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The Dehalogenation of Iodouracil by Cysteine. Intramolecular General-Acid Catalysis of Cysteine Addition to 5-Iodouracil[1]

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The rate-determining step of the cysteine-catalyzed deiodination of 5-iodouracil is the formation of 5-iodo-6-cysteinyl-5,6-dihydrouracil. The rate of the reaction depends upon the concentration of un-ionized 5-iodouracil and the following ionic species of cysteine; $^{-}OOC—(NH_3^+)—CH—CH_2—S^-$. Unlike the reaction of 2-mercapto-ethanol with 5-iodouracil, the cysteine reaction is not subject to catalysis by imidazolium ion and tris(hydroxymethyl)aminomethane hydrochloride. When the rates of cysteine reacting with 5-iodouracil are measured in both H_2O and D_2O,a large kinetic isotope effect is observed ($k_2^{H_2O}/k_2^{D_2O} = 4.10$), thus implicating the protonated α amino group of cysteine as an intramolecular general acid catalyst for the reaction. These results and possible mechanisms for the actual dehalogenation of the intermediate 5-iodo-6-cysteinyl-5,6-dihydrouracil are discussed in terms of a possible mechanism for enzymatic halopyrimidine dehalogenation.

In a recent communication from this laboratory, we reported that both cysteine and 2-mercaptoethanol promoted the rapid dehalogenation of 5-iodo- and 5-bromouracil (*1*). These results were not unexpected since it had been previously shown that both bisulfite buffers (*2*) and sodium hydrogen sulfide (*3*) dehalogenate 5-halouracils with marked rapidity. Simple thiols are thought to attack carbon 6 of the pyrimidine ring system as does sulfite ion (*4, 5*) to initially yield a 6-substituted dihydropyrimidine. Evidence for the formation of such a compound is taken from the fact that both bisulfite (*4, 5*) and simple thiols such as 2-mercaptoethylamine (*6*), glutathione (*7*), and cysteine (*8*) catalyze hydrogen–deuterium exchange at carbon 5 of various pyrimidine rings; however, only in the case of the intramolecular attack of the 5′ thiol of 5′-thiol-2′,3′-*O*-isopropylideneuridine on carbon 6 of the uracil ring system has such a thiol-substituted 5,6 dihydropyrimidine been isolated (*9*). Both 5-bromo- and 5-iodouracil are enzymatically dehalogenated (*10–13*) via a presumed mechanism which involved reduction of the 5,6 double bond, followed by spontaneous elimination of either HI or HBr. Recent studies in this laboratory have shown that neither the hydroxide ion nor the tris(hydroxymethyl)aminomethane-catalyzed elimination of HBr from 5-bromo-5,6-dihydrouracil is nearly rapid enough to account for the overall rate of enzymatic halouracil dehalogenation (*14*). Consequently, nucleophilic reactions between cysteine

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and 5-iodouracil may be directly related to the enzymatic mechanism of 5-iodo- and 5-bromouracil dehalogenation.

EXPERIMENTAL SECTION

Materials. Reagent grade inorganic salts were used as received. Water was deionized, glass distilled, and stored in polyethylene containers prior to the preparation of solutions. Deuterium oxide (99.7%, Matheson, Coleman, Bell) was glass distilled before use. Imidazole (Sigma Chemical Company, Grade I) was recrystallized three times from hot benzene (mp 89.5–90.5°C uncorrected, lit. 90–91°C corrected). 5-Iodouracil and tris(hydroxymethyl)aminomethane (Tris reagent grade) were from Sigma Chemical Company and were used as received. 2-Mercaptoethanol (Sigma Chemical Company) was vacuum distilled and stored under argon at 0°C prior to use. L-Cysteine hydrochloride and *S*-methylcysteine (Aldrich Chemical Company) were used as received.

