0.033 mole), 2,3-dichloroquinoxaline (6.0 g, 0.03 mole), methanol (200 ml), and water (20 ml) were heated under reflux for 2 hr, diluted with 150 ml of water, and filtered. The dried solid was recrystallized from 250 ml of dimethylformamide and dried at 140° (0.2 mm) for 2 hr to give 3.2 g (25%) of bright-yellow crystals, mp 298–320° dec.; IR: 2210 (C≡N), 1505, 1180, and 1120 (quinoxaline ring) cm^{-1}.

Anal.—Calc. for $C_{12}H_4N_4S_2$: C, 53.71; H, 1.50; N, 20.88; S, 23.90. Found: C, 54.07; H, 1.74; N, 20.5; S, 23.6.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 20, 1974, from the *Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy, Boston, MA 02115*

Accepted for publication August 5, 1974.

Abstracted from theses submitted by J. J. Lanzillo and Y. H. Lowe to the Massachusetts College of Pharmacy in partial fulfillment of the Doctor of Philosophy degree requirements.

The authors express their appreciation to The Gillette Co. for financial assistance.

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Bisulfite-Ion-Catalyzed Degradation of Fluorouracil

GERALD S. RORK and IAN H. PITMAN [x]

Abstract □ 5-Fluorouracil (I) reversibly adds bisulfite ion across its $>C_5{=}C_6<$ bond to form 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). The pH-independent equilibrium constant for this reaction was calculated to be 560 M^{-1} at ionic strength 1.00 M at 25°. Compound II was observed to be unstable in alkaline solution and reacted to yield uracil-6-sulfonic acid, fluoride ion, and α-fluoro-β-ureidopropionic acid-β-sulfonate (III), along with I (*via* a loss of HSO_3^-). In strongly alkaline conditions, *i.e.*, 1 M NaOH, III was observed to undergo a subsequent reaction to produce a compound believed to be α-fluoro-β-ureidoacrylic acid (V) or fluoromalonaldehydic acid (VII). These irreversible degradation reactions of II lead to a complete degradation of I in sodium bisulfite solutions. The similarity of this bisulfite-ion-catalyzed degradation of I to its hydrolytic degradation is discussed.

Keyphrases □ Fluorouracil—bisulfite-ion-catalyzed degradation □ Sodium bisulfite—role in degradation of 5-fluorouracil, nucleophilic addition □ Equilibrium—bisulfite ion addition to fluorouracil

It has been reported (1) that the antimetabolite 5-fluorouracil (I) undergoes a reversible, covalent addition of bisulfite ion (HSO_3^-) across its 5,6-carbon–carbon double bond to yield 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). Additional findings that II undergoes irreversible reactions in sodium bisulfite solutions leading to complete degradation of I (Scheme I) are now reported.

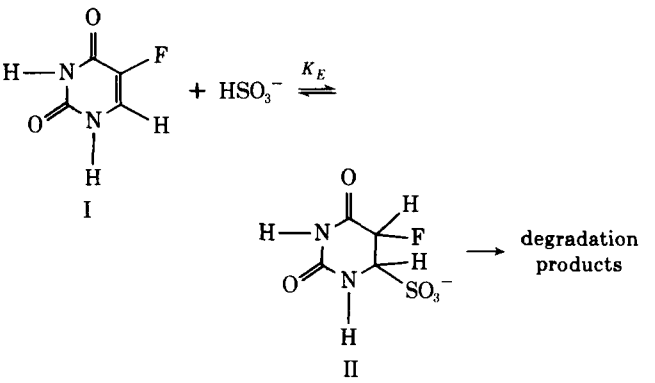
Although I is comparatively stable at room temperature in aqueous buffers that do not contain sodium bisulfite[1], it has been observed to degrade at higher temperatures and in alkaline conditions (2). It has been suggested that the first step in these reactions is covalent hydration of the 5,6-double bond of I to produce 5- or 6-hydroxy-5-fluoro-5,6-dihydrouracil, which then degrades to nonchromophoric products (*via* opening of the pyrimidine ring) (2).

The results of the present study are discussed in terms of similarities between the water-catalyzed and bisulfite-ion-catalyzed degradations of I.

EXPERIMENTAL

Materials and Equipment—Fluorouracil[2] was used as received. All water was redistilled from a Pyrex apparatus before use. All other chemicals were a reagent grade and were used without additional purification.

