Debromination of 5-Bromo-2′-deoxyuridine by Cysteine. Formation of Deoxyuridine and *S*-[5-(2′-Deoxyuridyl)]cysteine†

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ABSTRACT: Although the bromo residue of 5-bromo-2′-deoxyuridine (brUdRib) is generally regarded as chemically stable, brUdRib was found to undergo easy debromination on treatment with cysteine solution. 2′-Deoxyuridine and *S*-(5-deoxyuridyl)cysteine were the products of this reaction. The latter compound was isolated in a crystalline form and the structure established. No other product was detected by radiochromatography of [2-^{14}C]brUdRib following treatment with cysteine. By determining mole per cent of the three components in the reaction mixture spectrophotometrically as well as paper chromatographically, the rate of debromination and the product distribution were measured. The debromination followed pseudo-first-order kinetics and the two compounds were produced in parallel. The pseudo-first-order rate constant for the debromination at 37° was highest at pH 8.2; 4.9×10^{-2} min^{-1} with 0.25 M cysteine. While deoxyuridine formation was optimal at pH 8, the *S*-(5-deoxyuridyl)-cysteine formation was optimal at pH 9 where the ratio of the products in 0.25 M cysteine reaction was about 1. The rate of debromination linearly increased with increase of cysteine concentration, but the *S*-(5-deoxyuridyl)cysteine formation became much favored as the concentration of cysteine was lowered; in 0.01 M cysteine at pH 8.0, this product amounted to about 2-fold compared with deoxyuridine. 5-Bromouracil was also debrominated with cysteine to give uracil and *S*-(5-uracilyl)cysteine. Glutathione debrominated brUdRib at pH 8.1 and 37° to give deoxyuridine and, presumably, *S*-(5-deoxyuridyl)glutathione, although the rate constant was about $^{1}/_{20}$th of that with cysteine.

5-Bromo-2′-deoxyuridine (brUdRib)[1] is a chemical of considerable biological interest. It can be incorporated into DNA replacing thymine by 5-bromouracil residues, thereby inducing mutagenesis and sensitizing the DNA to uv light and ionizing radiation. In addition, brUdRib has various types of biological effect of unknown mechanism: brUdRib induces virus synthesis in mammalian cells (Teich *et al.*, 1973, and references cited therein), and there are many reports dealing with induction, as well as suppression, of differentiation of cells by brUdRib (for a review, see Wilt and Anderson, 1972).

While brUdRib is enzymatically dehalogenated following cleavage of the glycosidic linkage (Barrett and West, 1956; Pahl *et al.*, 1959; Kriss and Revesz, 1962), the bromine at the 5 position was generally thought to be chemically stable. brU is only slowly hydrolyzed with alkali at high temperature (Garrett *et al.*, 1968), and the bromo group of 5-bromouridine and brUdRib undergoes nucleophilic substitution with amines only under drastic conditions (Ueda, 1960; Visser, 1968). Recent findings that sulfur nucleophiles, NaSH (Szabo *et al.*, 1970) and Na_2SO_3 (Sander and Deyrup, 1972), can debrominate brU with marked rapidity prompted us to examine the effect of the amino acid cysteine on brUdRib. This paper deals with the reaction of brUdRib with cysteine which occurs under nearly physiological conditions giving deoxyuridine and *S*-(5-deoxyuridyl)cysteine as products (Scheme I).

Experimental Section

General. Water (distilled and deionized) used in kinetic studies had been boiled and nitrogen flushed while cooling. [2-^{14}C]brUdRib (56.5 Ci/mol) was a product of New England Nuclear. Before use, the sample was purified by paper chromatography (solvent 1) to remove a 6% impurity (radioactivity basis) which had an R_F value the same as deoxyuridine. Other reagents were commercial products used without further purification. Cysteine solutions were freshly prepared before use.

Paper chromatography was carried out ascendingly on Whatman 3MM paper. Chromatographic solvents were (1) 1-butanol–acetic acid–water (2:1:1, v/v); (2) isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); (3) 1-butanol–water (86:14, v/v); (4) isopropyl alcohol–concentrated HCl–water (75:17:8, v/v). Electrophoresis was done on Avicel plates at pH 1.6 (7% formic acid, 28 V/cm, 1 hr); at pH 6.9 (0.1 M sodium phosphate buffer, 28 V/cm, 1 hr); at pH 9.8 (0.05 M $NaHCO_3$–Na_2CO_3 buffer, 92 V/cm, 5 min). Ultraviolet spectra were recorded on Hitachi 124 spectrophotometer and 100-MHz nmr spectra on Jeol-NM-4H-100 and Jeol-JNM-PS-100 nmr spectrometers. ^{14}C radioactivity was measured with Packard Tri-Carb 3320 liquid scintillation spectrometer.

Velocity of Reaction of brUdRib with Cysteine. SPECTROPHOTOMETRIC METHOD. Reaction mixtures (2 ml) usually consisted of 0.010 M brUdRib, 0.05–0.25 M L-cysteine, and where indicated 0.1 M buffer. The pH was adjusted by the use of sodium acetate (pH 4.7) or potassium phosphate (pH 5.7–7.2) buffer. The pH's above 7.2 were adjusted with sodium hydroxide. The reaction mixtures were incubated under nitrogen atmosphere at 37°. The pH value fluctuation during the incubation period was less than ±0.04. Aliquots (25 μl) were withdrawn at desired intervals and diluted with 2.5 ml of 0.1 N HCl and the resulting solutions were subjected to uv spectrum measurement. From the optical densities at 262 (A_1), 278 (A_2), and 320 nm (A_3) thus determined, concentrations (mM) of brUdRib (c_1), deoxyuridine (c_2), and *S*-(5-deoxyuridyl)cysteine (c_3) were calculated using the following equations: $c_1 = -0.0614A_1 + 0.146A_2 - 2.38A_3$; $c_2 = 0.132A_1 -$

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[1] Abbreviations used are: brUdRib, 5-bromo-2′-deoxyuridine; brU, 5-bromouracil; m^{1}brU, the 1-methyl derivative.

