General Acid–Base Catalysis of the Reversible Addition of Bisulfite to 1,3-Dimethyluracil[1,2]

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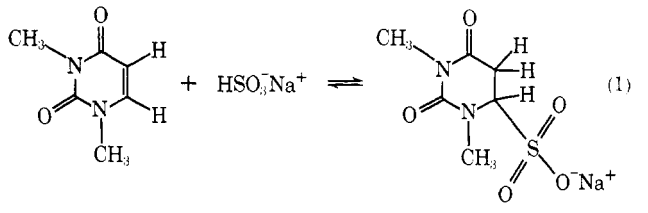
Abstract: The rates of the general base catalyzed desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate have been spectrophotometrically measured in a series of amine buffer systems at 25° and ionic strength 1.0 *M*. The second-order rate constants for general base catalysis by these amines follow the Brønsted relationship with $\beta = 0.87$; however, the value for hydroxide ion catalysis is about 200-fold less than would be predicted from the amine data. The second-order rate constant for ethanolamine catalysis of the desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate-*5-d* was measured in both deuterium oxide and water. In both solvents, the ratio of k_2^H/k_2^D was 4.1. The fact that a large deuterium isotope effect is observed when water is used as the solvent suggests that the mechanism for the desulfonation reaction involves rate-determining general base catalysis of proton transfer from C-5 of the dihydropyrimidine ring concomitant with expulsion of sulfite ion from C-6. The pH-independent equilibrium constant (K_{eq} = [1,3-dimethyl-5,6-dihydrouracil-6-sulfonate]/[1,3-dimethyluracil][bisulfite]$_{total}$) for bisulfite addition to the 6 position of 1,3-dimethyluracil is 45 ± 9.0 M^{-1} when measured in 30 and 70% neutralized potassium phosphate buffers at 25° and ionic strength 1.0 *M*. The analogies between this reaction and enzymatically catalyzed reactions which alkylate C-5 of pyrimidines are discussed.

Recently, several studies have shown that bisulfite adds reversibly to the 6 position of several physiologically important pyrimidines to yield the corresponding 5,6-dihydro-6-sulfonate derivatives.[4-7] Nucleophilic reactions of this type are important in biological systems since they alter the structure of nucleic acids and have been implicated as being mechanistically important in enzymatically catalyzed reactions which alkylate C-5 of several pyrimidine bases.[8,9] Because of our general interest both in the chemistry of dihydropyrimidines and in chemical systems which are models for enzymatically catalyzed reactions, we have kinetically examined the reaction of 1,3-dimethyluracil with sodium bisulfite (eq 1) to see whether or not the



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catalytic behavior of this system can be used to explain modes of catalysis by pyrimidine alkylating enzymes such as thymidylate synthetase, cytosine hydroxymethylase, and certain of the RNA methylases.

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(2) Submitted by R. W. E. to the faculty of the Department of Chemistry, University of Florida, in partial fulfillment of the requirements for the Bachelor of Science degree with Highest Honors.

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

(5) H. Hayatsu, Y. Wataya, and K. Kai, *ibid.*, **92**, 724 (1970).

(6) H. Hayatsu, Y. Wataya, K. Kai, and S. Iida, *Biochemistry*, **9**, 2858 (1970).

(7) H. Hayatsu and M. Inoue, *J. Amer. Chem. Soc.*, **93**, 2301 (1971).

(8) D. V. Santi and C. F. Brewer, *ibid.*, **90**, 6236 (1968).

(9) (a) T. I. Kalman, Abstracts, 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970, No. BIOL 59; (b) T. I. Kalman, *Biochemistry*, **10**, 2567 (1971).

Experimental Section

Materials. Reagent grade inorganic salts were used without further purification. Deionized carbon dioxide free water was used in all experiments. 1,3-Dimethyluracil and tris(hydroxymethyl)aminomethane (Tris ultra pure) were from Mann Research Laboratories and were used without further purification. Piperidine, glycine, ethanolamine, *n*-butylamine, and morpholine were from Eastman Organic Chemicals. These compounds were further purified either by distillation or recrystallization from ethanol as the hydrochlorides. Deuterium oxide (99.7%) was obtained from New England Nuclear Corporation and was glass distilled before use.

1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate was prepared under nitrogen by dissolving 1,3-dimethyluracil and sodium bisulfite in water to final concentrations of 3.03×10^{-2} and 0.500 *M*, respectively. Bisulfite addition was allowed to continue (about 25 hr) until ultraviolet absorption spectra indicated that all of the 1,3-dimethyluracil was converted to the corresponding 6-sulfonate. Such solutions were divided into aliquots, reflushed with nitrogen, sealed, and stored at −17° until used.