Potassium 5-fluoro-5,6-dihydrouracil-6-sulfonate monohydrate



Scheme I

[1] Fluorouracil is formulated for injection as a pH 9 solution (adjusted with sodium hydroxide) at a concentration of 50 mg/ml. Fluorouracil package insert, Roche Laboratories, Dec. 1972.

[2] Sigma Chemical Co.

was isolated from the reaction of I in a potassium sulfite solution at pH 5. Approximately 0.3 g of I was added to 4 ml of a saturated solution of potassium sulfite, which had been adjusted to pH 5 with concentrated hydrochloride acid. After stirring at room temperature for 1 hr, the mixture was heated to 95° and a clear solution was obtained.

White crystals formed on cooling and were collected by filtration and then washed with cold water and anhydrous ether. The NMR and UV spectra of this material are consistent with that previously reported (1) for the sodium salt of II, which was isolated in a different procedure.

Anal.—Calc. for $C_4H_6FKN_2O_6S$: C, 17.90; H, 2.24; N, 10.44. Found: C, 17.65; H, 2.26; N, 10.16.

Similarly, potassium 5-fluoro-1,3,5-trideutero-5,6-dihydrouracil-6-sulfonate with one molecule of D_2O of crystallization was isolated when the this procedure was followed using D_2O and DCl in place of H_2O and HCl.

Anal.—Calc. for $C_4HD_5FKN_2O_6S$: C, 17.58; D and H (as H), 2.20; N, 10.25. Found: C, 17.41; D and H, 2.17; N, 9.86.

Kinetic and Equilibria Measurements—The kinetics and equilibria of addition of HSO_3^- to I were measured by following the loss of absorbance of I in the 270–290-nm region that occurred after mixing solutions of I with aqueous buffer solutions containing sodium bisulfite. Unless otherwise stated, all solutions were measured at ionic strength 1.00 *M* (adjusted with potassium chloride) and at 25°.

Typically, 0.005–0.010 ml of a 0.1 *M* solution of I at pH 10 was added to a 1-cm cell. The cell contained 3.0 ml of an aqueous buffer containing sodium bisulfite and had been equilibrated at 25° in the cell compartment of a spectrophotometer. (The reference cell contained an identical solution and the instrument had been balanced at zero absorbance.) The cell was then inverted several times to ensure complete mixing, and the resultant loss in absorbance was followed as a function of time.

Observed first-order rate constants (k_{obs} values) were calculated from the slopes of semilogarithmic plots of the absorbance changes, $(A - A_\infty)$, *versus* time. Since II did not have any significant absorbance in the 270–290 region, the absorbance value at the start of the reaction A_0, and the absorbance value at equilibrium, A_∞, could be used to calculate an observed equilibrium constant for the addition of HSO_3^- to I. The equilibrium constants were calculated as described under *Results and Discussion*.

The kinetics of reactions of II in aqueous solutions were followed by mixing 0.005–0.010 ml of a 0.10 *M* solution of II in water with 3.0 ml of a particular buffer and observing the resultant absorbance changes that occurred in the UV spectrum of the solution.

A value of 6.50 ± 0.03 was calculated for the pKa of HSO_3^- from a potentiometric titration of sodium sulfite with standard hydrochloric acid solution at ionic strength 1.00 *M* (adjusted with potassium chloride) at 25°. The pKa of I was 7.71 ± 0.05 from spectrophotometric measurements at ionic strength 1.00 *M* at 25°.

Paper Chromatography—The paper chromatographic system used to separate the degradation products of the reaction of II in alkaline solution utilized 95% ethanol–acetic acid–water (80:10:10) as the developing solvent (ascending) on filter paper[3]. Strips 4 × 18 cm were cut, and duplicate spottings of the solution to be analyzed were made 1 cm from each edge of the strip and 1 cm from the bottom.

The developing solvent was allowed to ascend 10–12 cm and the strip was then allowed to dry. Spots were detected by three different methods: 1, illuminating by a 254-nm lamp; 2, spraying with a 5% methanolic potassium hydroxide solution, drying, and illuminating with a 254-nm lamp; and 3, spraying with a 3% solution of *p*-dimethylaminobenzaldehyde which was also 5% in hydrochloric acid.

Since duplicate spottings were made, the developed chromatogram was cut in half from top to bottom. Methods 1 and 2 were applied to one half, while Method 3 was applied to the other.