$0.0804A_2 - 0.450A_3$; $c_3 = 3.14A_3$. Molar extinction coefficients employed in the derivation of the equations were at 262, 278, and 320 nm, brUdRib, 5630, 9230, 0; deoxyuridine 10,200, 4300, 0; *S*-(5-deoxyuridyl)cysteine, 5740, 7610, 318.

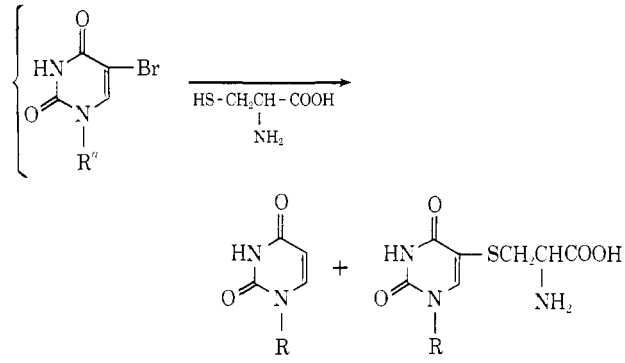
PAPER CHROMATOGRAPHIC METHOD. From the reaction mixtures described above, aliquots (30 μl) were taken and directly subjected to paper chromatography. Application of each aliquot on the chromatographic paper and drying with air stream (not heated) took about 3 min. After developing the paper with solvent 1, it was dried and each compound was eluted by soaking the paper in 0.01 N HCl overnight. From uv absorbances of the solutions the amounts of each component in the reaction mixture were calculated. The sum of the quantities of each compound recovered accounted for 85–95% of the brUdRib used in the reaction.

Reaction of Cysteine with [2-^{14}C]brUdRib. A reaction mixture, pH 9.25, containing 0.25 M cysteine, 0.010 M brUdRib, [^{14}C]brUdRib (4 μCi), and 0.25 N NaOH was incubated at 37°. Aliquots (10 μl) were withdrawn at desired reaction periods and chromatographed on narrow strips of Toyo filter paper No. 53. Uv-absorbing spots on the chromatograms were marked and the chromatograms were scanned for radioactivity on a Packard 7200 radiochromatogram scanner.

Molar absorption coefficient of *S*-(5-deoxyuridyl)cysteine was determined as follows. [^{14}C]*S*-(5-Deoxyuridyl)cysteine on the chromatogram was eluted and further purified by rechromatography (solvent 1) on Toyo filter paper No. 51A (acid washed). The compound was eluted with 0.01 N HCl from the paper, and radioactivity and uv spectrum were measured for the eluted compound. The same procedure was applied also for [^{14}C]deoxyuridine and [^{14}C]brUdRib on the chromatogram. Since the radioactivity per mole of each nucleoside should be equal and the molar absorption coefficient of deoxyuridine is 10,200 at 262 nm (Dunn and Hall, 1970), the molar absorption coefficient of *S*-(5-deoxyuridyl)cysteine at 278 nm was found to be 7610. The corresponding value for brUdRib at 278 nm determined by this method was 9230 which was identical with the value reported (Berens and Shugar, 1963).

Isolation of S-(5-Deoxyuridyl)cysteine. brUdRib (250 mg, 0.814 mmol) was dissolved in water (5 ml) containing some NaOH to adjust the pH to 8.7. The solution was heated at 80° and cysteine (256 mg, 2.11 mmol) was added in three portions, the pH being adjusted by NaOH at each addition. The heating was continued for about 10 min in total, and then the solution was concentrated to dryness under reduced pressure. Paper chromatographic analysis of the solution showed that the mole ratio, brUdRib:deoxyuridine:*S*-(5-deoxyuridyl)cysteine, was 0.03:2:1.3. The residue was dissolved in solvent 1 supplemented with some more acetic acid. After some insoluble material was removed by centrifugation, the solution was loaded on a column of cellulose (size, 2 × 61 cm) and the column was eluted with solvent 1. Deoxyuridine was eluted in the 100- to 140-ml fraction, cysteine in the 145- to 165-ml fraction, and *S*-(5-deoxyuridyl)cysteine in the 180- to 250-ml fraction. The last fraction was evaporated to dryness to give 200 mg of a lightly colored powder which contained a trace amount of cystine as revealed by cellulose thin-layer chromatographic analysis. The powder was dissolved in water and the slightly acidic solution was neutralized with aqueous NH_4OH. The solution was heated and diluted with ethanol and acetone to a slight turbidity. Upon cooling crystallization took place. The crystals were collected and recrystallized from 80% ethanol to give colorless leaflets: uv spectrum, λ_{max} 278 (pH 2), 276 (H_2O), 276 nm (pH 12). *Anal.* Calcd for $C_{12}H_{17}N_3O_7S \cdot 0.5H_2O$: C, 40.46; H, 5.09; N, 11.79. Found: C, 40.20; H, 4.99; N, 11.40. The ε value at 278 nm at pH 2 was found to be 7550 on weight basis which coincides within experimental error with the value obtained by radioactivity measurement (see above).

SCHEME I



[a] R = H, 2′-deoxy-β-D-ribofuranosyl.

