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Formation of S-[5-(2′-Deoxyuridyl)]thiol Compounds in the Dehalogenation of 5-Bromo- and 5-Iodo-2′-deoxyuridine with Cysteine Derivatives

Toshiyuki Chikuma, Kazuo Negishi, and Hikoya Hayatsu

Faculty of Pharmaceutical Sciences, University of Tokyo[1)]

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Dehalogenations of 5-bromo- and 5-iodo-2′-deoxyuridine by thiol compounds in aqueous solutions were studied. The thiols used were cysteine, cysteine methyl- and ethyl-esters, cysteamine, homocysteine, glutathione, 2-mercaptoethanol, and 2-mercaptopropionic acid. The products of these reactions and the rate constants were determined. At pH 8 and 37°, the dehalogenation rates by these thiols decreased in above-mentioned order. 5-Alkylthiouracil derivatives were found in products of the reactions between 5-bromo-2′-deoxyuridine and cysteine, cysteine ethyl ester, cysteamine, homocysteine and glutathione, as well as of the reaction between 5-iodo-2′-deoxyuridine and cysteine. This indicated that the S_N2 mechanism proposed in a previous paper (Y. Wataya, K. Negishi and H. Hayatsu, *Biochemistry*, **12**, 3992 (1973)) is generally operating in the dehalogenations of these compounds with thiols.

Keywords——5-bromo-2′-deoxyuridine; 5-iodo-2′-deoxyuridine; S-[5-(2′-deoxyuridyl)]thiol derivatives; dehalogenation; kinetic measurement; cysteine; cysteine ethyl ester; cysteamine; glutathione; cysteine derivatives

The sulfur-nucleophile-mediated dehalogenations of 5-halogenouracil are subjects of current investigation in several laboratories. Bisulfite[2)] and various thiol compounds, *i.e.*, NaSH,[3)] 2-mercaptoethanol,[4)] cysteine[4,5)] and glutathione,[5)] promote dehalogenation of 5-bromo- and 5-iodouracil derivatives under mild conditions in aqueous solution. These reactions have been taken as the chemical counterpart of the thymidylate synthetase-catalyzed dehalogenation of 5-bromo- and 5-iodo-2′-deoxyuridylate.[6)]

In the debromination of brUdRib[7)] with cysteine, the products are 2′-deoxyuridine and S-[5-(2′-deoxyuridyl)]cysteine.[5)] The formation of the latter compound implied that the reaction must involve a step of substitution of the halogen by the thiol group: the S_N2 mechanism in Figure 1.[5)] The rate-determining first step is the attack of thiolate anion to position 6 of the pyrimidine ring to form the 5,6-dihydro-5-halo-6-thio compound **1**. Evidence for the formation of **1** was recently provided by examination of a secondary isotope effect at position 6 of the halogenouracil.[8)] The intermediate **1** can then undergo either an S_N2 reaction to form **2**, or fission of a halonium ion[9)] to give the anionic species **3**. The intermediate **2** will be transformed into either **3** or the 5-substituted uracil derivative **4**.