RESULTS AND DISCUSSION

Kinetics and Thermodynamics of Addition of HSO_3^- to I—The reversible addition of HSO_3^- to I is believed[4] to involve attack by SO_3^{-2} at C-6, followed by protonation at C-5, in the forward direction (addition) and general and specific base-catalyzed removal of the C-5 proton, followed by elimination of SO_3^{-2}, in the reverse direction (desulfonation). A similar two-step process has been suggested to occur in the reversible addition of HSO_3^- to other uracil derivatives (3). The kinetic results of the present study closely parallel those obtained by Sedor *et al.*[4] and thus have not been included in detail.

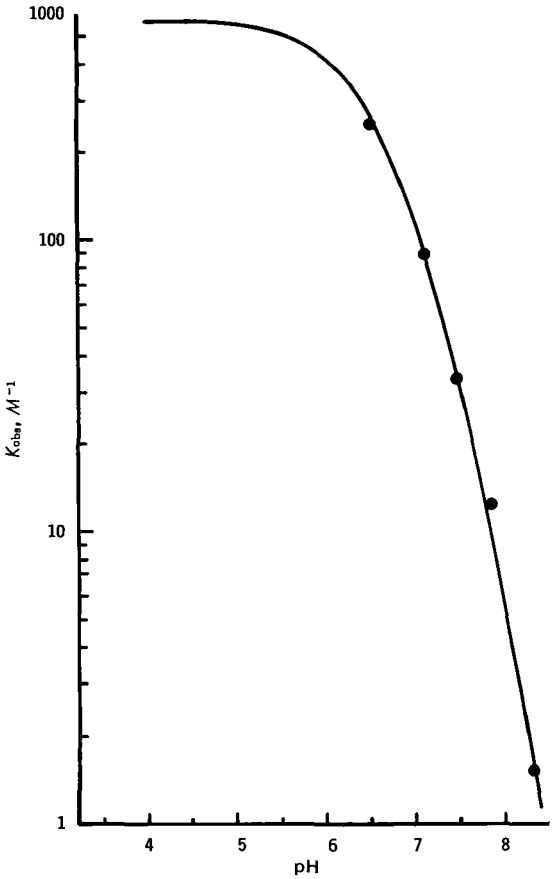


Figure 1—*Semilogarithmic plot of the observed equilibrium constants for addition of HSO_3^- to 5-fluorouracil, K_{obs} values versus pH. The solid line was calculated from Eq. 2 with K_E = 560 M^{-1}. All values were measured at ionic strength at 1.00 M and at 25°.*

Values of the observed equilibrium constant for addition of HSO_3^- to I (Scheme I) were calculated from the slopes of plots of $(A_0 - A_\infty)/A_\infty$ values *versus* the total sulfites concentration in solution, $[S_T] = [HSO_3^-] + [SO_3^{-2}]$, for a series of solutions at constant pH:

$$K_E = \frac{[II]}{[I][HSO_3^-]} \qquad \text{(Eq. 1}a\text{)}$$

$$K_{obs} = \frac{[II]}{[I_T][S_T]} = \frac{(A_0 - A_\infty)}{A_\infty[S_T]} \qquad \text{(Eq. 1}b\text{)}$$

where the term $[I_T]$ represents the concentration of 5-fluorouracil neutral molecule plus the concentration of 5-fluorouracil monoanion ($[I_T] = [I] + [I^-]$).

It is expected that II exists predominantly as a monoanion in the pH range investigated (6.5–8.3) since its pKa for ionization of an imino proton is approximately 10.0. The pKa of 5,6-dihydrouracil-6-sulfonate was previously observed to be 10.65 (3). The K_{obs} values can thus be expressed in terms of the pH-independent equilibrium constant, K_E, and the acid dissociation constants for HSO_3^-, $K_a^{HSO_3^-}$ and I, K_a^I, as shown in Eq. 2:

$$K_{obs} = K_E\left(\frac{[H^+]}{[H^+] + K_a^I}\right)\left(\frac{[H^+]}{[H^+] + K_a^{HSO_3^-}}\right) \qquad \text{(Eq. 2)}$$

[3] Whatman No. 2.
[4] F. A. Sedor, D. G. Jacobson, and E. G. Sander, to be published.