Isolation of S-(5-Uracilyl)cysteine. A solution of 5-bromouracil (500 mg) and cysteine (363 mg) in 15 ml of water (pH 9.0) was heated at 98° for 5 min. More cysteine (363 mg) was added and the pH was readjusted to 9 with NaOH. To ensure a complete reaction, the solution was allowed to stand overnight at room temperature. The pH was then lowered to 7.4 with HCl and cystine that precipitated was removed by filtration and the filtrate was allowed to stand at room temperature. Crystallization of the desired compound started to take place after about 30 min of the standing. The crystals were collected by filtration (142 mg) and recrystallized from water and then from 50% ethanol to fine needles: nmr spectra (sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal reference), (1) in 1 N NaOD: an octet of AB protons of an ABX system (δ_{H_A} 2.65 ppm and δ_{H_B} 2.97 ppm with $J_{AB} = 11.9$ Hz, $J_{AX} = 8.4$ Hz, $J_{BX} = 4.6$ Hz); a quartet of X proton ($\delta_{H_X} = 3.28$ ppm); a singlet of 6 H (7.94 ppm); (2) in 1 N DCl: an octet of AB protons of an ABX system (δ_{H_A} 3.24 ppm and δ_{H_B} 3.48 ppm with $J_{AB} = 8$ Hz, $J_{AX} = 5$ Hz, $J_{BX} = 6$ Hz); a triplet of X proton (δ_{H_X} 4.20 ppm); a singlet of 6 H (8.04 ppm). *Anal.* Calcd for $C_7H_9N_3O_4S \cdot H_2O$: C, 33.73; H, 4.45; N, 16.86; S, 12.86. Found: C, 33.49; H, 4.42; N, 16.85; S, 12.77.

In one experiment, D_2O was used instead of water in the reaction mixture and the product was purified by recrystallization from D_2O. This sample in 1 N NaOD showed the 8-ppm singlet signal (one proton) assignable to 6 H.

Results

Reaction of brUdRib with Cysteine and Identification of Products. When brUdRib was treated with cysteine in aqueous solution, it was decomposed with considerable rapidity at the pH region 7–9. The progress of the reaction was detected by uv spectrum change. The spectrum obtained by 17-hr reaction at pH 7.2 was almost identical with that of deoxyuridine. On the other hand, the spectral changes during the reaction at pH 9.1 were significantly different from those at pH 7.2. Thus, the absorbancy at 310- to 320-nm region increased gradually with time and no isosbestic point was observed in contrast to the pH 7.2 reaction where an isosbestic point was found at 270 nm.

The reaction mixtures were analyzed by paper chromatography run in solvent 1. The pH 7.2 reaction mixture gave one major spot having an R_F value identical with that of 2′-

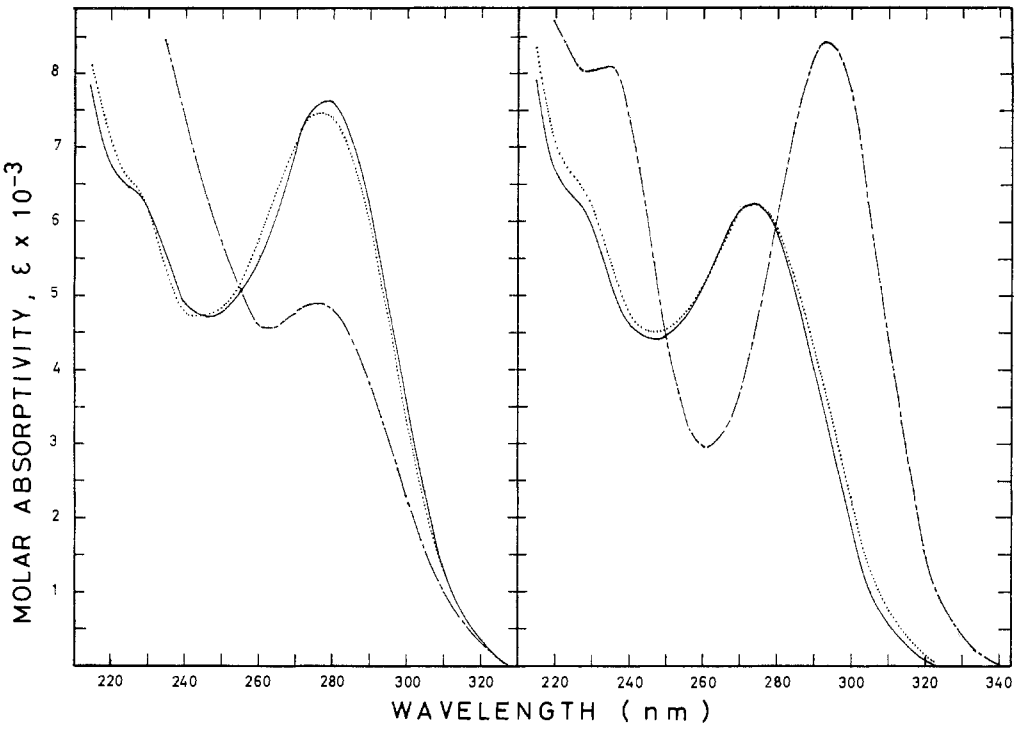


FIGURE 1: Ultraviolet spectra of *S*-(5-deoxyuridyl)cysteine (left) and *S*-(5-uracilyl)cysteine (right). ——, pH 2 (0.01 N HCl); · · · · ·, pH 7 (0.05 M potassium phosphate buffer); —·—·—·, pH 12 (0.01 N NaOH).

deoxyuridine. Uv spectra of the product in acid, neutral, and alkaline solutions were also identical with those of deoxyuridine. However, another uv-positive product with an R_F value smaller than that of deoxyuridine was observed on the chromatogram, although its amount was very small. In the pH 9.1 reaction mixture, the latter product was present in a greater amount, having a comparable absorption intensity to that of deoxyuridine on the chromatogram.

In order to investigate whether some non-uv-absorbing nucleosidic materials were present in the reaction mixture, [2-^{14}C]brUdRib was treated with cysteine at pH 9.25 and the reaction mixture was subjected to paper chromatography followed by radioactivity scanning. When the 6-hr reaction mixture was analyzed using chromatographic solvents 1–4, the radioactivity was present only in those areas corresponding to deoxyuridine and to the second product. R_F values of compounds are summarized in Table I. No other product was detected also in the initial stages, namely in the 30-min and 2-hr aliquots, of the reaction (run in solvent 1).