Kinetic measurements. The rates of cysteine and 2-mercaptoethanol addition across the 5,6 double bond of 5-iodouracil were spectrophotometrically monitored by measuring the decrease in either 290-nm absorbance (pH < 8.8) or 300- 310-nm absorbance (pH > 8.8) using either a Gilford 2000, Gilford 200, or a Zeiss PMQ II spectrophotometer each of which was equipped with a cell holder thermostated at 25°C. Reactions were initiated by the addition of 0.10 ml of 5.0×10^{-3} M 5-iodouracil to 2.90-ml solutions of the other reactants which had been equilibrated at 25°C in 3.0 ml capped cuvettes. Due to the extreme sensitivity of cysteine to oxidation, all stock solutions were deaerated with water-saturated argon prior to use. All transfers were performed under a constant stream of argon using argon-purged pipets, volumetric flasks, and cuvettes. To further prevent oxidation, all reaction mixtures contained 1×10^{-4} M EDTA·2 Na. Ionic strength was maintained at 1.0 M by either the addition of NaCl (cysteine) or $NaClO_4$ (2-mercaptoethanol). Pseudo first-order rate constants (k_{obsd}) were determined from semilogarithmic plots of $A_t - A_\infty$ against time and the relationship $k_{obsd} = 0.693/t_{1/2}$. Values of pH were measured after the completion of each reaction using a Radiometer PHM-26 pH meter equipped with a Radiometer GK2321C combination electrode.

Kinetic experiments in D_2O. All solutions were prepared in freshly distilled, deaerated deuterium oxide. Oxygen was excluded using argon as was previously described for the experiments in water. Values of pD were calculated from pH values determined as previously described using the relationship pD = pH + 0.40 (15).

Determination of pK_a *Values.* All acid dissociation constants were measured in both H_2O and D_2O at 25°C and ionic strength 1.0 M. For 5-iodouracil, the pK_a was determined spectrophotometrically at 303 nm from linear plots with slope equal to one of log ($A_{base} - A_{obsd}/A_{obsd} - A_{acid}$) against pH (*16*). In the case of cysteine, the apparent pK_a values for the thiol group and the protonated amine were determined by fractional neutralization of cysteine and *S*-methylcysteine using the Henderson-Hasselbach equation. The individual pK_a values for the thiol group and the protonated amine were then calculated using the apparent pK_a values and the equations derived by Grafius and Neilands (*17*).

Product analysis. As previously reported, uracil was found to be the pyrimidine product of these reactions (*1*).

RESULTS

The addition of cysteine and 2-mercaptoethanol to 5-iodouracil obeyed strict first-order kinetics in all reactions examined. Semilogarithmic plots of extent reaction plotted against time were linear for at least four, and in most cases, seven half-lives.

At constant external buffer concentration and pH, the pseudo first-order rate constants (k_{obsd}) for the reaction of cysteine and 5-iodouracil increased linearly as a function of the first power of total cysteine concentration. Figure 1 shows typical results at three different pH values using tris(hydroxymethyl)aminomethane (Tris) as an external buffer.

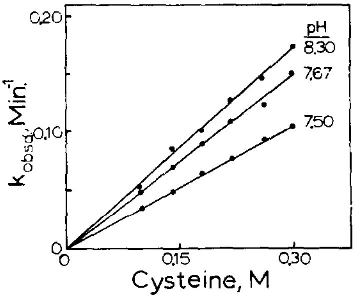


FIG. 1. Effect of increasing total cysteine concentration on the observed rate constants for cysteine addition to 5-iodouracil. Reaction mixtures contained 0.40 M Tris buffer and 1.67×10^{-3} M 5-iodouracil, 1×10^{-4} M EDTA, and varying amounts of cysteine, 25°C, $\mu = 1.0$ M.

To evaluate the nature of the reactive species and their contribution to the overall rate of the reaction, it was necessary to determine the pK_a values of both 5-iodouracil and cysteine in both H_2O and D_2O. These data determined at ionic strength 1.0 M and 25°C are summarized in Table 1. The values for the ionization of the various cysteine

TABLE 1

ACID DISSOCIATION CONSTANTS OF 5-IODOURACIL AND CYSTEINE IN EITHER H_2O OR D_2O AT 25°C AND IONIC STRENGTH 1.0 M

Compound	pK_a	
	H_2O	D_2O
5-Iodouracil	8.01	8.52
Cysteine[a]		
pK_a^2	8.33	8.58
pK_a^3	10.23	10.88
S-Methylcysteine	8.80	9.40
Cysteine[b] (calcd)	8.51	8.65

[a] Apparent pK_a values.