1) Location: *Bunkyo-ku, Tokyo 113, Japan.* Present address of T. Chikuma is Showa College of Pharmacy, *Setagaya-ku, Tokyo*, and that of K. Negishi and H. Hayatsu is Faculty of Pharmaceutical Sciences, Okayama University, *Tsushima, Okayama 700, Japan.*
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6) Y. Wataya and D.V. Santi, *Biochem. Biophys. Res. Commun.*, **67**, 818 (1975).
7) Abbreviations used are: brUdRib, 5-bromo-2′-deoxyuridine; iUdRib, 5-iodo-2′-deoxyuridine.
8) Y. Wataya and D.V. Santi, *J. Amer. Chem. Soc.*, **99**, 4534 (1977).
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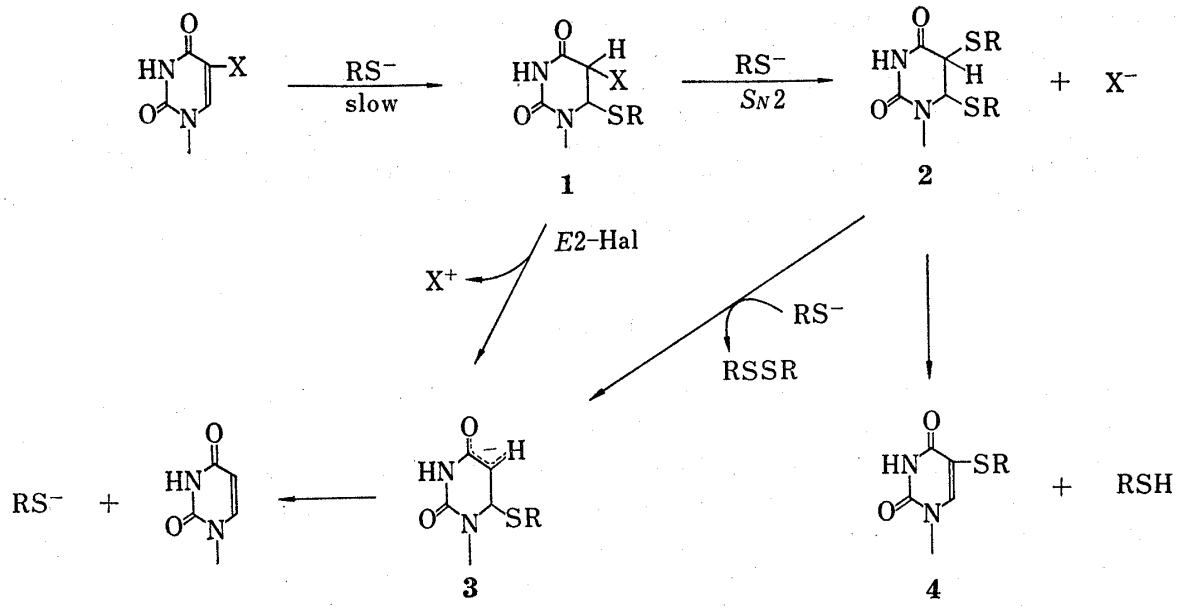


Fig. 1. Mechanism of the Dehalogenation with Thiols

An arising question is whether or not the above scheme is generally applicable to various thiols other than cysteine, and also not only to 5-bromouracil but also to 5-iodouracil. Another question related to this is what structural characteristics in the part of thiol determines the dehalogenating activity.

In this paper we describe results of investigation on the reactions between brUdRib (and iUdRib) and various thiols structurally related to cysteine.

Materials and Methods

General——Thiol compounds used were commercial products. They were checked for the SH-content by titration with iodine. L-Cysteine, L-cysteine methyl- and ethyl-esters, and DL-glutathione were 100% in SH-form; DL-homocysteine, 2-mercaptoethanol and 2-mercaptopropionic acid were 95% in SH-form; and cysteamine hydrochloride was 92% in SH-form. Accordingly, corrections were made for the concentrations of the thiol solutions, which were prepared freshly before use. BrUdRib was obtained from Sigma Chemical Co. and iUdRib from Kohjin Co. Ultraviolet absorbance was recorded on a Beckman Acta CIII spectrophotometer.

Kinetic Measurements and the Product Analysis by Paper Chromatography——A mixture consisting of 0.002 M brUdRib (or iUdRib), 0.1 M thiol compound and 0.1 M sodium phosphate was incubated under a nitrogen atmosphere at 37°. The pH was 8.0 and the volume of the mixture 3 ml. At desired periods portions of 0.2 ml of the mixture were taken, diluted with 4 ml of 0.1 N HCl and the absorbances of the resulting solutions at 290 nm were measured. In this measurement, a reference solution was used which was prepared from a control sample consisting of the thiol and the phosphate buffer. The value $(A_0-A_t)/(A_0-A_\infty)$ represented the fraction of the starting material remaining; A_0 is the absorbance at time zero, A_t that at time t, and A_∞ that at the time when the reaction came to completion. This calculation was based on the assumption that the two products, *i.e.* deoxyuridine and S-(5-deoxyuridyl)thiol, were formed in such a way that throughout the reaction-course the molar ratio of the two products were constant. That this was indeed so was previously shown for the reaction of brUdRib with cysteine,[5] and is also shown in the present study for the reaction of brUdRib with cysteine ethyl ester (see Table III).

