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The Addition of Bisulfite to 5-Fluorouracil. Evidence for a Change in Rate Determining Step[1]

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The kinetics of bisulfite addition to 5-fluorouracil were studied as a function of increasing concentrations of potential general acids. Values of $k_{obsd}/[SO_3^{=}]$ measured at 25°C and ionic strength 1.0 *M* increased linearly and then became invariant with increasing concentrations of either HSO_3^- or $(OHCH_2CH_2)_2N^+C(CH_2OH)_3$ HCl (BisTris$^+$HCl). A small kinetic hydrogen–deuterium isotope effect ($k_{HS}/k_{DS} = 1.10$) was observed for the general acid catalysed portion of the addition reaction. The kinetics of bisulfite elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate were studied in ethanolamine buffers. As previously observed with 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate, this reaction is subject to general base catalysis and exhibits a large kinetic hydrogen–deuterium isotope effect ($k_2^{H_2O}/k_2^{D_2O} = 3.8$). The kinetic results for the addition reaction are consistent with a multistep reaction pathway involving the initial formation of an oxyanion sulfite addition intermediate (II) which subsequently adds a proton and undergoes tautomerization to yield the final 5-fluoro-5,6-dihydrouracil-6-sulfonate product. Thus the elimination of bisulfite from 5-fluoro-5,6-dihydrouracil-6-sulfonate probably proceeds by an ElcB mechanism which involves, at relatively low concentrations of general base, rate determining general base catalyzed proton abstraction from carbon 5 to yield intermediate II followed by the rapid elimination of sulfite to yield 5-fluorouracil. These results may be related to both the enzymatically catalyzed dehalogenation of bromo- and iodouracil and the methylation of deoxyuridylate by thymidylate synthetase.

The addition of sulfur nucleophiles such as cysteine, 2-mercaptoethanol and bisulfite are important, biologically related reactions since they are thought to be involved in the thymidylate synthetase catalyzed methylation of deoxyuridylate by 5,10-methylene-tetrahydrofolate (*1–3*) and may be involved in the *in vivo* dehalogenation of 5-bromo- and 5-iodouracil (*4*). In a previous communication from this laboratory, we reported that bisulfite was eliminated from 1,3-dimethyl-5,6-dihyrouracil-6-sulfonate via a general base catalyzed reaction (*5*). Two mechanisms were considered for this process. The first was a concerted E2 elimination in which proton abstraction from carbon was concomitant with the elimination of sulfite while the other was a ElcB elimination which involved rate determining formation of a carbanion followed by rapid elimination of sulfite. Because of the previously observed stereo-selectivity of bisulfite addition across

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the 5,6 double bond of uracil (*6*, *7*), we favored the concerted E2 mechanism as being the most likely mechanism for the general base catalyzed elimination of sulfite from 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate. The corollary of this result would require that sulfite adds to carbon 6 of uracil via a concerted general acid catalyzed reaction with proton donation to carbon 5. The objective of this report is to show that bisulfite addition to 5-fluorouracil is a multistep process and hence proton donation from potential general acids is not concerted with the addition of sulfite to carbon 6 of the pyrimidine ring system.

EXPERIMENTAL SECTION

Materials. Reagent grade inorganic salts were used as received. Deionized water was glass-distilled prior to use. 5-Fluorouracil and Bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl)methane (Bis-Tris) from Hoffman-LaRoche and Nutritional Biochemicals, respectively, were used without further purification. Ethanolamine hydrochloride (Eastman) was recrystallized two times from ethanol and imidazole (Sigma) was recrystallized three times from benzene. Deuterium oxide (99.7%, Matheson, Coleman, Bell) was glass-distilled before use. 5-Fluoro-5,6-dihydrouracil-6-sulfonate was prepared by dissolution of 5-fluorouracil and sodium bisulfite in water at final concentrations of 3.0×10^{-2} *M* and 0.50 *M*, respectively. The reaction was allowed to proceed for 30 hr to insure complete reaction. Aliquots were then stored frozen under nitrogen until used (*5*). 5-Fluoro-5,6-dihydrouracil-6-sulfonate-5-*d* was prepared in an analogous fashion to the hydrogen compound by dissolving the solid reagents in deuterium oxide to the same final concentration. Argon was saturated with water prior to use.