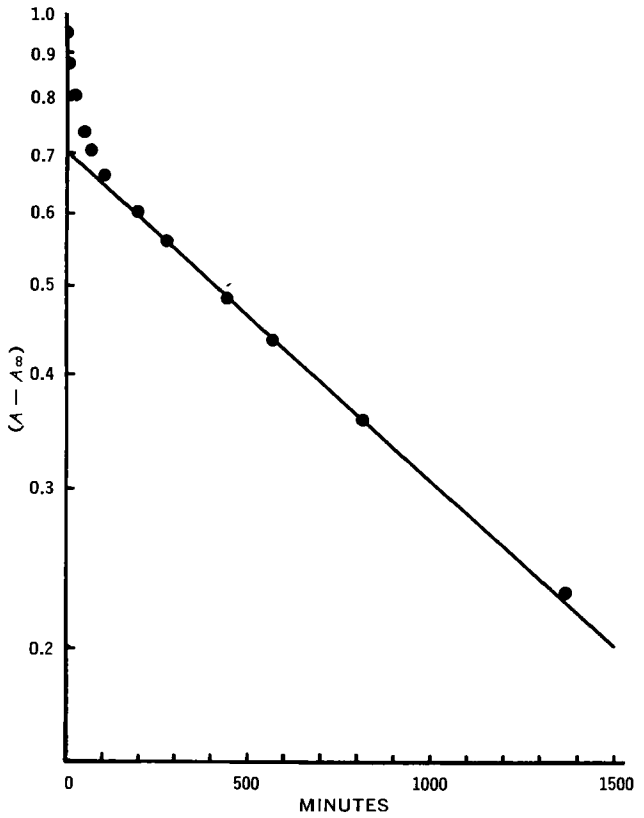


Figure 2—*Semilogarithmic plot* versus *time of the absorbance changes that occurred at 285 nm upon addition of I to a solution that was 0.233* M *in sodium sulfite and 0.3* M *in tromethamine buffer at pH 8.31, ionic strength 1.00* M, *25°.*

Figure 1 shows the equilibrium pH profile of K_{obs} values calculated from Eq. 1*b*. The values are plotted semilogarithmically *versus* pH, and the solid line was calculated from Eq. 2 using a value of $K_E = 560\ M^{-1}$, with $pKa^I = 7.71$ and $pKa^{HSO_3^-} = 6.50$. At constant total sulfite concentration, the equilibrium formation of II becomes less favorable as the pH is increased above 5. Equation 2 appears to describe this dependency adequately.

Degradation Products of II—At pH values below 8, only a first-order approach to equilibrium was observed when solutions of I were mixed with sodium bisulfite solutions. However, an additional reaction was observed in solutions containing tromethamine buffer and sodium sulfite at pH 8.31.

Figure 2 shows a semilogarithmic plot of the absorbance changes, $(A-{}^{I}A_{\infty})$, observed at 285 nm after a solution of I was mixed with a solution at pH 8.31, which was 0.233 *M* in sodium sulfite and 0.30 *M* in tromethamine buffer. These data suggested that II slowly degraded under these conditions. An observed first-order rate constant for this reaction was calculated to be 1.6×10^{-5} sec^{-1} from the terminal slope of Fig. 2. The intercept at zero time of this line was taken as the A_∞ value for the first reaction in calculating the observed equilibrium constant, K_{obs}, for the addition of HSO_3^- to I at this pH.

Further evidence that II underwent degradation reactions in alkaline solutions came from the observation that II and 5-deutero-II (5-*d*-II) did not yield quantitative amounts of I *via* a base-catalyzed desulfonation reaction. Figure 3 shows the UV spectrum of a 2.00×10^{-4} *M* solution of I in 0.01 *N* NaOH solution along with the UV spectra of 2.00×10^{-4} *M* solutions of II and 5-*d*-II in 0.01 *N* NaOH solution, which had been allowed to stand for 40 min at 25°. A first-order appearance of the absorbance due to I was observed for each of these two solutions, and the observed rate constants were calculated to be 5.5×10^{-3} sec^{-1} for II and 2.8×10^{-3} sec^{-1} for 5-*d*-II.

From the spectral data shown in Fig. 3, it was calculated that the yield of I was 45% from II and 17.5% from 5-*d*-II. The lower percentage yield of I from 5-*d*-II is believed to result from a decrease in the rate of the desulfonation reaction relative to a competing side reaction, which occurred when a deuterium was incorporated at C-5 of II. Primary kinetic deuterium isotope effects in the range of 4–5 have been observed in the desulfonation reaction of similar HSO_3^- adducts of uracils (3). Any deuterium isotope effect on the side reaction was apparently smaller than that on the desulfonation reaction. Thus, these data suggest that a side reaction competes with the desulfonation of II and that it results in the formation of a compound with virtually no absorbance in the 240–310-nm region.