[b] Calculated as described in the Experimental Section and Ref. 17. Represents the ionization of the following species:
$^{-}OOC—(NH_3^+)—CH—CH_2—SH \rightleftarrows {}^{-}OOC—(NH_3^+)—CH—CH_2—S^- + H^+$.

species and for *S*-methylcysteine are in reasonable agreement with the values determined by Grafius and Neilands (*17*) in 0.15 M NaCl; however, the value for 5-iodouracil is 0.24 pH units lower than the value previously reported by Berens and Shugar working in dilute (~0.05 M) buffers of unspecified ionic strength at 20–22°C (*18*). Using these pK_a values, the observed pH, the total cysteine concentration, and the equations derived by Williams *et al.* (*19*), it was possible to calculate the concentration of the ionic form of cysteine which could act as both a nucleophile and an intramolecular general acid, namely, $^{-}$OOC—(NH_3^+)—CH—CH_2—S^-(NH_3^+—Cys—S^-). Linear plots with zero intercepts of k_{obsd} against [NH_3^+—Cys—S^-] were then constructed using data obtained over several pH units. The slopes of these lines represent the

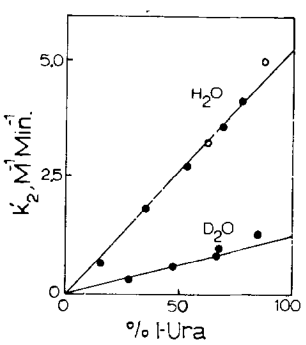


FIG. 2. Dependence in both H_2O and D_2O of the apparent second-order rate constants for $^{-}$OOC (NH_3^+)—CH—CH_2—S^- addition across the 5,6 double bond of 5-iodouracil on the percentage of un-ionized 5-iodouracil (I-Ura) present at any particular pH value. Open circles represent data obtained in imidazole rather than Tris buffers.

apparent second-order rate constants (k_2') for the reaction of NH_3^+—Cys—S^- with the total concentration of all species of 5-iodouracil. Plotting these apparent second-order rate constants (k_2') as a function of the percentage un-ionized 5-iodouracil results in a linear relationship (Fig. 2) which can be extrapolated to 100% to yield $k_2^{\mathrm{H_2O}} = 5.25$ M^{-1}min^{-1}, the true second-order rate constant for the reaction of un-ionized 5-iodouracil with NH_3^+—Cys—S^-.

The effect of pH on the rate of cysteine reacting with 5-iodouracil was measured at constant total cysteine concentration (0.167 M) by plotting the zero intercepts (k_0) of plots of k_{obsd} versus either the concentration of Tris$^+$–HCl or imidazolium ion. The results shown in Fig. 3 are compatible with a rate law (Eq. 1) which involves cysteine, NH_3^+—Cys—S^-, reacting with un-ionized 5-iodouracil (5-IUra). Using the appropriate pK_a values for cysteine (Table 1) and the equations of Williams et al. (*19*), it is then

$$\text{Rate} = k_2^{\mathrm{H_2O}}[\text{5-IUra}]\,[\mathrm{NH_3^+\!-\!Cys\!-\!S^-}] \qquad (1)$$

possible to derive Eq. 2.

$$\frac{\text{Rate}}{[\text{5-IUra}]_{\text{total}}} = k_{\text{obsd}} = k_0 = k_2^{\mathrm{H_2O}}\,\alpha\left(\frac{[\text{cysteine}]_{\text{total}}}{1 + \frac{[\mathrm{H^+}]}{K_a} + \frac{K_c}{[\mathrm{H^+}]} + \frac{K_b}{K_a}}\right) \qquad (2)$$

This equation which describes k_0 as a function of $k_2^{H_2O}$, $[H^+]$, the total concentration of all cysteine species, the fraction un-ionized 5-iodouracil (α), and a series of dissociation constants (K_a, K_b, and K_c[4]) for the ionization of the various cysteine species was used to calculate the solid line shown in Fig. 3. In the ascending limb of this figure, this equation gives an excellent fit with the experimental data. The deviation observed in the descending limb is probably associated with the uncertainties in determining the acid dissociation constants of the various species of cysteine using S-methylcysteine as a model compound (*17*).

