5-Nitro-2'-deoxyuridine 5'-Phosphate: A Mechanism-Based Inhibitor of Thymidylate Synthetase

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Abstract: 5-Nitro-2'-deoxyuridine 5'-phosphate (**2**) was found to be a mechanism-based affinity label for thymidylate synthetase purified from amethopterin resistant *Lactobacillus casei*. The inhibition proceeds in two steps via the initial formation of the noncovalent complex **3** ($K_i = 5 \times 10^{-7}$ M) which is converted to the covalent complex **4**. The net dissociation constant for the overall sequence is 6.5×10^{-10} M, a value predicted from model studies of the bimolecular reaction of 2-mercaptoethanol with 5-nitrouridine or **2**.

Thymidylate synthetase (EC 2.1.1.45) is a viable and clinically effective target enzyme for the control of cell growth.[1] It is unique in several respects, the foremost being that it is the key enzyme in the synthesis of thymidine 5'-phosphate, an essential precursor for the synthesis of DNA. Additionally, since the product thymidine 5'-phosphate does not have any recognized metabolic role other than that leading to a precursor for DNA synthesis, inhibition of this enzyme should not interfere with normal metabolism of cells in the G_1 or resting phase.

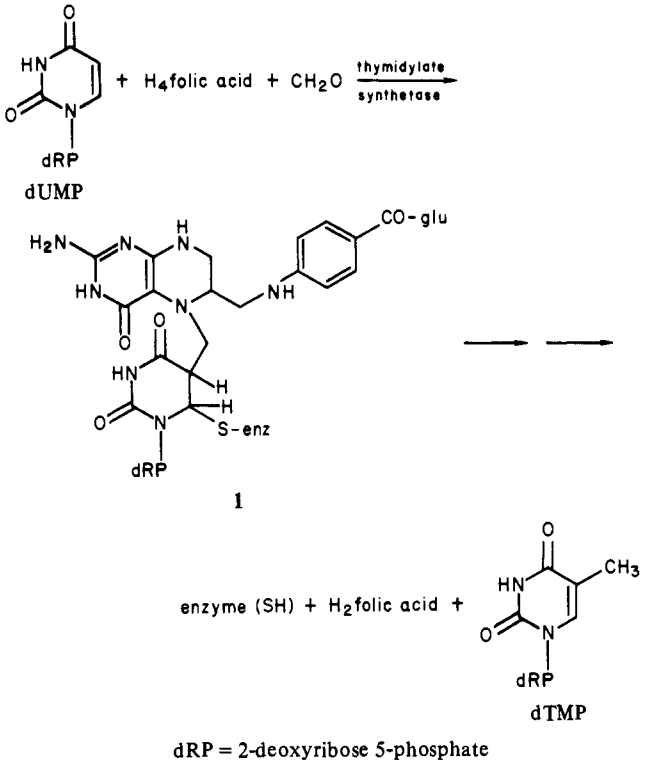
The mechanism of the enzymatic reaction can be viewed in two steps (Scheme I): alkylation of carbon 5 of the pyrimidine ring of the substrate 2'-deoxyuridine 5'-phosphate (dUMP) by the one-carbon carrier, N_5,N_{10}-methylenetetrahydrofolic acid (5,10-CH_2H_4folate) to give **1** followed by an internal redox reaction to give the product, dTMP, and the oxidized cofactor, 7,8-dihydrofolic acid. Substantial evidence from model studies[2-4] using 5-fluoro-2'-deoxyuridine 5'-phosphate has been reported that implicates a pathway whereby addition of a nucleophilic group (cysteine-SH) of the enzyme to carbon 6 of the pyrimidine ring in the substrate could precede or be concerted with alkylation at carbon 5 by the cofactor.[5] Model studies also support a hydride transfer in the final step to give the products.[6]

Substrate analogues reported to be potent inhibitors of this enzyme are 5-fluoro-, 5-(trifluoromethyl)-,[7] 5-mercapto-,[8] 5-formyl-,[9] and 5-(α-bromoacetyl)-2'-deoxyuridine 5'-phosphate.[10] The 5-fluoro,[7] 5-(trifluoromethyl),[7] 5-formyl, and 5-(α-bromoacetyl)[10] analogues are irreversible inhibitors of this enzyme. Considering the mechanistic features of the enzymatic addition to carbon 6, a classical Michael addition reaction, the substitution of a nitro group at carbon 5 in the pyrimidine ring of the substrate should enhance the proposed covalent addition of the enzyme thiol to give the covalent complex. On this basis 5-nitro-2'-deoxyuridine was prepared[11] and found to be an effective antiviral agent.[12] The same authors suggested, on the basis of labeling studies, that the antiviral activity could arise after in vivo conversion of the nucleoside to the 5'-nucleotide (**2**) and inhibition of thymidylate synthetase. Two preliminary reports confirm that compound **2** is a potent inhibitor of thymidylate synthetase derived from amethopterin resistant *Lactobacillus casei*.[13,14] The reported inhibition constants (K_i) were 0.029 and 0.023 μM.

The mechanism proposed for this inhibition formulated addition of the enzyme nucleophile to carbon 6 of the inhibitor in the noncovalent complex **3** to give the reversible covalent complex **4** (Scheme II).[14] The adduct should be a reasonably strong acid by virtue of the stabilization afforded **4** as the conjugate anion.

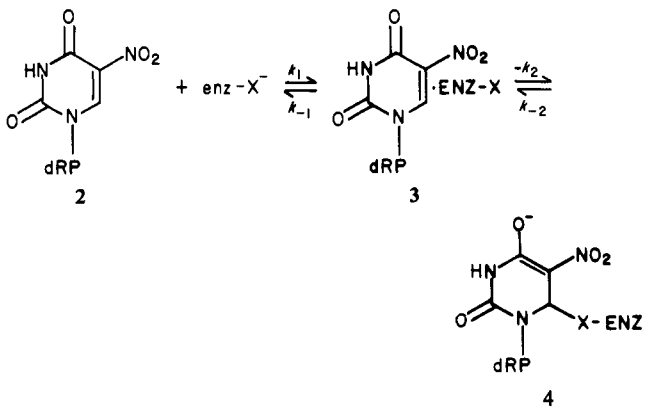
Support for the mechanism of the reaction of **2** with thymidylate synthetase was obtained from model studies of the bimolecular reaction of mercaptoethanol (**5**) with 5-nitrouridine (**6**) and the title compound **2**. Treatment of a neutral aqueous (^2H_2O) solution of 5-nitrouridine (**6**) with an excess of **5** resulted in an upfield shift of the pyrimidine carbon-6 proton NMR resonance of **6**

Scheme I



dRP = 2-deoxyribose 5-phosphate

Scheme II



dRP = 2'-deoxyribose 5'-phosphate

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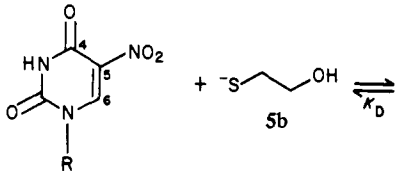
Table I. ^{13}C-NMR Assignments for 5-Nitrouridine (6) and the Adduct 7 Formed by Reaction of 6 with 2-Mercaptoethanol (5)[a]

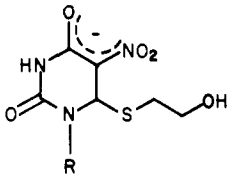
carbon	compd 6	adduct 7
C_2	154.3	156.2
C_4	162.3	162.8
C_5	128.4	112.9
C_6	149.0	64.3
$C_{1'}$	93.8	92.4
$C_{2'}$	77.3	74.3
$C_{3'}$	70.8	72.7
$C_{4'}$	86.5	86.2
$C_{5'}$	62.0	62.1
CH_2SH	29.0	42.8
CH_2OH	65.8	

[a] Shifts are reported in parts per million relative to Me_4Si; C refers to the pyrimidine ring and C' to the sugar. The spectra were measured by using a ^2H_2O–dioxane solution of 6 before and after the addition of 10 equiv of 2-mercaptoethanol (5). The pH of the solution was maintained at 6.8 by the addition of NaO^2H.

