

teractions will also be present when the intermediate enamine is formed by decarboxylation.

Experimental Section

The reagents and instrumentation employed in this study are listed in the accompanying paper.⁶

Preparation of 2-Substituted 3-Methylbenzothiazolium Salts. 2-Substituted benzothiazoles¹⁹ were made by heating *o*-aminobenzenethiol with an equivalent amount of the appropriate carboxylic acid or acid anhydride in a bomb at temperatures ranging from 120 to 165 °C.²⁰ They were quaternized with methyl iodide.²¹ The 2-hydroxymethyl iodide was used as such, mp 227–228 °C (lit.²⁰ mp 219 °C), but the others were converted to perchlorates. Perchlorate salts were prepared either by dissolving the iodides in a warm saturated solution of magnesium perchlorate in absolute ethanol or by dissolving in a mixture of ethyl acetate, absolute ethanol, and 70% perchloric acid (36:8:5 by volume). Perchlorate salts crystallized on cooling and were recrystallized from ethanol. Melting points and analyses were recorded in Table II. Analyses were made by Atlantic Microlab, Inc. Chemical shifts (τ , D₂O) of *N*-methyl, *C*-methyl, and CH protons observed during kinetic runs are: C₂H₅, 5.74, 8.36, and 6.45; (CH₃)₂CH, 5.69, 8.37, and 6.00; CH₂OH, 5.78 and 4.55; CH₃CHOH, 5.69, 8.23, and 4.18, respectively.

Kinetics of Hydrogen-Deuterium Exchange. Details are similar to those in the accompanying article.⁶ Stock solutions of formic acid and sodium formate were employed. Ionic strength was maintained at 1 M using KCl (with iodides) or NaCl (with perchlorate salts). Reactions were followed for 2–3 half-lives except for the slowest runs involving 0.1 M DCl where only 1 half-life was observed. In addition to substrates being examined separately, a run was also made on a pair of substrates with similar reactivities in the same mixture.

Following a kinetic run pD measurements were made at 25.0 °C on both heated and unheated reaction mixtures. Differences were of the order 0.03 except in the case of two runs with IV at the lowest buffer concentrations; one of these also contained hydroxyethyl compound. Curiously, in these cases the difference in pD was 0.12; the pD of the unheated sample was recorded. This makes the free base concentration uncertain by about 15%. A pH reading was converted to pD by adding 0.40.²²

Control Studies to Determine the Stabilities of Substrates. Each compound was heated in formate buffer in H₂O for a period corresponding to 10–20 half-lives for isotope exchange. No decomposition was detected by NMR; pH differences between heated and unheated samples were no greater than 0.03.

Acknowledgment. This work was kindly supported by Grant AM-17442 from the National Institutes of Arthritis, Metabolism, and Digestive Diseases.

References and Notes

- (1) L. O. Krampitz, *Annu. Rev. Biochem.*, **38**, 213 (1969).
- (2) J. Hine, L. G. Mahone, and C. L. Liotta, *J. Am. Chem. Soc.*, **89**, 5911 (1967).
- (3) A. Streitwieser, Jr., and F. Mares, *J. Am. Chem. Soc.*, **90**, 2444 (1968).
- (4) J. Hine and P. D. Dalsin, *J. Am. Chem. Soc.*, **94**, 6998 (1972).
- (5) A. A. Bothner-By and C. Sun, *J. Org. Chem.*, **32**, 492 (1967); J. Hine, K. G. Hampton and B. C. Menon, *J. Am. Chem. Soc.*, **89**, 2664 (1967); F. G. Brodwell, M. VanderPuy, and N. R. Vanier, *J. Org. Chem.*, **41**, 1885 (1976).
- (6) J. A. Zoltevicz and J. K. O'Halloran, *J. Org. Chem.*, preceding article in this issue.
- (7) 2-(1-Hydroxyethyl)thiamin⁸ and related derivatives^{9,10} have been shown to undergo general-base-catalyzed deprotonation of the type considered here. Generally Tris buffers were employed but no attempt was made to dissect pseudo-first-order rate constants into individual component terms associated with buffer base and lyate ion.
- (8) J. J. Mielal, R. G. Votaw, L. O. Krampitz, and H. Z. Sable, *Biochim. Biophys. Acta*, **141**, 205 (1967).
- (9) J. Suchy, J. J. Mielal, G. Bantle, and H. Z. Sable, *J. Biol. Chem.*, **247**, 5905 (1972); A. A. Gallo and H. Z. Sable, *ibid.*, **251**, 2564 (1976).
- (10) J. J. Mielal, G. Bantle, R. G. Votaw, I. A. Rosner, and H. Z. Sable, *J. Biol. Chem.*, **246**, 5213 (1971).
- (11) J. J. Christensen, R. M. Izatt, and L. D. Hansen, *J. Am. Chem. Soc.*, **89**, 213 (1967).
- (12) E. S. Hand and W. P. Jencks, *J. Am. Chem. Soc.*, **97**, 6221 (1975).
- (13) J. R. Owen, *Tetrahedron Lett.*, 2709 (1969); J. Metzger, H. Larive, E. J. Vincent, and R. Dennilaular, *J. Chim. Phys.*, **60**, 944 (1963).
- (14) J. Hine and N. W. Flachskam, *J. Am. Chem. Soc.*, **95**, 1179 (1973); W. J. Hehre and W. A. Lathan, *J. Chem. Soc., Chem. Commun.*, 771 (1972).
- (15) M. J. S. Dewar and P. Rona, *J. Am. Chem. Soc.*, **91**, 2259 (1969).
- (16) J. Crosby, R. Stone, and G. E. Lienhard, *J. Am. Chem. Soc.*, **92**, 2891 (1970); J. Crosby and G. E. Lienhard, *ibid.*, **92**, 5707 (1970).
- (17) M. Sax, P. Pulsinelli, and J. Pletcher, *J. Am. Chem. Soc.*, **96**, 155 (1974); W. Shin, J. Pletcher, G. Blank, and M. Sax, *ibid.*, **99**, 3491 (1977).
- (18) R. G. Yount and D. E. Metzler, *J. Biol. Chem.*, **234**, 738 (1959).
- (19) F. M. Hamer, R. J. Rathbone, and B. S. Winton, *J. Chem. Soc.*, 954 (1947); J. Metzger and H. Plank, *Bull. Soc. Chim. Fr.*, 1692 (1956); V. M. Zubarovsky, *Zh. Obshch. Khim.*, **21**, 2199 (1951); R. Gugliedmitt, E. J. Vincent, J. Metzger, J. Berger, and R. Garnier, *Bull. Soc. Chim. Fr.*, 4195 (1967).
- (20) V. M. Zubarovsky, *J. Gen. Chem. USSR*, **21**, 2295 (1951); *Chem. Abstr.*, **48**, 6640h (1952).
- (21) J. Metzger, H. Larive, R. Dennilaular, R. Baralle, and C. Gaurat, *Bull. Soc. Chim. Fr.*, 2868 (1964); M. Azzaro and J. Metzger, *ibid.*, 1575 (1964).
- (22) A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, **40**, 700 (1968).

