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The Dehalogenation of the 5-Halo-5,6-Dihydrouracils

Uracil Formation from 5-Iodo- and 5-Bromo-5,6-Dihydrouracil

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The elimination of halide ion from either 5-bromo- or 5-iodo-5,6-dihydrouracil to yield uracil is a slow reaction which, in the case of 5-iodo-5,6-dihydrouracil, is 400 times slower than the enzymatic release of $^{125}I^-$ from 5-[^{125}I]iodouracil. The elimination of HBr from 5-bromo-5,6-dihydrouracil is subject to general base catalysis by tris(hydroxymethyl)aminomethane ($k_2^{\text{Tris base}} = 11 \times 10^{-4}\ M^{-1}\ \text{min}^{-1}$, 37°C, ionic strength 1.0 *M*). At pH values near and above physiological, both the bromo- and iododihydropyrimidines are subject to hydrolysis of the dihydropyrimidine ring, a reaction which parallels halide elimination to yield uracil. The resulting 2-halo-3-ureidopropionate then cyclizes via intramolecular attack of the ureido oxygen atom to yield halide ion and 2-amino-2-oxazoline-5-carboxylic acid as final products. In dilute hydroxide ion, the kinetics of 5-bromo-5,6-dihydrouracil hydrolysis (25°C, ionic strength 1.0 *M*) show a change in rate-determining step as a function of increasing hydroxide ion concentration, a result which, as in the case of 5,6-dihydrouracil, can be explained in terms of the formation of a tetrahedral addition intermediate. The data are discussed relative to enzymatically catalyzed halopyrimidine dehalogenation.

INTRODUCTION

The deoxynucleosides of the halogenated uracils are important antiviral agents which, upon removal of the deoxyribose ring, are rapidly dehalogenated *in vivo* to a variety of products, including halide ions and uracil (*1–6*). The pathway for the dehalogenation of the halogenated uracils is not understood; however, a number of workers has speculated (*1–3*) that dehalogenation might occur via reduced nicotinamide adenine trinucleotide (NADPH)-linked dihydrouracil dehydrogenase-catalyzed reduction of the uracil's 5,6 double bond followed by spontaneous elimination of a proton and halide ion to yield uracil as the final pyrimidine product (Eqs. [1] [2]). In 1970, Cooper and Greer (*7, 8*) presented evidence supporting the involvement of dihydrouracil dehydrogenase in 5-iodouracil (I-Ura) dehalogenation (Eq. [1]). They showed the existence of an enzyme(s) in high-speed rat liver supernatant solutions which catalyzed $^{125}I^-$ release from ^{125}I-Ura, required NADPH for activity, and was inhibited by known inhibigors of dihydrouracil dehydrogenase (*9*). In 1901, Fischer and Roeder (*10*) found that HBr was eliminated from 5-Br-6-methyl-5,6-dihydrouracil in dilute hydroxide, conditions which should also hydrolyze the dihydropyrimidine ring to yield the corresponding β-ureidocarboxylic acid (*11, 12*). Barrett and West (*1*) also demonstrated the elimination of HBr from 5-Br-5,6-dihydrouracil at pH 7.4, 37°C.

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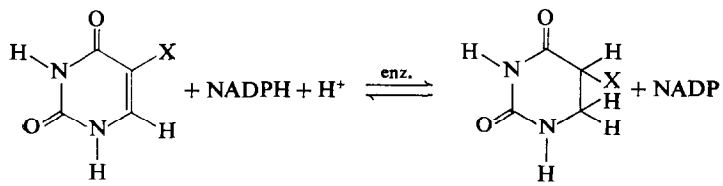

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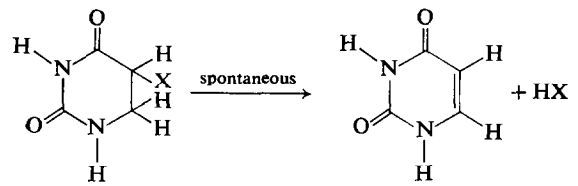

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The objectives of this report are to show that the nonenzymatically catalyzed rates of halide elimination from either 5-iodo-5,6-dihydrouracil (I-DHU) or 5-bromo-5,6-dihydrouracil (Br-DHU), shown in Eq. [2], are too slow to account for the physiologically important dehalogenation of the parent halogenated pyrimidines and that, at alkaline pH, the dominant reaction of the halodihydrouracils is the hydrolysis of the dihydropyrimidine ring.