Scheme III



6, R = ribose
2, R = 2-deoxyribose 5-phosphate



7, R = ribose
8, R = 2-deoxyribose 5-phosphate

normally observed at δ 9.5. The reaction did not occur below a pH of 5. The adduct 7 formed in the reaction was reversible; acidification of the neutral mixture restored the carbon-6 proton resonance. In addition, chromatography of the adduct mixture using elution solvents without the mercaptan 5 gave the starting nucleoside 6 as the sole product.

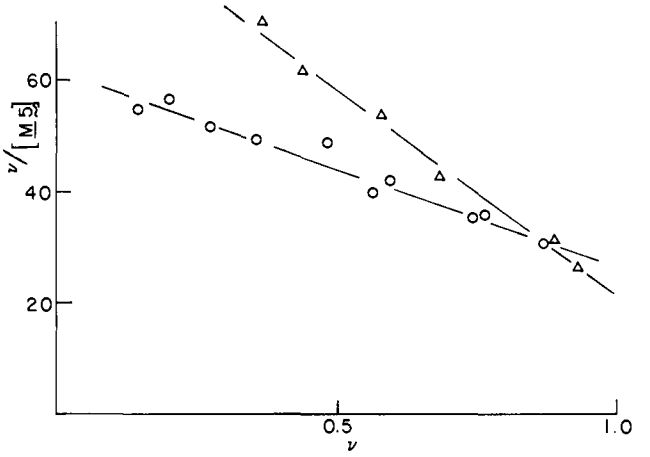


Figure 1. Scatchard plot of the ratio of the mole fraction of adduct [7 or 8] formed/concentration of 2-mercaptoethanol [5] (ν/5) vs. mole fraction of adduct formed (ν): 5-nitro-2'-deoxyuridine 5'-phosphate (2) plus 2-mercaptoethanol, O; 5-nitrouridine (6) plus 2-mercaptoethanol, Δ.

The ^{13}C NMR spectral assignments of the nucleoside 6 are compared to that of the reaction product 7 in Table I. The assignments for the nucleoside 6 are in agreement with those reported for 5-nitrouracil[15] and related nucleosides[16] with the correction for the $C_{2'}$ and $C_{3'}$ assignments.[17] Addition of mercaptoethanol (5) to the buffered solution of 6 resulted in significant upfield shifts for carbon 5 (−13.5 ppm) and carbon 6 (−84.7 ppm). Triplett and co-workers,[18] in a study of the addition of bisulfite to carbon 6 of nucleosides, note similar shifts in the resonance of these carbons. Of particular interest is their report that carbon 6 of a series of bisulfite adducts of nucleosides and nucleotides was assigned in the same region (65–69 ppm) as carbon 6 in the adduct 7. If the mercaptan adds to the conjugated carbonyl represented by carbons 4–6 as depicted in Scheme III, such an upfield shift for carbons 5 and 6 should be observed. Bannister and Kagan[19] characterized a similar reaction product, addition to carbon 6, from the intramolecular cyclization of 5'-deoxy-5'-mercaptouridine.

Previous studies of the nucleophilic addition of 2-mercaptoethanol to carbon 6 of 1,3-dimethyl-5-nitrouracil by Pitman and co-workers[20] demonstrated that the anion of 2-mercaptoethanol (5b) gave a reversible adduct. We noted a shift in the ultraviolet absorption spectrum from 311 to 332 nm accompanied by a 64% increase in extinction coefficient when 5-nitrouridine (6), buffered at pH 6.8, was treated with an excess of 2-mercaptoethanol (5). Stop-flow kinetic analysis of the reaction could not be used for determination of the velocity of the forward reaction since the reaction was completed in less than 1 ms, the minimum time scale that could be analyzed.

Kalman[21] examined a similar equilibration wherein glutathione catalyzed proton exchange at carbon 5 of uridine. In the rate-determining step it was observed that the anion of glutathione reacted with the unionized form of uridine. These findings, coupled with those of Pitman and co-workers[20] and our observed adduct formation between 5-nitrouridine (6) and 2-mercaptoethanol (5), support the mechanism of the reaction as being the nucleophilic addition of the anion of 2-mercaptoethanol (5b) to the neutral

(1) (a) Danenberg, P. V. *Biochim. Biophy. Acta* **1977**, *497*, 73–92. (b) Friedkin, M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1973**, *38*, 235–292.
(2) Byrd, A.; Dawson, W. H., Ellis, P. D.; Dunlap, R. B. *J. Am. Chem. Soc.* **1978**, *100*, 7478–7486.
(3) Santi, D. V.; McHenry, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1855–1875.
(4) Danenberg, P. V.; Langenbach, R. J.; Heidelberger, C. *Biochemistry* **1974**, *13*, 926–933.
(5) Bellisario, R. L.; Maley, G. F.; Galivan, J. H.; Maley, F. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1848–1852.
(6) Wilson, R. S.; Mertes, M. P. *Biochemistry* **1973**, *12*, 2879–2886.
(7) Reyes, P.; Heidelberger, C. *Mol. Pharmacol.* **1965**, *1*, 14–30.
(8) Kalman, T. I.; Bardos, T. J. *Mol. Pharmacol.* **1970**, *6*, 621–630.
(9) Santi, D. V.; Sakai, T. T. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1320–1325.
(10) Brouillette, C. B.; Chang, C. T.-C.; Mertes, M. P. *J. Med. Chem.* **1979**, *22*, 1541–1544.
(11) Huang, G. F.; Torrence, P. F. *J. Org. Chem.* **1977**, *42*, 3821–3824.
(12) DeClercq, E.; Descamps, J.; Huang, G. F.; Torrence, P. F. *Mol. Pharmacol.* **1978**, *14*, 422–430.
(13) Mertes, M. P.; Chang, C. T.-C.; DeClercq, E.; Huang, G. F.; Torrence, P. F. *Biochem. Biophys. Res. Commun.* **1978**, *84*, 1054–1059.
(14) (a) Matsuda, A.; Wataya, Y.; Santi, D. V. *Biochem. Biophys. Commun.* **1978**, *84*, 654–659. (b) Wataya, Y.; Matsuda, A.; Santi, D. V. *J. Biol. Chem.* **1980**, *255*, 5538–5566.
(15) Ellis, P. D.; Dunlap, R. B.; Pollard, A. L.; Seidman, K.; Cardin, A. D. *J. Am. Chem. Soc.* **1973**, *95*, 4398–4403.
(16) Dorman, D. E.; Roberts, J. D., *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 19–26.
(17) Mantsch, H. H.; Smith, I. C. P. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 808–815.
(18) (a) Triplett, J. W.; Smith, S. L.; Layton, W. J.; Digenis, G. A. *J. Med. Chem.* **1977**, *20*, 1594–1597. (b) Triplett, J. W.; Digenis, G. A.; Layton, W. J.; Smith, S. L. *J. Org. Chem.* **1978**, *43*, 4411–4414.
(19) Bannister, B.; Kagan, F. *J. Am. Chem. Soc.* **1960**, *82*, 3363–3368.
(20) Pitman, I. H.; Cho, M. J.; Rork, G. S. *J. Am. Chem. Soc.* **1974**, *96*, 1840–1843.
(21) Kalman, T. I. *Biochemistry* **1971**, *10*, 2567–2573.