Kinetic measurements. The rates of bisulfite addition to the 5,6 double bond of 5-fluorouracil were monitored spectrophotometrically by following the decrease in absorbance at 280 nm, corresponding to disappearance of 5-fluorouracil. Reaction mixtures containing all components except 5-fluorouracil were prepared in stoppered argon flushed 3-ml cuvettes. After equilibration at 25°C, reactions were initiated by the rapid addition of 0.10 ml of 5.0×10^{-3} *M* 5-flourouracil. Ionic strength was maintained at 1.00 *M* by addition of NaCl. Absorbance measurements were made on a Zeiss PMQII spectrophotometer equipped with a cell holder thermostatted at 25°C.

The rates of bisulfite elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate were measured in ethanolamine buffers (25°C, $\mu = 1.0$ *M*) by following the increase in 5-fluorouracil absorbance at 275 nm which occurs when concentrated stock solutions of the 5-fluorouracil-bisulfite adduct are 300-fold diluted into previously equilibrated buffer solutions (*5*). As in the case of the addition reaction, absorbance measurements were made using a Zeiss PMQII spectrophotometer equipped with cell holders thermostatted at 25°C.

Following the completion of each kinetic run, the pH of the reaction mixtures was determined using a Radiometer PHM-26 pH meter equipped with a Radiometer GK 2321C combination electrode.

Pseudo first-order rate constants were determined from linear, semilogarithmic plots of extent reaction against time using the relationship $k_{obsd} = 0.693/t_{1/2}$.

The rates of both the addition of bisulfite to 5-fluorouracil and the elimination of bisulfite from 5-fluoro-5,6-dihydrouracil-6-sulfonate were measured at ionic strength 1.0 *M* and 25°C using deuterium oxide as solvent. Reaction conditions were identical with those used when water was the solvent. Values of *pD* were calculated using pH values determined as previously described and the relationship $pD = \text{pH} + 0.40$ (*8*).

RESULTS

Both the addition of bisulfite to 5-fluorouracil and its elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate followed strict first-order kinetics in all reactions examined. Semilogarithmic plots of extent reaction versus time were linear for at least three and in most cases five half-lives.

Bisulfite addition to 5-fluorouracil. The kinetics of bisulfite addition to 5-fluorouracil were evaluated by measuring pseudo first-order rate constants (k_{obsd}) as a function of increasing bisulfite buffer concentration using various sulfite–bisulfite buffer ratios. Plots of k_{obsd} against increasing bisulfite buffer were not linear (Fig. 1). At the lower

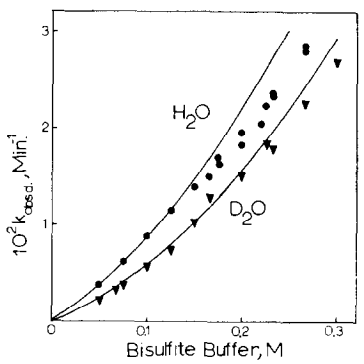


FIG. 1. Nonlinear dependence of the pseudo first-order rate constants for the addition of bisulfite to 5-fluorouracil on increasing concentrations of bisulfite buffer (40% bisulfite), ionic strength 1.0 *M*, 25°C. The curves were calculated with the rate constants described in the text using Eq. 1.

concentrations of total buffer, the values of k_{obsd} increased with a greater than first power dependence on total bisulfite while at the higher concentrations of bisulfite buffer, the relationship between k_{obsd} and total buffer concentration approached a first-order dependence.

To help elucidate the nature of the reaction and to evaluate the apparent third-order rate constants (k_{HS}) for bisulfite acting as a potential general acid catalyst, plots of $k_{obsd}/[SO_3^=]$ against $[HSO_3^-]$ were constructed using data obtained for a series of sulfite–bisulfite buffer ratios. Figure 2 shows a representative series obtained in buffers which were 40% bisulfite. The slope and intercept of the ascending limb of this plot represents k_{HS} and k_o, the apparent third-order rate constant for bisulfite catalysis of sulfite addition to 5-fluorouracil and the apparent second-order rate constant for sulfite addition to 5-fluorouracil in the absence of bisulfite, respectively. This latter rate constant (k_o) contains potential terms for catalysis by hydronium ion and water. At the higher buffer

concentrations, the reaction appears to become independent of the concentration of bisulfite as indicated by the horizontal area of Fig. 2. Consequently, in these higher regions of bisulfite buffer concentration, the bisulfite independent second-order rate constant (k_S) for sulfite reacting with 5-fluorouracil was simply evaluated from the slopes of linear plots of k_{obsd} versus $[SO_3^=]$. Values of k_{HS}, k_o, and k_S obtained in this manner were 0.93 M^{-2} min^{-1}, 0.11 M^{-1} min^{-1} and 0.17 M^{-1} min^{-1}, respectively.