Figure 4 illustrates the changes in the UV spectrum that occurred with time in a solution that was 1.67×10^{-4} *M* in II and 0.01 *N* in sodium hydroxide. An isosbestic point was observed at 256 nm during the reaction of II to I for at least seven half-lives (15 min). These spectral data are also consistent with a reaction scheme in which a side reaction occurs parallel to the desulfonation reaction and leads to the formation of a nonchromophoric product. The slow increase in absorbance in the 260-nm region, resulting in loss of the isosbestic point, will be discussed below.

NMR spectroscopy and paper chromatography were utilized to investigate the nature of the degradation products formed from 5-*d*-II in alkaline solutions. The deuterated derivative was studied since it gave a higher percentage of products other than I, as mentioned previously.

The NMR spectra of Compounds II (spectrum A) and 5-*d*-II (spectrum B) in D_2O are shown in Fig. 5. After the pD of the solution of 5-*d*-II in D_2O was increased to approximately 12 with a NaOD solution, the NMR spectrum of the resultant solution, spectrum C, indicated that the singlet at 4.9 ppm (due to 5-*d*-II) had disappeared and that signals due to I (doublet centered at 7.6 ppm, $J = 5$ Hz) and an unidentified compound (doublet centered at 5.25 ppm, $J = 29$ Hz) had developed.

The formation of a bright-yellow color when an acidic solution of *p*-dimethylaminobenzaldehyde was added to a portion of this solution suggested that some opening of the pyrimidine ring had occurred. Neither 5-*d*-II nor I gave a positive test with this reagent, which is specific for primary (*versus* secondary) amines, amides, and ureido functions (4).

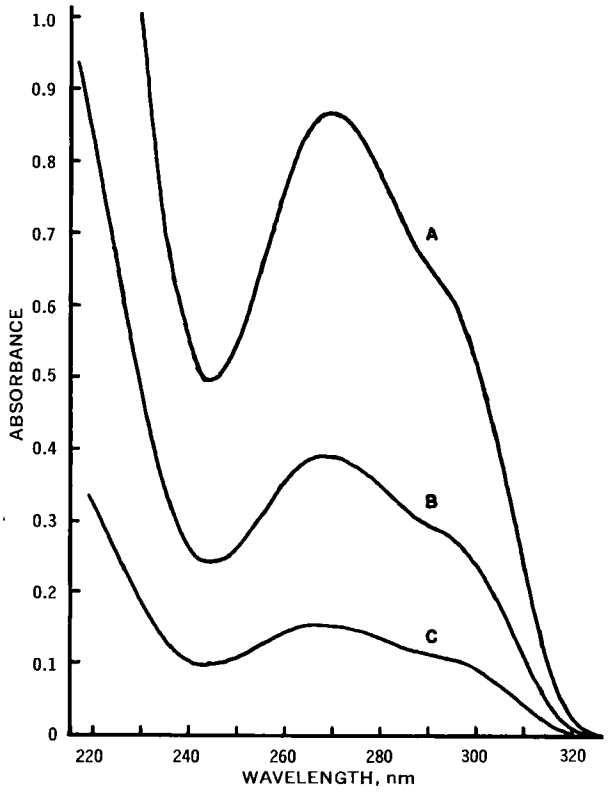


Figure 3—*UV spectra of 2.00 × 10⁻⁴* M *solutions of II (B) and 5-d-II (C) in a 0.01* N *NaOH solution measured 40 min after the solutions were made. The spectrum of a 2.00 × 10⁻⁴* M *solution of I in 0.01* N *NaOH solution is shown for comparison (A).*