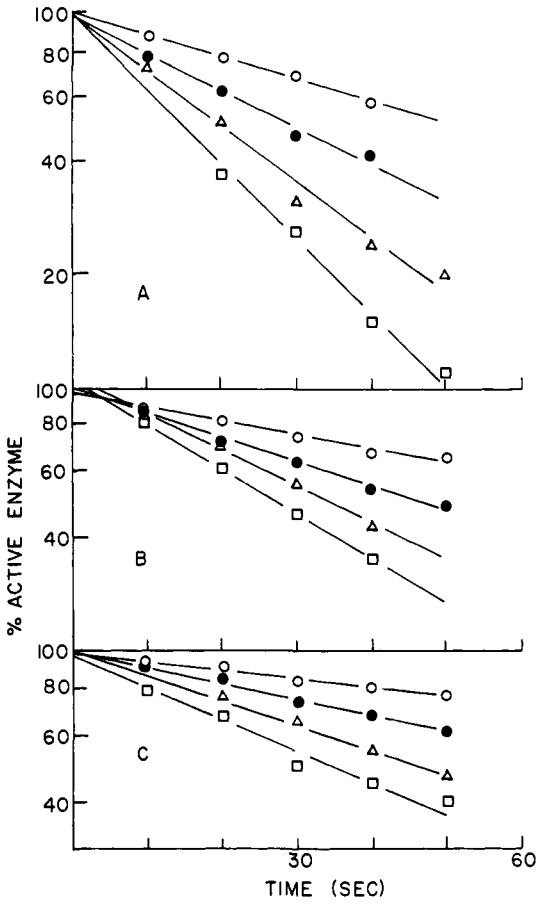


Figure 2. Plot of the log of the percentage of active thymidylate synthetase (specific activity = 3.2 μM/(min mg)) vs. time (s) in the presence of varying concentrations of inhibitor 5-nitro-2′-deoxyuridine 5′-phosphate (**2**): 0.05 μM, ○; 0.1 μM, ●; 0.15 μM, △; 0.2 μM, □; A, no substrate; B, substrate (6 μM); C, substrate (15 μM).

pyrimidine ring of 5-nitrouridine (**6**) (Scheme III).

The dissociation constant of the adduct **7** formed from the reaction of 2-mercaptoethanol (**5**) with 5-nitrouridine (**6**) was calculated from the increase in absorption at 335 nm associated with the addition of varying amounts of 2-mercaptoethanol. The value for $K_{D_{obsd}}{}^{7}$ was 0.013 M at pH 6.8 where $K_{D_{obsd}}{}^{7}$ is defined in eq 1 and $[\mathbf{6}]_T$ and $[\mathbf{5}]_T$ represent total concentrations of the

$$K_{D_{obsd}}{}^{7} = [\mathbf{6}]_T[\mathbf{5}]_T/[\mathbf{7}] \quad (1)$$

neutral and anionic forms of 5-nitrouridine (**6**) and 2-mercaptoethanol (**5**).

Graphic analysis of the double reciprocal plot of the dependence, fraction of adduct (v) vs. concentration of **5** and a Scatchard plot (Figure 1) of (v)/[**5**] vs. the mole fraction of adduct (v) indicated a 1:1.3 adduct or binding of **5** at more than a single site on **6** to give **7**. The dissociation constant $K_{D_{obsd}}$ was determined from the equation $v/[5] = n/K_D - v/K_D$ where n equals the number of independent equivalent binding sites for **5** in the adduct.

Since previous studies on similar models show a pH dependence, incorporation of these considerations into eq 2 represents the pH

$$K_D{}^{7} = [\mathbf{5b}][\mathbf{6}]/[\mathbf{7}] \quad (2)$$

dependence on the dissociation constant of the adduct **7**. Substitution into eq 1 gives the expression for $K_{D_{obsd}}$ in eq 3 where K_a and $K_a{}^{5}$ are the ionization constants for the nucleoside **6** and 2-mercaptoethanol (**5**).

$$K_{D_{obsd}} = \left(1 + \frac{K_a}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_a{}^{5}}\right)K_D \quad (3)$$

The ionization constant for the nucleoside **6** was determined by using the spectrophotometric shift of **6** in acid at 304 nm to the λ_{max} in base at 321 nm for the anionic form. From these measurements an apparent pK_A of 6.5 was determined for 5-nitrouridine **6**. In view of the report by Fox and co-workers[22] that noted an unusual shift in the alkaline solution spectrum of **6** which precluded an accurate pK_a determination, we also titrated **6** and found a pK_a of 6.6. A pK_a of 6.50 as determined at 1 M ionic strength for 5-nitro-2′-deoxyuridine has been reported.[14b]

The reported[23] value of 1.9×10^{-10} M for the ionization of 2-mercaptoethanol ($K_a{}^{5}$) was used in the determination of the $K_D{}^{7}$. Accordingly the $K_D{}^{7}$ for the dissociation of the anionic adduct **7** to give the neutral nucleoside **6** and the anionic form of 2-mercaptoethanol (**5b**) (eq 2) is 6.2×10^{-6} M.

Similar model studies also were done by using 5-nitro-2′-deoxyuridine 5′-phosphate (**2**). Treatment of **2** with varying concentrations of 2-mercaptoethanol (**5**) at pH 6.8 gave a shift in the ultraviolet absorption from 303 to 326 nm with an isoabsorptive point at 313 nm. This bathochromic shift was reversible on acidification. Analysis of the data using varying concentrations of **5** either by a double recriprocal plot or a Scatchard plot (Figure 1) gave an observed dissociation constant ($K_{D_{obsd}}$) of 0.028 M for the adduct **8**. The data from the plot show a value of 1.7 for the number of independent binding sites for **5** in the adduct **8**. The pK_a of **2** was determined by examination of the ultraviolet spectra at pHs ranging from 4.95 to 10.3. A complex pattern was observed for the spectral shift in acid from 304 nm to the alkaline spectral maximum (pH 10.3) at 323 nm. It is evident from the shift in the isoabsorptive point from 264 (acid) to 277 nm (pH 8.9) that another species is formed. An apparent pK_a of 7.1 was calculated for **2** from the spectra changes in the pH range of 4.9–8.4. Wataya and co-workers[14b] recently determined the pK_a of **2** to be 6.83 at 1 M ionic strength.