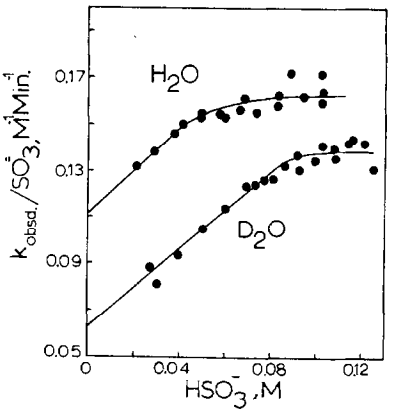


FIG. 2. Dependence of the apparent second-order rate constants for sulfite attack on 5-fluorouracil on the concentration of bisulfite at 25°C, ionic strength 1.0 *M*. Bisulfite buffers were 40% bisulfite.

To further evaluate the behavior of the addition of sulfite ion to 5-fluorouracil, we conducted kinetic studies at constant total bisulfite concentration using Bis–Tris as a variable external buffer system. Figure 3 shows the result of plotting $k_{obsd}/[SO_3^=]$ as a

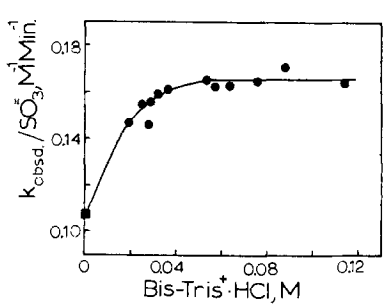


FIG. 3. Dependence of the apparent second-order rate constants for sulfite attack on 5-fluorouracil on the concentration of Bis–Tris$^+$HCl, pH 6.91, 25°C, ionic strength 1.0 *M*. Bisulfite was included in all reaction mixtures at a final concentration of 0.050 *M*. The point (■) at [Bis–Tris$^+$ HCl] = 0 was determined in 0.050 *M* bisulfite buffer, pH 6.91.

function of increasing Bis–Tris$^+$HCl at pH 6.91. Although it was not experimentally possible to obtain reliable rate constants below about 0.02 *M* Bis–Tris$^+$HCl, the behavior of the reaction as a function of increasing potential general acid appears to be the same as is the case with bisulfite. At lower concentrations, the rate constants appear to depend on Bis–Tris$^+$HCl concentration, but at the higher concentrations this dependence becomes nonexistent.

To gain some insight concerning the mode of proton transfer in the bisulfite catalyzed part of the overall reaction, pseudo first-order rate constants were measured as a function of increasing bisulfite buffer using deuterium oxide as solvent. Figure 2 shows the results of these experiments in buffer systems containing 40% bisulfite. The slope of the ascending limb of this plot yields an apparent third-order rate constant in D_2O (k_{DS}) equal to 0.84 M^{-2} min^{-1}. Thus, the ratio $k_{HS}/k_{DS} = 1.10$. This small isotope effect indicates that in the general acid catalyzed portion of the overall addition reaction proton transfer is predominantly to an electronegative atom such as the oxygen on carbon 4 rather than to the carbon atom at position 5 of the pyrimidine ring system.

Bisulfite elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate. In an attempt to correlate the kinetic data for the addition of bisulfite to 5-fluorouracil with the previously proposed mechanisms for bisulfite elimination from 1,3-dimethyl-5,6-dihydro-

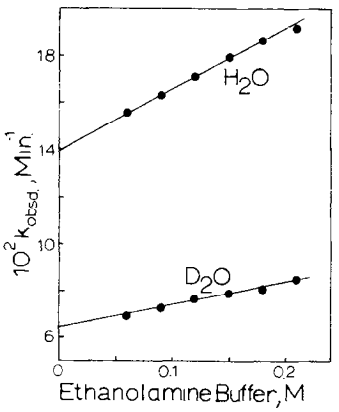


FIG. 4. Observed rate constants in both H_2O and D_2O for bisulfite elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate in ethanolamine buffers. Fraction ethanolamine free base equal to 0.60, 25°C, ionic strength = 1.0 *M*.