Kinetics and Mechanism of the Deamination of 1-Methyl-5,6-dihydrocytosine

Sanford Slae and Robert Shapiro*

Department of Chemistry, New York University, New York, New York 10003

Received August 30, 1977

Kinetic studies of the deamination of 1-methyl-5,6-dihydrocytosine (MDC) have been carried out in acidic and basic aqueous solutions at 37 °C, $\mu = 1.0$ (ionic strength). General-base catalysis was observed under acidic but not basic conditions. The Brønsted relationship for this reaction showed $\beta = 0.19$. No dependence on hydroxide ion concentration was demonstrable under alkaline conditions. Activation enthalpies and entropies were measured for this reaction in the absence of general catalysts in acidic and basic media for the range 20–47 °C. Direct hydroxide ion attack on the protonated substrate is a plausible mechanism for the reaction in alkaline media. An alternative mechanism involving participation of water as a proton-transfer agent in the transition state with either formation or reaction of the tetrahedral intermediate as the rate-determining step is also consistent with all of the kinetic data.

The deamination of cytosine to uracil by bisulfite^{1,2} has been applied widely, as a synthetic method in nucleoside chemistry and as a tool for the modification of nucleic acids.³ Mutations are induced in bacteria and viruses upon treating them with high concentrations of bisulfite and acidic pH.⁴ The mutagenic specificity observed indicates that the mutations

are caused by cytosine deamination within DNA.⁴ The possibility exists that environmental bisulfite and sulfur dioxide may constitute a mutagenic hazard⁵ to the general public. To evaluate this hazard, it is necessary to be able to extrapolate deamination rates to low bisulfite concentrations at neutral pH.

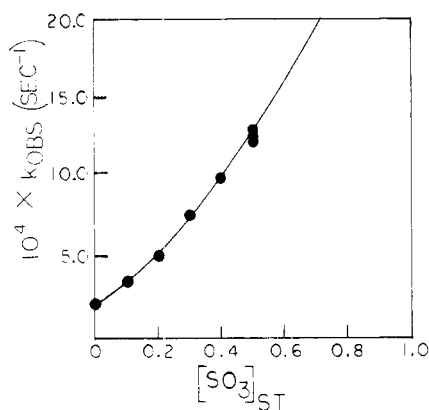
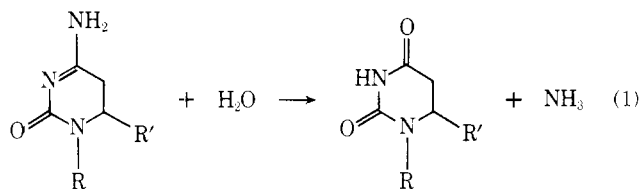


Figure 1. Deamination of MDC, 37 °C, $\mu = 1.0$, pH 4.40. Dependence of observed rate constant on stoichiometric sulfite concentration. Curvature due to varying pyrosulfite ion catalysis.

A number of aspects of the deamination mechanisms have been explored.^{6,7} A remaining point of uncertainty has been the nature of the buffer catalysis involved in the deamination of the dihydrocytosine intermediate in the reaction scheme (1a \rightarrow 2a).



- 1a, R = β -D-ribofuranosyl; 2a, R = β -D-ribofuranosyl;
 R' = SO_3^- R' = SO_3^-
 b, R = CH_3 , R' = H b, R = CH_3 ; R' = H

In order to explore the mechanism of this reaction without the complications arising from the possible loss of bisulfite with 1a and 2a, we have conducted a detailed study of the deamination of a model compound, 1-methyl-5,6-dihydrocytosine. Preliminary observations on this system were reported earlier.^{7,8}

Results

Kinetics of the Deamination of MDC in Acidic Media.

The kinetics of the deamination of 1-methyl-5,6-dihydrocytosine, MDC, to 1-methyl-5,6-dihydrouracil (1b \rightarrow 2b) at 37 °C, $\mu = 1.0$, were measured in the presence of 0.5 M HCl and appropriate buffers (pH range 4.40–7.52). In this range from 8 to 100% of the substrate ($\text{p}K_a = 6.40 \pm 0.03$) is protonated. Excellent first-order kinetic plots were obtained and this reaction was found to be subject to general base catalysis. The catalytic coefficients were measured for a series of nine bases. Corrections were made for the mole fraction of substrate protonated in accordance with eq 4 since the neutral substrate demonstrated no general base catalysis.

$$d[\text{P}]/dT = k[\text{HSub}^+][\text{B}] = k\chi_{\text{HSub}^+}[\text{Sub}]_{\text{ST}}[\text{B}] \quad (2)^9$$

$$\frac{1}{[\text{Sub}]_{\text{ST}}} \frac{d[\text{P}]}{dt} = k\chi_{\text{HSub}^+}[\text{B}] = k_{\text{obsd}} \quad (3)$$

$$\frac{k_{\text{obsd}}}{[\text{B}]\chi_{\text{HSub}^+}} = \frac{k'_{\text{obsd}}}{\chi_{\text{HSub}^+}} = k \quad (4)$$

For sulfate ion, acetate ion, Tris, and imidazole, the catalytic coefficient, $k_{\text{obsd}}/[\text{B}]$, was obtained directly from the slope of a plot of k_{obsd} against $[\text{B}]$. This procedure was followed for a series of buffered solutions of varying base concentration at constant pH and ionic strength. In the case of the sulfite and phosphate buffers, more than one catalytic species is present,