EXPERIMENTAL SECTION

Materials. Deionized, glass-distilled water was used to prepare all reaction mixtures and solutions. Inorganic reagents obtained from Fisher Chemical Company were used as received. Tris(hydroxymethyl)aminomethane (Tris) and 5,6-dihydrouracil were from Sigma Chemical Company. 5-bromo-5,6-dihydrouracil (Br-DHU) was synthesized by direct bromination of 5,6-dihydrouracil, according to the method of Zee-Cheng *et al.* (*13*). 5-Iodo-5,6-dihydrouracil (I-DHU) was synthesized from NaI and Br-DHU in anhydrous acetone, according to the method of Rork and Pitman (*14*).

Ultraviolet absorption spectra. Ultraviolet spectra were recorded using either a Cary 14, Cary 15, or Zeiss PMQII spectrophotometer, each of which was equipped with a cell holder thermostated at either 25 or 37°C.

Kinetic experiments. The kinetics of uracil formation from both Br-DHU and I-DHU were followed by spectrophotometrically measuring the increase in A_{260nm} which occurs when either 1.54×10^{-4} *M* Br-DHU or 1.67×10^{-4} *M* I-DHU is incubated with increasing concentrations of Tris buffers of varying percent Tris-free base, 37°C, ionic strength 1.0 *M* (maintained with KCl).

The rates of Br-DHU ring opening were also measured spectrophotometrically at 260 nm by observing the rapid decrease in absorbance that occurs when either 5.95×10^{-4} or 6.15×10^{-4} *M* Br-DHU is allowed to react with increasing concentrations of KOH (0.167–66.67 m*M*), 25°C, ionic strength 1.0 *M*.

Following the completion of each kinetic run in Tris buffer, the pH was measured at 37°C using a Radiometer PHM-26 pH meter equipped with a Radiometer GK2321C combination electrode.

Psuedo-first-order rate constants were evaluated from linear (at least three half-

lives), semilogarithmic plots of extent of reaction against time, using the relationship $k_{obsd} = 0.693/t_{\frac{1}{2}}$.

Product identification. To determine the final products of the reaction of Br-DHU in Tris buffers, 5.01 mmoles of Br-DHU and 5.4×10^{-2} mmoles of 2-[^{14}C]Br-DHU were incubated in 1 liter of Tris buffer (0.2 *M*, 40% free base, pH 8.21) for 13 days at 37°C. Extent of reaction was monitored spectrophotometrically following uracil formation at 257 nm. When A_{257} was virtually constant over a 3-day period, the reaction mixture was lyophilized to dryness, redissolved in 65 ml of water, brought to pH 9.6 with the careful addition of ammonium hydroxide, and applied to a 1.5×110-cm Dowex 1-X8-formate ion-exchange column. The column was washed with 250 ml of H_2O to remove Tris base and unreacted Br-DHU followed by elution with a linear 800-ml 0–1.0 *M* formic acid gradient. Fractions (5 ml) were collected and assayed for both absorbance at 257 nm and radioactivity, using a Beckman liquid scintillation counter. Desired fractions were pooled and lyophilized to dryness. The resulting samples were then compared to known compounds using melting point, infrared spectra, ultraviolet spectra, and mobility on thin-layer chromatographic systems as criteria. Recovery of radioactivity, uncorrected for quenching or counter efficiency, was 100 ± 10%.

RESULTS

Uracil Formation from Br-DHU and I-DHU

Uracil formation from both Br- and I-DHU was measured spectrophotometrically as a function of increasing concentrations of Tris buffers of varying fraction neutralization. Representative spectra measured as a function of time for both I- and Br-DHU, incubated in 0.80 *M* Tris buffer, pH 7.56, 37°C, are shown in Figs. 1 and 2, respectively.

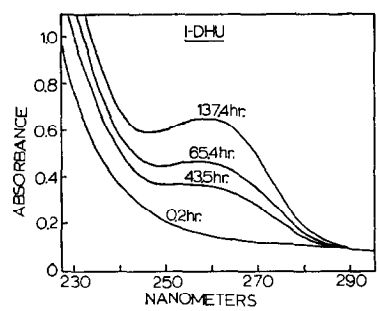


FIG. 1. Ultraviolet absorption spectra of reaction mixtures containing 1.67×10^{-4} *M* 5-iodo-5,6-dihydrouracil in 0.80 *M* Tris buffer, pH 7.56, 37°C, ionic strength 1.0 *M*. Blank solutions contained all reaction components except the iododihydropyrimidine.