The pH-independent dissociation constant ($K_D{}^{8}$) of the adduct **8** to give the nucleotide **2** and the anion of 2-mercaptoethanol (**5b**) as formulated in Scheme III was calculated from the $K_{D_{obsd}}{}^{8}$ to be 2.2×10^{-5} M (eq 3). This compares favorably with that determined by Wataya et al.[14b] for the same model (3.44×10^{-5} M).

The inhibition of thymidylate synthetase by the title compound was reported in two independent studies[13,14] to be competitive with substrate. Incubation of the inhibitor with the enzyme, furthermore, showed loss of enzyme activity; however, the kinetics of inactivation while apparently first order under the conditions of the experiments were not first order with respect to the concentration of the enzyme–inhibitor complex.[14]

It is commonly found that affinity labeling reagents interact initially to form a reversible noncovalent complex. Subsequently this complex proceeds to give the covalent complex.[24,25] This is the case with 5-(α-bromoacetyl)-2′-deoxyuridine 5′-phosphate, a mechanism-based irreversible inhibitor that shows the expected rate saturation effect and substrate protection.[10] Moreover, the rate of enzyme inactivation by this compound is first order in the concentration of the reversible complex.

Since this pattern of inactivation was not observed with higher concentrations of 5-nitro-2′-deoxyuridine 5′-phosphate (**2**), further studies were undertaken to elucidate the mechanism of the reaction. The points in question relate to the mechanism of the reaction as formulated in Scheme II.

Enzyme inactivation studies were done by using thymidylate synthetase at a specific activity of 3.2 μM/(min/mg) (radioisotope assay) purified from amethopterin resistant *Lactobacillus casei*. In the preliminary report of the inhibition of thymidylate synthetase a spectrophotometric assay was used for enzyme activity.[13] This assay monitors the change in absorbance at 340 nm, a measure of the formation of 7,8-dihydrofolic acid.

An alternative assay described by several authors for detection of low levels of enzyme is a radioisotope measurement that clearly

(22) Wempen, I.; Doerr, I. L.; Kaplan, L.; Fox, J. J. *J. Am. Chem. Soc.* **1960**, *82*, 1624–1629.
(23) Irving, J.; Nelander, L.; Wadso, I. *Acta Chem. Scand.* **1964**, *18*, 769–787.
(24) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245–3249.
(25) Petra, P. H. *Biochemistry* **1971**, *10*, 3163–3170.

Table II. The Kinetics of Inactivation of Thymidylate Synthetase by 5-Nitro-2'-deoxyuridine 5'-Phosphate (2) in the Absence and Presence of Substrate 2'-Deoxyuridine 5'-Phosphate[a]

	k_{obsd}, s^{-1}		
[μM of 2]	[S] = 0	[S] = 6 μM	[S] = 15 μM
0.05	0.013	0.0085	0.0054
0.10	0.023	0.016	0.010
0.15	0.034	0.023	0.016
0.20	0.044	0.028	0.019

[a] This enzyme preparation was determined to have a V_{max} of 170 pmol/min and a K_m of 4.9 μM; the concentration during preincubation was 0.015 μM.

is more sensitive.[26,27] This assay is an analysis of tritium in ^3H_2O formed as the product of the conversion of [5-^{3}H]-2'-deoxyuridine 5'-phosphate to thymidine 5'-phosphate, a direct measurement of product formation. For application of this method to kinetic analysis it was noted that the velocity of the reaction at substrate concentrations below the K_m was linear for almost 1 min in an assay using 10-s points. Given this time constraint a 30-s radioisotope assay was developed that is sufficiently sensitive for low levels of substrate, is useful for analysis of potent inhibitors, and clearly is conservative of materials. The assay concentrations of reactants in the radioisotope method were scaled up to those required for the spectrophotometric assay and the two methods compared directly. In agreement with the results of Crusberg and co-workers[28] we observed a slightly decreased rate which those authors attributed to an isotope effect. With use of the radioisotope assay for these studies, the K_m determined from the double reciprocal plot of velocity vs. substrate concentration was 4.9 μM; the V_{max} from this plot was 170 pmol of ^3H_2O formed per minute. Dunlap et al.,[29] Crusberg et al.,[28] and Daron and Aull,[30] using the spectrophotometric assay procedure, reported K_m's ranging from 5.2 to 0.7 μM. During these studies it was found that the optimum conditions for maximum velocity required dialysis of the concentrated enzyme with 2-mercaptoethanol for 24 h.

Incubation of the enzyme (~0.015 μM) in a solution containing 2-mercaptoethanol, buffer, and salts for varying periods of time followed by assay under conditions for maximum velocity (substrate concentration $10K_m$) showed little inactivation of the enzyme. The rapid enzyme inactivation by **2** required the development of an assay for maximum velocity after incubation periods of 10, 20, 30, 40, and 50 s. A plot of the log of the percentage of remaining active enzyme vs time as seen in Figure 2 is linear during the initial reaction period. The reciprocals of the observed pseudo-first-order rate constants for the inactivation reaction (Table II) were found to be proportional to the reciprocal of the concentration of 5-nitro-2'-deoxyuridine 5'-phosphate (**2**) (Figure 3).

The results in Table II show a rate saturation effect at inhibitor concentrations in the range of 0.05–0.2 μM. The ratio $k_{obsd}/[2]$ decreases from 0.26 to 0.22 s^{-1} μM^{-1} as inhibitor concentration increases in the absence of substrate. In agreement with the report by Wataya and co-workers[14b] we also found higher concentrations of inhibitor to be saturating. The most common mechanism for the inactivation is the reaction of the inhibitor with the enzyme to give a rate-limiting complex which is converted to the covalent complex **4** (Scheme II).

The expression that relates the velocity of the inactivation process as developed by Kitz and Wilson[24] is shown in eq 4. While

$$1/k_{obsd} = \left[\frac{K_i[S]}{K_m k_2} + \frac{K_i}{k_2}\right]\frac{1}{[I]} + \frac{1}{k_2} \quad (4)$$