The pH of the reaction mixture was checked both at time zero and after the incubation was over, and the change of the pH value was less than ±0.1 in all the kinetic runs studied. Good pseudo-first-order kinetics were generally observed up to two half-lives of the starting material.

When the reactions proceeded to completion, 0.40 ml each of the reaction mixtures was subjected to ascending paper chromatography (solvent 1: 1-butanol-acetic acid-water, 2: 1:1, v/v; or solvent 2: isobutyric acid-0.5 N NH_4OH, 10: 6, v/v). *Rf* values of compounds in these solvent systems: compound, *Rf* (solvent system): brUdRib, 0.75 (1), 0.62 (2); iUdRib, 0.75 (1), 0.62 (2); deoxyuridine, 0.64 (1), 0.57 (2). *Rf* values of S-[5-(2′-deoxyuridyl)]thiol compounds are given in Table IV. Compounds on the chromatograms were visualized by ultraviolet (UV) light. These compounds were eluted by soaking the paper in 0.01 N HCl overnight and the quantities were determined by measuring the ultraviolet absorption of the eluants against

appropriate references. By this procedure, the formation of the S-[5-(2′-deoxyuridyl)]thiol compound can easily be detected to an extent as little as 1% of the starting material. In the determination of this type of compound, the molar extinction coefficient previously reported for S-[5-(2′-deoxyuridyl)]cysteine[5] was used for calculation. Molar extinction coefficients employed in the quantitation were thus 9230 (278 nm) for brUdRib, 10200 (262 nm) for 2′-deoxyuridine, and 7610 (278 nm) for S-[5-(2′-deoxyuridyl)]thiols.

The pH-rate Profile of the Deiodination of iUdRib——The pH-rate profile was examined using 0.002 M iUdRib and 0.05 M cysteine (or 0.05 M cysteine ethyl ester) as the reagent. Buffers used were 0.1 M sodium phosphate at pH<8 (down to 5.95), 0.1 M tris-HCl at pH 8—9, and 0.1 M sodium carbonate at pH>9 (up to 10.1).

Results

BrUdRib and iUdRib were treated with excess cysteine, cysteine methyl- and ethyl-esters, cysteamine, homocysteine, glutathione, 2-mercaptoethanol and 2-mercaptopropionic acid at pH 8 and 37°, and the apparent pseudo-first-order rate constants for the disappearance of the halogenodeoxyuridine were determined. The results presented in Table I show that cysteine was the most powerful among the thiols tested. The order of the effectiveness was cysteine>cysteine ethyl ester~cysteine methyl ester>cysteamine>homocysteine>glutathione>2-mercaptoethanol>2-mercaptopropionic acid. This order of reactivity was not parallel with the ease of dissociation of the agent's SH group. For example, the SH groups of cysteine alkyl esters, as well as that of cysteamine, are more dissociated than that of cysteine at the pH of the reaction mixture, pH 8, and yet these reagents were less reactive than cysteine.

Table I. The Pseudo-first-order Rate Constants fo the Dehalogenations of 5-Halogeno-2′-deoxyuridine by Thiols

Thiol Compounds			Sub-strate	$k_{obsd} \times 10^3$ (min^{-1})[a]	Relative rate		Ratio in k_{obsd}
Name	Structure	pK_{SH}			B	I	I/B
Cysteine	$HOOCCH(NH_2)CH_2SH$	8.5	B[b]	15.4	1.0		1.8
			I[c]	27.7		1.0	
Cysteine methyl ester	$CH_3OOCCH(NH_2)CH_2SH$	7.45	B	6.9	0.45		2.5
			I	17.3		0.62	
Cysteine ethyl ester	$C_2H_5OOCCH(NH_2)CH_2SH$	7.45	B	8.6	0.56		2.2
			I	18.7		0.68	
Cysteamine	$H_2NCH_2CH_2SH$	8.35	B	3.4	0.22		3.5
			I	12.0		0.43	
Homocysteine	$HOOCCH(NH_2)CH_2CH_2SH$	9.14	B	1.44	0.09		6.6
			I	9.5		0.34	
Glutathione	$HOOCCH(NH_2)CH_2CH_2CONH$-$CH(CONHCH_2COOH)CH_2SH$	9.12	B	0.70	0.05		8.3
			I	5.8		0.21	
2-Mercapto-ethanol	$HOCH_2CH_2SH$	9.72	B	0.51	0.04		7.3
			I	3.7		0.13	
2-Mercapto-propionic acid	$HOOCCH_2CH_2SH$	10.84	B	0.22	0.02		8.1
			I	1.9		0.07	