Table I. Sulfite Catalyzed Deamination of MDC, 37 °C, $\mu = 1.0$ M

pH	$\chi_{\text{HSub}^+}^a$	$10^3 \times k_{\text{obsd}}/\chi_{\text{HSub}^+},$ $\text{M}^{-1} \text{s}^{-1}$	$10^3 \times k_{\text{calcd}},^c$ $\text{M}^{-1} \text{s}^{-1}$
4.40	0.990	1.455 ^b	1.43
5.00	0.962	1.44 ^b	1.48
5.40	0.909	1.46 ^b	1.575
6.00	0.715	1.945	1.88
7.30	0.112	3.25	3.52
7.45	0.0814	3.88	3.615

^a χ indicates mole fraction. ^b k_{obsd} from slope determination based on lowest two concentrations of $(\text{SO}_3)_{\text{ST}}$ at this pH as explained in the text. ^c k_{calcd} based on $k_{\text{HSO}_3^-} = 1.41 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$, $k_{\text{SO}_3^{2-}} = 3.90 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$, and calculated values of χ_{HSub^+} , $\chi_{\text{HSO}_3^-}$, $\chi_{\text{SO}_3^{2-}}$ at each pH.

and the resulting overall rate effect of any stoichiometric concentration is due to the contribution of several bases. Separation of the total catalytic effect of each "phosphate" solution into the component rates due to monohydrogen phosphate and dihydrogen phosphate ions was achieved according to

$$\frac{k_{\text{obsd}}}{[\text{PO}_4]\chi_{\text{HSub}^+}} = \frac{k'_{\text{obsd}}}{\chi_{\text{Sub}^+}} = k = k_{\text{HPO}_4^{2-}}\chi_{\text{HPO}_4^{2-}} + k_{\text{H}_2\text{PO}_4^-}\chi_{\text{H}_2\text{PO}_4^-} \quad (5)$$

k is measured at two different pH values and $\chi_{\text{HPO}_4^{2-}}$ and $\chi_{\text{H}_2\text{PO}_4^-}$ are calculated from the known $\text{p}K_a$ for H_2PO_4^- for these conditions. The two simultaneous equations are solved for the two unknowns, $k_{\text{HPO}_4^{2-}}$ and $k_{\text{H}_2\text{PO}_4^-}$.

The above treatment proved unsuccessful in the case of sulfite ion. Sulfite ion buffers in the pH region 4.40 to 6.00 contain more than two catalyzing species. Spectrophotometric studies of acidified aqueous solutions of sulfite ion revealed the presence of pyrosulfite ion ($\lambda_{\text{max}} 255 \text{ nm}$, $\epsilon = 1.98 \times 10^3 \text{M}^{-1}$) in accordance with the equilibrium $2\text{HSO}_3^- \rightleftharpoons \text{S}_2\text{O}_5^{2-} + \text{H}_2\text{O}$, $K_{\text{eq}} = [\text{S}_2\text{O}_5^{2-}]/[\text{HSO}_3^-]^2$. The value of K_{eq} was measured spectrophotometrically to be $(2.16 \pm 0.08) \times 10^{-1} \text{M}^{-1}$ at 37 °C, $\mu = 1.0$. This compares well with a literature value¹⁰ of $2.20 \times 10^{-1} \text{M}^{-1}$, 25 °C, $\mu = 0.9$. As the concentration of pyrosulfite ion depends on the square of the concentration of bisulfite ion, its mole fraction, and therefore its relative catalytic effect, vary with bisulfite ion concentration, even at constant pH. This perturbing effect of pyrosulfite ion on the kinetics of this reaction gives rise to curvilinear plots when the observed rate constant is plotted against the stoichiometric sulfite ion concentration (Figure 1). The slopes of these plots increase with increasing stoichiometric sulfite concentration as expected.¹¹

To simplify the situation, the sulfite rate data were treated in the following manner: The slopes of k_{obsd} against stoichiometric sulfite concentration (as defined by the two points of lowest concentration) were measured and used to estimate the effective rate contribution of bisulfite and sulfite ions alone. When these values of $k'_{\text{obsd}}/\chi_{\text{HSub}^+}$ were then treated in the same manner as the data for phosphate ion, the resulting rate constants for sulfite and bisulfite ions showed excellent agreement with the experimental rate slopes at six different pH values, as shown in Table I.

When the catalytic effects of sulfite and bisulfite ions were subtracted from the overall reaction rate observed at the more concentrated stoichiometric sulfite solutions in the pH 4.40, 5.00, and 5.40 buffers, the enhancement of reaction rate due to pyrosulfite ion was obtained. This rate information was combined with the calculated pyrosulfite ion concentration and the mole fraction of protonated substrate for the above conditions to permit calculation of the catalytic coefficient

Table II. Pyrosulfite Ion Catalyzed Deamination of MDC, 37 °C, $\mu = 1.0$ M

pH	χ_{HSub^+}	$10^3 \times k_{\text{obsd}}, \text{s}^{-1}$	$10^3 \times k_{\text{sulfite}}^a, \text{s}^{-1}$	$[\text{S}_2\text{O}_5^{2-}]^b$	$10^3 \times (k_{\text{obsd}} - k_{\text{sulfite}}) / \chi_{\text{HSub}^+} [\text{S}_2\text{O}_5^{2-}], \text{M}^{-1} \text{s}^{-1}$
4.40 ^c	0.990	1.27	0.89	0.050	7.68
5.00 ^d	0.962	1.42	0.94	0.050	9.88
5.40 ^e	0.909	2.11	1.42	0.040	9.52

^a k_{sulfite} is the expected catalytic effect of sulfite and bisulfite ions alone for this stoichiometric sulfite concentration. ^b Calculated from $[\text{S}_2\text{O}_5^{2-}] = 0.216[\text{HSO}_3^-]^2$ at this stoichiometric sulfite concentration. ^c $[\text{SO}_3]_{\text{ST}} = 0.50$ M. ^d $[\text{SO}_3]_{\text{ST}} = 0.50$ M. ^e $[\text{SO}_3]_{\text{ST}} = 0.685$.