The second product was positive in ninhydrin test and behaved as a cation at pH 1.6 and as an anion (more negatively charged than uridine) at pH 9.8 as detected by electrophoresis (Table II) indicating that it bears a cysteine residue in its structure. This product was isolated in a crystalline form by a preparative experiment including work-up with cellulose column chromatography. The compound was stable under aerobic conditions indicating that it does not possess free SH. Elemental analysis and uv absorption spectrum of this material (Figure 1) strongly suggested that it is *S*-(5-deoxyuridyl)-cysteine. The uv spectra resembled those of *S*-methyl-5-mercaptodeoxyuridine (Kotick *et al.*, 1969). Uridine substituted at position 5 with amino nitrogen is known to exhibit substantially different spectra (Ueda, 1960; Visser, 1968). The structure was further confirmed by experiments carried out with brU. Thus, when brU was treated with cysteine, products were uracil and a ninhydrin-positive compound analogous to *S*-(5-deoxyuridyl)cysteine as detected by paper

TABLE I: R_F Values of Compounds.

Compound	Solvent 1	2	3	4
brUdRib	0.65	0.53	0.40	0.73
brU	0.65		0.43	0.67
2′-Deoxyuridine	0.54	0.59	0.23	0.67
Uracil	0.54		0.27	0.57
S-(5-Deoxyuridyl)cysteine	0.28	0.17	0	0.17
S-(5-Uracilyl)cysteine	0.26		0	0.14
Cysteine	0.47		0 to ~0.03	0.45
Cystine	0.20		0	
S-(5-Deoxyuridyl)glutathione	0.30			

TABLE II: Relative Mobilities in Electrophoresis.[a]

Compound	pH 1.6	6.9	9.8
S-(5-Deoxyuridyl)cysteine	+0.45	0	−0.53
S-(5-Uracilyl)cysteine	+0.50	−0.1	−0.62
Cysteine	+0.80	0	−0.82
Uridine 5′-phosphate	−0.67	−1.0[b]	−1.0[b]
Uridine	0[b]	0[b]	−0.21
Cytidine	+1.0[b]	+0.1	0[b]

[a] See text for experimental conditions. The values marked + indicate migration toward the cathode, and − toward the anode. [b] Standards based on which mobilities of other compounds were determined.

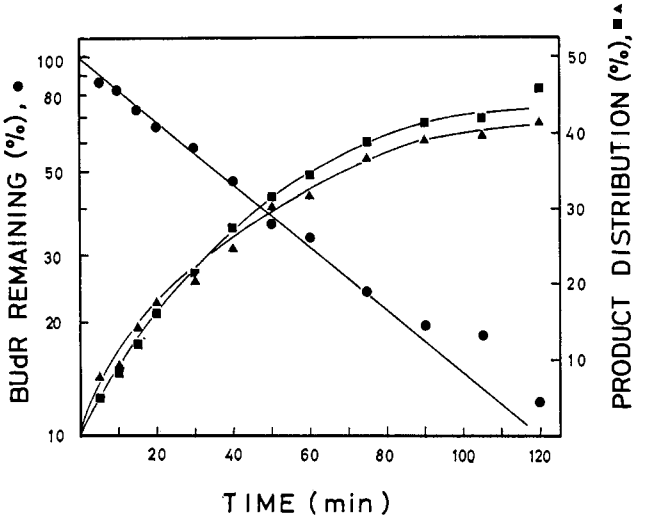


FIGURE 2: Time course of the reaction of brUdRib (0.010 M) with 0.25 M cysteine at pH 9.1 and 37°. ●, ■, ▲ represent mole per cent of brUdRib, deoxyuridine, and *S*-(5-deoxyuridyl)cysteine, respectively, at each reaction time.

chromatography. The latter product was isolated in crystals (see Figure 1, Tables I and II for uv, R_F and mobility in electrophoresis). The uv spectra resembled those of *S*-methyl-5-mercaptouracil (Bardos and Kalman, 1966). Nmr spectrum of this material was very clear-cut and the signals were unambiguously assignable to the protons of *S*-(5-uracilyl)cysteine (see Experimental Section). The assignment of the 8-ppm singlet to the proton at position 6 but not 5 was confirmed by the presence of the same signal of equivalent strength in a sample of *S*-(5-uracilyl)cysteine prepared by a reaction of brU with cysteine in D_2O. Therefore, an isomeric structure *S*-(6-uracilyl)cysteine is impossible.

Velocity of Debromination and Product Distribution. Based on the results described above, the reaction of brUdRib with cysteine was followed spectrophotometrically. At several pH values examined pseudo-first-order kinetics were obtained regarding the decrease of brUdRib. An example of the time course is shown in Figure 2. Effect of pH on the rate constants of the debromination and on the product distribution is given in Figure 3. The debromination was most rapid at pH 8.2 and the rate decreased sharply at both sides of this pH value. While the deoxyuridine formation was maximum at pH about 8, the *S*-(5-deoxyuridyl)cysteine formation was most favorable at pH approximately 9. Besides the spectrophotometric measurement, paper chromatographic analysis of the reaction mixture was performed in order to confirm the product distribution, though its use was limited because the reaction can proceed during application of the solution on the chromatographic paper. This effect, however, should not be great at the pH regions where the rates of the debromination were moderate, and the data obtained were actually coincident with those obtained spectrophotometrically (Figure 3).

