(d, 1, C_{6H}, J_{H-F} = 7.0 Hz). Anal. ($C_8H_9FN_2O_3$) C, H, N.

Optical Resolution of Thf-FU (3). (1) **Formation of Diastereoisomers with Brucine.** A saturated solution of the Thf-FU (10 g, 0.05 mol) in EtOH at 70 °C and a saturated solution of brucine dihydrate (21.5 g, 0.05 mol) in EtOH at 70 °C were mixed and the resulting solution stood at room temperature until crystallization was complete. The solid was recrystallized from EtOH at 70 °C to give 12.5 g (42.1%) of a pure diastereoisomer (**4**): mp 187–188 °C; $[\alpha]^{23}_D$ −46.0° (*c* 0.5, $CHCl_3$). Anal. ($C_{31}H_{35}FN_4O_7$) C, H, N. Concentration of the mother liquor gave a solid, which was recrystallized from EtOH at 70 °C to give 10.7 g (36.0%) of another pure diastereoisomer (**5**): mp 162.5–164 °C; $[\alpha]^{23}_D$ −89.6° (*c* 0.5, $CHCl_3$). Anal. ($C_{31}H_{35}FN_4O_7$) C, H, N.

(2) Recovery of *R* and *S* Isomers from the Diastereoisomers. (a) Treatment with Silica Gel. The crystals (**4**, 11.9 g) were dissolved in $CHCl_3$–EtOH (5:1, v/v) and applied to a column of silica gel (500 g) equilibrated with the same solvent. Elution with the same solvent gave 3.8 g (95%) of the *R*-(+) isomer of Thf-FU (**3a**): mp 174–175.5 °C; $[\alpha]^{23}_D$ +70.0°, $[\alpha]^{23}_{436}$ +182.0° (*c* 0.5, $CHCl_3$); UV λ_{max}^{pH2} 271 nm (ε 9100); UV λ_{max}^{pH7} 270 nm (ε 8800); UV λ_{max}^{pH12} 270 nm (ε 7000); ^{1}H NMR (pyridine-d_5) δ 3.73 and 4.10 [m(2), 2, $C_{4'H}$], 6.13 (qd, 1, $C_{1'H}$), 7.72 (d, 1, C_{6H}, J_{H-F} = 7.0 Hz). Anal. ($C_8H_9FN_2O_3$) C, H, N. The same treatment of crystals of **5** gave 3.7 g (92.5%) of the *S*-(−) isomer of Thf-FU (**3b**): mp 175–177 °C; $[\alpha]^{23}_D$ −70.0°, $[\alpha]^{23}_{436}$ −187.0° (*c* 0.5, $CHCl_3$); UV λ_{max}^{pH2} 271 nm (ε 9000); UV λ_{max}^{pH7} 270 nm (ε 8700); UV λ_{max}^{pH12} 270 nm (ε 6900); ^{1}H NMR (pyridine-d_5) δ 3.73 and 4.10 [m(2), 2, $C_{4'H}$], 6.12 (qd, 1, $C_{1'H}$), 7.72 (d, 1, C_{6H}, J_{H-F} = 7.0 Hz). Anal. ($C_8H_9FN_2O_3$) C, H, N.

(b) Treatment with Dilute HCl. A solution of the crystals (**4**, 5.9 g) in EtOH (150 mL) was mixed with dilute HCl keeping the pH at 3.5–4.5 at room temperature. The mixture was stirred for 1 h, then the solvent was removed in vacuo, and the residue was treated with water (30 mL)–$CHCl_3$ (90 mL). The $CHCl_3$ layer was separated, washed with water, and evaporated to dryness. Recrystallization of the residue from EtOH gave 1.8 g (90.1%) of the *R* isomer **3a**. The same treatment of crystals of **5** gave 1.7 g (85.6%) of the *S* isomer **3b**.

Antibacterial Activity. The minimum inhibitory concentrations (MIC) of the present compounds against various bacteria were determined in defined medium[15] (Muller Hinton medium) using the standard plate dilution method[16] with incubation at 37 °C for 24 h.