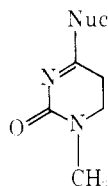
Table III. Base Catalysis of the Deamination of MDC, 37 °C, $\mu = 1.0$ M

Base	pK_a^a	$[\text{B}]_{\text{ST}}, \text{mol}$	pH	$10^3 \times k'_{\text{obsd}} / \chi_{\text{HSub}^+}^b, \text{M}^{-1} \text{s}^{-1}$
SO_4^{2-}	1.16	0.1–0.3	4.40	0.183 ± 0.009
HSO_3^-	1.47	0.1–0.7	4.40–7.45	1.41 ^d
H_2PO_4^-	1.70	0.2–0.75	4.98–5.33	0.423 ^d
$\text{S}_2\text{O}_5^{2-}$?	0.1–0.7	4.40–5.40	9.0 ± 1.2^d
CH_3CO_2^-	4.60	0.2–1.0	5.05	0.299 ± 0.027
HPO_4^{2-}	6.31	0.2–0.75	4.98–5.33	2.18 ^d
SO_3^{2-}	6.55 ^c	0.2–0.7	4.40–7.45	3.90 ^d
Imidazole	6.92	0.1–0.8	5.40	1.50 ± 0.28
Tris	8.24	0.05–0.3	6.58–7.52	1.76 ± 0.33

^a pK_a for these conditions as determined from the best values or estimates from 33–39. ^b χ denotes mole fraction. ^c Measured in this work. ^d Correction for χ_{HSub^+} included in calculation of k_{obsd} as explained in the text.

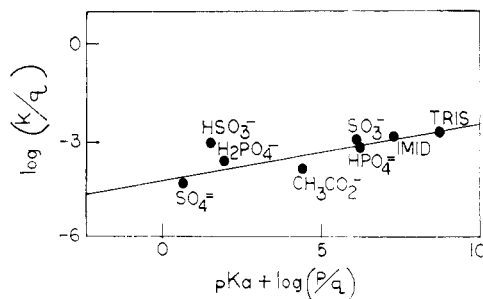
of pyrosulfite ion for MDC deamination (Table II). The resulting value of $k_{\text{S}_2\text{O}_5^{2-}} = (9.0 \pm 1.2) \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$ indicates the large catalytic effect of pyrosulfite ion. Although pyrosulfite ion is a weaker base than sulfite ion,¹² it is a stronger catalyst in this reaction:¹³ $(k/q)_{\text{S}_2\text{O}_5^{2-}} / (k/q)_{\text{SO}_3^{2-}} = 1.4$.

The catalytic coefficients for all nine bases studied are summarized in Table III. Bisulfite ion demonstrates a large positive deviation from the Brønsted relation¹³ defined by the other bases (Figure 2)¹⁴ and the best Brønsted relation defined by the other bases gives $\log(k/q) = (0.19 \pm 0.03)(\text{pK}_a + \log p/q) - (4.31 \pm 1.9)$. A comparison of the relative rate constants of imidazole and biphosphate ion, $(k/q)_{\text{IM}} / (k/q)_{\text{HPO}_4^{2-}} = 2.06$, shows clearly that nucleophilic catalysis, which is characterized by large ratios of imidazole to biphosphate rate constants¹⁶ (1000:1), is not present in this system; therefore, nu-



cleophilic attack by general bases to form an intermediate, which undergoes subsequent rapid hydrolysis to form the final product, can be excluded from this mechanism.

The Kinetics of the Deamination of MDC in Basic Media. For MDC deamination of studies at 37 °C, $\mu = 1.0$ in the pH range 8.55–8.95, the extent of protonation of the substrate is approximately 0.3–0.9%. When the catalytic coefficients for general bases present and water are taken into account, it becomes apparent that less than 1% of the observed rate (Table IV) is due to the reaction path observed in acid.

**Figure 2. Brønsted relationship for base-catalyzed MDC deamination, 37 °C, $\mu = 1.0$, acidic conditions. Based on Table III.****Table IV. Deamination of MDC in Basic Media, Presence of General Bases, $\mu = 1.0$ M, 37 °C**

pH	Base	Concn range, mol	$10^5 \times k_{\text{obsd}}, \text{s}^{-1}$
8.55	Tris	0.1–0.6	6.39 ± 0.30
	SO_3^{2-}	0.05–0.10	6.40 ± 0.38
8.95	Tris	0.2–0.4	6.59 ± 0.13
	SO_3^{2-}	0.1	6.55
	Imidazole ^a	0.1–0.6	5.88 ± 0.27

^a In the presence of 0.2 M Tris.

Table V. Rate Constants for the Deamination of MDC in Acidic and Basic Media at Various Temperatures, $\mu = 1.0$ M

Temp, °C	$10^5 \times k_{\text{obsd}}, \text{s}^{-1}$	
	pH 8.55–8.95 ^a	pH 0.4 ^b
20.0	1.10	1.81 ± 0.09
37.0	6.49 ± 0.1	14.1 ± 0.5
47.0	13.7 ± 0.5	36.6 ± 1.5

^a In the presence of 0.2–0.4 M Tris. ^b In the presence of 0.4 M HCl.

The kinetic results clearly show that (within experimental error) the observed deamination rate is insensitive both to the pH of the medium and the presence and concentration of added bases such as imidazole, Tris, and sulfite ion. The neutral substrate therefore suffers neither specific nor general base catalysis upon deamination in alkaline media. The possibility of a reaction of the protonated substrate with hydroxide ion will be considered subsequently.

Activation Parameters. Measurement of MDC deamination rate constants in acidic and basic media at several temperatures (Table V, Figure 3) allowed the calculation of activation parameters for these reactions. These were found to be: $\Delta H^\ddagger = 20.7 \pm 0.8$ kcal/mol, $\Delta S^\ddagger = -11.0 \pm 2.6$ eu for the reaction of protonated MDC in acidic media, and $\Delta H^\ddagger = 17.0 \pm 1.1$ kcal/mol, $\Delta S^\ddagger = -23.2 \pm 3.6$ eu for the neutral substrate in basic media. It appears that the relative speed of the reaction in acidic media is due to the compensation by a favorable activation entropy for an unfavorable enthalpy.

The Reaction with Water. The measured rate constants for the reactions of protonated and neutral substrates with water can be used to calculate the expected reaction rate at zero buffer concentration for a series of rate studies at various pH values according to the formula,

$$k_{\text{obsd}}^{\text{intercept}} = k_{\text{HSub}^+} \chi_{\text{HSub}^+} + k_{\text{Sub}} \chi_{\text{Sub}}$$

where $k_{\text{HSub}^+} = 1.41 \times 10^{-4} \text{s}^{-1}$ and $k_{\text{Sub}} = 6.49 \times 10^{-5} \text{s}^{-1}$ at 37 °C, $\mu = 1.0$. A comparison of the calculated and observed values of these rate constants is presented in Figure 4. The observed reaction rates are well described by this relationship.