1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate-*5-d* was prepared in an analogous manner to the compound with a hydrogen atom at C-5 by dissolving both 1,3-dimethyluracil and sodium bisulfite in deuterium oxide to the same final concentrations. Previous work in deuterium oxide by Shapiro, *et al.*,[4] and Hayatsu, *et al.*,[6] indicated that nucleophilic addition of bisulfite to the 6 position of uracil causes stereospecific addition of a deuterium atom to C-5. More recently, Kai, *et al.*,[10] have used this method to both deuterate and tritiate C-5 of cytidine and cytidine 5′-phosphate.

Spectra. Ultraviolet absorption spectra of 1,3-dimethyluracil, 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate, and the desulfonation reaction products were obtained at room temperature with a Cary-14 recording spectrophotometer.

Kinetic Measurements. The rates of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate desulfonation were followed by measuring the increase in 1,3-dimethyluracil absorbance at 270 mμ after dilution (300-fold) of concentrated sulfonate solutions (see Materials) into buffer solutions. The reaction mixtures containing all components except the sulfonate were prepared in capped 3-ml cuvettes and equilibrated at 25° followed by initiation with 0.01 ml of the concentrated sulfonate solution. The ionic strength was maintained at 1.0 *M* by the addition of KCl unless otherwise noted. Absorbance measurements were made on either a Zeiss PMQII or a Gilford 2000 spectrophotometer, both of which were equipped with cell holders thermostated at 25°. Pseudo-first-order rate constants (k_{obsd}) were determined from linear semilogarithmic plots of the extent of

(10) K. Kai, Y. Wataya, and H. Hayatsu, *J. Amer. Chem. Soc.*, **93**, 2089 (1971).

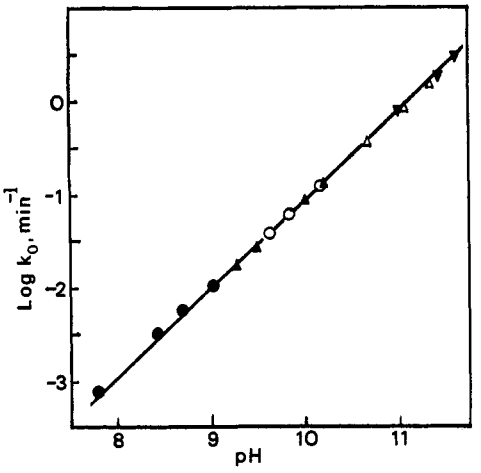


Figure 1. The dependence on pH of the rates of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate desulfonation at 25° and ionic strength 1.0 *M*. Values of k_0 were obtained from the intercepts of plots of k_{obsd} against buffer concentration. Buffers used were: ●, tris(hydroxymethyl)aminomethane; ○, glycine; ▲, ethanolamine; △, *n*-butylamine; and ▼, piperidine.

reaction, $A_\infty - A_t$, against time and the relationship $k_{obsd} = 0.693/t_{1/2}$. Values of k_{obsd} obtained in this manner were generally reproducible to within ±5%.

Measurements of pH were made immediately, following each kinetic run using a Radiometer PHM-26 pH meter equipped with a Sargent S-30070-10 combination electrode. Hydroxide ion concentration was calculated from the observed pH, $K_W = 10^{-14}$, and a hydroxide ion activity coefficient of 0.67.[11]

Product Analyses. The reaction products and completeness of the desulfonation reaction were determined by diluting a sulfonate solution which initially contained 3.03×10^{-2} *M* 1,3-dimethyluracil plus 0.500 *M* sodium bisulfite 300-fold in 0.10 *M* potassium hydroxide, and measuring its absorption spectrum using 0.500 *M* sodium bisulfite diluted to the same extent in 0.10 *M* potassium hydroxide as a blank. The magnitude of the absorbance at 270 mμ of this difference spectrum was equal to 95% of the 270-mμ absorbance obtained from a spectrum of authentic 1.01×10^{-4} *M* 1,3-dimethyluracil measured under the same conditions. Exactly the same results were obtained when these experiments were conducted in 0.16 *M n*-butylamine buffer (65% amine).

Equilibrium Constant. The observed equilibrium constant for the addition of sodium bisulfite to 1,3-dimethyluracil was spectrophotometrically measured in 0.30 *M* potassium phosphate buffers (30 and 70% K_2HPO_4) at 25° and ionic strength 1.0 *M* using essentially the same methods as used by Jencks and associates to measure the equilibrium constants for hemiacetal formation from carbonyl compounds and nucleophiles.[12] Because it was necessary in certain cases to wait 7 days to ensure that equilibrium was reached, the tubes containing the reaction mixtures were carefully flushed with nitrogen and capped to prevent evaporation. To prevent heavy metal catalyzed oxidation reactions, each reaction mixture was made 1×10^{-4} *M* in ethylenediaminetetraacetic acid.