Antitumor Activity in Vivo. Male Donryu rats weighing 120 ± 5 g were inoculated subcutaneously in the inguinal region with 5×10^6 cells of AH-130 carcinoma or Yoshida sarcoma per rat. Intraperitoneal administration of a drug (90 mg/kg) was started 24 h later and continued daily for 7 days in test groups of six animals each. On the tenth day after the last inoculation the tumor was weighed and compared with tumors in the control group.

Degradation of the Thf-FU Isomers by the Microsome Fraction of Mouse Liver. The microsome fraction was prepared by a standard method from mouse liver and suspended in 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl at a concentration equivalent to that of a 25% liver homogenate. A mixture of 0.8 mL of this suspension, 0.1 mL of 50 mM NADPH, and the Thf-FU isomer (200 μg) was adjusted to 1 mL with the same buffer–salt mixture. This solution was incubated at 37 °C for 4 h and then extracted with $CHCl_3$ (10 vol × 2). The aqueous layer was separated and neutralized. FU was determined by assaying the antibacterial activity of the sample against *Staphylococcus aureus* 209P by the thin-layer-cup method.[17]

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Carbon-13 Nuclear Magnetic Resonance Investigations into the Interactions of Bisulfite with Pyrimidine Nucleosides and Nucleotides

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Carbon-13 NMR is utilized to demonstrate the attack of bisulfite anion on uridine, 5-fluorouridine, and uridine 5′-monophosphate. The attack produces a pair of diastereomeric adducts similar in structure to those seen in the uracil series. Intensity data from the equilibrium system give an estimate for the individual equilibrium constants. Thymidine and thymidine 5′-monophosphate show no evidence of nucleophilic attack by bisulfite. This evidence indicates that bisulfite addition to nucleosides and nucleotides models the enzymatic methylation of uridine by the enzyme thymidylate synthetase better than the uracil bisulfite system.

Nucleophilic attack at carbon-6 of the uracil ring is postulated as an integral part of the mechanism of pharmacologically important enzymatic reactions such as the conversion of 2′-deoxyuridine 5′-monophosphate to

Table I. Carbon-13 Chemical Shifts (ppm) for the Pyrimidines and Their Bisulfite Adducts[a]

Compd	C_2	C_4	C_5	C_6	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
Uridine	152.40	168.05	103.68	142.76	90.12	70.58	74.69	85.42	62.38
Thymidine	152.75	167.31	112.62[a]	137.71	85.26	38.77	71.00	86.53	61.75
5'-UMP	152.00	167.45	103.59	142.95	89.53	71.07	74.98	84.95[b] [d]	64.24[c] [d]
5'-TMP	152.64	167.39	112.51[d]	138.49	85.84	39.37	72.17	86.79[e] [d]	64.87[f] [d]
5-Fluorouridine	152.81	165.11[g] [d]	142.35[h] [d]	125.56[i] [d]	90.29	70.38	74.74	85.00	61.78
Uridine HSO_3^- A	153.88	172.91	33.57 (t)	65.90 (d)	92.66 (d)	70.77 (d)	72.74 (d)	84.74 (d)	62.69 (t)
Uridine HSO_3^- B	154.55	172.91	33.57 (t)	67.18 (d)	94.31 (d)	70.29 (d)	74.30 (d)	84.06 (d)	62.38 (t)
5'-UMP HSO_3^- A	153.70	172.93	33.28	66.77	92.45	71.17	74.89	83.08[j] (d)	64.82[k] (d)
5'-UMP HSO_3^- B	154.58	172.93	33.66	65.88	94.69	70.28	72.45	83.48[j] [d]	65.57[h] [d]
5-Fluorouridine HSO_3^- A	155.16	169.58[l] [d]	83.36[m] [d]	67.30[n] [d]	91.23	70.98	75.02	84.51	62.22
5-Fluorouridine HSO_3^- B	153.34	169.58[l] [d]	83.36[m] [d]	66.38[o] [d]	93.78	69.83	72.81	83.91	61.76