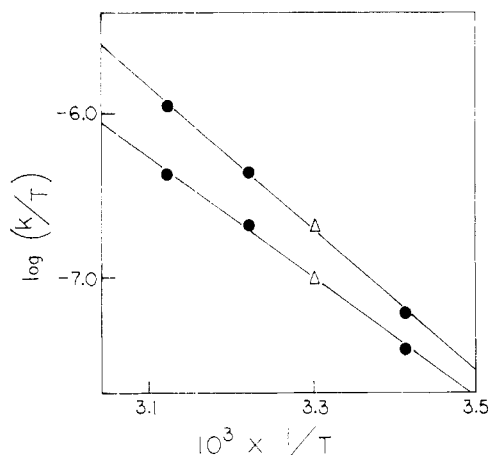
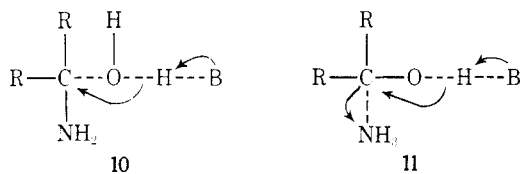


Figure 3. Dependence of background deamination rate constant on temperature for MDC, $\mu = 1.0$, acidic and basic conditions: (●) data from this work; (Δ) data from ref 7.

Discussion

Mechanism of MDC Deamination. Acidic Conditions.

The dependence of the MDC deamination rate on the concentration of the protonated substrate, $[\text{HSub}^+]$, the presence of general base catalysis reported here, and the absence of general acid catalysis reported previously⁷ are consistent with the mechanism presented in Scheme I: $3 \rightarrow 4 \rightarrow 6 \rightarrow 7 \rightarrow 8$ where either tetrahedral intermediate formation, $4 \rightarrow 6$, or reaction, $7 \rightarrow 8$, may be rate determining. The general base would be present in either transition state 10 or 11.



Mechanism of MDC Deamination. Basic Conditions.

The mechanism for MDC deamination in basic conditions must explain the following facts: (1) the reaction rate is pH independent in the range studied, 8.5–9.0; (2) no general base catalysis is observed; (3) the activation entropy is negative and large.

Several reaction mechanisms need to be considered in analyzing the reaction in basic media. Intermediate formation, if rate determining, could occur in two ways. In mechanism A, direct nucleophilic attack of hydroxide ion on the protonated substrate forms the reaction intermediates (Scheme I: $3 \rightarrow 4 \rightarrow 6 \rightarrow 8$ where $4 \rightarrow 6$, OH^- is slow), while in mechanism B, attack by water occurs on the neutral substrate without base assistance (Scheme I: $3 \rightarrow 6 \rightarrow 8$, where $3 \rightarrow 6$ is slow). Rate-determining product formation from the intermediate is another possible mechanism that is consistent with the kinetic results (Scheme I: $6 \rightarrow 8$, slow), as in mechanism C. Since all three mechanisms yield the same rate law, which can be shown by the steady state approximation to be of the form,

$$k_{\text{obsd}} = \frac{1}{[\text{Sub}]_{\text{ST}}} \frac{d[\text{P}]}{dT} = kK\chi_{\text{Sub}}$$

they all predict lack of rate dependence on base concentration.

The catalytic properties of this reaction are consistent with all three suggested mechanisms. The change from general base catalysis of the deamination reaction in acidic media to no base dependence in basic media can be understood as arising from a change in mechanism from base-assisted reaction of

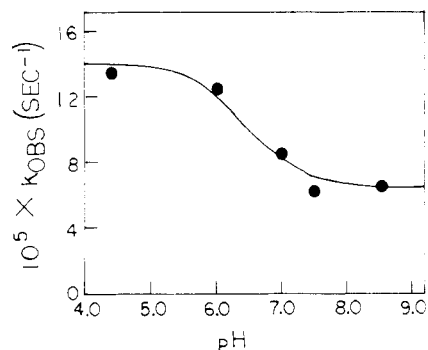
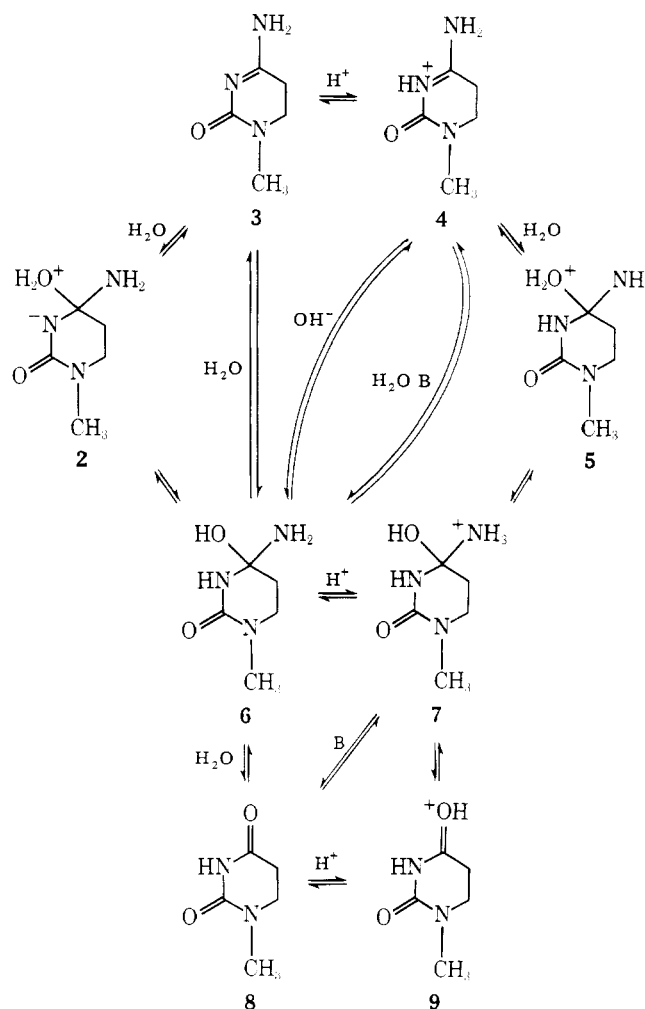


Figure 4. pH dependence of the observed intercepts of general base catalyzed MDC deamination, 37 °C, $\mu = 1.0$. The points represent experimental determinations. The curve was calculated according to the equation, $k_{\text{obsd}} = 1.41 \times 10^{-4} (\text{s}^{-1}) \chi_{\text{HSub}^+} + 6.49 \times 10^{-5} (\text{s}^{-1}) \chi_{\text{Sub}}$ as explained in the text.