Kinetic Measurements in Deuterium Oxide. The rates of the ethanolamine-catalyzed desulfonation reaction were measured in deuterium oxide at 25° and ionic strength 1.0 *M*. Ethanolamine–hydrochloride was twice dissolved in deuterium oxide and taken to dryness under vacuum. All solid reagents were prepared in freshly distilled deuterium oxide. 1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate-*5-d* was prepared as described in Materials. Values of pD were calculated using pH values determined as previously described and the relationship pD = pH + 0.40.[13]

Results

The desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate was found to follow strict first-order kinetics in every reaction examined. Semilogarithmic plots of $A_\infty - A$ against time were linear for at least 3 and in most cases 5 half-lives.

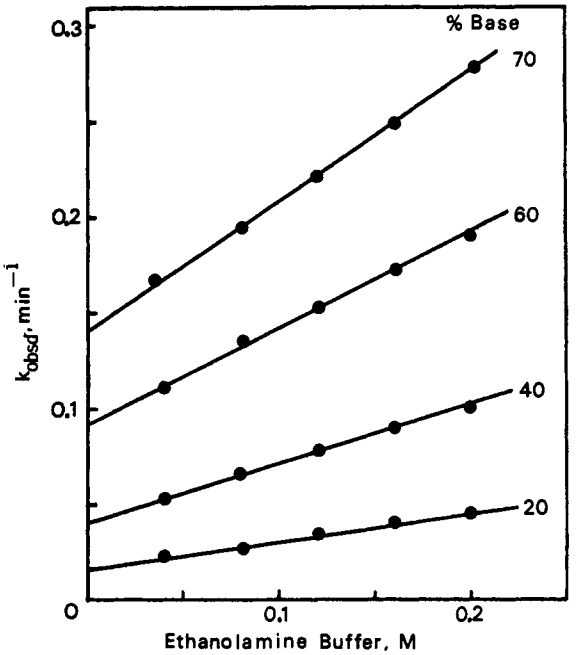


Figure 2. The rates of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate desulfonation in ethanolamine buffers at 25°, ionic strength 1.0 *M*.

Catalysis by Hydrogen Ion, Hydroxide Ion, and Water. The pH dependencies of the observed first-order rate constants in the absence of potential general acids and bases (k_0) are shown in Figure 1. Values of k_0 were determined from the intercepts of plots of k_{obsd} against total buffer concentration (Figure 2). The pH–rate profile shows that in the pH region examined the reactions are catalyzed by hydroxide ion and that catalysis by hydrogen ion and water does not contribute to the rate of the reaction. Using these data a second-order rate constant based on hydroxide ion activity was determined to be $k_2' = 880\ M^{-1}\ min^{-1}$. The solid line in Figure 1 which gives a satisfactory fit with the experimental points was calculated using this rate constant assuming that the contributions to catalysis by both hydrogen ion and water were either negligible or equal to zero.

Catalysis by General Acids and Bases. The effectiveness of amines and their conjugate acids as catalysts for the desulfonation of 1,3-dimethyl 5,6-dihydrouracil-6-sulfonate was measured by obtaining values of k_{obsd} in increasing buffer concentrations at several different buffer ratios. Figure 2 shows the results of a typical experiment with ethanolamine buffers. The slopes of the lines in Figure 2 were used to determine the apparent second-order rate constants (k_{app}) for buffer catalysis. Table I shows a compilation of the values of k_{app} and the various buffer systems used. The second-order rate constants for the basic species of the buffer (k_2) were determined either from the intercepts of plots of k_{app} against the percentage of free amine in the various buffers tested or more simply by dividing k_{app} by the fraction of free amine in the buffer. In all cases, no catalysis could be detected by the acidic component of the buffer since all intercepts at 0% amine were equal to zero. Table II shows the values of k_2 for catalysis by the six amines tested.

(11) J. F. Kirsch and W. P. Jencks, *J. Amer. Chem. Soc.*, **86**, 833 (1964).
(12) (a) E. G. Sander and W. P. Jencks, *ibid.*, **90**, 6154 (1968); (b) B. M. Anderson and W. P. Jencks, *ibid.*, **82**, 1773 (1960).
(13) P. K. Glascoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

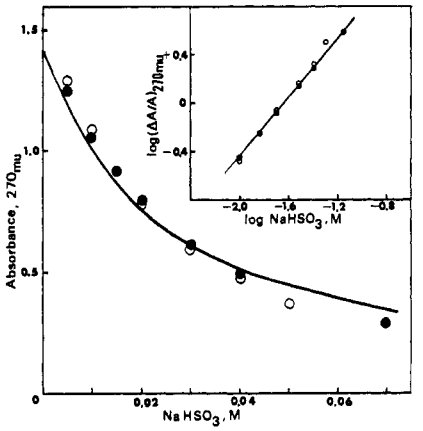


Figure 3. The decrease in absorbance at 270 mμ resulting from the addition of sodium bisulfite to 1,3-dimethyluracil at 25° in 0.30 *M* potassium phosphate buffers (○, 30% K_2HPO; ●, 70% K_2HPO_4), ionic strength 1.0 *M*. The insert shows a logarithmic plot of $(\Delta A/A)_{270\ m\mu}$ against total sodium bisulfite concentration.