Dependence of the debromination rate on concentration of cysteine was determined at pH 8.0 and a linear correlation was obtained as shown in Figure 4. This figure also shows that the product distribution varied with the change in concentration of cysteine: the ratio, *S*-(5-deoxyuridyl)cysteine to deoxyuridine, markedly increased as the concentration of cysteine was lowered, and the production of the former compound was much more favored than deoxyuridine at 0.01 M cysteine concentration. The product distribution in the 0.01 M cysteine solution was also confirmed by paper chromatographic anal-

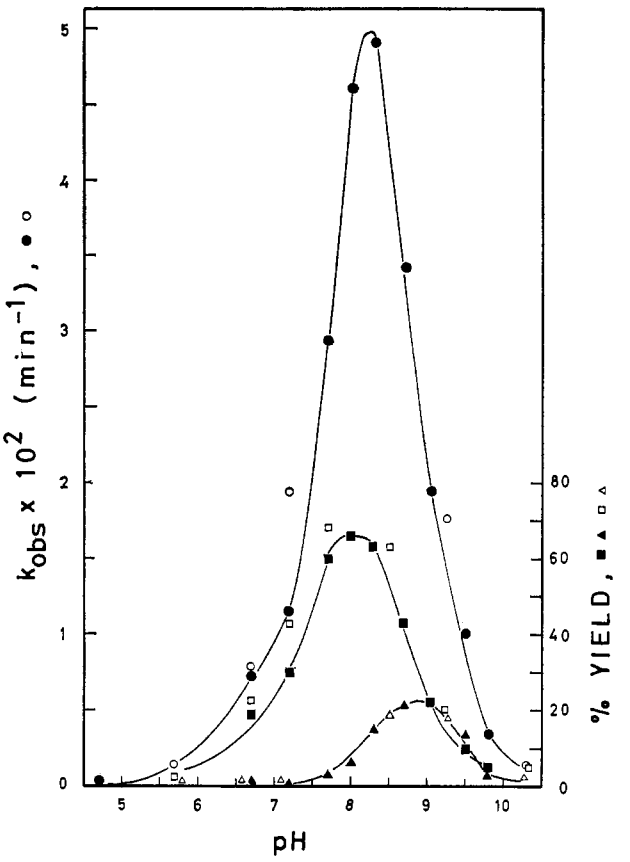


FIGURE 3: pH dependence of the reaction velocity and product distribution. The reactions were with 0.010 M brUdRib in 0.25 M cysteine at 37°. Solid symbols represent values determined spectrophotometrically and open symbols those determined paper chromatographically. Per cent yields are values at 30-min reaction: (■,□) deoxyuridine; (▲,△) *S*-(5-deoxyuridyl)cysteine.

ysis: the ratio found for the 6-hr reaction was 1.9. This 5-mercaptouracil derivative can be formed even at lower pH value. Thus, incubation of a mixture containing 0.010 M cysteine, 0.4 mM brUdRib and 0.2 M potassium phosphate buffer for 30 hr at pH 7.2 and 37° resulted in the formation of 31% *S*-(5-deoxyuridyl)cysteine and 39% deoxyuridine.

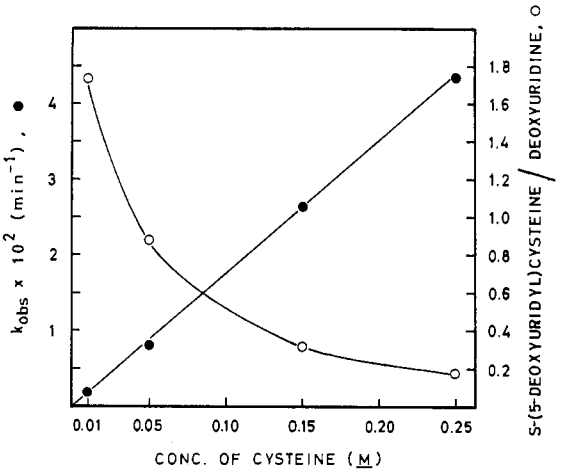
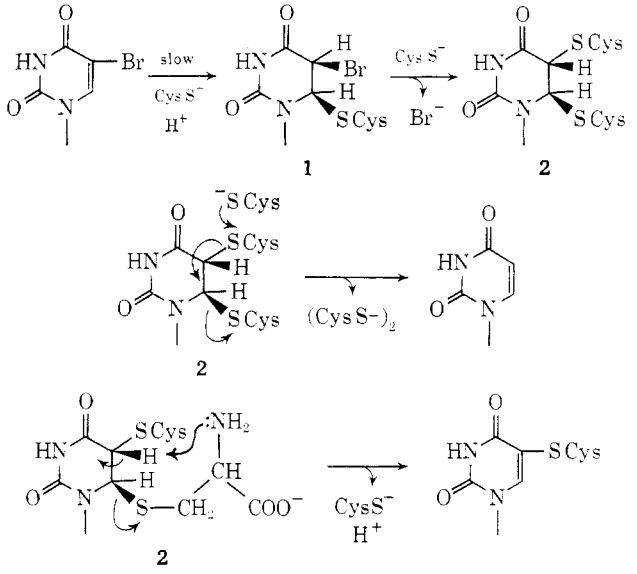


FIGURE 4: Dependence of the rate and the product distibution on cysteine concentration. Incubation was at pH 8.0 and 37°. In the reaction with 0.01 M cysteine, 0.0004 M brUdRib was used instead of 0.01 M brUdRib in all other reactions; 0.2 M potassium phosphate buffer was present in all the reaction mixtures.

SCHEME II



The fact that the yield of *S*-(5-deoxyuridyl)cysteine exceeded that of deoxyuridine is important for elucidating the mechanism of the reaction, so that we confirmed it by carrying out a reaction using a higher concentration of brUdRib. Thus, a solution containing 0.01 M brUdRib and 0.005 M cysteine (pH 8.1) was incubated at 37° and $^1/_4$-equivalent (to brUdRib) amounts of cysteine were added from time to time (six times) in order to maintain the cysteine concentration at low values. After incubation for 23 hr in total, the reaction was almost complete and paper chromatographic analysis showed that the product ratio *S*-(5-deoxyuridyl)cysteine: deoxyuridine was 2.4:1.