a) Conditions of the reaction: 0.002 M substrate, 0.1 M thiol and 0.1 M sodium phosphate at pH 8.0 and 37°.
b) 5-Bromo-2′-deoxyuridine.
c) 5-Iodo-2′-deoxyuridine.

The dehalogenations of iUdRib were more rapid than those of brUdRib regardless of the thiol compound used. The difference in the rates for iUdRib and brUdRib was greater for thiol agent of lower dehalogenating activity. As a result, the rate of the dehalogenation of iUdRib is relatively insensitive to the kind of thiol used, compared with that of brUdRib.

When these reactions came to completion, or near completion, they were subjected to paper chromatographic analysis for products. As Table II shows, S-[5-(2′-deoxyuridyl)]thiol derivatives were formed along with 2′-deoxyuridine in the debrominations of brUdRib with

Table II. Analysis of Products formed by Treatment of 5-Bromo- and 5- Iodo-2′-deoxyuridine with Thiols[a]

Thiol	Substrate	Time of reaction (hr)	Product		Starting material remaining (mol %)
			2′-Deoxyuridine (mol %)	S[b] (mol %)	
Cysteine	B[c]	20	75.7	24.3	0
	I[d]	20	100	0	0
Cysteine methyl ester	I	24	100	0	0
Cysteine ethyl ester	I	23	100	0	0
Cysteamine	B[e]	20	87.6	8.4	4
	I	27	100	0	0
Homocysteine	B	24	73.5	26.5	0
	I	24	100	0	0
Glutathione	B	25	82	18	0
	I	25	100	0	0
2-Mercaptoethanol	B	48	88.3	0	11.7
	I	24	100	0	0
2-Mercaptopropionic acid	B	48	74.8	0	25.2
	I	24	100	0	0

a) The reaction mixture consisted of 0.002 M brUdRib (or iUdRib), 0.1 M thiol and 0.1 M sodium phosphate. The pH was 8.0 and the incubation temperature 37°.
b) S-[5-(2′-deoxyuridyl)]thiol derivatives.
c) 5-Bromo-2′-deoxyuridine.
d) 5-Iodo-2′-deoxyuridine.
e) The thiol concentration in this experiment was 0.05 M.

Table III. Distribution of Products in the Reaction of 5-Bromo-2′-deoxyuridine with Cysteine Ethyl Ester[a]

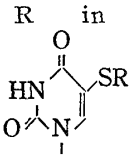
Time of reaction (hour)	Starting material remaining (mol %)	Products (mol %)			(ii) + (iii) / (i)
		2′-Deoxyuridine (i)	S-[5-(2′-Deoxyuridyl)]cysteine ethyl ester (ii)	S-[5-(2′-Deoxyuridyl)]cysteine (iii)	
2	26.7	61.2	10.9	1.3	0.20
22	0	83.1	5.4	11.5	0.20

a) The reaction mixture was 0.01 M in 5-bromo-2′-deoxyuridine and 0.1 M in cysteine ethyl ester. The incubation was at 37° and pH 8.0. Eighty microliters of the reaction mixture were chromatographed on paper using solvent system 1, and the products on the chromatogram were quantitated as described in text.

cysteine, cysteamine, homocysteine and glutathione. The 5-alkylthiouracil derivative was not detectable in the reactions of 2-mercaptoethanol and 2-mercaptopropionic acid. The deiodination of iUdRib with the thiols gave only 2′-deoxyuridine as product. However, deiodination of iUdRib with a dilute solution of cysteine did yield S-[5-(2′-deoxyuridyl)]cysteine: thus, on treatment of 0.002 M iUdRib with 0.01 M cysteine at pH 9 and 37° for 21 hr, 2% S-[5-(2′-deoxyuridyl)]cysteine was produced along with 98% 2′-deoxyuridine. This observation was consistent with the previous report[5] that the product ratio, [S-5-deoxyuridylcysteine]/[deoxyuridine], increases with the decrease of the cysteine-concentration.