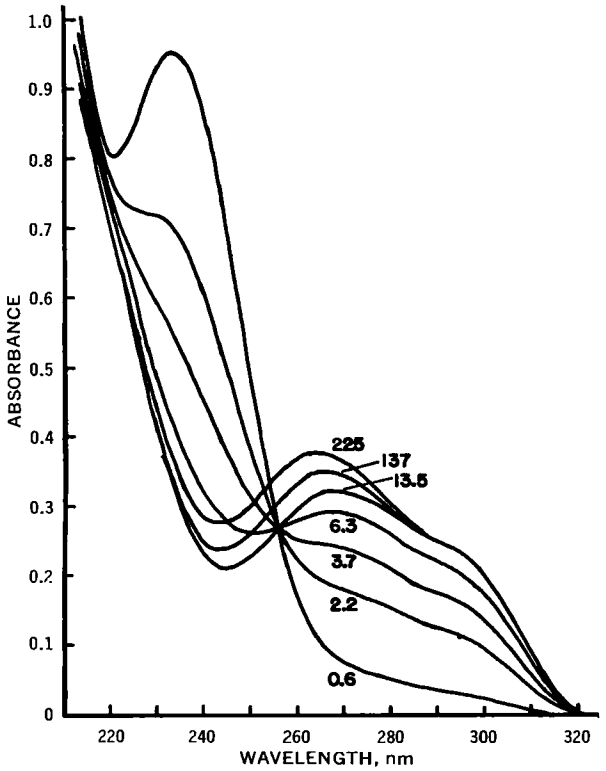
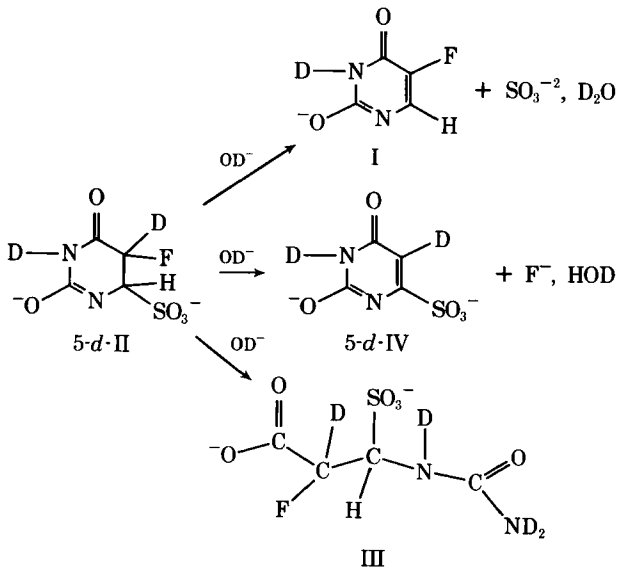


Figure 4—*UV spectral changes that occurred upon addition of II to a 0.01* N *NaOH solution to yield a 1.67* $\times$ 10^{-4} M *solution. The time, in minutes, after the addition of II is appropriately labeled for each scan.*

It is known (5, 6) that 5,6-dihydrouracils hydrolyze at the 3,4-linkage in alkaline solution to yield β-ureidopropionic acids, which readily develop a similar yellow color with this reagent. It is expected that 5-*d*-II would undergo a similar 3,4-ring opening since the fluorine at C-5 would activate the C-4 carbonyl group toward attack by OH^-. In the paper chromatographic analysis of this solution, as described under *Experimental*, only one spot (R_f 0.07) was observed to develop a yellow color when sprayed with an acidic solution of *p*-dimethylaminobenzaldehyde. This spot was detectable with a 254-nm UV lamp only after spraying with a 5% methanolic potassium hydroxide solution.

Apparently, the component exhibited in this low R_f spot and the doublet at 5.25 ppm in spectrum C of Fig. 5 is α-deutero-α-fluoro-β-ureidopropionic acid-β-sulfonate (III), which was formed *via* base-catalyzed 3,4-ring opening of 5-*d*-II (Scheme II). Compounds II and 5-*d*-II were not observed to have measurable vicinal H—F couplings, apparently because of the specific orientation of the C_5—F and C_6—H bonds.



Scheme II

The large coupling of 29 Hz, assigned to III, is typical of vicinal H—F couplings on saturated carbon (7). Opening of the ring would allow rotation about the carbon–carbon bond, providing a different orientation of H and F. Thus, 3,4-ring opening could drastically change the coupling constant of the C_6—H and not markedly change its chemical shift.

Two other spots were observed in the paper chromatographic analysis: one at R_f 0.50 and one at R_f 0.16. The spot at R_f 0.50 was detected with a 254-nm UV lamp and the R_f value was identical to that of authentic 5-fluorouracil (I). The spot at R_f 0.16 was also detectable with a 254-nm UV lamp but was less intense. This spot was cut from the chromatogram and eluted with a small volume of water.