[a] The chemical shift of the C-5 methyl carbon is 12.40 ppm; letters in brackets denote observed multiplicity; letters in parentheses denote multiplicity observed in off-resonance decoupled or coupled spectra; t = triplet; d = doublet. [b] J_{CP} = 8.0 Hz. [c] J_{CP} = 3.6 Hz. [d] The chemical shift of the C-5 methyl carbon is 12.62 ppm. [e] J_{CP} = 8.5 Hz. [f] J_{CP} = 4.2 Hz. [g] J_{CF} = 236.6 Hz. [h] J_{CP} = 3.6 Hz. [i] J_{CF} = 35.5 Hz. [j] J_{CP} = 8.0 Hz. [k] J_{CP} = 3.6 Hz. [l] J_{CF} = 20.9 Hz. [m] J_{CF} = 194.5 Hz. [n] J_{CF} = 37.6 Hz. [o] J_{CF} = 36.5 Hz.

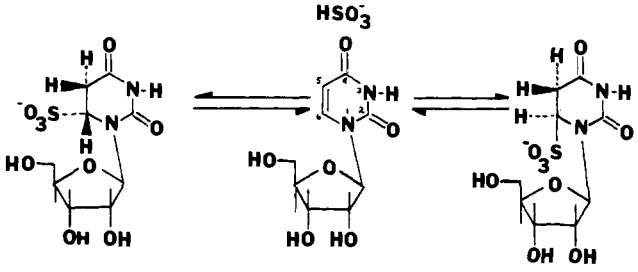


Figure 1. Formation of a pair of diastereomers upon addition of bisulfite to carbon-6 of uridine.

thymidylate by the enzyme thymidylate synthetase. This is the target enzyme for the well-known antitumor agents, 5-fluorouracil, 2'-deoxy-5-fluorouridine 5'-monophosphate, and 5-trifluoromethyluracil.[2]

Nucleophilic attack of bisulfite on uracil and uridine is assumed to be a valid model for this enzymatic process. While the literature is replete with reactions of bisulfite with uracil,[3-10] it is relatively devoid of investigations concerning bisulfite additions to nucleosides and nucleotides.[11-13] In this work natural abundance ^{13}C NMR is utilized to (a) demonstrate the formation of diastereomeric bisulfite adducts of uridine (Figure 1) which are similar in structure to the uracil bisulfite adduct;[14] (b) demonstrate that uridine 5'-monophosphate (5'-UMP) and 5-fluorouridine form similar adducts; (c) provide a method for calculation of equilibrium constants for these additions; and (d) demonstrate the failure of thymidine and thymidine 5'-monophosphate (5'-TMP) to form bisulfite adducts in detectable quantities under identical conditions. This evidence strengthens the hypothesis that the nucleophilic attack of bisulfite anion (or the sulfite dianion) on carbon-6 of the pyrimidine ring of the nucleoside or nucleotide is a valid model for the more complex enzymatic process with thymidylate synthetase.

In such a model system the initiating step should be a process that is favored (i.e., the initiating step of this mechanism is a nucleophilic attack at carbon-6 of the substrate). Further, after the methylation has occurred the intermediate should be an unstable species and collapse to product, regenerating the enzymic nucleophile. The relative stabilities of the adducts within the model system (bisulfite interacting with uridine and thymidine) support such a hypothesis.

Experimental Section

Source of Materials. 5-Fluorouridine was obtained from Calbiochem Co., San Diego, Calif., and used without further purification. Uridine, uridine 5'-monophosphate, thymidine, and thymidine 5'-monophosphate were obtained from Aldrich Chemical Co., Milwaukee, Wis., and used without further purification.

Nuclear Magnetic Resonance Spectra. NMR spectra were measured on 1.0-mL solutions in 8-mm sample tubes with a Varian Associates CFT-20 spectrometer. Standard operating conditions were pulse width = 7 μs (45° flip angle); aquisition time for a 4K FID = 0.512 s; total cycle time = 0.512+ s (no delay); sweep width = 4000 Hz (200 ppm); accumulated transients = 10000; exponential smoothing and apodization selected to optimize spectra, and complete proton decoupling. Spectra were measured in aqueous solution referenced to dioxane ^{13}C as 1348.8 Hz from Me_4Si. When needed, off-resonance partially decoupled spectra or high-resolution coupled spectra were obtained. The ambient probe temperature was 31 °C. The lock signal was provided by a capillary tube containing D_2O.