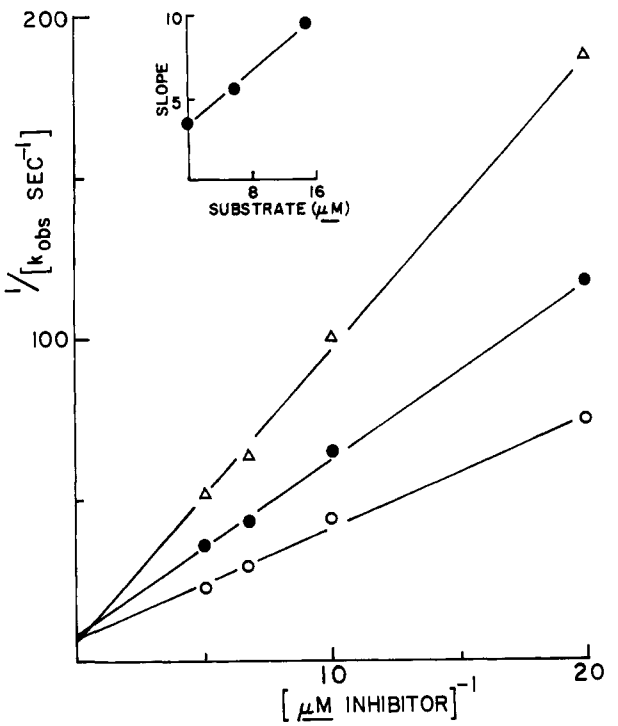


Figure 3. Double-reciprocal plot of the observed rate constant for inactivation of thymidylate synthetase (specific activity 3.2 μM/(min mg)) vs. concentration of added inhibitor 5-nitro-2'-deoxyuridine 5'-phosphate (**2**) in the absence (○) and presence of added substrate 2'-deoxyuridine 5'-phosphate (6 μM, ●; 15 μM, △). Inset in Figure 3 is a replot of the slope vs. concentration of substrate.

this expression is commonly used to describe irreversible inhibitors, it can be used to analyze this data since the reverse reaction, k_{-2}, is much slower than k_2[14b] (Scheme II). A plot of the reciprocals of the observed rate constant for inactivation vs. the inhibitor concentration in the absence of substrate gave the rate constant for inactivation (k_2) of 0.146 s^{-1} and a calculated inhibition constant (K_i) of 5×10^{-7} M (Figure 3). If compound **2** is interacting at the active site to give the reversible complexes of enzyme and **2** noted in Scheme II, then the addition of the substrate for this enzyme should afford protection by decreasing the amount of available enzyme and subsequently decrease the observed rate of inactivation (k_2) in accord with the relationship in eq 4. With the two substrate concentrations (Table II, Figure 3), the calculated K_m for substrate was determined by a replot of the slope vs. substrate concentration (inset, Figure 3). The value for the K_m was 9×10^{-6} M; this compares reasonably well with the observed K_m of 4.9×10^{-6} M.

Previous K_i determinations[13,14] from double-reciprocal plots of the initial velocity of the reaction vs. substrate concentration at various levels of inhibitor gave values of 0.029 and 0.023 μM for **2**. However, considering the rapid rate of enzyme inactivation by this inhibitor and the fact that enzyme will be lost during the assay by covalent inactivation these values for dissociation of the noncovalent complex could be low under the conditions of the assay.[31] For these reasons a rapid assay method (15 s) was used to develop data for a Dixon plot (Figure 4) of the reciprocal of the velocity vs. inhibitor concentration at varying levels of substrate according to eq 5. This method of plotting is useful for distinguishing competitive and noncompetitive inhibition reactions.[32] By this analysis α is diagnostic for the type of inhibition; an α

$$\frac{1}{v} = \left[\frac{1 + \alpha K_m/[S]}{\alpha K_i V_{max}}\right]\frac{1}{[I]} + \frac{1}{V_{max}}\left[1 + \frac{K_m}{[S]}\right] \quad (5)$$

(26) Lomax, M. I. S.; Greenberg, G. R. *J. Biol. Chem.* **1967**, *242*, 109–113.
(27) Roberts, D. *Biochemistry* **1966**, *5*, 3546–3548.
(28) Crusberg, T. C.; Leary, R.; Kisliuk, R. L. *J. Biol. Chem.* **1970**, *245*, 5292–5296.
(29) Dunlap, R. B.; Harding, N. G. L.; Huennekens, F. M. *Biochemistry*, **1971**, *10*, 88–97.
(30) Daron, H. H.; Aull, J. L. *J. Biol. Chem.* **1978**, *253*, 940–945.
(31) Tipton, K. F. *Biochem. Pharmacol.* **1973**, *22*, 2933–2941.
(32) Segel, I. H. "Enzyme Kinetics"; Wiley-Interscience: New York, 1976; Chapter 3.

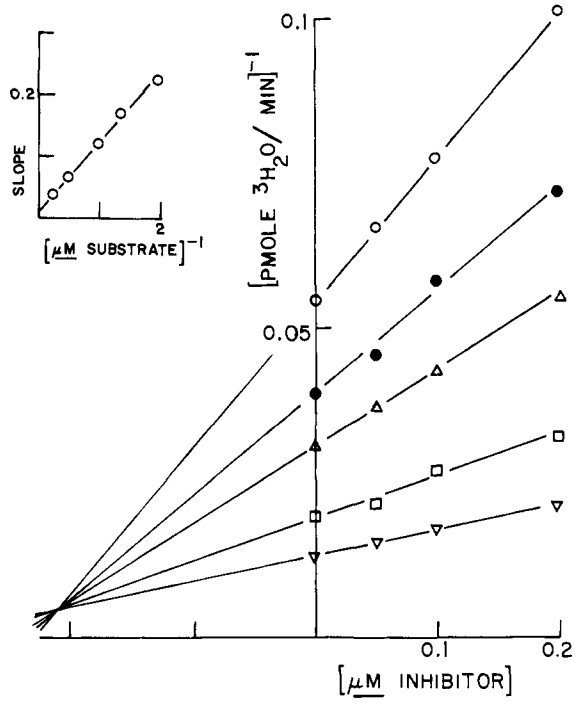


Figure 4. Dixon plot of the reciprocal of the velocity of thymidylate synthetase catalysis (pmol of ^3H_2O/min) vs. concentration of inhibitor 5-nitro-2′-deoxyuridine 5′-phosphate (**2**) at varying levels of added substrate 2′-deoxyuridine 5′-phosphate: 0.5 μM, ○; 0.7 μM, ●; 1.0 μM, △; 2 μM, □; 4 μM, ▽. The specific activity of enzyme used in this analysis was 1.1 μM/(min/mg). Inset in Figure 4 is a replot of the slopes from Figure 4 vs. the reciprocal of the concentration of substrate 2′-deoxyuridine 5′-phosphate.

> 1 found for the line intersect above the horizontal axis indicates competitive inhibition. The V_{max} for this enzyme preparation was 130 pmol/min and the K_m was 3.0 μM. From a replot of the slopes vs. the reciprocal of the substrate concentrations (inset, Figure 4) the K_i for **2** was calculated to be 2.1×10^{-7} M. This value is in reasonable agreement with the K_i determined from the inactivation studies (5×10^{-7} M); however, it is 10 times greater than the value estimated from the double-reciprocal plots reported in the preliminary studies.[13,14]

Discussion

Although the chemical nature of the intermediates in the thymidylate synthetase catalyzed reaction are unknown, the mechanism studies[3-5] describing the initial events in the reaction strongly support the pathway whereby an enzyme-bound thiol (anion) adds to carbon 6 of the substrate. Model studies using carbon-5 proton exchange[21] and nucleophilic addition to carbon 6 of 5-substituted uracil and uridine models[18-21,33] also follow this pathway.