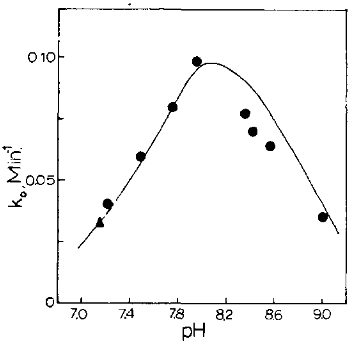
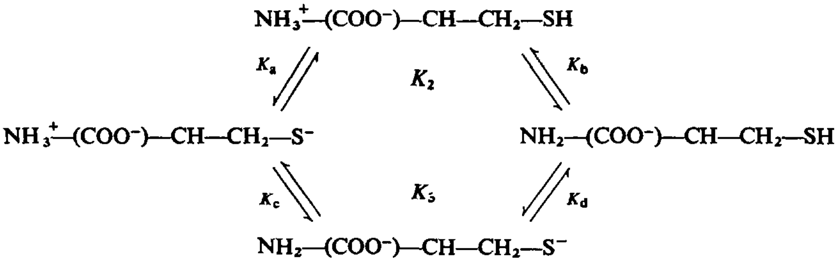


FIG. 3. The effect of pH on the rate of cysteine addition across the 5,6 double bond of 5-iodouracil, 25°, $\mu = 1.0$ M. Values of k_0 were obtained from the zero intercepts of plots of k_{obsd} against increasing tris (hydroxymethyl) aminomethane concentration (Fig. 2). The closed triangle indicates data obtained in imidazole rather than Tris buffers. The solid line was calculated using Eq. 2.

The effectiveness of both Tris and imidazole buffer systems as potential catalysts for the addition of cysteine to 5-iodouracil was evaluated by measuring k_{obsd} at constant cysteine concentration, pH, and ionic strength as a function of increasing buffer concentration. In the case of Tris buffers, observed rate constants were measured between 0.20 and 0.53 M total buffer at six different buffer ratios (pH 7.22–9.03), while in the case of imidazole buffer, the total buffer concentration range was 0.200–0.450 M at pH = 7.15. The results of these experiments indicate that k_{obsd} does not increase

[4] Value of K_a, K_b, K_c, and K_d are defined and calculated by the following scheme and equations of Grafius and Neilands (17). From this representation, $K_2 = K_a + K_b$, $K_3 = K_c + K_d$, and $K_2K_3 = K_aK_c = K_bK_d$. Thus, using the values of p$K_a{}^2$ and p$K_a{}^3$ for cysteine (Table 1) and assuming that the pK_a for



S-methylcysteine (Table 1) represents the ionization designated by K_b, K_a and K_c can be calculated from the above equations. This treatment results in values in H_2O of 3.09×10^{-9} M, 1.59×10^{-9} M, and 8.90×10^{-11} M, for K_a, K_b, and K_c, respectively. Values for K_a, K_b, and K_c in D_2O were calculated in the same manner.

with increasing buffer concentration. Consequently, it can be concluded that neither Tris$^+$–HCl nor imidazolium ion catalyze this reaction.

This lack of catalysis by Tris buffers is in direct contrast to similar data obtained for 5-iodouracil and 2-mercaptoethanol which does not contain a protonated amine group. Figure 4 shows the results of plotting k_{obsd} against increasing Tris$^+$–HCl concentration at two different buffer ratios. The equality of the slopes at both pH 8.12 and 7.85 indicate that the protonated amine is the buffer species responsible for catalysis of 2-mercaptoethanol addition to 5-iodouracil.

The reaction between cysteine and 5-iodouracil was examined for possible hydrogen-deuterium isotope effects by measuring k_{obsd} as a function of increasing cysteine concentration at several pD values using D_2O as solvent (Fig. 2). Extrapolation to 100% un-ionized 5-iodouracil results in a second-order rate constant ($k_2^{D_2O}$) for the reaction of ND_3^+—Cys—S^- with un-ionized 5-iodouracil equal to 1.28 $M^{-1}min^{-1}$. Thus, the ratio $k_2^{H_2O}/k_2^{D_2O} = 4.10$.