The products in the debromination of brUdRib with cysteine ethyl ester were analyzed at two stages of the reaction course. As Table III shows, the initial formation of S-[5-(2′-deoxyuridyl)]cysteine ethyl ester was followed by gradual hydrolysis of the ester group to give S-[5-(2′-deoxyuridyl)]cysteine. The fact the ratio 5-alkylthiouracils to uracil was constant throughout the reaction (see the last column in Table III) is consistent with this sequence of events in this reaction.

Table IV. Properties of S-[5-(2′-Doexyuridyl)]thiol Compounds

R in 	Ultraviolet absorption[a] λ max (nm)	Ultraviolet absorption[a] λ min (nm)	Mobility in paper electrophoresis[b] (cm)	*Rf* value in paper chromatography[c] *Rf*	Solvent
$-CH_2CH(NH_2)COOH$	278	250	0	0.33	1
				0.44	2
$-CH_2CH(NH_2)COOC_2H_5$	276	254	−2.3	0.33	2
$-CH_2CH_2NH_2$	278	249	−2.5	0.31	1
$-CH_2CH_2CH(NH_2)COOH$	275	248	0	0.39	2
$-CH_2CH[NHCOC_2H_4CH-(NH_2)COOH]CONHCH_2COOH$	280	259	+2.4	0.23	2

a) In 0.01 N HCl; *b*) Run at 7 V/cm for 90 min, at pH 7 (0.03 M sodium phosphate). Uridine 5′-phosphate traveled +3.2 cm, and glycine ethyl ester − 5.1 cm under these conditions.
c) Solvents are given in text. All of the compounds gave positive reaction with ninhydrin.

Properties of the various S-[5-(2′-deoxyuridyl)]thiol derivatives are summarized in Table IV and they are all consistent with the structures assigned to these substances.

Previous studies on the pH-rate profile of the cysteine-mediated debromination of brUdRib have indicated that the reactive species in this reaction are thiolate anion RS^- and the undissociated brUdRib molecule.[5] The fact that the rate of the reaction was proportional to the concentration of cysteine was consistent with this view, and therefore it was assumed that the rate-determining step was the attack of the thiolate anion to position 6 of the undissociated brUdRib molecule (see Fig. 1). The availability of two structurally similar thiols having different pK_{SH} values, *i.e.* cysteine and its alkyl ester, would allow to provide further support for this reaction mechanism. The effect of the concentration of cysteine and cysteine ethyl ester on the rate of deiodination of iUdRib at pH 7.5 and 37° was therefore investigated. With both thiols, the rate increased linearly with the increase of the thiol concentration (Fig. 2).

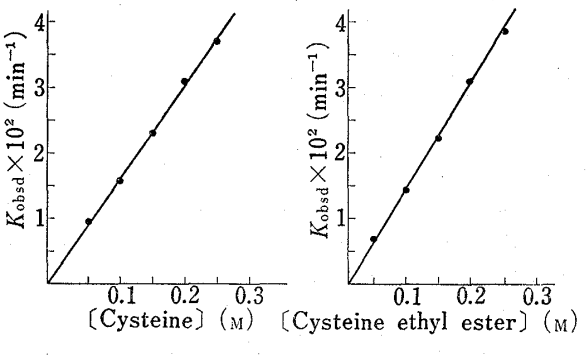


Fig. 2. The Rate of Deiodination of 5-Iodo-2′-deoxyuridine as a Function of the Concentration of Cysteine and Cysteine Ethyl Ester

Reaction conditions : 0.002 M 5-iodo-2′-deoxyuridine at pH 7.5 and 37°.