Sample Preparation. Samples were prepared by dissolving the compound in water to form a molar solution. To 1 mL of this solution sodium bisulfite (105 mg, 1.0 mmol) was added and the pH adjusted to 6.9 with sodium hydroxide. The sample was allowed to stand at room temperature for 24 h to equilibrate.

Calculation of Equilibrium Constants. The equilibrium constants for the system shown in Scheme I were calculated using eq 1 and 2 where A is the more stable bisulfite adduct, B is the

$$K_{eq(A)} = [A]_e/[Urid]_e[HSO_3^-]_e \quad (1)$$

$$K_{eq(B)} = [B]_e/[Urid]_e[HSO_3^-]_e \quad (2)$$

less stable bisulfite, and Urid is the unreacted uridine.

The values of $[A]_e$, $[B]_e$, and $[Urid]_e$ can be obtained from eq 3 and the analogous equations for $[B]_e$ and $[Urid]_e$ where I_a, I_b,

$$[A]_e = I_a/(I_a + I_b + I_c) \times [Urid]_T \quad (3)$$

and I_c are the signal intensities corresponding to the adducts and starting materials. $[Urid]_T$ is the total uridine in the system ($[Urid]_T = [Urid]_e + [A]_e + [B]_e$). The calculation of $[HSO_3^-]_e$ was done using eq 4 in which $b = 1 + K_{a_2}/[H^+] + [H^+]/K_{a_1}$, a =

$$[HSO_3^-]_e = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \quad (4)$$

$2K$, and $c = [A]_e + [B]_e - [HSO_3^-]_T$ where K_{a_1} and K_{a_2} are the dissociation values for sulfurous acid, and K is the equilibrium constant for the formation of pyrosulfite.[16]

Results and Discussion

Measurement of the ^{13}C NMR spectrum of uridine in water provides a spectrum identical with that previously reported[15] in which all the signals have been assigned (Table I). Measurement of the ^{13}C NMR of the same sample (Figure 2, upper) after the addition of sodium

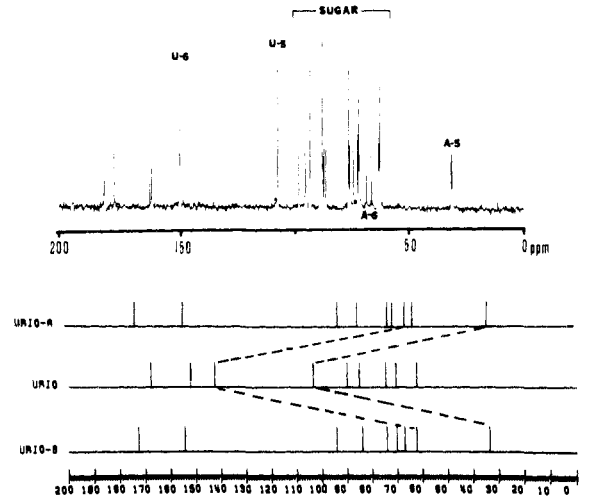


Figure 2. Schematic plot (lower) of the ^{13}C NMR spectra (upper) of the three substances in the reaction mixture: uridine, uridine bisulfite adduct A, and uridine bisulfite adduct B.

Table II. ^{13}C NMR Intensity Data for the Uridine Series

Compd	Intensity at carbon-1'	% intensity
Uridine	62	59.6
Uridine HSO_3^- A	23	22.0
Uridine HSO_3^- B	20	19.4
Uridine PO_4^{2-}	48	30.8
Uridine PO_4^{+} HSO_3^- A	55	35.3
Uridine PO_4^{2-} HSO_3^- B	53	33.9
5-Fluorouridine	85	39.5
5-Fluorouridine HSO_3^- A	71	33.0
5-Fluorouridine HSO_3^- B	59	27.4

bisulfite shows the same uridine spectrum superimposed on the spectra of a pair of diastereomeric uridine bisulfite adducts (Figure 2, lower). It is significant that the signals arising from C_5 and C_6 of the adducts (33.57, 33.76, and 65.90 ppm, respectively) are in agreement with those previously reported for the bisulfite adduct of uracil.[14] The presence of two hydrogens on C-5 and one on C-6 was verified by off-resonance decoupled and coupled spectra. The presence of two signals for C-6 and C-5 of the adducts as well as the appearance of two new signals for each of the five carbon atoms of the ribose group, all of unequal intensity, demonstrates the expected differential formation of two diastereomeric adducts of uridine (Figure 1).[17] It is not possible to specify which signals correspond to which diastereomer, but the reasonable assumption has been made that the stronger signal of each pair corresponds to one diastereomer (labeled A) and the weaker signal of each pair corresponds to the other diastereomer (labeled B) (Table I).