Although trimethylamine greatly accelerated the cysteine-catalyzed hydrogen isotope exchange at position 5 of uridylic acid (Wataya *et al.*, 1972, 1973), it did not enhance the rate of the debromination: the rate with 0.01 M brUdRib and 0.15 M cysteine at pH 8.0 in the presence of 0.5 M trimethylamine was 2.35×10^{-2} min^{-1}. EDTA or hydroquinone which might affect the rate by preventing possible autoxidation of cysteine was also without effect: the rate in 0.25 M cysteine (pH 8.9) was 3.01×10^{-2} min^{-1} with 0.1 mM EDTA addition and 3.01×10^{-2} min^{-1} with 0.1 mM hydroquinone. Product distributions in these three reactions were also similar to that in the absence of the agents. In order to eliminate the possibility that visible light is catalyzing the reaction, one experiment was carried out in the dark. No retardation of the reaction was observed in the absence of light, for the reaction at pH 7.8 and 37° with 0.15 M cysteine. Stability of *S*-(5-deoxyuridyl)-cysteine toward cysteine treatment was examined. When *S*-(5-deoxyuridyl)cysteine was treated with 0.25 M cysteine at 37° and at pH 7 and 9 for 18 hr, no degradation of the compound was observed as checked by uv spectra.

Debromination of brUdRib with glutathione was studied by paper chromatographic analysis. When 0.01 M brUdRib was treated with 0.25 M glutathione at pH 8.1 and 37°, brUdRib remaining was found to be as follows: 95% at 30 min; 82% at 90 min; 12% at 19 hr. The rate of the debromination can be estimated to be about $^1/_{20}$th of the rate of corresponding cysteine reaction. Products in the 19-hr reaction mixture were deoxyuridine (79%) and another compound, presumably *S*-(5-deoxyuridyl)glutathione (9%), the per cent being calculated on the assumption that the latter compound has the same molar extinction coefficient at its λ_{max} as *S*-(5-deoxyuridyl)cysteine. This product was ninhydrin positive, and in 0.01 N HCl λ_{max} was 280 nm and λ_{min} 240 nm which were very close to those of *S*-(5-deoxyuridyl)cysteine. The R_F value of this product in solvent 1 was also similar to that of *S*-(5-deoxyuridyl)cysteine (Table I).

Discussion

The easy debromination of brUdRib with cysteine to give deoxyuridine and *S*-(5-deoxyuridyl)cysteine was not unexpected because m^{1}brU is known to react at room temperature with NaSH in dimethyl sulfoxide giving 1-methyluracil and 1-methyl-5-mercaptouracil disulfide as products (Szabo *et al.*, 1970). Production of a compound similar to *S*-(5-uracilyl)-cysteine was also reported in the uv irradiation of a mixture of 5-iodouracil and cysteamine and the compound was tentatively assigned the structure *S*-(5-uracilyl)cysteamine (Rupp and Prusoff, 1965). It should be noted that uv irradiation of a mixture of cysteine and uracil results also in covalent bond formation between SH and uracil to give, in this case, 5-*S*-cysteine-6-hydrouracil (Smith and Aplin, 1966).

Sulfur nucleophiles are known to add across the 5,6 double bond of uracil to give 5,6-dihydro-6-substituted uracil derivatives. For example, bisulfite with uracil forms 5,6-dihydrouracil-6-sulfonate (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970a,b); and 5′-deoxy-5′-mercaptoisopropylideneuridine gives 6,5′-*S*-cyclo-5,6-dihydroisopropylideneuridine by an intramolecular addition of the 5′-SH group to the 5,6 double bond (Chambers and Kurkov, 1963). This type of addition compounds exhibits enhanced reactivity at the 5 position: bisulfite- and cysteine-catalyzed hydrogen isotope exchanges of uridine are believed to occur through such addition compound (Wataya and Hayatsu, 1972; Wataya *et al.*, 1972, 1973); bisulfite causes rapid dehalogenation of 5-halogenouracil by forming the adduct, 5,6-dihydro-5-halouracil-6-sulfonate (Sander and Deyrup, 1972). Therefore it is reasonable to suppose that cysteine forms a similar 1,2-addition compound (**1**) with brUdRib.

Since the velocity of the debromination of brUdRib is first order to the concentration of cysteine regardless of the large change in the product distribution (Figure 4), it seems that the initial attack of cysteine to brUdRib is the rate determining slow step and subsequent steps leading to deoxyuridine and *S*-(5-deoxyuridyl)cysteine are rapid. From Figure 2 it is apparent that the two products are formed parallelly; *S*-(5-deoxyuridyl)cysteine is not an intermediate for the production of deoxyuridine. If the adduct **1** undergoes nucleophilic substitution with cysteine S$^-$ accompanying a Walden inversion at C-5 as has been proposed in the reaction of brU with NaSH (Szabo *et al.*, 1970), it would give the trans adduct **2** (Scheme II). Trans elimination of **2** forming cystine and deoxyuridine may be initiated by the attack of cysteine S$^-$ as illustrated in the scheme, which provides an explanation for the more favorable formation of deoxyuridine by higher concentration of cysteine. On the other hand, formation of *S*-(5-deoxyuridyl)cysteine requires abstraction of the hydrogen at position 5 of the adduct **2**. This may be a process analogous to the cysteine-catalyzed hydrogen–deuterium exchange of uridine which is optimal also at pH 9 and is believed to occur through an intramolecular electron transfer by the amino group (pK_a for HS-R-$NH_3^+ \rightleftarrows$ HS-R-NH_2 of cysteine is 8.85 (Elson and Edsall, 1962)) (Wataya *et al.*, 1972, 1973). It should be noted that the cysteinyl residue at the 6 position is located cis to the hydrogen at the 5 position, making this interaction sterically possible.