In the case of Br-DHU, the reaction was followed to completion (551 hr), while, with I-DHU, these exceptionally slow reactions were terminated at 137.4 hr. Each of the spectra presented in Figs. 1 and 2 shows increasing absorption at 260 nm indicative of uracil formation, while, in the case of I-DHU, there is also an increase in end absorbance

below 240 nm due to the formation of iodide ion which has an absorption maximum at 225 nm (Fig. 1).

To measure uracil formation from both Br- and I-DHU under the same conditions that I-Ura was enzymatically dehalogenated (*8*), the initial velocities of uracil formation (A_{260}) were measured in reaction mixtures which contained, per milliliter 0.20 μmole of halopyrimidine and 10 μmole of NaH_2PO_4 (pH 7.40). Use of an extinction coefficient for uracil equal to 8.2×10^3 M^{-1} cm^{-1} (*15*) allows calculation of the nanomoles of uracil formed per hour per milliliter of reaction mixture. These values, based on data

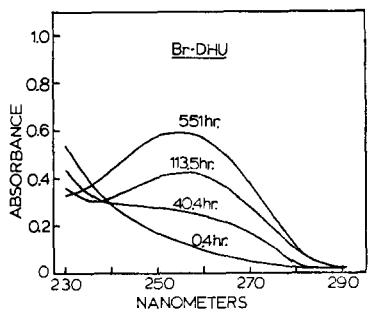


FIG. 2. Ultraviolet absorption spectra of reaction mixtures containing 1.54×10^{-4} *M* 5-bromo-5,6-dihydrouracil in 0.80 *M* Tris buffer, pH 7.55, 37°C, ionic strength 1.0 *M*. Blank solutions contained all reaction components except the bromodihydropyrimidine.

collected during the first 21 hr where the 260-nm absorbance increase appears linear with time, are about 0.06 and 0.26 nmole hr^{-1} ml^{-1} for Br-DHU and I-DHU, respectively.

To confirm the fact that the increase in absorption seen at 260 nm was due to uracil formation, reaction mixtures containing both Br-DHU and I-DHU were allowed to

TABLE 1

ULTRAVIOLET SPECTRAL DATA[a]

Pyrimidine derivative	λ_{max} (nm)	Uracil product[b] (%)	Ratio (A/A_{260})			
			240 nm	250 nm	270 nm	280 nm
Uracil	258	—	0.50	0.85	0.69	0.17
Rx: Br-DHU	258	29	0.45	0.82	0.69	0.19
Rx: I-DHU	258	59	0.95	0.90	0.68	0.18
—[c]	(258)	(59)	(0.50)	(0.88)	(0.67)	(0.20)

[a] For the products of either 5-bromo- or 5-iododihydrouracil reacting with 60% free base Tris buffer, pH 8.28, 37°C, ionic strength 1.0 *M*, followed by acidification. Following the reaction at pH 8.28, 0.50 ml of 4.68 *M* HCl was added to 2.5 ml of each reaction mixture and the spectra were recorded at room temperature. In the case of I-DHU, acidified reaction mixtures and blanks were threefold diluted prior to recording the spectra.

[b] Calculated using the following relationship: % uracil product = $[(A_{260}/\varepsilon^{Ura}) \div (X\text{-DHU})] \times 100$, where A_{260}, ε^{Ura}, and (X-DHU) represent the absorbance of the acidified reaction mixtures, the 260-nm extinction coefficient of uracil at pH = 0 (15), and the molar concentration of halodihydro-pyrimidine in the reaction mixtures.

[c] Data in parentheses obtained after reaction mixtures and blanks passed through Dowex 1-Cl^- to remove iodide ion.

react to completion at 37°C in 0.80 *M* Tris buffer, pH 8.28, followed by acidification. Difference spectra were then recorded and the spectral ratios compared to spectra of authentic uracil treated under the same conditions (Table 1). These ratios agree fairly well with those of the authentic uracil spectra except in the case of the I-DHU where I^- makes the ratios obtained at 240 and 250 nm artificially high. To circumvent this problem, iodide ion was removed from aliquots of the I-DHU reaction mixtures prior to recording the spectra. This treatment gave spectral ratios for the ultraviolet-absorbing I-DHU product which are comparable to authentic uracil. These spectra were also used to calculate the percentage of conversion of both Br- and I-DHU to uracil. In 0.8 *M* Tris buffer, pH 8.28, 37°, this conversion was 29 and 59% for Br-DHU and I-DHU, respectively, arguing that another reaction, most likely the hydrolysis of the 5,6-dihydropyrimidine ring, is also occurring under these conditions although the *p*-