Reaction of 5'-UMP with bisulfite in aqueous solution at pH 6.9 was followed in exactly the same manner. First the spectrum of 5'-UMP was measured (Table I) and then the spectrum of the reaction mixture following addition of sodium bisulfite. The spectrum of the reaction solution is similar to that of uridine except that the relative intensities of the adduct signals are greater than those seen in the uridine system (Table II). The reaction of 5-fluorouridine[18] with bisulfite was measured in the same manner (Table I).

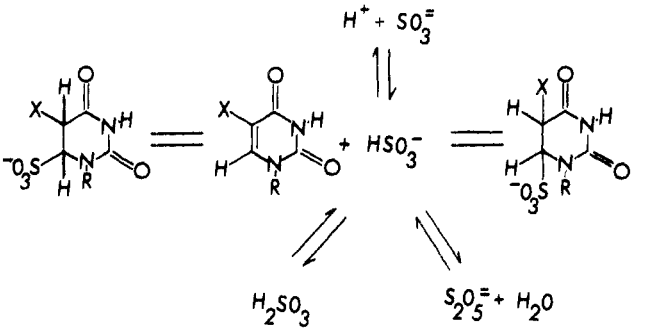
While ^{13}C NMR intensity data obtained in pulse FT experiments are not necessarily proportional to the amounts of material giving rise to the observed signals, unless special experimental conditions are met, it is possible to use routine intensity data in certain restricted

Table III. Equilibrium Constants for the Uridine Series in Aqueous Bisulfite Solution

Reaction	I_u[a]	I_a[a]	I_b[a]	K_a	K_b	Ratio K_a/K_b
Uridine + HSO_3^-	62	23	20	1.30	1.14	1.14
5-Fluorouridine + HSO_3^-	85	71	59	4.13	3.38	1.22
Uridine PO_4^{2-}	48	55	53	7.20	6.92	1.04

[a] I_u = intensity of uridine, 5-fluorouridine, or 5'-UMP. I_a = intensity of adduct A. I_b = intensity in adduct B.

Scheme I



circumstances. In this case, it is reasonable to assume that T_1 values and variations in intensity are essentially the same for some of the carbons, notably C_1' which shows well-resolved nonoverlapping signals, in the starting uridine and in the adducts (Table II). This permits an estimation of the equilibrium constants for the formation of the diastereomeric adducts of uridine, 5-fluorouridine, and 5'-UMP. After correcting for the appropriate equilibria involving HSO_3^-,[16] we obtain the equilibrium constants presented in Table III for the system presented in Scheme I. The values are, at best, good to 5% but do provide an indication of the order of magnitude involved.[19] It is significant that the equilibrium constants for 5-fluorouridine are greater than those of uridine by a factor of 4.

The reaction of thymidine and 5'-TMP with bisulfite in aqueous solution at pH 6.9 was followed as described above (Table I). Shapiro et al.[10] have produced evidence that bisulfite does add to thymidine. However, Hayatsu et al.[20] and Klotzer[21] report that they were unable to generate such adducts. Neither the nucleoside nor the nucleotide demonstrates any spectral evidence of bisulfite adducts, even when subjected to high concentrations of sodium bisulfite and measured over a pH range from 3 to 8.