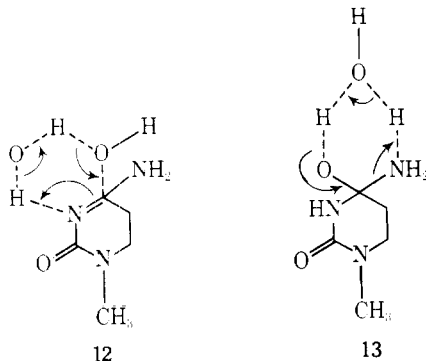
Scheme I. Mechanism of MDC Deamination in Aqueous Solutions



the protonated substrate with water in acidic media to nucleophilic attack of hydroxide ion on the protonated substrate in basic media. This change is due to the large increase in $[\text{OH}^-]$. The base-catalyzed reaction with water decreases with decreasing χ_{HSub^+} during this change in conditions and becomes kinetically less favorable with respect to mechanism A under basic conditions.

If the transition state of mechanisms B and C involved the participation of a second water molecule functioning as a proton-transfer agent, then the catalytic change from acidic

to basic media may be explained in the following manner. If the formation of the intermediate is rate determining as in mechanism B, then the assistance of this second water molecule in the transition state 12 would remove the need for base



catalysis. A similar explanation can be used for mechanism C, where the reaction of the intermediate is rate determining, as in the transition state of structure 13. The uncatalyzed reaction of water with the neutral substrate becomes faster than the base-catalyzed water reaction of the protonated substrate as the reaction conditions become basic, since the χ_{HSub^+} decreases markedly during this transition.

The mechanism discussed here must also be examined in connection with the observed reaction activation entropy, -23 eu. This negative and large entropy is consistent with each of the above mechanisms. For mechanism B, a large, negative ΔS^\ddagger is expected, due to the participation of the proton-transfer water molecule in the transition state. Thus, three molecules ($\text{Sub} + 2\text{H}_2\text{O}$) go to one moiety in the transition state and there is a loss of translational freedom of motion for two molecules. In addition, there is a generation of scattered partial charges at various polar centers in a highly structured and concerted transition state. These factors are all expected to produce a more negative entropy.¹⁷ The participation of water as a proton-transfer agent in the transition state of mechanism C would also be consistent with the observed ΔS^\ddagger .

Bell et al.^{18,19} have found in their studies of the hydration of 1,3-dichloroacetone in aqueous acetone mixtures that the activation entropy of the reaction depended on the number of water molecules participating in the transition state. Based on the kinetic order with respect to water, and kinetic isotope effects, it was determined that in addition to the reacting water molecule, extra water molecule(s) are contained in the transition state for the reaction in the absence of general acid catalysts. The transition state for the benzoic acid catalyzed hydration reaction contained one less extra water molecule. This finding was supported by the activation parameters which showed the acid-catalyzed reaction having higher activation enthalpies ($\delta\Delta H^\ddagger = 3.83$ kcal/mol) and entropies ($\delta\Delta S^\ddagger = 17.6$ eu)²⁰ than the uncatalyzed reaction with water. This compares with MDC deamination where the base catalyzed reaction in acidic media has higher activation parameters ($\delta\Delta H^\ddagger = 3.7$ kcal/mol, $\delta\Delta S^\ddagger = 12$ eu) than the uncatalyzed reaction in basic media. Thus, the results reported here can similarly be explained by the involvement of an additional water molecule in the transition state for the reaction in basic media (12, 13) in place of the general base present in the transition state for MDC deamination in acidic media (10, 11).

On the basis of the experimental ΔS^\ddagger , one might be tempted to exclude mechanism A which involves a transition state of much greater charge dispersion than the ground state and, therefore, a predicted activation entropy closer to zero. However, kinetic studies of related systems such as Schiff bases^{21a} have demonstrated activation entropies of -30 eu for

reactions proceeding through a nucleophilic attack by hydroxide on the protonated substrate (imine) in basic media.

Bisulfite Ion Catalysis in Deamination Reactions. Our results demonstrate that bisulfite ion is not exceptionally stronger than other catalysts in the deamination of 1-methyldihydrocytosine. It is effective as a general base catalyst for deamination of the protonated substrate, but many other buffers also catalyze the reaction. There is no buffer catalysis for the deamination of the unprotonated substrate. If the above mechanisms also hold for the deamination of dihydrocytosine 1a to 2a, then bisulfite plays no unique role in that deamination either. The important role for bisulfite in the conversion of cytosine to uracil is the formation of the adduct 1a from cytosine. If one were extrapolating to low bisulfite concentrations in calculating cytosine deamination rates, and if a constant concentration of another buffer effective in catalyzing the deamination step were present, one would then expect the deamination rate to fall in direct proportion to the bisulfite concentration (rather than to the square of the concentration).

Amidine Hydrolysis. The mechanistic considerations presented here are fully consistent with kinetic studies of related amidines. Deamination rates of MDC, 1,3-dimethyl-5,6-dihydrocytosine (DDC), and 1-cyclohexyl-*N*(4)-dimethyl-5,6-dihydrocytosine (CDDC) were previously measured⁸ in acidic and basic media at 20 °C, $\mu = 0.01$, and the pK_a and observed deamination rate constants in acidic and basic media for these compounds respectively were: for MDC 6.62, $2.78 \times 10^{-5} \text{ s}^{-1}$, $8.35 \times 10^{-6} \text{ s}^{-1}$; for DDC 8.05, $2.50 \times 10^{-5} \text{ s}^{-1}$, $9.73 \times 10^{-5} \text{ s}^{-1}$; for CDDC 6.40, $2.78 \times 10^{-5} \text{ s}^{-1}$, $8.35 \times 10^{-6} \text{ s}^{-1}$. Although MDC deaminates at a slower rate in basic than acid media under these conditions, DDC exhibits a rate enhancement by a factor of almost 4 as the pH is raised from 7 to 9, after which the rate remains constant. This compound, which for structural reasons must deaminate by a mechanism not involving the water-bridged transition state, structure 5, may demonstrate a nucleophilic hydroxide ion attack on protonated substrate, similar to mechanism A, thereby accounting for rate acceleration in basic media. Alternatively, the different pH profiles can be attributed to the variation in pK_a of the substrates.^{21b,c}