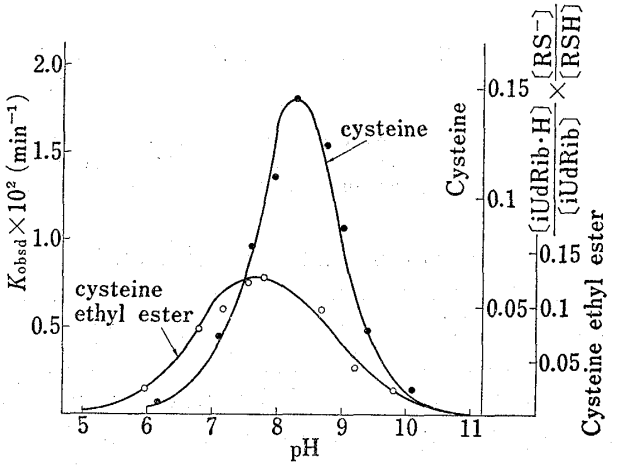


Fig. 3. Deiodination of 5-Iodo-2′-deoxyuridine with Cysteine and Cysteine Ethyl Ester as a Function of pH

The curves, which are theoretical ones, were drawn on the basis of the calculations detailed in text. The scale on the right-hand side represents the product of the fractions of the undissociated 5-iodo-2′-deoxyuridine (iUdRib) and of the dissociated thiol; [iUdRib–H] representing the concentration of undissociated iUdRib, [iUdRib] that of total iUdRib, $[RS^-]$ that of dissociated thiol, and [RSH] that of total thiol.

The pH-rate profiles for the deiodination of iUdRib with cysteine and cysteine ethyl ester were examined at a constant reagent-concentration of 0.05 M, and the results are given in Fig. 3. The optimum pH values were 8.35 for the deiodination with cysteine and 7.9 for that with cysteine ethyl ester. The curves drawn in the Figure represent theoretical pH-rate profiles, calculated on the assumption that the reactive species were the undissociated iUdRib and the thiolate anion. Excellent fits between the experimental points and the theoretical curves are seen for both the cysteine- and the cysteine ethyl ester-reactions. The derivation of the curves were performed as follows. Thus, k_{obsd} must be proportional to $([iUdRib\cdot H]/[iUdRib_{total}])\times([cys\ S^-]/[cys_{total}])$, where $[iUdRib\cdot H]$ represents the concentration of undissociated iUdRib, $[iUdRib_{total}]$ that of total iUdRib, $[cys\ S^-]$ that of the thiolate form of cysteine, and $[cys_{total}]$ that of total cysteine. Using the pK_a value 8.2 of iUdRib,[10] one obtains;

$$[iUdRib\cdot H]/[iUdRib_{total}] = 1/(1+10^{pH-8.2}) \quad (1)$$

Cysteine dissociates in a complicated manner as shown in Fig. 4. Species bearing a thiol anion are *B* and *D*. Since [D] is much smaller than [B] at pH below 9, only *B* is regarded as being involved in the reaction. Using the values, pK_A 8.50, pK_B 8.85, pK_C 10.35, and pK_D 10.00,[11] equation (2) was derived.

$$[B]/[cys_{total}] = 1/(1+10^{8.50-pH}+10^{8.50-8.85}+10^{pH-10.35}) \quad (2)$$

From eq. (1) and (2), the value of $([iUdRib\cdot H]/[iUdRib_{total}])\times([cys\ S^-]/[cys_{total}])$ can be calculated at a given pH, and, employing an appropriate scale, the curve shown in Fig. 3 was obtained.