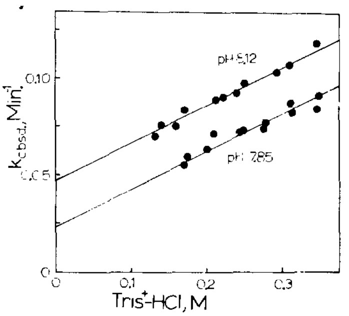
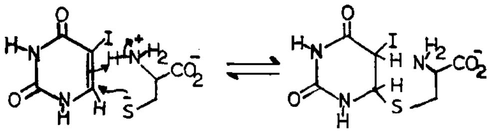


FIG. 4. Dependence of the observed rate constants for 2-mercaptoethanol addition across the 5,6 double bond of 5-iodouracil on increasing concentrations of tris (hydroxymethyl) aminomethane hydrochloride (Tris$^+$–HCl). Reaction mixtures contained 0.95 M 2-mercaptoethanol, 1.67×10^{-4} M 5-iodouracil, 1×10^{-4} M EDTA, and increasing concentrations of Tris buffers, 25°C, $\mu = 1.0$ M.

In the case of the buffer-catalyzed addition of 2-mercaptoethanol to 5-iodouracil, potential hydrogen-deuterium isotope effects were evaluated in Tris buffer (63% Tris$^+$–HCl). The slopes of data plotted as in Fig. 4 were used to evaluate $k_2^{H_2O}$ and $k_2^{D_2O}$. The resulting ratio ($k_2^{H_2O}/k_2^{D_2O} = 1.13$) indicates that considerably less zero point energy is lost due to deuterium transfer in the 2-mercaptoethanol reaction than is the case in the reaction of cysteine.

DISCUSSION

The analysis of the kinetic data, the hydrogen-deuterium kinetic isotope effect of 4.10, and the lack of effective catalysis by potential external general acids are most compatible with two possible mechanisms for the reaction of cysteine with 5-iodouracil (Eqs. 3 and 4). Based on the fact that the overall reaction has a first order dependence on cysteine concentration and the magnitude of the hydrogen-deuterium isotope effect each of these mechanisms requires that the formation of 5-iodo-6-cysteinyl-5,6-dihydrouracil is the rate-determining step in the overall reaction. No clear distinction



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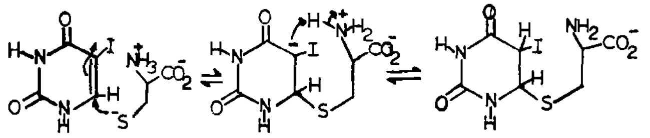

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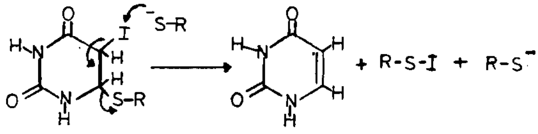
can be made between these two mechanisms; however, based on the observed trans stereochemistry for the analogous addition of bisulfite across the 5,6 double bond of uracil (*20, 21*), the concerted mechanism shown in Eq. 3 might be favored.

The presence of the large hydrogen-deuterium isotope effect for addition of cysteine to 5-iodouracil ($k_2^{H_2O}/k_2^{D_2O} = 4.10$) compared to the much smaller ratio for the Tris$^+$–HCl catalyzed addition of 2-mercaptoethanol ($k_2^{H_2O}/k_2^{D_2O} = 1.13$) would indicate that in the case of cysteine, the protonated α-amino group is acting as an intramolecular general acid catalyst of proton transfer to carbon-5 of the pyrimidine ring system. This interpretation relative to the site of proton transfer for the cysteine reaction is supported by the fact that isotope effects of similar magnitude are observed for the general base-catalyzed desulfonation of 1,3 dimethyl-5,6-dihydrouracil-6-sulfonate (*22*) and the bisulfite-catalyzed hydrogen–deuterium exchange reaction at carbon-5 of uridine (*5*). In the case of the Tris$^+$–HCl-catalyzed addition of 2-mercaptoethanol to 5-iodouracil, the much smaller isotope effect ($k_2^{H_2O}/k_2^{D_2O} = 1.13$) might be considered as being compatible with a reaction involving proton transfer to a more electronegative atom such as the oxygen atom on carbon-4 of the iodouracil ring system. Thus, it probably can be concluded that the site of proton transfer in the intramolecular catalyzed cysteine reaction is different from that of the intermolecular catalyzed addition of 2-mercaptoethanol to 5-iodouracil. This result might argue that the addition of bisulfite across the 5,6 double bond of uracil is not the microscopic reverse of the general base-catalyzed desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate (*22*). Work currently in progress should help resolve this question.