The optimum at pH 8.2 for the debromination may be explained by the attack of cysteine S^- (pK_a = 8.5 (Elson and Edsall, 1962)) to neutral brUdRib (pK_a = 7.9 (Berens and Shugar, 1963)). The fact that although glutathione possesses SH with similar dissociation (pK_a = 8.6 (Wallenfels and Streffer, 1966)) its reaction with brUdRib is considerably slower than cysteine, may be due to the bulkiness of the reagent compared with cysteine. Although alternative mechanisms are conceivable, the one described above certainly provides satisfactory explanations for all the experimental results obtained.

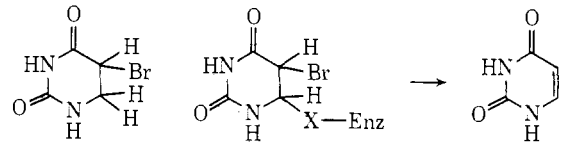
During preparation of the present manuscript, Sedor and Sander (1973) published preliminary data on debromination of brU with cysteine. They obtained pseudo-first-order kinetics measuring the decrease in $A_{290\,nm}$ and isolated uracil as a product by mixed-bed resin column chromatography. However, *S*-(5-uracilyl)cysteine was not found apparently because any of the product would have been retarded on the column if indeed it was formed.

The results described above suggest that the bromo residue in brUdRib may not be chemically stable under conditions brUdRib is used in biological studies. Cells contain free cysteine and glutathione. In mammalian tissues the cysteine concentration is 0.3×10^{-4} to 1×10^{-4} M and the glutathione concentration is 0.5×10^{-3} to 1×10^{-2} M (Gaitonde, 1967). In addition, culture medium usually contains low concentrations of cysteine due to added serum (Gaitonde, 1967). Various effects of brUdRib on mammalian cells with unestablished mechanism are known. There is toxic effect of brUdRib (Littlefield and Gould, 1960); virus synthesis is induced by brUdRib (Teich *et al.*, 1973, and references cited therein); stimulation (Schubert and Jacob, 1970) or suppression (Bischoff and Holtzer, 1970) of differentiation is effected by brUdRib; some enzymes are depressed by this agent (Stellwagen and Tomkins, 1971). These effects were demonstrated by treatment of the cells with varying concentrations of brUdRib (usually 10^{-4}–10^{-5} M) for several days or more. The possibility therefore exists that some of the effects of brUdRib might have been caused after degradation of brUdRib with sulfhydryl compounds.

We consider that enzymatic debromination of brU should now be reexamined carefully in the light of the present findings. brU is considered to be hydrogenated by dihydrouracil dehydrogenase to give 5,6-dihydro-5-bromouracil (**3**) which is then spontaneously converted into uracil liberating HBr (Barrett and West, 1956; Pahl *et al.*, 1959; Kriss and Revesz, 1962). However, literature shows that **3** is not so unstable. The half-time for uracil formation from **3** at pH 7.4 and 37° is about 48 hr (Barrett and West, 1956) (see Scheme III). Further, in the dehalogenation of 5-iodouracil with a liver enzyme preparation, 5,6-dihydro-5-iodouracil was not detected by paper chromatographic analysis of the incubation mixture (Cooper and Greer, 1970). Therefore the working hypothesis seems possible that the enzyme decomposes brU by nucleophilic addition at the 5,6 double bond followed by a rapid debromination of the enzyme-bound substrate (**4**), as is shown in the chemical model reported in the present paper.

The uv-catalyzed reaction between 5-iodouracil and cysteamine mentioned earlier was proposed (Rupp and Prusoff, 1965) to be a possible mechanism of the protective action of cysteamine type compound (Hotz, 1963) against sensitization of DNA to irradiation by incorporated brU. The protective effect of thiols including cysteine was usually observed by irradiation of brU–DNA system for a short period in dilute thiol solutions (Freifelder and Freifelder, 1966), and therefore the dark reaction reported in the present paper would not have contributed greatly to the observed effect. However, in some cases where a higher concentration of cysteine and longer reaction periods were employed (Hotz and Mueller, 1961), care must now be taken to interpret the results. It is of general interest to examine reaction of cysteine with brU–DNA.

SCHEME III



3 4

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References

Bardos, T. J., and Kalman, T. I. (1966), *J. Pharm. Sci. 55*, 606.

Barrett, H. W., and West, R. A. (1956), *J. Amer. Chem. Soc. 78*, 1612.

Berens, K., and Shugar, D. (1963), *Acta Biochim. Polon. 10*, 25.

Bischoff, R., and Holtzer, H. (1970), *J. Cell Biol. 44*, 134.

Chambers, R. W., and Kurkov, V. (1963), *J. Amer. Chem. Soc. 85*, 2160.

Cooper, G. M., and Greer, S. (1970), *Cancer Res. 30*, 2937.

Dunn, D. B., and Hall, R. H. (1970), *in* Handbook of Biochemistry, Selected Data for Molecular Biology, Sober, H. A., Ed., 2nd ed, Cleveland, Ohio, The Chemical Rubber Co., p G-57.

Elson, E. L., and Edsall, J. T. (1962), *Biochemistry 1*, 1.

Freifelder, D., and Freifelder, D. R. (1966), *Mutat. Res. 3*, 177.

Gaitonde, M. K. (1967), *Biochem. J. 104*, 627.

Garrett, E. R., Nestler, H. J., and Somodi, A. (1968), *J. Org. Chem. 33*, 3460.

Hayatsu, H., Wataya, Y., and Kai, K. (1970a), *J. Amer. Chem. Soc. 92*, 724.

Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. (1970b), *Biochemistry 7*, 2858.

Hotz, G. (1963), *Biochem. Biophys. Res. Commun. 11*, 393.

Hotz, G., and Mueller, A. (1961), *Z. Naturforsch. B 15*, 450.

Kotick, M. P., Szantay, C., and Bardos, T. J. (1969), *J. Org. Chem. 34*, 3806.

Kriss, J. P., and Revesz, L. (1962), *Cancer Res. 22*, 254.

Littlefield, J. W., and Gould, E. A. (1960), *J. Biol. Chem. 235*, 1129.