In the present study we have found that mercaptoethanol (**5**) forms a reversible adduct with both 5-nitrouridine (**6**) and 5-nitro-2′-deoxyuridine 5′-phosphate (**2**). Evidence for the structure of the adduct is derived from analogy to similar systems[21,33] and ^{13}C NMR studies of the adduct **7** formed from mercaptoethanol (**5**) and the nucleoside **6**. Both carbons 5 and 6 of the pyrimidine ring of the nucleoside **6** shift upfield when **6** is treated at pH 7.8 with the thiol **5**. The only other significant changes in the ^{13}C spectra are the unusual shifts noted for $C_{2'}$ (−3 ppm) and $C_{3'}$ (+1.9 ppm) of the ribose ring in the adduct **7**.

Similar ultraviolet spectral changes are observed when either the nucleoside **6** or the nucleotide **2** are treated with the thiol at pH 6.8. The adducts formed in both cases are reversible on treatment with acid. These observations together with the findings by Pitman and co-workers[20] and those of Kalman[21] support the mechanism in Scheme III as a reasonable pathway for adduct formation in these models.

Wataya et al. also studied adduct formation in the same model.[14b] At pH 7.4 they observed a similar shift in the ultraviolet maxima from 316 to 332 nm on treating **2** with 2-mercaptoethanol which was used to estimate the apparent equilibrium constant of 50 M^{-1} whereas at pH 6.8 we determined the same value to be 40 M^{-1}.

One unusual finding in these studies is that the results of the equilibration studies for formation of the adducts **7** and **8** indicate from graphic analysis that there is more than one independent binding site (n) for the thiol **5** in each adduct. Careful inspection of the ultraviolet spectral changes going from **6** to the adduct **7** shows a shift in the isoabsorptive point from 316 to 319 nm when mercaptoethanol concentrations are 10^4 greater than those of the nucleoside **6**. A more subtle but similar shift from 313 to 315 nm was noted in the model reaction of the nucleotide **2** and high concentrations of **5**. The points for both plots in Figure 1 were obtained from the spectral changes common to the initial isoabsorptive point and calculate for values of $n = 1.3$ for adduct **7** and 1.7 for adduct **8**.

One factor that could account for the unexplained stoichiometry in the reaction is that compound **2** is reported to be unstable in solution.[14b] It is reasonable that some decomposition of **2** occurred during these studies. This is further supported by the complex ultraviolet pattern we observed in the pK_a determination of **2** wherein a second isoabsorptive point is evident.

The pH-independent dissociation constants (K_D) for the adducts in Scheme III (eq 3) were 6.2×10^{-6} and 2.2×10^{-5} M, respectively, for the adducts **7** and **8**. The protonated forms of adducts **7** and **8** are not expected to contribute significantly to the equilibrium because of the unique stabilization afforded the anionic species.[14b,20] A similar value (3.4×10^{-5} M) was reported for the dissociation of **8** by using the pK_a values of 6.80 for **2** (determined at 1 M ionic strength) and 9.5 for 2-mercaptoethanol.[14b]

Sander and Jencks[34] have described a linear free energy relationship, $\log K_{eq} = \Delta\gamma + A$, for the addition of nucleophilic reagents to aldehydes where Δ is a measure of the sensitivity of the carbonyl compound, γ is a measure of the relative ability of a nucleophile to covalently add to the carbonyl, and A is constant for a given reaction series. Pitman and co-workers[20] examined adduct formation by nucleophilic addition to carbon 6 of 1,3-dimethyl-5-nitrouracil and found that this relationship is valid for 1,4 addition to the carbonyl. In their studies the log of the product of the pH-independent equilibrium constant (K_{eq}) and the ionization constant of the nucleophilic reagent (K_a) was linearly related to the γ value of the conjugate acid of the anion. Although it is recognized that the correlation has limited application, the results suggest that the reactivity of the α,β-unsaturated carbonyl group in **2** or **6** (carbons 4–6) toward nucleophilic addition is dependent on the γ value of the nucleophile.

Within the series where the same carbonyl group is used (**2**) the pH-independent dissociation constant (K_D^8) from the model studies should allow for an estimation of the equilibration of the covalent adduct **4** to give the anionic form of the enzyme and the inhibitor **2** (Scheme II). The assumption made in this analysis is that 2-mercaptoethanol (**5**) is a reasonably good model for the catalytic cysteine residue at the enzyme active site and that it adequately represents the nucleophilic character. Sander and Jencks[34] reported the γ value for 2-mercaptoethanol was similar to that of thioglycolate (0.53 vs. 0.35). Accordingly, a calculated value for the pH-independent dissociation constant ($K_{D_{calcd}}^4$) of the enzyme adduct can be derived from eq 6 where K_D^8 ($2.2 \times$

$$\log (K_a^5/K_D^8) = \log (K_a^{ESH}/K_{D_{calcd}}^4) \qquad (6)$$

10^{-5} M) is the pH-independent dissociation constant for the breakdown of the model adduct **8** and K_a^5 (1.9×10^{-10} M) and

(33) Chikuma, T.; Negishi, K.; Hayatsu, H. *Chem. Pharm. Bull.* **1978**, *26*, 1746–1752 and references therein.

(34) Sander, E. G.; Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 6154–6162.

(35) Monroe, W. A.; Lewis, C. A., Jr.; Dunlap, R. B. *Biochem. Biophys. Res. Commun.* **1978**, *80*, 355–360.

K_a^{ESH} are the respective acid dissociation constants of the conjugate acids of the thiol anions of mercaptoethanol (**5**) and the enzyme active site cysteine.

Dunlap and co-workers,[35] from studies on pH dependence of inactivation of thymidylate synthetase by sulfhydryl group reagents, concluded that the K_a of the catalytic cysteine residue at the active site is approximately 1×10^{-8} M. From eq 6 the calculated pH-independent constant for the breakdown of the covalent adduct formed from the inhibitor **2** and the enzyme anion is 1×10^{-3} M. The model reaction therefore predicts that the formation of the covalent bond between the anionic form of the enzyme and the inhibitor is 1000 times faster than its cleavage. If, as the model reactions suggest, the calculated dissociation constant *in the absence of the association step* is 1×10^{-3} M, a contribution of ~4 kcal to the overall equilibrium in Scheme II is estimated for the covalent bond-forming reaction. The contribution to the equilibria by the enzyme–inhibitor association step (k_{-1}/k_1) was determined from the Dixon plot to be 2.1×10^{-7} M, a contribution of ~9 kcal to the overall sequence in Scheme II. On the basis of the model studies and the observed K_i (2.1×10^{-7} M), the net reaction for dissociation of **4** to give the inhibitor **2** and the anionic enzyme form should be the product of the two equilibration steps ($k_{-1}k_{-2}/k_1k_2$), a calculated value for K_{net} of 2.1×10^{-10} M.

Support for the mechanism of inactivation being dependent on the concentration of the complex **3** (Scheme II) is derived from the rate saturation effect at higher concentrations of **2** and the protection against inactivation by **2** afforded by the substrate. The K_i as determined from the inactivation studies was 5×10^{-7} M. Wataya and co-workers[14b] using isotope dilution techniques recently demonstrated that formation of the covalent adduct **4** was reversible. Furthermore, the net rate constant for dissociation of **4** to give **2** and the anionic form of the enzyme (k_r) was reported to be 0.15×10^{-3} s^{-1}.