The UV spectrum of this solution strongly suggested that the compound was uracil-6-sulfonic acid (IV) (present as a sulfonate salt). This identification was based on the reported (8) spectral data of authentic IV [pKa_2 = 7.2; λ_{max} 264 nm (ϵ 8470 cm^{-1} M^{-1}) in 0.1 *N* HCl and λ_{max} 293 nm (ϵ 9190 cm^{-1} M^{-1}) in 0.1 *N* NaOH]. The spectrum showed λ_{max} 264 nm at pH <5, which then shifted to longer wavelengths with an isosbestic point at 277 nm as the pH of the solution was increased. At pH 11–12, λ_{max} 293 nm was observed; the ratio of the 293-nm peak to the 264-nm peak (at pH 5) was 1.06. This value is in good agreement with the literature value of 1.09.

A strong, positive test for the presence of fluoride ion was obtained for this solution, whose NMR spectrum and paper chroma-

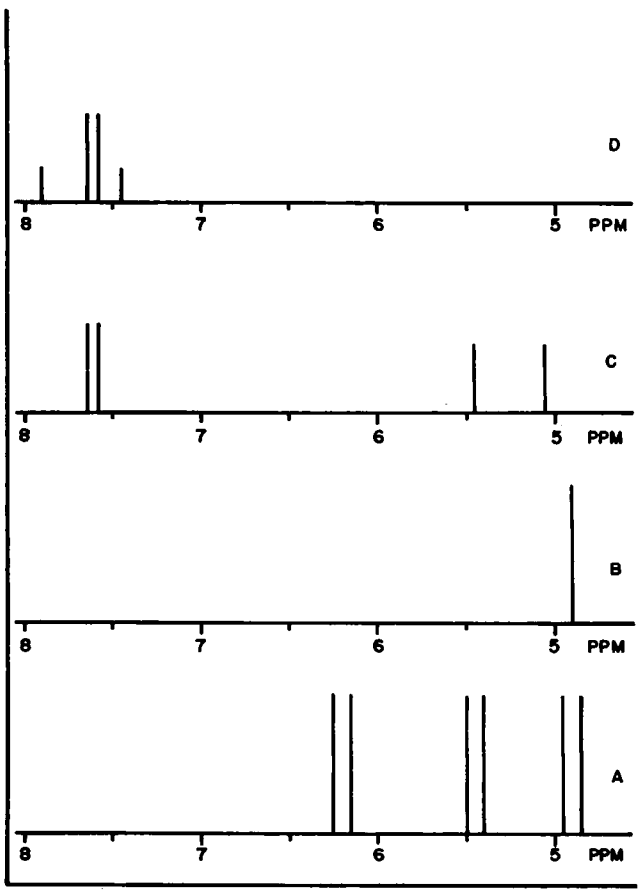
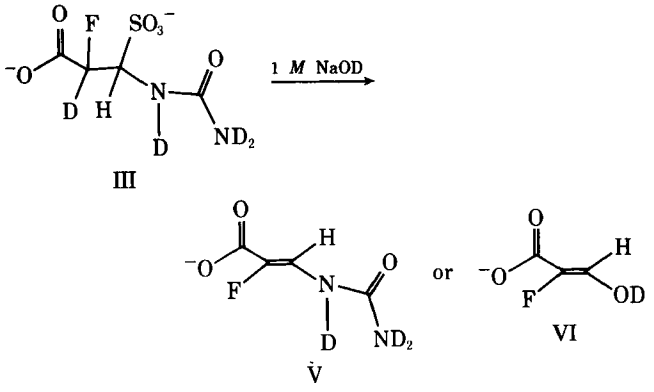


Figure 5—*NMR spectral data for a solution of II in D_2O (A) and a solution of 5-d-II in D_2O (B). Spectrum C was obtained several minutes after addition of sufficient NaOD solution in D_2O to bring the pD of Solution B to approximately 12–13. Spectrum D was measured several minutes after the addition of additional NaOD solution (40% NaOD in D_2O) to Solution B.*



Scheme III

tographic behavior has just been discussed, when it was treated with a zirconium–alizarin solution (9). Neither 5-*d*-II, I, nor any of the solution components gave a positive test with this reagent. Thus, it appears that the spot at R_f 0.16 is due to 5-*d*-IV, formed *via* HF elimination (Scheme II). This compound would obviously not have appeared in spectrum C of Fig. 5 since it contains no hydrogen substituents. It apparently is a relatively minor product in these reactions.