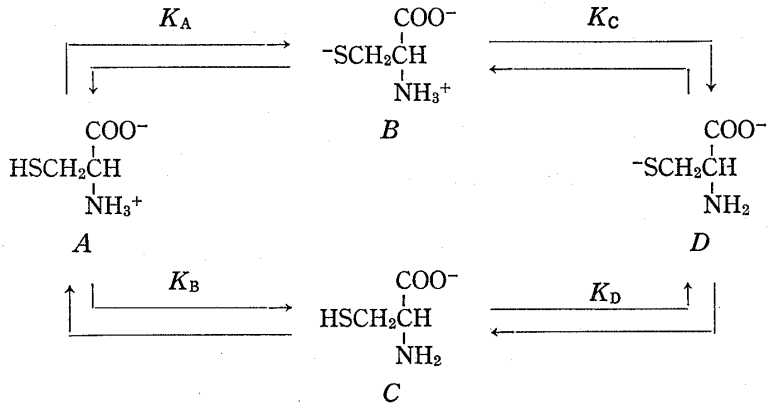


Fig. 4. Dissociations of Cysteine

Similar calculations were made for the cysteine ethyl ester-mediated reaction. In this case, however, the species corresponding to *D* in Fig. 4 was also taken into account, because the pK_C in Fig. 4 for the ester is close to the pH values at which the reactions were run. Assuming that the reactivity of species *D* is equal to that of *B*, and using the values, pK_A 7.45, pK_B 6.77, pK_c 8.41, and pK_D 9.09,[11] the values for $([iUdRib\cdot H]/[iUdRib_{total}])\times(([B]+[D])/[cys\ ethyl_{total}])$ were claculated, and the curve was drawn.

The second order rate constants for the reaction between undissociated iUdRib and thiolate anion were calculated and the values that are pH-independent are given in Table V. The thiolate of cysteine is two times more reactive than that of cysteine ethyl ester.

Since the structural difference between the reactive species of cysteine and its ester is only the presence of the carboxylate anion in the former, the possible effect of externally

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Table V. Second Order Rate Constants for the Dehalogenation of 5-Iodo-2′-Deoxyuridine by Thiolate Anions

Thiol compound	k_2 ($l \cdot mol^{-1} \cdot min^{-1}$)[a]
Cysteine	2.53±0.18
Cysteine ethyl ester	1.23±0.09

a) $k_2 = k_{obsd} \cdot [iUdRib_{total}]/[iUdRib \cdot H] \cdot [RS^-]$, where $[iUdRib_{total}]$ represents the concentration of total iUdRib, [iUdRib·H] that of undissociated iUdRib, and $[RS^-]$ that of thiolate anion.

added carboxylate anion to the cysteine ethyl ester-iUdRib reaction was examined. It was found that the presence of either 0.5 M sodium acetate or 0.5 M N-acetylglycine in the reaction mixture of 0.002 M iUdRib and 0.05 M cysteine ethyl ester at pH 7.8 and 37° did not make any difference in k_{obsd} value from those in its absence.

Discussion

The formation of 5-thiouracil derivatives was previously reported for the dehalogenations of 5-bromouracil (or its deoxyriboside) with hydrogen sulfide,[3] cysteine,[5] and glutathione.[5] The present study indicates that the reactions of brUdRib with cysteine alkyl esters, cysteamine and homocysteine give the 5-thiouracil compounds and that this type of product can also be formed in the dehalogenation of iUdRib with cysteine. It can therefore be concluded that the S_N2 mechanism illustrated in Fig. 1 is a general one operating in dehalogenations of brUdRib and iUdRib with thiols.

Since there is a strong indication that the $E2$-Hal mechanism (Fig. 1) is also operating,[9] both mechanisms $E2$-Hal and S_N2 seem to be simultaneously taking place. IUdRib, in constrast to brUdRib, forms only a very little amount of 5-thiolated product, indicating that the iodo compound undergoes mainly the $E2$-Hal process.

The reason why the thiolate anions of cysteine alkyl esters and cysteamine are less reactive than that of cysteine is worthy of consideration. The difference between these two kinds of thiolate anions is the presence of an anionic carboxylate group in cysteine and its absence in the others. In cysteine molecule, the –I effect of the protonated amino group which can weaken the nucleophilicity of the thiolate will be cancelled intramolecularly by the presence of the carboxylate anion. The absence of this cancellation in the other thiolates will account for their weaker nucleophilicity. The lack of any rate enhancement by the externally added carboxylates supports the intramolecular nature of the effect.

In summary, this study has shown that (1) iUdRib is dehalogenated more rapidly than brUdRib regardless of the kind of cysteine derivatives used, and even with thiols of low reactivity toward brUdRib, iUdRib is easily dehalogenated; (2) Significant portion of the product is the 5-alkylthiouracil derivative for the dehalogenation of brUdRib, whereas in the dehalogenation of iUdRib, this type of compound is formed only in trace amounts, if any.