Equilibrium Measurements. The equilibrium constant for the addition of sodium bisulfite to 1,3-dimethyluracil (eq 1) was determined at 25° and ionic strength 1.0 *M* by measuring the absorbance decrease at 270 mμ which occurred when 1.70×10^{-4} *M* 1,3-dimethyluracil was allowed to react to equilibrium with increasing concentrations of sodium bisulfite in 0.30 *M* potassium phosphate buffers (30 and 70% K_2HPO_4). The insert in Figure 3 shows the data plotted using eq 2 in which *A* and Δ*A* are proportional to the equilibrium concentrations of 1,3-dimethyluracil and its 6-sulfonate, respectively. From this logarithmic plot, the apparent equilibrium constant, $K_{eq} = 45\ M^{-1}$, was determined from the relationship $\log K_{eq} = -\log [NaHSO_3]_{total}$ at $\log (\Delta A/A)_{270\ m\mu} = 0$. The solid line in Figure 3 was calculated using this value and the equation $K_{eq} = (\Delta A/A)_{270\ m\mu}(1/[NaHSO_3]_{total})$. The data do not fit this calculated curve with the precision achieved with nucleophiles and carbonyl compounds.[12] Consequently, $K_{eq} = 45\ M^{-1}$ is probably accurate to within ±20%. The reason for this lack of precision is most probably due to side reactions of the bisulfite during the relatively long time required to reach equilibrium.

$$\log (\Delta A/A)_{270\ m\mu} = \log K_{eq} + \log [NaHSO_3]_{total} \quad (2)$$

Table I. Values of k_{app} for the Buffer-Catalyzed Desulfonation of 1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate at 25° and Ionic Strength 1.0 *M*

Base	% base	Concn range, *M*	k_{app},[a] M^{-1} min^{-1}
Tris(hydroxymethyl)-aminomethane	20	0.100–0.350	0.0102
	50	0.100–0.350	0.0230
	65	0.100–0.350	0.0300
Morpholine	26.5	0.113–0.339	0.036
	55.6	0.113–0.339	0.091
	64.6	0.113–0.339	0.101
Glycine	40	0.100–0.300	0.210
	50	0.100–0.300	0.260
	70	0.100–0.300	0.400
Ethanolamine	20	0.040–0.240	0.160
	30	0.080–0.160	0.230
	40	0.040–0.240	0.320
	60	0.040–0.240	0.490
	70	0.040–0.240	0.640
n-Butylamine	33.2	0.097–0.339	1.85
	50.5	0.097–0.339	2.90
	64.5	0.097–0.339	3.50
Piperidine	20	0.025–0.100	8.7
	40	0.050–0.125	18.1
	50	0.050–0.125	20.8

[a] Determined from the slopes of plots of k_{obsd} against buffer concentration (Figure 2). Values of k_{obsd} which were used to obtain k_{app} were reproducible to ±5.0%.

Table II. Second-Order Rate Constants for the General Acid–Base Catalyzed Desulfonation of 1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate at 25° and Ionic Strength 1.0 *M*

Base	p[a]	q[a]	pK_a[b]	k_2,[c] M^{-1} min^{-1}
Tris(hydroxymethyl)-aminomethane	3	1	8.42	0.052
Morpholine	2	1	8.89	0.153
Glycine	3	1	9.82	0.538
Ethanolamine	3	1	9.84	0.820
n-Butylamine	3	1	11.06	5.57
Piperidine	2	1	11.56	43.5
Hydroxide Ion	2	1	15.74	590[d]

[a] Statistical corrections (ref 14). [b] Apparent pK_a values of the conjugate acids were determined at ionic strength 1.0 *M* and 25° from either the pH of half-neutralized solutions or the degree of neutralization and the pH. [c] Second-order rate constants for general base catalysis were obtained either by dividing the apparent second-order rate constants for buffer catalysis (k_{app}) by the fraction of base in each buffer or from the intercepts of plots k_{app} against per cent base in each buffer. Values of k_{obsd} from which k_{app} and k_2 were derived were reproducible to ±5.0%. [d] For hydroxide ion concentration, based on $K_w = 1 \times 10^{-14}$, the measured pH and an activity coefficient of 0.67 for hydroxide ion.[11]