uracil-6-sulfonate (*5*), pseudo first-order rate constants for this reaction were measured by 300-fold dilution of concentrated solutions of 5-fluoro-5,6-dihydrouracil-6-sulfonate into increasing concentrations of ethanolamine buffer (pH 10.0). Figure 4 shows the results of these studies using both water and deuterium oxide as solvents. As was the case of the 1,3-dimethyluracil adduct (*5*), bisulfite elimination from 5-fluoro-5,6-dihydrouracil-6-sulfonate is subject to ethanolamine buffer catalysis. Furthermore, the magnitude of the ratio of the second-order rate constants ($k_2^{H_2O}/k_2^{D_2O} = 3.8$) for ethanolamine buffer catalysis, measured in either water or deuterium oxide and determined from the slopes of the data shown in Fig. 4, indicate that proton abstraction is from carbon rather than a more electronegative atom such as oxygen. Thus, in the bisulfite elimination reaction, the site of proton transfer in the rate determining step is likely different than that of the addition reaction.

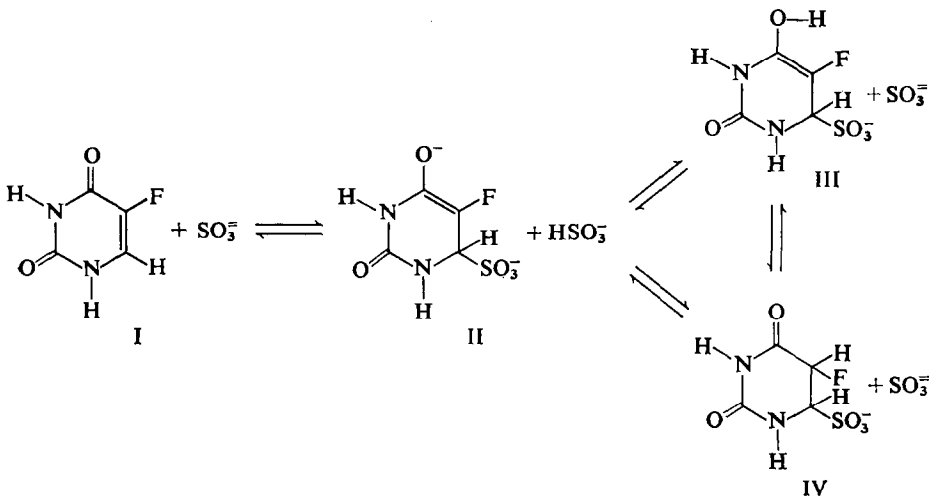
DISCUSSION

The kinetic data for the addition of bisulfite to 5-fluorouracil indicate that the rate of the reaction changes from a first-order to a zero-order dependence with increasing

concentration of potential general acid (i.e., either bisulfite or Bis–Tris$^+$HCl). This phenomenon is shown in Figs. 2 and 3 and can be further substantiated by the fact that the curve shown in Fig. 1, which gives an excellent fit to the experimental data at low but not at high concentrations of bisulfite buffer, can be calculated using Eq. (1). In this equation, k_{HS}, k_o, α, $1 - \alpha$ and Bis_{tot} represent the

$$k_{obsd} = k_{HS}\alpha(1 - \alpha)[\text{Bis}_{\text{tot}}]^2 + k_o(1 - \alpha)[\text{Bis}_{\text{tot}}] \quad (1)$$

previously described rate constants evaluated from data such as that shown in Fig. 2, the fraction bisulfite, the fraction sulfite, and the total concentration of bisulfite buffer, respectively. This change in the order of the reaction with respect to increasing concentrations of either bisulfite or Bis–Tris$^+$HCl cannot be explained by the ionization of a proton on the pyrimidine substrate, since pH was held constant and hence indicates a multistep reaction pathway in which the rate-determining step of the reaction changes with increasing concentration of potential general acid. Such evidence has been previously interpreted to implicate the formation of tetrahedral addition intermediates between acyl compounds and nucleophilic reagents which then react further to yield products (*9–11*). An analogous pathway for the addition of bisulfite to 5-fluorouracil is shown in Scheme 1. In this pathway, the second step of the reaction (II → III), which represents proton transfer from bisulfite to an oxyanion sulfite addition intermediate, would appear to be rate determining at the lower concentrations of general acid. The idea that III rather than IV is the initial product of this step is supported by the fact that a small kinetic hydrogen–deuterium isotope effect ($k_{HS}/k_{DS} = 1.10$) is observed for the general acid catalyzed portion of the addition reaction. Thus, the initial site of proton transfer is most likely the oxygen on carbon 4 rather than carbon 5 of the ring

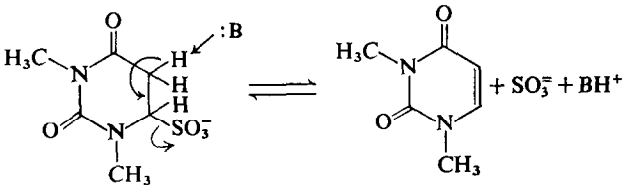


SCHEME 1

system. To account for the final 5-fluoro-5,6-dihydrouracil-6-sulfonate product (IV) of the overall reaction (*12*), a tautomerization (III ⇄ IV) involving proton transfer

from the oxygen atom to carbon 5 would be required to yield the thermodynamically stable product.