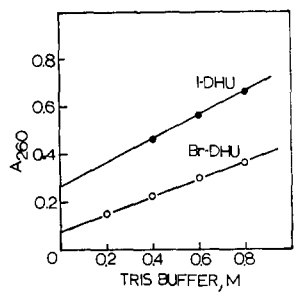


FIG. 3. Relationship between increasing Tris buffer concentration and uracil formation from 5-iodo- and 5-bromo-5,6-dihydrouracil at 37°C, ionic strength 1.0 *M*. Open circles (o) show Br-DHU in 60% free base Tris buffers, pH 8.28, and closed circles (●) show I-DHU in 20% free base Tris buffers, pH 7.56. Absorption measurements for Br-DHU were recorded after complete reaction when A_{260} nm was no longer increasing with time, while those for I-DHU were measured at $t = 137.4$ hr.

dimethylaminobenzaldehyde spot test did not yield a yellow spot which had the same intensity as that usually observed under the same conditions for a compound having a free ureido group.

The magnitude of the final 260-nm absorption due to uracil formation is linearly related to Tris buffer concentration. This dependence is illustrated in Fig. 3 for Br-DHU in pH 8.28 Tris buffers (complete reaction) and I-DHU in pH 7.56 Tris buffers ($t = 137.4$ hr).

The pseudo-first-order rate constants for uracil formation from Br-DHU were measured as a function of increasing concentrations of Tris base in buffers which were both 45.0 and 64.3% Tris base (Fig. 4). The fact that parallel lines were observed for both buffer ratios argues that the free amine is the catalytically active buffer component. Hence, the elimination of HBr from Br-DHU is likely subject to general base catalysis of proton transfer. The slope of these lines (Fig. 4) represents the second-order rate constant (k_2) for Tris base catalysis of HBr elimination from Br-DHU and has a value of $11 \times 10^{-4}\ M^{-1}\ \text{min}^{-1}$,

Incubating Br-DHU in buffers of increasing pH increases the rate of absorbance increase at 260 nm, but decreases the total 260-nm absorbance observed at times

sufficient to ensure complete reaction. This result indicates that, besides HBr elimination to yield uracil, Br-DHU is subject to another reaction which predominates at higher pH values. Since the hydrolysis of both dihydrouracil and 1,3-dimethyldihydrouracil to the corresponding β-ureido acids is a hydroxide-dependent, non-buffer-catalyzed reaction (*12*), the hydrolysis of Br-DHU was kinetically studied at ionic

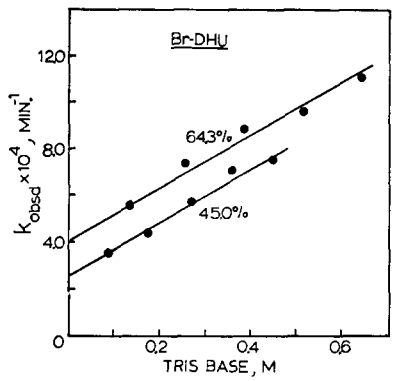


FIG. 4. Effect of increasing concentrations of Tris base on the observed pseudo-first-order rate constants for uracil formation from 5-bromo-5,6-dihydrouracil, 37°C, ionic strength 1.0 *M*. Percentages refer to the percentage of Tris base in the buffers employed.

strength 1.0 *M*, 25°C, as a function of increasing hydroxide concentration. Figure 5 shows the results of these studies. At lower hydroxide ion concentrations, there is a greater than first-order dependence on hydroxide because the slope of the line arbitrarily drawn through the first seven points has a slope of 1.3, which is greater than the

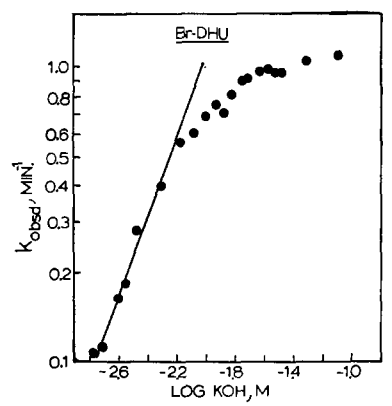


FIG. 5. Logarithmic relationship between the pseudo-first-order rate constants for 5-bromo-5,6-dihydrouracil hydrolysis and increasing KOH concentration, 25°C, ionic strength 1.0 *M*. Reaction mixtures contained either 5.95 or 6.15 $\times$ 10^{-4} *M* Br-DHU.

value of 1.0 which would be predicted for a reaction which is first order in hydroxide ion concentration. As the hydroxide ion concentration is increased up to about 0.10 *M*, the observed rate constants become almost invariant with hydroxide ion concentration.