The lack of external buffer catalysis for the addition of cysteine to 5-iodouracil also supports the idea of intramolecular catalysis of proton transfer for this reaction. Deboer et al. (*23*) studying the elimination of water from the photohydrates of both cytidine and cytidine monophosphate, have observed external buffer catalysis for the reaction of cytidine but not of cytidine monophosphate. These workers have explained these results in terms of intramolecular general base catalysis of proton transfer from carbon 5 by the 5′-monophosphate anion, a highly warranted conclusion based on the earlier work of Fikus et al. (*24*) with cytidine 5′-dimethyl phosphate. Based on these results, it can probably be concluded that the lack of observable buffer catalysis for the addition of cysteine to 5-iodouracil is due to the presence of an effective intramolecular general acid catalyst, the protonated α-amino group.

The fact that the addition of cysteine to 5-iodouracil appears to be rate-determining does not allow these kinetic data to elucidate the mechanism of the dehalogenation of the resulting 5-iodo-6-cysteinyl-5,6-dihydrouracil to yield uracil as the final pyrimidine product. Several promising possibilities which involve the participation of additional thiol molecules do, however, exist.[5] The first of these (Eq. 5) involves the attack of a



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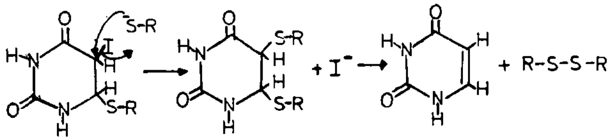
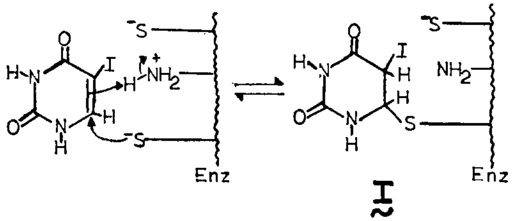
second molecule of thiol anion to yield a sulphenyl iodide (*25*). These are known compounds which further react with both thiols and water to give disulfides (R–S–S–R), iodide and mixtures of sulfinic (RSO_2H), and sulfonic acids (RSO_3H).

A second possibility involves a nucleophilic substitution reaction between carbon 5 of the dihydropyrimidine ring system and another molecule of thiol anion (Eq. 6)[6]. This reaction involves the formation of a 5,6 dithiol-substituted dihydropyrimidine, which



(6)

must then eliminate the two thiol groups, perhaps by an internal oxidation reaction involving the formation of a disulfide (R–S–S–R). This reaction has been proposed to be involved in NaSH dehalogenation of 5-bromouracil (*3*) and must be considered because



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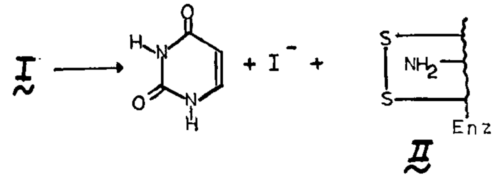

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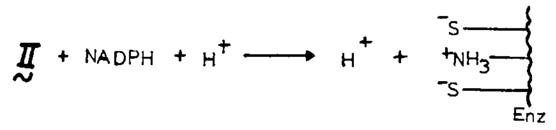

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[5] A third equally attractive hypothesis was suggested by a referee. This mechanism involves the attack of a thiol anion on the sulfur atom of 5-iodo-6-cysteinyl-5,6-dihyrouracil to yield uracil, I^- and a disulfide.

[6] *Note added in proof.* In recent communication, Wataya, *et al.* (26) have reported that cysteine debrominates 5-bromo-2′-deoxyuridine to yield both deoxyuridine and S-[5-(2′-deoxyuridyl)] cysteine.

of its analogy to known reactions which alkylate thiol groups, such as the reaction of cysteine with iodoacetamide (*25*).

The mechanism for the enzymatic dehalogenation of 5-iodo and 5-bromouracil is presumed to involve enzymatically catalyzed triphosphopyridine nucleotide-linked reduction of the 5,6 double bond, followed by spontaneous elimination of either HBr or HI to yield the uracil products (*10–13*). Considering the fact that the elimination of HBr from 5-bromo-5,6-dihydrouracil is far too slow to account for enzymatic dehalogenation (*10*, *14*), then pathways such as the one shown in Eqs. 7–9 might be considered feasible for enzymatic halopyrimidine dehalogenation.

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