Pahl, H. B., Gordon, M. P., and Ellison, R. R. (1959), *Arch. Biochem. Biophys. 79*, 245.

Rupp, W. D., and Prusoff, W. H. (1965), *Biochem. Biophys. Res. Commun. 18*, 158.

Sander, E. G., and Deyrup, C. A. (1972), *Arch. Biochem. Biophys. 150*, 600.

Schubert, D., and Jacob, F. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 247.

Sedor, F. A., and Sander, E. G. (1973), *Biochem. Biophys. Res. Commun. 50*, 328.

Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Amer. Chem. Soc. 92*, 422.

Smith, K. C., and Aplin, R. T. (1966), *Biochemistry 5*, 2125.
Stellwagen, R. H., and Tomkins, G. M. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1147.
Szabo, L., Kalman, T. I., and Bardos, T. J. (1970), *J. Org. Chem. 35*, 1434.
Teich, N., Lowy, D. R., Hartley, J. W., and Rowe, W. P. (1973), *Virology 51*, 163.
Ueda, T. (1960), *Chem. Pharm. Bull. 8*, 455.
Visser, D. W. (1968), *in* Synthetic Procedures in Nucleic Acid Chemistry, Vol. 1, Zorbach, W. W., and Tipson, R. S., Ed., New York, N. Y., Interscience, p 409.
Wallenfels, K., and Streffer, C. (1966), *Biochem. Z. 346*, 119.
Wataya, Y., and Hayatsu, H. (1972), *Biochemistry 11*, 3583.
Wataya, Y., Hayatsu, H., and Kawazoe, Y. (1972), *J. Amer. Chem. Soc. 94*, 8927.
Wataya, Y., Hayatsu, H., and Kawazoe, Y. (1973), *J. Biochem. (Tokyo) 73*, 871.
Wilt, F. H., and Anderson, M. (1972), *Develop. Biol. 28*, 443.

Studies on the Fluorescence of the Y Base of Yeast Phenylalanine Transfer Ribonucleic Acid. Effect of pH, Aminoacylation, and Interaction with Elongation Factor Tu†

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ABSTRACT: The fluorescence spectrum and peak emission intensity of the Y base in yeast $tRNA^{Phe}$ is unchanged upon aminoacylation of $tRNA^{Phe}$. In both $tRNA^{Phe}$ and phenylalanyl-$tRNA^{Phe}$ the Y base fluorescence intensity at the emission maximum depends on the presence of Mg^{2+} and varies with pH. These results indicate that aminoacylation does not lead to changes of the tertiary structure or environment at the anticodon region of $tRNA^{Phe}$ which affect the fluorescence properties of the Y base.

The rate of deacylation of phenylalanyl-$tRNA^{Phe}$ is significantly retarded when the molecule interacts with EF-Tu and GTP to form a ternary complex. On the other hand, the fluorescence properties of the Y base in the ternary complex are not significantly different from those in uncomplexed phenylalanyl-$tRNA^{Phe}$. These observations suggest that the EF-Tu may bind to the phenylalanyl-$tRNA^{Phe}$ at its 3′ terminus. This conclusion is consistent with the results of others on the structural requirements in phenylalanyl-$tRNA^{Phe}$ and other aminoacylated-tRNAs for EF-Tu and GTP recognition.

Yeast $tRNA^{Phe}$ contains a highly fluorescent modified guanine nucleotide, commonly referred to as the Y base, located adjacent to the 3′ end of the anticodon (RajBhandary *et al.*, 1967). Even before the structure of this intrinsic chromophore had been elucidated (Nakanishi *et al.*, 1970), a number of laboratories had made extensive use of its unusual optical properties to study the conformation of yeast $tRNA^{Phe}$. These studies suggested that the fluorescence of the Y base is a very sensitive indicator of the local conformation at the anticodon region of $tRNA^{Phe}$ and is strongly dependent on external conditions, especially Mg^{2+} ion concentration (Eisinger *et al.*, 1970; Beardsley *et al.*, 1970). Robison and Zimmerman (1971) demonstrated that the increase in the Mg^{2+}-induced fluorescence of yeast $tRNA^{Phe}$ is almost completely reversed at temperatures which do not alter the secondary structure, but influence the tertiary structure of the tRNA. Such studies led to the conclusion that the highly fluorescent, Mg^{2+} stabilized, conformation is the biologically active state.

Utilizing a variety of evidence, Levitt (1969) and Cramer *et al.* (1969) proposed tentative tertiary structures for $tRNA^{Phe}$ in which, within the constraints of the basic cloverleaf arrangement, the 3′ end of the molecule and the anticodon-containing loop are maximally separated. This aspect of the proposed model was confirmed by singlet–singlet energy transfer experiments which utilized the Y base as the energy donor and a number of acceptor chromophores attached to the 3′ end of $tRNA^{Phe}$ (Beardsley and Cantor, 1970). The most recent model of the tertiary structure of $tRNA^{Phe}$ which is based on X-ray crystallographic studies at 4-Å resolution (Kim *et al.*, 1973) also shows a large separation, 82 Å, between the anticodon and amino acid acceptor end of the molecule.

In this study our objective was to utilize the fluorescence of the Y base to follow the conformational state of the anticodon-containing region of $tRNA^{Phe}$ as the molecule proceeds through certain steps of the protein-synthesizing cycle. More specifically, we have investigated some of the fluorescence properties of the Y base in $tRNA^{Phe}$ after aminoacylation with phenylalanine and after ternary complex formation of Phe-$tRNA^{Phe}$ with the protein elongation factor Tu[1] and GTP (for a review of the function of EF-Tu, see Lucas-Lenard and Lipmann, 1971). Our results indicate that the fluorescence properties of the Y base are the same in $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$. These results are discussed in terms of the pro-

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[1] Abbreviation used is: EF-Tu, elongation factor Tu.