Solvent Effects. The effects of either increasing or decreasing solvent polarity on the desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate are shown in Table III. These data indicate that the values of k_{obsd} after correction for pH fluctuations are not dramatically affected by increasing potassium chloride concentration but become increasingly smaller with increasing ethanol concentration. Consequently, the rate increases observed with increasing buffer concentration were not due to solvent effects since ionic strength was

Table III. Effects of Ionic Strength and Ethanol Concentration on the Rate of Desulfonation of 1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate at 25°

Added solvent[a]	Concn, *M*	pH	k_{obsd},[b] min^{-1}
None		10.85	0.19
Ethanol	0.41	10.84	0.20
	1.03	10.81	0.13
	1.44	10.79	0.08
	2.05	10.78	0.04
KCl	0.13	10.86	0.20
	0.33	10.93	0.24
	0.47	10.96	0.24
	0.67	11.01	0.23
	0.80	11.04	0.24

[a] All reaction mixtures contained 0.01 ml of the sulfonate solution and were 0.16 *M* *n*-butylamine buffer, 50% free base. [b] Approximate values since these data were corrected for differences in pH using a plot of log k_0 *vs.* pH (Figure 1).

(14) (a) S. W. Benson, *J. Amer. Chem. Soc.*, **80**, 5151 (1958); (b) D. Bishop and K. L. Laidler, *J. Chem. Phys.*, **42**, 1688 (1965); (c) R. P. Bell and P. G. Evans, *Proc. Roy. Soc. Ser. A*, **291**, 297 (1966).

maintained at 1.0 M and the maximum concentration of buffer employed was 0.350 M or below (Table I).

Deuterium Isotope Effects. The results of measuring in both deuterium oxide and water the second-order rate constants for ethanolamine catalysis of the desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate with either a deuterium or a hydrogen at C-5 are shown in Table IV. These data indicate that when a deuterium is substituted for a hydrogen atom at C-5, there is a large hydrogen–deuterium kinetic isotope effect which is independent of using either water or deuterium oxide as the solvent. No isotope effect is observed when the rates are measured in deuterium oxide using the sulfonate synthesized with a hydrogen atom at position 5. These results support the buffer catalysis data because they indicate there is a loss of zero-point energy due to a deuterium being transferred in the transition state of the rate-determining step of the reaction and hence a mechanism involving general catalysis of proton transfer.

Table IV. Effects of Either Deuterium Oxide or Water on the Second-Order Rate Constants for General Base Catalysis by Ethanolamine on the Desulfonation of 1,3-Dimethyl-5,6-dihydrouracil-6-sulfonate Substituted with Either H or D at C-5 at 25° and Ionic Strength 1.0 M

Solvent	Atom at C-5	k_2,[a] M^{-1} min^{-1}	k_2^H/k_2^D
H_2O	−H	0.82	1.00
	−D	0.20	4.10
D_2O	−H	0.88	0.94
	−D	0.20	4.10

[a] Values of k_{obsd} from which k_2 were derived were reproducible to ±5.0%.

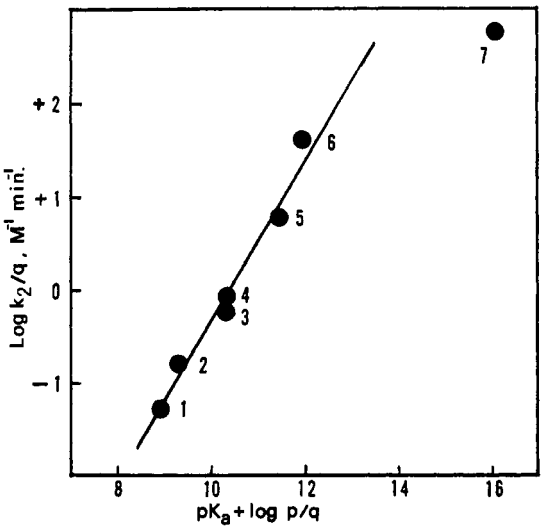
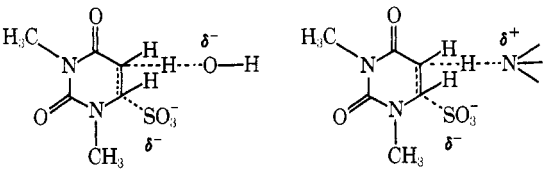


Figure 4. The relationship between the second-order rate constants for general base catalysis of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate desulfonation and the pK_a of the general base, corrected for statistical effects. Data points are numbered as follows: (1) tris(hydroxymethyl)aminomethane; (2) morpholine; (3) glycine; (4) ethanolamine; (5) n-butylamine; (6) piperidine; (7) hydroxide ion.