These data argue for a multistep reaction pathway with a change in rate-determining step, which manifests itself as the kinetics of Br-DHU hydrolysis are studied as a function of increasing hydroxide ion concentration.

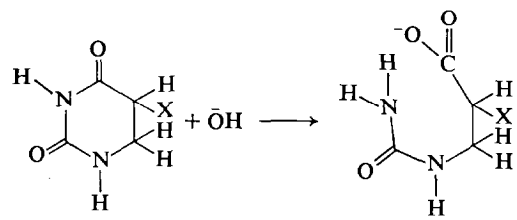
Since the spectral data indicated that only part of the 5-halo-5,6-dihydrouracil yields uracil as a product, a complete identification of products was undertaken using 2-[^{14}C]Br-DHU to aid in the detection of nonchromophoric products. After the reaction was almost complete (13 days, 37°C, pH 8.21), reaction mixtures were lyophilized, redissolved in water, brought to pH 9.6, and chromatographed on Dowex 1-X8-formate using water and a linear formic acid gradient as eluants. This ion-exchange chromatographic method yielded three major radioactive peaks. One peak, which eluted with water almost in the column void volume, comprised about 10% of the total radioactivity applied to the column and was assumed to be unreacted Br-DHU. It was not possible to characterize this peak as Br-DHU since it eluted along with the Tris base and, hence, continued to react under these conditions. The other two radioactive peaks, one of which absorbed strongly at 257 nm, were not sharply resolved by formic acid elution; however, pooling of the leading and trailing fractions of these peaks allowed characterization of essentially pure compounds. The white, fluffy powder obtained from the leading edge of the first peak was found to have a melting point (213°C), infrared spectra, and cellulose thin-layer chromatographic behavior (solvent, 1-butanol : acetic acid : H_2O, 60 : 20 : 20, v/v/v) identical with 2-amino-2-oxazoline-5-carboxylic acid (**I**), a compound which has been completely characterized by Kim *et al.* (*16*). The crystalline material obtained from the trailing edge of the peak which contained both radioactivity and ultraviolet absorbance was identified as uracil by its ultraviolet spectrum measured in 0.01 *M* HCl ($\lambda_{max} = 257$ nm, $\varepsilon_{257\,nm} = 7.9 \times 10^3\ M^{-1}\ cm^{-1}$, $A_{240}/A_{260} = 0.48$, $A_{280}/A_{260} = 0.16$; authentic uracil standard: $\lambda_{max} = 257$ nm, $\varepsilon_{257\,nm} = 8.3 \times 10^3\ M^{-1}\ cm^{-1}$, $A_{240}/A_{260} = 0.48$, $A_{280}/A_{260} = 0.17$).

DISCUSSION

Uracil formation from either Br-DHU or I-DHU at 37°C and near physiological pH (7.56) is a slow reaction which cannot explain the rapid release of $^{125}I^-$, observed by Cooper and Greer (*8*), upon incubation (37°C, pH 7.4, 1.0-ml final volume) of 0.20 μmole of ^{125}I-Ura with NADPH, glucose 6-phosphate, and a rat liver supernatant solution. These workers did not observe the accumulation of uracil as the pyrimidine product, a result likely caused by the fact that rat liver supernatant solution contains the enzymes responsible for uracil catabolism. If, however, one assumes the pathway for halopyrimidine dehalogenation, shown in Eqs. [1] and [2], then 1 : 1 stoichiometry should exist between $^{125}I^-$ and uracil formation in the enzymatic system. Examination of Cooper and Greer's (*8*) data shows that the enzymatically catalyzed rate of $^{125}I^-$ formation from ^{125}I-Ura is about 100 nmoles hr^{-1} ml^{-1} in reaction mixtures initially containing 0.20 μmole of ^{125}I-Ura, 10 μmoles of phosphate buffer (pH 7.4), 0.5 μmole of NADPH, 10 μmoles of glucose 6-phosphate, and 0.10 ml of rat liver supernatant solution per milliliter, 37°C. This should correspond to the formation of an equal amount of uracil during the same period of time. Calculation of the initial rate of uracil