The kinetics of the general base catalyzed elimination of bisulfite from 5-fluoro-5,6-dihydrouracil-6-sulfonate (IV) are similar to those for 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate (*5*). Both reactions are subject to general base catalysis by ethanolamine and both exhibit relatively large kinetic hydrogen–deuterium isotope effects ($k_2^{H_2O}/k_2^{D_2O} = 3.8 - 4.10$) which indicate that the site of proton abstraction is most likely carbon 5 rather than the more electronegative oxygen atom. Previously (*5*), we had interpreted similar and more extensive data for elimination of bisulfite from 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate as being consistent with either a concerted E2 elimination (Eq. 2) in which proton transfer from carbon 5 is concomitant with sulfite elimination, or with an ElcB mechanism (Eq. 3) which involves rate determining carbanion formation followed by a rapid elimination of sulfite. Based on the fact that the overall addition and elimination of a proton and sulfite across the 5,6 double bond of



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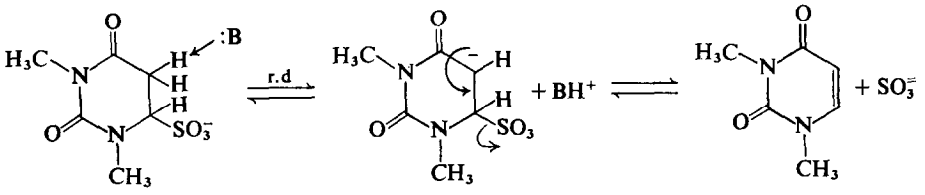

(3)

both uracil (*6*, *7*) and 5-fluorouracil (*12*) appears to be a stereoselective process, we favored but could not prove that the concerted E2 process was the most likely mechanism for the elimination reaction. This hypothesis then requires that sulfite addition to the uracil ring system proceeds via a concerted general acid catalyzed mechanism, a conclusion which is not warranted at least in the case of 5-fluorouracil because of the multistep nature of the reaction pathway and the small kinetic hydrogen–deuterium isotope effect. Consequently, we are left with the conclusion that the most likely mechanism for the reversible addition of bisulfite to 5-fluorouracil involves the formation of an intermediate oxyanion (II) followed by general acid catalyzed proton transfer to the oxygen on carbon 4 (III) with subsequent tautomerization to yield the final thermodynamically most stable product. The reverse reaction, the elimination of $SO_3^=$ from 5-fluoro-5,6-dihydrouracil-6-sulfonate, would then involve rate determining general base catalyzed proton abstraction from carbon 5 to yield the anion intermediate (II)

directly, followed by rapid elimination of sulfite to give 5-fluorouracil.[4] Other recent studies from this laboratory (*4*), involving the dehalogenation of 5-iodouracil by thiol compounds, such as cysteine and 2-mercaptoethanol, would support these conclusions. In the case of 2-mercaptoethanol reacting with 5-iodouracil, kinetic data indicate that the general acid catalyzed addition of the thiol anion to the pyrimidine ring system rather than the dehalogenation of the intermediate 6-thiol dihydropyrimidine is rate determining. This general acid catalyzed thiol addition reaction also exhibits a small kinetic hydrogen–deuterium isotope effect ($k_2^{H_2O}/k_2^{D_2O} = 1.13$); thus, as in the case of bisulfite addition to 5-fluorouracil, proton donation is probably not directly to carbon 5 of the pyrimidine ring system.

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[4] G. S. Rork and I. H. Pitman, Pharmaceutical Chemistry Department, University of Kansas, studying the elimination of bisulfite from both 5,6-dihydrouracil-6-sulfonate and 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate in morpholine buffers up to 1.0 *M* total morpholine buffer concentration, have observed a nonlinear relationship between k_{obsd} and morpholine buffer concentration. Their evidence supports an ElcB mechanism for bisulfite elimination from these compounds. We wish to thank Dr. Pitman for communicating these results to us prior to publication.