Discussion

The second-order rate constants for the general base catalyzed desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate follow a Brønsted plot with a slope, β, of 0.87 (Figure 4). This relationship was established with amines; however, the second-order rate constant for hydroxide ion catalysis is about 200-fold less than the value predicted from the amine data. Since β is quite large, indicating a high degree of proton transfer in the transition state, it is probably only because of the lower catalytic reactivity of hydroxide ion that general catalysis by amines can be observed. Analogous results have been observed in the general acid catalyzed formation and breakdown of the hemiacetals of hydrogen peroxide and p-chlorobenzaldehyde in which α is equal to or close to one.[15] In this reaction, general acid catalysis can only be observed because the rate constant for proton catalysis falls below the line established with carboxylic acids. The specific reasons why hydroxide ion is less efficient as a general base catalyst than its basicity relative to the basicity of a series of amines would predict are not easily rationalized; however, lower reactivity of hydroxide ion relative to other bases has been previously observed in other reactions which involve proton abstraction from carbon.[16–18] It is known that general acid and general base catalysts in which the chemical nature of the proton donating or accepting atom are different often do not fit the same Brønsted relationship.[19] Kresge, *et al.*[20] have recently proposed that the Brønsted α (or β) only approximates the degree of proton transfer for a series of general catalysts and that the actual extent of proton donation or removal in the transition state depends upon the chemical nature of the individual catalyst. In this desulfonation reaction, hydroxide ion is the only base catalyst tested which has negative charge. Consequently, proton abstraction by hydroxide ion concomitant with the expulsion of sulfite ion would result in the development of additional negative charge in the transition-state **1** and hence might have less transition-state stability than would be expected in the transition-state **2** of the amine-catalyzed reactions in which there is no development of additional negative charge. An alternative view might be that in the amine-catalyzed reaction there is additional transition-state stability due to the interaction of the developing negative and positive charges.



The data presented for general base catalysis of the desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate are consistent with three kinetically indistinguish-

(15) E. G. Sander and W. P. Jencks, *J. Amer. Chem. Soc.*, **90**, 4377 (1968).

(16) R. P. Bell, "Acid-Base Catalysis," Oxford University Press, London, 1941, p 92.

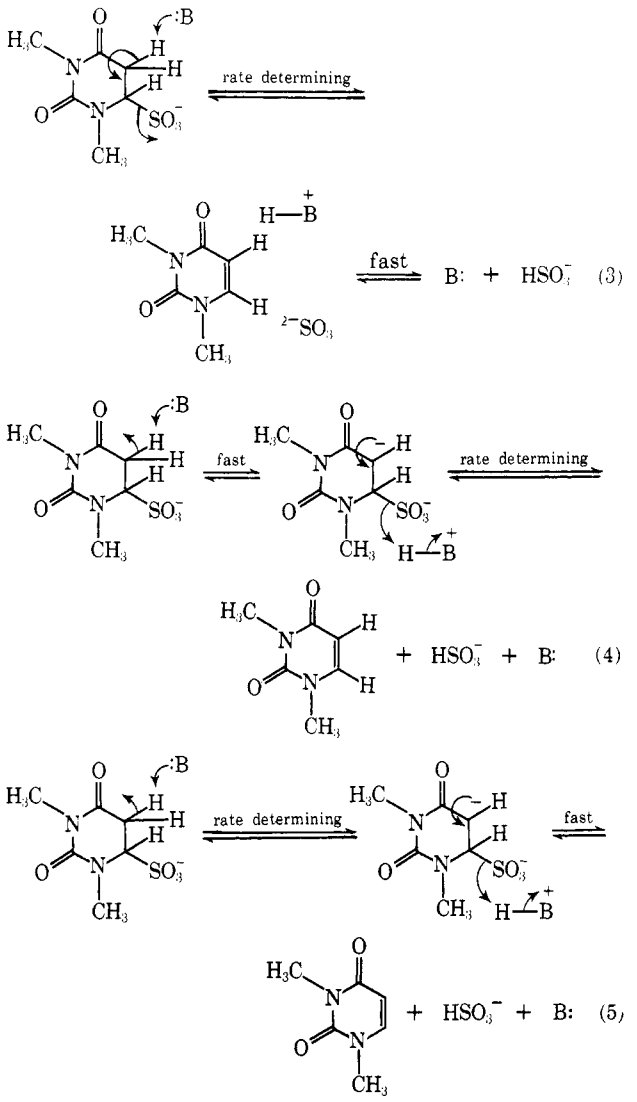
(17) R. P. Bell, D. H. Everett, and H. C. Longuet-Higgins, *Proc. Roy. Soc., Ser. A*, **186**, 443 (1946).

(18) M. L. Bender and A. Williams, *J. Amer. Chem. Soc.*, **88**, 2502 (1966).

(19) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, pp 170–182.

