at position 6 of dUMP or its analogues by the thiol group of the active-site cysteine of the enzyme renders the 5-position susceptible to attack (Scheme I), ultimately resulting in the formation of the ternary complex.[1-3] Furthermore, it has been established that analogues with electron-withdrawing substituents at C-5 have greater affinity for the enzyme in the presence of the cofactor than for the enzyme alone.[15,16] The better inhibition observed with the spin-labeled analogue 2 than with 3 may be attributed to the electron-withdrawing properties of the C-5 substituent of the former. The absence, in 4, of the N-3 proton, which is known to affect binding,[15,16] and/or its geometry could account for its lack of inhibition.

ESR titrations of the C-5-spin-labeled anlogues with the enzyme and the resulting Scatchard plots indicate similar binding constants for the formation of the reversible binary complex for both compounds 2 and 3 (Table I). These association constants are similar to the K_{assoc} reported[12] ($K_{assoc} = 2.4 \times 10^5\ M^{-1}$) for the binary complex formation between the potent inhibitor FdUMP and the enzyme, indicating that these C-5 analogues bind to the active site of the enzyme in a similar manner. The presence of the bulky spin-label substituents does not seem to affect the formation of the binary complex. Furthermore, the C-4-spin-labeled analogue 4 did not bind to the enzyme, and hence no binary complex was formed with this analogue. These results suggest a model for the active-site binding of the C-5-substituted analogues where the substituents are pointed toward the opening of the active site (Figure 5). From the calculated values,[26] it can be surmised that the depth of the active site crevice is at least 12 Å. The "dip-stick" method can thus be used to determine the active site size by using spin probes of varying tether lengths, as has been done with other enzymes[27,28] and in the case of double-stranded DNA using spin-labeled nucleotides.[26]

Several studies have indicated one binding site per enzyme for dUMP and FdUMP in phosphate buffers,[29,32] while studies carried out in Tris buffers indicated two binding sites for FdUMP.[29,33] This anomaly has been attributed to the effects of the different ions present in these buffers by these investigators. In addition, sequencing studies indicate two identical subunits.[34] A sequential model of interaction of the subunits has been proposed[32] to account for this apparent discrepancy, where the second binding site remains unavailable for binding until the first site has been completely filled with both the nucleotide and the cofactor. The experimental value of one binding site for the binary complex obtained with the C-5-spin-labeled analogues in Tris buffer would seem to indicate that the interaction of these analogues with the enzyme also follows a similar sequential model with only one site being initially available for the binary complex formation.

This set of spin-labeled dUMP analogues thus enables the influence of the structural features of the analogues to be evaluated at the different steps of the enzymatic mechanism. The importance of the N-3 proton in binding is confirmed by the studies with analogue 4. The ESR binding and inhibition studies with the C-5 analogues give direct evidence that the effect of the electron-withdrawing substituents on binding is minimal for the binary complex formation, but is instead exerted during the formation of the ternary complex.

Conclusions

We have established the binding of spin-labeled analogues to the active site of the enzyme and the potential for using spin-labeled analogues and ESR spectroscopy to investigate the depth and topography of the active site. These studies have shown the active site to be at least 12 Å in depth. Similar association constants for the substrates in the binary complex indicate similar binding modes. The differential affinity of the analogues for the enzyme in the ternary complex is attributed to the varying electron-withdrawing abilities of the substituents.

Experimental Section

All UV measurements were obtained with a Gilford 250 scanning spectrometer equipped with an automatic cuvette changer. All ESR spectra were obtained with a Varian E-104 Century Series spectrometer that was interfaced with an Apple II+ microcomputer, and ^1H NMR spectra were obtained with a Nicolet NTC 300 FT instrument. Purity of the spin-labeled nucleotides were ascertained with a Bioanalytical HPLC system using a μ Bondapak C18 column. Preparative paper chromatography on the nucleotides was done by using an absolute ethanol–1 M ammonium acetate (7:3, v/v) solvent system. The synthesized nucleotides were purified with DEAE-Sephadex columns using a linear gradient of 0.05–0.4 M ammonium bicarbonate. The extinction coefficients of the spin-labeled nucleoside monophosphates were determined by phosphate analysis.[35] The purified enzyme was activated prior to ESR studies by preincubating the enzyme in 100 mM Tris buffer, pH 7.4, with 30 mM DTT for 30 min at room temperature.

5-(3-Aminoallyl)-2′-deoxyuridine 5′-Monophosphate (AA-dUMP) (1). The method by Langer et al.[17] for the synthesis of the corresponding triphosphate was used. Thirty-six milligrams

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(0.1 mmol) of dUMP and 160 mg (0.5 mmol) of mercuric acetate in 10 mL of 0.1 M sodium acetate buffer, pH 6.0, were stirred for 4 h at 50 °C. After cooling and addition of 39 mg (9 mmol) of LiCl, the solution was extracted several times with ethyl acetate, and the Hg–dUMP was precipitated with cold 95% ethanol. This was then dissolved in 5 mL of 0.1 M sodium acetate, pH 5.0, and 0.6 mL of fresh allylamine (0.6 mmol) and 68 mg (2 nucleotide equivalents) of potassium tetrachloropalladate(II) were added and allowed to react for 20 h. After removal of the excess metal ions with a Dowex (Na^+ form) column, the products were resolved with a DEAE-Sephadex column. Further purification was obtained by paper chromatography (R_f 0.216). The yield was 35%. UV (pH 7.0): λ_{max} 288 nm (ϵ 7000); λ_{max} 240 nm (ϵ 10 500); λ_{min} 264 nm (ϵ 5100). ^1H NMR (D_2O): δ 8.0 (s, C6-H), 2.2 (m, C2′-H), 3.6 (d, CH_2 allylamine), 4.0 (s, C5′-H), 4.2 (s, C4′-H), 4.5 (s, C3′-H), 6.2 (m, C1′-H), 6.4 (m, vinyl H).