UV spectral data (Fig. 4) indicated that the nonchromophoric product of the side reaction mentioned, presumably Compound III, slowly underwent a subsequent reaction in alkaline conditions to produce an unknown compound with a strong chromophore in the 240–280-nm region. When the R_f 0.07 spot was eluted with water and the resultant solution was made 1 *M* in sodium hydroxide, a peak with λ_{max} 260 nm slowly developed over 1 hr.

Similarly, when the solution whose NMR spectrum is shown as spectrum C in Fig. 5 was made strongly alkaline (by addition of several drops of 40% NaOD in D_2O to the sample tube), the doublet at 5.25 ppm was lost and a doublet centered at 7.67 ppm, with a coupling constant of 27 Hz, was observed as shown in spectrum D.

These UV and NMR spectral changes that occurred in strongly alkaline conditions are apparently due to a degradation of III to either α-fluoro-β-ureidoacrylic acid (V) or fluoromalonaldehydic acid (VI) (Scheme III). Compound VI has been reported (10) as a degradation product of 5-fluoro-6-hydroxy-5,6-dihydrouracil (VII) in 1 *M* NaOH solution, along with urea and a small amount (6%) of I.

In a 1 *M* NaOH solution, VII was reported to have a strong chromophore (13,300 cm^{-1} M^{-1}) with a λ_{max} of 260 nm. This UV behavior seems very similar to that observed for the alkaline degradation product of the R_f 0.07 spot. The fact that the doublet shown in spectrum D of Fig. 5 is very similar in chemical shift to the C_6—H of I suggests a structure such as that of V or VI. The large observed coupling constant of 27 Hz is consistent with reported *trans*-H—F vicinal couplings on carbon–carbon double bonds (7).

A comparison of the reaction sequences in Schemes I and II with the postulated (2) mechanism of degradation of I in aqueous buffers that did not contain sodium bisulfite indicates that the irreversible degradation of I in both cases is most likely initiated by covalent addition of a nucleophilic reagent to I to yield a 5,6-dihydrouracil. In the former case, HSO_3^- is the nucleophilic reagent; in the latter case, it is water.

Because of the structural similarities of 5-fluoro-6-hydroxy-5,6-dihydrouracil and II, it seems likely that the magnitude of their rate constants for irreversible degradation would be similar. The irreversible degradation reactions postulated for both compounds involve attack of OH^- at their C-4 carbonyl groups and elimination of HF across their 5,6-bonds. Hence, the rate at which I undergoes irreversible degradation would be determined largely by the magnitudes of the rate and equilibrium constants for addition of HSO_3^- or H_2O to I.

Previous studies (11, 12) have indicated that HSO_3^- adds much more rapidly than H_2O to >C=N— and >C=O groups and that the magnitudes of the equilibrium constants for these reactions are much larger for HSO_3^- addition than for H_2O addition. A similar relationship is expected to apply to additions of these species to >C=C< bonds. Hence, it is not surprising that I is irreversibly degraded much more rapidly in solutions that contain sodium bisulfite than it is in water.

Consequently, it is expected that whenever the 5,6-double bond of 5-fluorouracil is saturated, the resultant compound will be unstable with respect to the degradation reactions discussed. The antimetabolic properties of 5-fluorouracil are believed to be due to an inhibition of the enzyme thymidylate synthetase. In a recent study of the binding of 5-fluoro-2-deoxyuridylate to this enzyme in the presence of 5,10-methylenetetrahydrofolate, the formation of a 5,6-substituted-5-fluoro-5,6-dihydrouracil structure in the enzyme–substrate complex was proposed (13). Based on the results of the present study, a binding of this type may also lead to increased rates of degradation of the pyrimidine ring structure or possibly to loss of HF across the 5,6-bond.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 10, 1974, from the *Pharmaceutical Chemistry Department, School of Pharmacy, University of Kansas, Lawrence, KS 66044*

Accepted for publication August 9, 1974.

Supported in part by a National Institutes of Health Grant (5-ROI GM 18348) and facilitated by the award to Ian H. Pitman of a Public Health Service Career Development Award (IK4-GM-70,100).

The authors acknowledge the technical assistance of Miss Debby Scales. They also thank Dr. E. G. Sander of the Biochemistry Department of the University of Florida for communicating the results of kinetic studies of the addition of HSO_3^- to 5-fluorouracil prior to the publication of his manuscript.

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