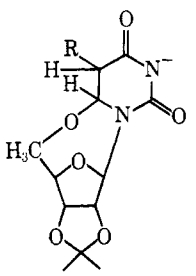
(20) A. J. Kresge, S. Slae, and D. W. Taylor, *J. Amer. Chem. Soc.*, **92**, 6309 (1970).

able mechanisms all of which involve the expulsion of sulfite ion *via* simple carbon–sulfur bond cleavage (eq 3–5).[21] The first of these mechanisms (eq 3) is concerted and represents general base–specific acid catalysis of the desulfonation reaction and according to



the principle of microscopic reversibility specific base–general acid catalysis for the addition of sulfite ion to 1,3-dimethyluracil. This mechanism (eq 3) is favored for the following reasons. (a) The data presented in Table IV indicate that the ethanolamine buffer catalyzed desulfonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulfonate-*5-d* is 4.1-fold slower than the corresponding reaction of the sulfonate with a hydrogen at C-5. This result was obtained when the reaction rates of both compounds were measured either in water or deuterium oxide. Kinetic deuterium isotope effects of similar magnitude have been observed for acetate anion catalysis of the enolization of deuterated ketones, reactions which also involve rate-determining proton abstraction from carbon adjacent to a carbonyl function.[22,23] If the mechanism shown in eq 4 is operative (*i.e.*, rate-determining proton donation from the conjugate acid of the amine to the sulfite ion leaving group) then no deuterium isotope effect should be observed because the deuterium atom on the electronegative nitrogen would rapidly exchange with the solvent water. The fact that no deuterium isotope effect is observed when the rate of desulfonation is measured in deuterium oxide using the sulfonate with a hydrogen at C-5 confirms this conclusion. Thus the mechanism presented in eq 4 can be rejected. (b) The mechanism shown in eq 5 indicates that in both directions the reaction involves either rate-determining proton abstraction or proton donation to a carbanion intermediate *via* general catalysis. If this mechanism were operative, it is doubtful that both the addition and elimination of bisulfite ion to uracil would have the strict trans stereochemistry previously observed both by Shapiro, *et al.*,[4] and Hayatsu, *et al.*[6]

The nucleophilic addition of bisulfite to pyrimidines has been implicated as being mechanistically important in enzymes such as thymidylate synthetase, cytosine hydroxymethylase, and certain of the transfer ribonucleic acid methylases. Santi and Brewer[8] have reported that electrophilic substitution reactions of uracil furanosides analogous to those catalyzed by thymidylate synthetase proceed exclusively by rate-determining nucleophilic attack of an ionized hydroxyl group on the furanoside to yield a cyclic dihydronucleoside intermediate **3**. Kalman,[9] based on the fact that glutathione catalyzes hydrogen–deuterium exchange at C-5 of uridine, has proposed that thiol-containing



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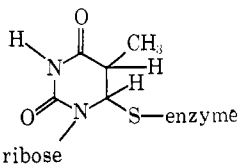

4

enzymes which catalyze electrophilic substitution reactions of *S*-adenosylmethionine with pyrimidine bases do so *via* nucleophilic attack by the thiol on C-6 of the pyrimidine ring. This proposed enzymatic mechanism involves the formation of a covalently bonded dihydropyrimidine–enzyme intermediate **4** which must react further to expel the enzyme leaving group to yield the methylated nucleotide product. Similar data on the influence of thiols on hydrogen–deuterium exchange at C-5 of uridine have also been reported by Heller.[24]

If enzymes such as thymidylate synthetase have catalytic mechanisms involving the formation of intermediates such as **4**, then the study of reversible nucleophilic reactions such as the addition of bisulfite to 1,3-dimethyluracil might help to understand the more complex enzymatic reactions. Our data indicate that general acid catalyzed proton donation to C-5 con-

(21) Another mechanism suggested by a referee which cannot be completely eliminated by these data involves rate-determining proton abstraction from C-5 to yield a bicyclic intermediate which has sulfur bridging C-5 and -6 of the dihydropyrimidine ring system. This mechanism is less favored than direct expulsion of sulfite ion concomitant with proton abstraction (eq 3) because of the specific stereochemistry observed for the overall reaction[4,6] and the fact that reversible formation of such an intermediate would be unfavorable due to the amount of strain involved in the formation of the three-membered ring.