5-[3-[[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolinyl)carbonyl]-amino]allyl]-2′-deoxyuridine 5′-Monophosphate (pDUAP) (2). Compound 1 was converted to the Na^+ form by ion exchange on a Dowex column. Seven milligrams (0.012 mmol) of 1 in 1 mL of 0.1 M sodium borate buffer, pH 9.35, was reacted with 35 mg (0.125 mmol) of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy-*N*-hydroxysuccinimide ester dissolved in a minimum amount of *N*,*N*-dimethylformamide. The reaction products were resolved on a DEAE-Sephadex column and 2 was collected. Further purification was obtained by means of HPLC (yield 60%). Chromatography of pDUAP on Whatmann No. 1 gave a R_f 0.76 with EtOH–1 M $AcONH_4$ (7:3, v/v). Bacterial alkaline phosphatase digestion of pDUAP gave DUAP with a R_f 0.24 on silica gel (10% MeOH–$CHCl_3$). UV (pH 7.0): λ_{max} 240 nm (ϵ 14 100); λ_{max} 290 nm (ϵ 8000); λ_{min} 270 nm (ϵ 6300). ^1H NMR (D_2O): δ 7.7 (s, C6-H), 1.3, 1.4 (s, CH_3 pyrroline), 2.2 (m, C2′-H), 5.9 (s, C1′-H), 6.2 (m, vinyl protons allylamine), 3.6 (CH_2 allylamine). Other sugar protons were as expected between δ 3.9 and 4.2.

4-[[[*N*-(2,2,6,6-Tetramethyl-1-oxy-4-piperidyl)carbamoyl]methyl]thio]-2′-deoxyuridine 5′-Monophosphate (pls⁴dU) (4). 4-Thio-dU was synthesized from dU according to published procedures[19,20] and was then phosphorylated to the monophosphate by the method of Van Boom et al.[21] The spin label was introduced by the reaction of 4-S-dUMP with a 5-fold molar excess of α-iodoacetamido tempo,[22] and compound 4 was isolated by paper chromatography, followed by DEAE-Sephadex column chromatography (yield 25%); pls⁴dU is the dehydroxy analogue of pls⁴ U, which was previously described.[36] UV (pH 7.0): λ_{max} 303 nm (ϵ 11 700). ^1H NMR (D_2O): δ 8.1 (d, C6-H), 6.55 (d, C5-H) 6.05 (m, C1′-H), 2.2 (m, C2′-H), 3.7-4.4 (m, sugar protons), 1.8 (d, CH_2 piperidine), 1.2, 1.3 (s, CH_3 piperidine).

Other Compounds. pDUTT (3) was synthesized according to published procedures.[18]

Purification of Enzyme. Crude thymidylate synthetase from *Lactobacillus casei* (New England Enzyme Center) was purified according to the procedure of Beaudette et al.[30] Dilute enzyme solutions were concentrated with an Amicon Ultrafiltration Cell, Model 12, with Diaflo ultrafilters. Protein concentrations were determined by the Lowry method[37] and by the UV method using a molar extinction coefficient[31] of 1.08×10^{-5} M^{-1} cm^{-1}. Native polyacrylamide gels (7%) showed a single protein band for the purified enzyme.

Enzyme Assay. The enzymatic activity was determined by the spectrophotometric method of Wahba and Friedkin[23] at 25 °C. The assay mixture contained 100 mM β-mercaptoethanol, 15 mM HCHO, 25 mM Mg^{2+}, 35 mM Tris buffer, pH 7.4, 0.7 mM EDTA, varying amounts of dUMP and inhibitors, and 0.6 unit of enzyme in a total volume of 1.0 mL. The reaction was initiated by the addition of the enzyme to the reaction mixture.

ESR Binding Studies. The reaction mixture contained 50 mM Tris buffer, pH 7.4, 2 mM EDTA, 15 mM DTT, 6.6 μM pDUAP or 5.7 μM pDUTT, and 125 μg (9.9 μM) of the activated enzyme in a total volume of 180 μL. ESR spectra were recorded before and after the addition of the enzyme at normal gain settings, and immobilized nitroxide spectra were recorded at 10 times higher gain and twice the modulation amplitude. The height of the center peak (h_0) of the ESR spectrum at normal gain settings was utilized as a direct measure of the concentration of the unbound, free analogue in the reaction mixture. Competition experiments were performed by adding 20-fold molar excess of FdUMP to the reaction mixtures containing the bound species.

For the Scatchard plot, the reaction mixture contained 50 mM Tris buffer, pH 7.4, 2 mM EDTA, 15 mM DTT, 5.6 μM activated enzyme, and 2 in a concentration range of 3.7–37 μM or analogue 3 in a concentration range of 3.2–32 μM in a total volume of 215 μL. Control experiments to determine the nonspecific loss of signal were performed in the presence of 20-fold molar excess of FdUMP in the same reaction mixtures.

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