Deamination studies of the photohydrate of 3'-cytidylic acid, a closely related compound,²² are also consistent with the results reported here for MDC. The pK_a was measured to be 5.56 at 0 °C, $\mu = 0.05$. Deamination rate constants demonstrated a pH dependence resembling that of Figure 4. There was a decrease in rate constant by a factor of 2.5 on going from pH 4.0 to pH 8.0. The activation parameters which can be derived from the original rate data are $\Delta H^\ddagger = 16.2 \pm 0.1$ kcal/mol, $\Delta S^\ddagger = -19.4 \pm 1$ eu (pH 4.0) and $\Delta H^\ddagger = 17.3 \pm 0.1$ kcal/mol, $\Delta S^\ddagger = -15.5 \pm 1$ eu (pH 8.0). No general base catalysis was observed for the deamination reaction under basic conditions.

Diarylformamidine hydrolysis has been studied under acidic and basic conditions in 20% aqueous dioxane mixtures.^{23,24} It was found that, in acidic media, the reaction demonstrated general base catalysis ($\beta = 0.29$), electron withdrawing groups accelerate the reaction rate ($\rho = 3.78$ at 40 °C), and the activation entropy was -22 ± 3 eu. The reaction rate in concentrated solutions of strong acids varied directly with hydronium ion concentration and water activity and inversely with h_0 . It was suggested that a general base and at least two molecules of water are present in the transition state.

Similar rate results were obtained for diarylacetylamidine hydrolysis in 20% aqueous dioxane. Since diarylacetylamidines are 1000–2500 times slower in this reaction than diarylformamidines (due to an increase of 4.5 kcal/mol in ΔH^\ddagger), it was reasoned that nucleophilic attack of water on the pro-

tonated substrate was most likely to be the rate-determining step.

Extensive hydrolysis studies have been made on the amidines 1,3-diphenyl-2-imidazolium chloride (DPIC)²⁵ and *N*^{5,10}-methenyltetrahydrofolic acid (MTF).²⁴ Brønsted correlations for the reaction pathways first order in base and second order in base were measured at 25 °C, $\mu = 1.0$, in the pH range 7–11. It was argued that the reaction mechanism involved rate-determining reaction of the tetrahedral intermediate. Direct spectral evidence for the existence of the tetrahedral intermediate in this reaction was obtained at pH 12.²⁶ In the case of MTF at high buffer concentrations, the formation of the tetrahedral intermediate became rate determining.

In conclusion, it can be said that amidine hydrolysis occurs through a sequence of steps closely balanced in individual rate constants so that the identity of the rate-determining step is highly dependent on substituents, pH, and the presence of general bases.

Experimental Section

Materials. 1-Methyl-5,6-dihydrocytosine (MDC) was synthesized by the method of Chang and Lewis.²⁷ Sigma Trizma base and Trizma HCl were used in the preparation of solutions of Tris(hydroxymethyl)aminomethane, (Tris). Aldrich Reagent grade imidazole was recrystallized three times from benzene before use (mp 88.5–89.0 °C, lit. mp 90–1 °C). All other materials were commercially available reagent grade and were used as supplied. Water used in kinetic and equilibrium measured was double distilled in Pyrex vessels and degassed with nitrogen.

Apparatus. Measurements of pH were made on a Radiometer Model 22 pH meter equipped with a glass electrode. Potentiometric titrations were performed on a Radiometer assembly, including Titrator II, Autoburette ABU 12, pH meter 26, and Titrigraph SBR 2C, having a constant temperature bath jacketing the titration vessel. Ultraviolet absorbance measurements were recorded on a Cary 15 recording spectrophotometer.

Kinetics of the Deamination of 1-Methyl-5,6-dihydrocytosine (MDC). Reaction mixtures were kept in the Cary 15 spectrophotometer thermostated cell compartment, and the variation of ultraviolet absorbance at 245 nm was recorded as a function of time. Reduced path lengths of 0.1 to 0.3 mm were employed when appropriate. Temperatures were measured with a thermometer calibrated by the National Bureau of Standards and were stable to within ± 0.1 °C. The slope of the plot of $\ln(\text{OD} - \text{OD}_\infty)$ vs. time was analyzed by the method of least squares on a Hewlett Packard 3000 computer. Catalytic coefficients of general bases were obtained by rate measurements in aqueous buffers of varying catalyst concentration and constant pH and ionic strength. When aqueous solutions of bisulfite ion were used, 10^{-3} M hydroquinone was present as a radical inhibitor.

Determination of the pK_a of MDC and Bisulfite Ion. The pK_a values for the dissociation of MDC were determined to be 6.40 ± 0.03 (potentiometric titration) and 6.49 ± 0.02 (spectroscopic method) at 37 °C, $\mu = 1.0$, and the first value was considered to be less subject to errors and used in subsequent calculations. The pK_a for bisulfite ion dissociation was measured potentiometrically to be 6.55 under the same conditions.

Determination of the K_{eq} for Pyrosulfite Ion Formation. The equilibrium constant, K_{eq} , for pyrosulfite ion formation from bisulfite ion in acidic media was measured spectrophotometrically to be $(2.16 \pm 0.08) \times 10^{-1} \text{ M}^{-1}$ at 37 °C, $\mu = 1.0$, where $K_{eq} = [\text{S}_2\text{O}_5^{2-}]/[\text{HSO}_3^-]^2$. This was based on a value of $\epsilon = 1.98 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$, $\lambda_{\text{max}} 255 \text{ nm}$.¹⁰ The value reported here compares well with $2.2 \times 10^{-1} \text{ M}^{-1}$ at 25 °C $\mu = 0.9$, based on this value of ϵ .^{10,28} This constant is known to be very dependent on ionic strength, changing from 0.076 ($\mu = 0$) to 0.34 ($\mu = 2.0$) at 25 °C.¹⁰

Acknowledgment. This research was supported by Grant ES 01033 from the National Institutes of Health, U.S. Public

Health Service. Robert Shapiro has been a Research Career Development Awardee (GM-50188) of the National Institutes of Health.