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(23) F. A. Long and D. Watson, *J. Chem. Soc.*, 2019 (1958).
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comitant with nucleophilic attack of sulfite ion on C-6 of the pyrimidine ring system can occur. This would seem quite analogous to nucleophilic attack by a thiol at the active site of an enzyme on C-6 concomitant with methyl donation to C-5 from an electrophile such as either *S*-adenosylmethionine or the cationic imine ($N^+{=}CH_2$) shown by Kallen and Jencks[25] to be an intermediate in the condensation of formaldehyde with tetrahydrofolate. Also the collapse of an intermediate such as **4** to yield the methylated pyrimidine involves proton abstraction from C-5. Since enzymes must catalyze reactions under constant and physiological conditions of pH, the enzyme most likely catalyzes the breakdown of the enzyme–dihydropyrimidine intermediate **4** *via* a general base catalyzed mechanism. This presumption is also in accord with our data with the sodium bisulfite–1,3-dimethyluracil system.

Another important biological aspect of the reaction of bisulfite ion with pyrimidines is the finding that the 5,6-dihydrocytosine-6-sulfonate is rapidly deaminated under physiological conditions of temperature and pH to yield uracil.[4,6] Thus, bisulfite can be considered mutagenic as has been demonstrated by Hayatsu and Miura who have shown that bisulfite induces mutations in phage λ.[26]

(25) R. H. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5851 (1966).

(26) H. Hayatsu and A. Miura, *Biochem. Biophys. Res. Commun.*, **39**, 156 (1970).

Studies on Polypeptides. L. Synthesis of a Protected Tritricontapeptide Hydrazide Corresponding to Positions 48–80 of the Primary Structure of Ribonuclease T_1[1-4]

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Abstract: Syntheses are described of two *N*-benzyloxycarbonylpeptide *tert*-butoxycarbonylhydrazides which correspond to positions 48–65 and 66–80 of the proposed primary structure of the enzyme ribonuclease T_1. Evidence is presented to indicate that these materials are sequentially homogeneous. These fragments were condensed to form an *N*-benzyloxycarbonyltritricontapeptide *tert*-butoxycarbonylhydrazide corresponding to positions 48–80 of the sequence of the enzyme. Advantages and disadvantages of the stepwise synthesis of peptides in solution are discussed.

For reasons discussed in previous communications,[1,3] we have subdivided the peptide chain of ribonuclease T_1 (ribonucleate guanine nucleotido-2′-transferase (cyclizing) 2.7.7.26) into a series of fragments (Figure 1) which serve as the starting materials for attempts to construct the entire primary structure of the enzyme *via* fragment condensation. Thus far, we have described details pertaining to the synthesis of fragments B, C, and D and to their condensation to form the partially protected hexatricontapeptide BCD (Figure 2) and have provided evidence for the sequential and stereochemical homogeneity of this compound.[1] The present investigation concerns the preparation of the partially protected tritricontapeptide EF (Figure 2) which corresponds to positions 48–80 of the enzyme's peptide chain.

Four protected peptide hydrazides, subfragments E_1, E_2, F_1, and F_2 (Figure 1), were prepared mainly by stepwise methods in solution[5,6] according to the routes illustrated in Schemes I–IV. Following suitable deprotection, subfragments E_1 and E_2 were linked to form fragment E; subfragments F_1 and F_2 were similarly

(1) See J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971), for paper XLIX in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation. The early phases of this investigation were supported by the Research Laboratories, Edgewood Arsenal, Contract No. DA-18-035-AMC-307 (A). The opinions expressed are those of the authors and do not reflect endorsement by the contractor.

(3) Preliminary communications of some of the results presented in this article have appeared: N. Yanaihara, C. Yanaihara, G. Dupuis, J. Beacham, R. Camble, and K. Hofmann, *J. Amer. Chem. Soc.*, **91**, 2184 (1969); K. Hofmann, *Peptides, Proc. Eur. Peptide Symp., 10th, 1969*, 130 (1971); H. T. Storey and K. Hofmann, *ibid., 12th, 1971*, in press.

(4) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M = aminopeptidase M; DCC = *N,N′*-dicyclohexylcarbodiimide; DMSO = dimethyl sulfoxide; DMF = dimethylformamide; EC = ethylcarbamoyl; EtOH = ethanol; F = formyl; *i*-PrOH = isopropyl alcohol; MeOH = methanol; N_3 = azide; NMM = *N*-methylmorpholine; OCP = 2,4,5-trichlorophenyl ester; ONHS = *N*-hydroxysuccinimido ester; *O-t*-Bu = *tert*-butyl ester; TEA = triethylamine; TFA = trifluoroacetic acid; THF = tetrahydrofuran; tlc = thin layer chromatography; X = *tert*-butoxycarbonylhydrazide; Y = benzyloxycarbonylhydrazide; Z = benzyloxycarbonyl. In order to simplify the designation of complex peptide derivatives, the following nomenclature is used: subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF = the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazides of subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF. Subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF *tert*-butoxycarbonylhydrazides = the amino-deprotected *tert*-butoxycarbonylhydrazides. Subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF hydrazides = the unprotected hydrazides of the *N*-benzyloxycarbonyl subfragments and fragments.

(5) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

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