An analysis of the available data allows for a determination of the individual rate constants using the methology for partition analysis described by Cleland.[36] Accordingly, the mechanism in Scheme II reduces to the form shown in eq 7 where k_f represents

$$K_{net} = \frac{k_r}{k_f} = \frac{k_{-1}k_{-2}}{k_1k_2} = \frac{[2]\ [ES^-]}{[4]} \qquad (7)$$

the net rate constant of the forward reaction and k_r the net rate constant for the breakdown of the covalent adduct **4** to give the anionic form of the enzyme and **2**. From the data available the K_i is 5×10^{-7} M (inactivation studies), k_2 is 0.146 s^{-1} and the net rate constant (k_r) for the reverse reaction as reported by Wataya et al.[14b] is 0.15×10^{-3} s^{-1}.

The net rate constant for the forward reaction (k_f) can be calculated from the inactivation studies since the product $k_f[I] = k_{obsd}$. At an inhibitor concentration of 1×10^{-7} M the observed rate of formation of **4** was 0.023 s^{-1}. Thus, $k_f = 0.023 \times 10^7$ M^{-1} s^{-1}. Substitution of the appropriate values for k_f, k_r, $k_{-1}/k_1(K_i)$ and k_2 gives a value for k_{-2} of 0.19×10^{-3} s^{-1}. The ratio of k_{-2}/k_2 is 1.3×10^{-3} and agrees with the calculated value from the model studies that predicted the rate of covalent bond formation (k_2) to be 1000 times faster than cleavage (k_{-2}). The net dissociation constant for the reaction in Scheme II is 6.5×10^{-10} M.

It would appear from these studies that the early models of Kalman[21] and Pitman's[20] groups are a valid interpretation of the enzyme inhibition mechanism as proceeding through the sequence of reaction of **2** with the enzyme anion to give the reversible covalent complex **4** as noted in Scheme II.

Previous studies also have addressed the separate steps in the mechanism of thymidylate synthetase catalysis. A value of 10^{-5} M is reported for dissociation of the noncovalent complex formed from this enzyme and 5-fluoro-2'-deoxyuridine 5'-phosphate as measured in the absence of cofactor.[37,38] In another study the dissociation constant for the noncovalent complex of thymidylate synthetase and 5,6-dihydro-2'-deoxyuridine 5'-phosphate was 10^{-4} M. Saturation of the ring double bond in this example precluded covalent bond formation.[39]

Resolution of the association step and the covalent bond forming step in the sequence in Scheme II recently was reported in a study involving a series of 5-substituted styryl derivatives of 2'-deoxyuridine 5'-phosphate.[40] Covalent addition of the enzyme nucleophile to carbon 6 in these compounds should be sensitive to electronic effects contributing to stabilization of the resulting conjugate anion[41] as represented by structure **4**. Accordingly, substitution on the phenyl ring in 5-styryl derivatives should enhance covalent bond formation if the substituent is electron withdrawing (*m*-nitro, $\sigma = +0.71$; *p*-nitro, $\sigma = +0.78$) and should be less favored if the substituent is electron donating (*m*-amino, $\sigma = -0.16$). We found that there was no significant difference in the K_i of these derivatives (0.2–0.6 μM) when the 3- and 4-nitrostyryl, styryl, and 3-aminostyryl[42] derivatives were examined as inhibitors of thymidylate synthetase.[40] Prolonged incubation did not affect the reaction, and the K_i, which represents the initial association complex of inhibitor and enzyme, is comparable to that found for 5-nitro-2'-deoxyuridine 5'-phosphate.

Experimental Section

UV and NMR spectra were recorded by using a Cary Model 219 and Varian Model EM-360 or T-60 and a Brucker WP-80. Thymidylate synthetase purified from methotrexate resistant *Lactobacillus casei* purchased from the New England Enzyme Center, Tufts University, was purified and used at a specific activity of 1.1 and 3.2 μmol of TMP formed per min per mg of protein using the radioisotope assay. The enzyme was activated by dialysis for 24 h at 4 °C against 0.1 M potassium phosphate (pH 6.8) containing 50 mM mercaptoethanol. The substrate [5-^{3}H]-2'-deoxyuridine 5'-phosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, CA, and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis, MO, to give a specific activity of 500 μCi/μmmol. The cofactor, *dl*-tetrahydrofolic acid, was also purchased from Sigma Chemical Co. The synthesis of the inhibitor 5-nitro-2'-deoxyuridine 5'-phosphate has been described.[11]

Enzyme Assay. The enzyme was assayed by modification of the radioisotope assays described by Roberts[27] and Lomax and Greenberg.[26] The solution, 0.1 mL, contained 25 mM mercaptoethanol, 0.22 mM *dl*-tetrahydrofolic acid, 6.75 mM formaldehyde, 5 mM sodium bicarbonate, 3 mM magnesium chloride, 0.12 mM EDTA, 6 mM tris–acetate buffer, pH 6.8, 5 μL of the diluted enzyme solution, substrate, and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate [5-^{3}H]-2'-deoxyuridine 5'-phosphate was used at a specific activity of 500 μCi/μmol. The assays were started by the addition of the enzyme to the complete mixture and then incubated at 30 °C. Incubation was stopped at 15 s by the addition of 50 μL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added and the solution vortexed and allowed to stand 15 min. The suspension was filtered through a glass wool plugged Pasteur pipette, and 0.1 mL of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts. Velocity is reported in the adjusted value of picomoles of ^3H_3O formed per minute in the assay.

Preincubation Studies. The enzyme (~1.5×10^{-8} M) was preincubated at 30 °C in 50 μL of solution containing 5 mM 2-mercaptoethanol, 6 mM magnesium chloride, 0.24 mM EDTA, 12 mM tris–acetate buffer, pH 6.8, and varying concentrations of inhibitor. Substrate protection was evaluated by including the indicated concentration of substrate at a specific activity of 500 μCi/μmol. After incubation for the indicated time period, the assay for remaining active enzyme was started by the addition of 50 μL of a solution containing buffer and other components of the assay to give the same concentrations as noted in the enzyme assay. A high substrate concentration (50 μM) was used in these assays to afford

(36) Cleland, W. W. *Biochemistry* **1975**, *14*, 3220–3224.
(37) Galivan, J. H.; Maley, G. F.; Maley, F. *Biochemistry* **1976**, *15*, 356–362.
(38) Santi, D. V. *J. Med. Chem.* **1980**, *23*, 103–111.
(39) Park, J. S.; Chang, C. T.-C.; Mertes, M. P. *J. Med. Chem.* **1979**, *22*, 319–321.
(40) DeClercq, E.; Balzarini, J.; Descamps, J.; Bigge, C. F.; Chang, C. T.-C.; Kalaritis, P.; Mertes, M. P. *Biochem. Pharmcol.* **1981**, *30*, 495–502.
(41) Wataya, Y.; Santi, D. V.; Hansch, C. *J. Med. Chem.* **1977**, *20*, 1469–1473.
(42) Bigge, C. F.; Hasson, M. E.; Chang, C. T.-C.; Mertes, M. P. unpublished.

reasonably high velocity and to competitively reduce the enzyme inactivation by the inhibitor during the assay. The assay was run for 30 s and treated as described in the enzyme assay section. Inactivation of the enzyme was measured by comparing the velocity at time zero to that at the indicated incubation times. Under the conditions of the assay the uninhibited enzyme retained 96% of the initial activity after 4 min of incubation.