formation using reaction mixtures containing 0.20 μmole of I-DHU and 10 μmoles of sodium phosphate buffer (pH 7.4) per milliliter, 37°C, showed that only 0.26 nmole hr^{-1} ml^{-1} was formed, a rate which is about 400 times too slow to account for the rate of $^{125}I^-$ release from ^{125}I-Ura, observed by Cooper and Greer (*8*). Consequently, the nonenzymatically catalyzed elimination of halide ion from the 5-halo-5,6-dihydropyrimidines is likely not involved in enzymatic halopyrimidine dehalogenation.

Incubation of either Br-DHU or I-DHU in buffer systems of near physiological pH does not result in uracil as the only major product, a conclusion warranted by the fact that A_{260} values at complete reaction are not sufficient to account for the initial concentration of 5-halodihydrouracil and by the fact that 2-amino-2-oxazoline-5-carboxylic acid (I) is an insoluble product. Thus, in addition to E-2 elimination of HX ($X = -Br$, $-I$) to yield uracil, the halodihydrouracil is hydrolyzed to a ureidocarboxylic acid (Eq. [3]), which subsequently cyclizes via intramolecular nucleophilic attack of the



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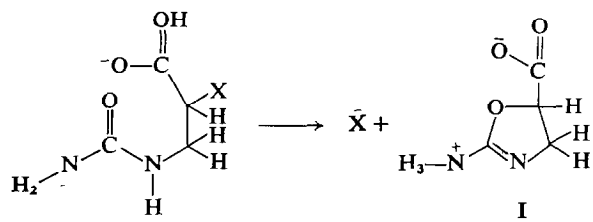
oxygen atom on the halogen-containing carbon atom to yield the oxazoline and halide ion as products (Eq. [4]). A similar reaction involving intramolecular attack of the



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ureido nitrogen has been shown by Fox's laboratory (*17*, *18*) to be involved in the dehalogenation of 2′, 3′,-*O*-isopropylidene-5-halouridine in strongly alkaline medium, and Hegarty and Bruice (*19*) have shown that nucleophilic reactions involving ureido groups may proceed by both N and O attack.

The hydrolysis of Br-DHU in dilute hydroxide at 25°C is a reasonably rapid reaction; hence, during the course of these reactions, uracil formation is too negligible to be observed at 260 nm. Like 5,6-dihydrouracil (12), the alkaline hydrolysis of Br-DHU is a multistep reaction, as evidenced by the fact that the reaction undergoes a change from a greater than first-order to an almost zero-order dependence on increasing concentration of hydroxide ion. As with the unsubstituted dihydrouracils, this kinetic behavior likely represents the formation and subsequent collapse of a tetrahedral addition intermediate.

The work of Cooper and Greer (*7*, *8*) points toward the conclusion that NADPH-linked dihydrouracil dehydrogenase is responsible for the formation of halodihydro-

pyrimidines in the overall dehalogenation of the halouracils. If the halodihydropyrimidines are intermediates in this overall process and the noncatalyzed elimination of halide ion is too slow to account for the overall reaction rate, then how are these compounds dehalogenated? The first and most obvious possibility is that an enzyme present in rat liver supernatant solutions is responsible for the catalysis of this elimination reaction to yield uracil as a product. The second possibility involves catalysis of the ring-opening reaction of the dihydropyrimidine ring system (Eq. [3]) by dihydropyrimidine amidohydrolase followed by either spontaneous or enzyme-catalyzed formation of five-membered ring products, such as **I**, along with halide ion.

Finally, perhaps dihydrouracil dehydrogenase (Eq. [1]) is not involved in the initial step of halopyrimidine dehalogenation. Cooper and Greer (*7*, *8*) clearly showed an NADPH requirement for $^{125}I^-$ formation from ^{125}I-Ura. This requirement for reducing equivalents could be required for the reduction of an enzyme system which dehalogenates the halopyrimidines directly and proceeds by a mechanism similar to that proposed by us for the cysteine-promoted dehalogenation of both Br- and I-Ura (*20*, *21*). Work currently in progress in our laboratory should help evaluate these possibilities.

ACKNOWLEDGMENTS

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