Registry No.—MDC, 696-10-6.

References and Notes

- R. Shapiro, R. E. Servis, and M. Welcher, *J. Am. Chem. Soc.*, **92**, 1422 (1970).
- H. Hayatsu, Y. Nataya, and K. Kai, *J. Am. Chem. Soc.*, **92**, 724 (1970).
- H. Hayatsu, *Progr. Nucleic Acid Res. Mol. Biol.*, **16**, 75 (1976).
- R. Shapiro, *Mutat. Res.*, **39**, 149 (1977).
- Science*, **187**, 502 (1975).
- M. Sono, W. Wataya, and H. Hayatsu, *J. Am. Chem. Soc.*, **95**, 4745 (1973).
- R. Shapiro, V. Difate, and M. Welcher, *J. Am. Chem. Soc.*, **96**, 906 (1974).
- D. M. Brown and M. J. E. Hewlins, *J. Chem. Soc. B*, 2050 (1968).
- $d[P]/dT$ represents the rate of product formation; χ represents the mole fraction of a given species of the overall stoichiometric mixture of related materials, e.g., $\chi_{\text{HSO}_3^-}$ represents the mole fraction of protonated substrate; ST denotes stoichiometric; Sub denotes substrate; k_{obsd} is the observed rate constant; and k_{calcd} is the calculated rate constant as described in the text.
- D. G. Bourne, T. Higuchi, and I. N. Pitman, *J. Pharm. Sci.*, **63**, 865 (1974).
- Previously reported data⁷ for sulfite ion catalyzed deamination of MDC, 30 °C, $\mu = 1.0$, pH 4.4, 5.0, needs to be corrected for pyrosulfite catalysis under these conditions.
- HS_2O_5^- is completely dissociated under conditions where HSO_3^- is only partially ionized.¹⁰
- k is the rate constant and q and p are respectively the statistical correction for the number of basic sites in the catalyst and acidic sites in the conjugate acid. See, for example, W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, N.Y., 1969.
- In a kinetic study of the general base-catalyzed deamination of the related amidine, 1,3-diphenyl-2-imidazolium chloride,¹⁵ it was found that HPO_4^{2-} had an exalted catalytic effect due to a possible bifunctional acid-base catalytic role. This special effect may explain bisulfite ion's enhanced catalytic properties here as well as those of pyrosulfite ion.
- D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **89**, 7088 (1967).
- S. L. Johnson, *Adv. Phys. Org. Chem.*, **5**, 237 (1967).
- L. Schaleger and F. A. Long, *Adv. Phys. Org. Chem.*, **1**, 1 (1963).
- R. P. Bell and P. E. Sorenson, *J. Chem. Soc., Perkin Trans. 2*, 1740 (1972).
- R. P. Bell and D. G. Horne, *J. Chem. Soc., Perkin Trans. 2*, 1371 (1972).
- Since the values reported here indicated a decreasing ΔS^\ddagger with increasing mole fraction of water,¹⁸ a value of $\delta\Delta S^\ddagger$ less than 17.6 eu would be expected for one mole fraction of water.
- (a) R. K. Chaturvedi and E. H. Cordes, *J. Am. Chem. Soc.*, **89**, 1230 (1967); (b) E. H. Cordes and W. P. Jencks, *ibid.*, **85**, 2843 (1963); (c) T. C. Plichter, S. Kochler, and E. H. Cordes, *J. Am. Chem. Soc.*, **90**, 7072 (1968).
- H. E. Jones, J. C. LeBlanc, and K. B. Freeman, *J. Mol. Biol.*, **13**, 849 (1965).
- R. H. DeWolfe and R. M. Roberts, *J. Am. Chem. Soc.*, **75**, 2942 (1953); R. H. DeWolfe, *ibid.*, **82**, 1585 (1960).
- D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **89**, 7098 (1967).
- R. H. DeWolfe and J. R. Keefe, *J. Org. Chem.*, **27**, 493 (1962).
- D. R. Robinson, *J. Am. Chem. Soc.*, **92**, 3138 (1970).
- C. C. Cheng and L. R. Lewis, *J. Heterocycl. Chem.*, **1**, 260 (1964).
- Calculated values of K_{eq} are subject to uncertainties in ϵ since the pyrosulfite concentration is calculated from the observed absorbance by the expression $[\text{pyrosulfite}] = (\text{OD}_{\text{pyrosulfite}}/\epsilon_{\text{pyrosulfite}})$. For example, had an alternative value of $\epsilon = 4 \times 10^3$ been used,²⁹ the calculated K_{eq} would have been $(1.07 \pm 0.04) \times 10^{-1} \text{ M}^{-1}$. Values of ϵ and K reported earlier^{29–32} have been criticized¹⁰ and the best available value was used here.
- R. M. Golding, *J. Chem. Soc.*, 3711 (1960).
- G. P. Arkhipova and I. I. Chistyakova, *Zh. Prikl. Khim. (Leningrad)*, **44**, 2193 (1971).
- T. E. Eriksen and J. Lind, *Acta Chem. Scand.*, **26**, 3325 (1972).
- E. Hayon, A. Treinen, and J. Wilf, *J. Am. Chem. Soc.*, **94**, 47 (1972).
- A. Albert and E. D. Serjeant, "Ionization Constants of Acids and Bases", Methuen and Co., London, 1962.
- G. Kortum, W. Vogel, and K. Andrussov, "Dissociation Constants of Organic Acids in Aqueous Solutions", Butterworths, London, 1961.
- D. D. Perrin, "Dissociation Constants of Inorganic Acids and Bases In Aqueous Solutions", Butterworths, London, 1965.
- D. D. Perrin, "Dissociation Constants of Organic Acid and Bases In Aqueous Solutions", Butterworths, London, 1965.
- L. G. Sillen and A. E. Martell, "Stability Constants of Metal-Ion Complexes", 2nd ed., The Chemical Society, London, 1964.
- L. G. Sillen and A. E. Martell, "Stability Constants of Metal-Ion Complexes", Supplement No. 1, The Chemical Society, London, 1971.
- H. A. Sober, Ed., "Handbook of Biochemistry", Chemical Rubber Publishing Co., Cleveland, Ohio 1970.