If the nucleophilic addition of bisulfite across the $>C_6{=}C_5<$ bond of the uracil ring is a model for the enzymatic process, as previously postulated, then uridine and 5'-UMP should be even better models for the enzymatic system. In enzyme systems which follow mechanisms involving covalent bond catalysis, the stability of the substrate–enzyme covalent complex should be large relative to that of the product–enzyme complex. Indeed, the model substrate, uridine, is found to have equilibrium constants of 1.30 and 1.14 L/mol, while 5'-UMP is found to have equilibrium constants of 7.20 and 6.92 L/mol. Conversely in the cases of both model products (thymidine and 5'-TMP), there is no indication of bisulfite addition (at a minimum the equilibrium constants are too small to be measured by this method). This model system supports the mechanistic pathway previously described. The initial step for the enzymatic process is nucleophilic attack (the cysteine thiol has been suggested as the nucleophile[2,10]) resulting in a dihydrouridine (or dihydrouridylate) species (Figure 3). The model system demonstrates that such addition is favored as indicated by the equilibrium con-

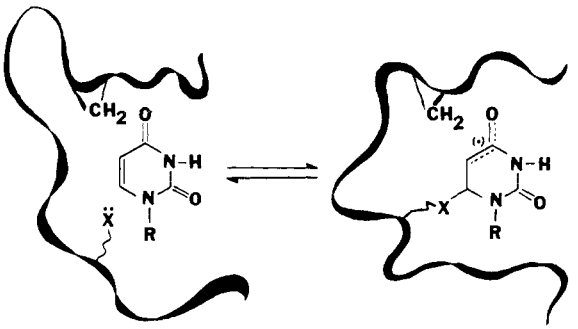


Figure 3. Enzyme nucleophilic attack resulting in formation of a dihydrouridylate species.

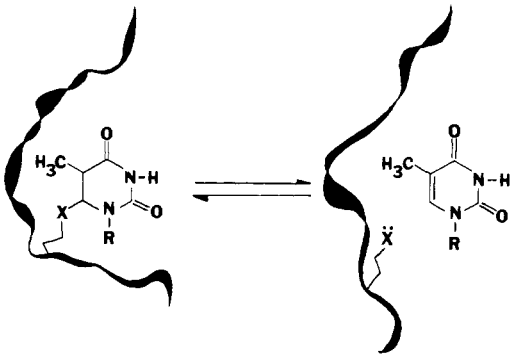


Figure 4. Elimination of the nucleophile releasing thymidylate and regenerating the enzyme.

stant for bisulfite addition. Subsequent methylation by the folate coenzyme would result in a dihydrothymidine (or dihydrothymidylate) species (Figure 4). In the model system this species is not favored. Thus, after methylation the equilibrium would favor the free thymidine, and the complex would dissociate, regenerating the nucleophilic moiety on the enzyme and the product (thymidine).

Clearly a molecule which forms a more stable covalent complex with the enzyme will act as a competitive inhibitor of that enzyme. The equilibrium constants for 5-fluorouridine are 4.13 and 3.38 L/mol, a factor of 4 greater than the equilibrium constants for uridine.

Carbon-13 NMR has been utilized to demonstrate the existence of the bisulfite adducts of uridine, 5-fluorouridine, and 5′-UMP as pairs of diastereomers of differing stabilities. Each diastereomer has a unique carbon-13 NMR spectrum resulting from stereochemical differences within the adducts. It is expected that the assignment of the chemical shifts of the diastereomers in the model system will allow the facile examination of the enzymatic process resulting in the elucidation of the stereospecific enzymatic pathway. Further, the data strongly support this model system and suggest that 5′-UMP HSO_3^- more closely models the enzymatic process than does uracil HSO_3^-.

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(17) The steric differences result because nucleophilic attack can occur from above or below the plane of the double bond. Thus carbon-6 becomes optically active, and since the nearby sugar is also optically active, the products are distinguishable diastereomers.
(18) As noted earlier the diastereomers differ only in the orientation of the substituents at carbon-6. In the case of the 5-halouridines, four diastereomers are possible; however, this was observed only in the case of 5-chlorouridine. Only two diastereomers are observed upon bisulfite addition to 5-fluorouridine and 5-bromouridine (J. W. Triplett, Ph.D. Thesis, University of Kentucky, Lexington, Ky., 1977).
(19) The value for K_{eq} of the total reaction ($[A]_e + [B]_e =$ [product]) calculated from these data was found to match that calculated using data presented by Shapiro et al.[10]
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