Determination of Adduct Formation. The ultraviolet spectra were recorded for solutions containing 5-nitro-2'-deoxyuridine 5'-phosphate [**2**] at a concentration of 5.4×10^{-5} M, 0.024 M tris–acetate buffer (pH 6.8), 0.012 M magnesium chloride, and 0.5 mM ethylenediaminetetraacetic acid, and concentrations of 2-mercaptoethanol (**5**) varying from 0 to 3.6×10^{-2} M concentration. Changes in the absorbance at 335 nm were used to determine the mole fraction of adduct **8** formation. Similarly, changes in absorbance at 335 nm were observed for 6.8×10^{-5} M solutions of 5-nitrouridine (**6**) treated in the same manner.

Acid dissociation constants (K_a apparent) for **2** and **6** were determined by ultraviolet absorption changes of solutions of varying pH and by titration (**6**).

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Communications to the Editor

Reaction of CS_2 with Metal Cluster Carbonyls of the Iron Triad: Synthesis and X-ray Structure of $[Fe_5(CO)_{13}S_2(CS)]$ Containing a Six-Electron-Donor Thiocarbonyl Group

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The thiocarbonyl ligand (CS) has been observed to bond to metal centers in the linear μ_1,[1] the bridging μ_2[1,2] and the triply bridging, μ_3, modes[3] (Figure 1a–c). It may also bridge two metal centers linearly by donation to one metal atom from the carbon and to the second from the sulfur[4] (Figure 1d). There are also examples of coordination to four metal atoms.[3,5] In $[\{(\eta^5\text{-}C_5H_5)Co\}_3(\mu_3\text{-}S)(\mu_3\text{-}CSCr(CO)_5)]$,[3] the Co_3 triangle is capped by the carbon while the sulfur donates to an independent chromium center (Figure 1e), and in $[Fe_4(CO)_{12}(CS)S]$[5] the carbon atom caps an Fe_3 triangle with the sulfur bonded to the fourth iron atom which is terminally bonded to the triangular Fe_3 unit (Figure 1f). In these bonding modes the thiocarbonyl ligand may be considered to donate two or four electrons to the metal unit.

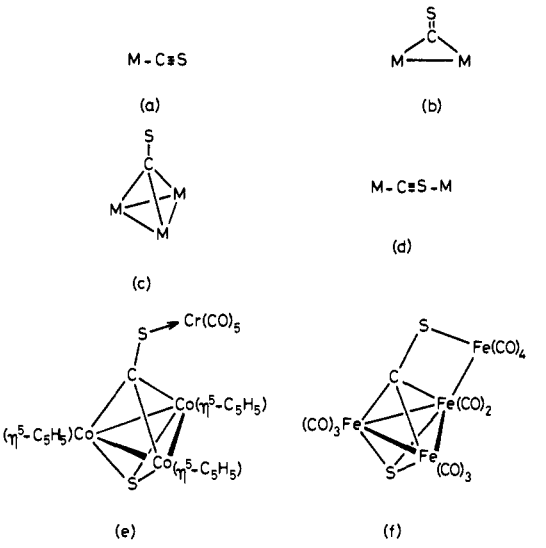

Figure 1. Modes of bonding of the thiocarbonyl ligand.

We now report the preparation and characterization of the complex $[Fe_5(CO)_{13}S_2(CS)]$ in which a further bonding mode for the thiocarbonyl ligand has been established. In this complex the thiocarbonyl group is bonded to four iron atoms arranged in a square and may be formally regarded as a six-electron donor. A further point of interest is the Fe_5 unit which is unusual in that it contains a square plane of four iron atoms with the fifth iron atom terminally bonded to this (Figure 2).

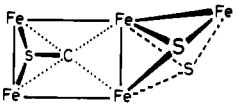

Figure 2. The cluster core in $[Fe_5(CO)_{13}S_2(CS)]$.

The reaction between $[Fe_3(CO)_{12}]$ and excess CS_2 in hexane solution (80 °C, under a 10-atm CO/Ar (1:1) pressure, 18 h) gave a mixture of products. After removal of the solvent, the mixture was dissolved in CH_2Cl_2 and separated by TLC using silica plates and hexane as eluant. Four products **1**–**4** were obtained. Component **1** was found to be $[Fe_3(CO)_9S_2]$,[6] component **2** was identified as $[Fe_4(CO)_{12}(C_2S_4)]$, component **4** was $[Fe_4(CO)_{12}(CS)S]$,[5] and component **3** has now been identified as $[Fe_5(CO)_{13}S_2(CS)]$.

The reaction conditions employed and the product distribution are similar to those reported[5] previously for the preparation of $[Fe_4(CO)_{12}(CS)S]$, except that the reaction time is longer. Under these conditions lower yields of **2** and **4** and higher yields of **1** and **3** (although **3** is obtained in only 2% yield) are obtained. Crystallization was achieved by the slow cooling of a benzene/hexane solution giving black platelets. Spectroscopic and analytical data have been obtained for **3**,[7] and full details of the molecular structure have been established through single-crystal X-ray structure analysis[8,9] (Figure 3).

(1) Butler, I.S. *Acc. Chem. Res.* **1977**, *10*, 359. Yaneff, P.V. *Coord. Chem. Rev.* **1977**, *23*, 183.
(2) Efraty, A.; Arneri, R.; Huang, M. H. A. *J. Am. Chem. Soc.* **1976**, *98*, 639. Dunker, J. W.; Finer, J. S.; Clardy, J.; Angelici, R. J. *J. Organomet. Chem.* **1976**, *114*, C49. Wnuk, F. A.; Angelici, R. J. *Inorg. Chem.* **1977**, *16*, 1173.
(3) Werner, H.; Leonhard, K. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 627. Werner, H.; Leonhard, K.; Kolb, O.; Rottinger, E.; Vahrenkamp, H. *Chem. Ber.* **1980**, *113*, 1654.
(4) Dombek, B. D.; Angelici, R. J. *J. Am. Chem. Soc.* **1974**, *96*, 7568. *Inorg. Chem.* **1976**, *15*, 2403.
(5) Broadhurst, P. V.; Johnson, B. F. G.; Lewis, J.; Raithby, P. R. *J. Chem. Soc., Chem. Commun.* **1980**, 812.
(6) Wei, C. H.; Dahl, L. F. *Inorg. Chem.* **1965**, *4*, 493.
(7) IR ν(CO) (cyclohexane solution) 2091 (w), 2062 (s), 2041 (vs), 2034 (ms), 2027 (w), 2022 (w), 2010 (m), 1977 (w); ν(CS) (Nujol mull) 921 (m) cm^{-1}. Anal. Calcd C, 22.34; H, 0.00. Found: C, 22.62% H, 0.12. Mass spectrum; *m/e* 752.

0002-7863/81/1503-3198$01.25/0