Mechanism of Hydrolysis of Halogenated Nitrosoureas

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Abstract □ The hydrolysis kinetics of halogenated nitrosoureas were investigated using chlorozotocin as a model. Evidence is presented to show that the hydrolytic reaction of halogenated nitrosoureas between pH 3 and 8 is a summation of spontaneous water and hydroxide-ion-catalyzed reactions; the later reaction is the sum of two parallel reactions. The relative contribution of each reaction changes with pH and results in different product distributions. Dianionic phosphate (HPO_4^{2-}) increased the amount of free chloride-ion production without significantly altering the hydrolysis rate. A mechanism is proposed to explain this behavior. The role of hydrolytic decomposition products produced at physiological pH on the biological activity of nitrosoureas is discussed.

Keyphrases □ Nitrosoureas, halogenated—kinetics and mechanism of hydrolysis, effect of pH □ Hydrolysis—kinetics and mechanism, halogenated nitrosoureas, effect of pH □ Kinetics—hydrolysis of halogenated nitrosoureas, effect of pH □ Mechanism—hydrolysis of halogenated nitrosoureas, effect of pH

Nitrosoureas are a mainstay of cancer chemotherapy, and several of them have undergone extensive clinical trials. Nevertheless, the mechanism of action of nitrosoureas (Table I) is not well understood. The difficulty in explaining their mechanism is related mainly to the instability of nitrosoureas in plasma, which gives rise to many possible reactive intermediates.

A useful approach to the understanding of these reactive intermediates has been chemical kinetic studies at or near physiological pH (1–8). A systematic kinetic study of nonhalogenated nitrosoureas (*i.e.*, where the N-1 substituent is a simple alkyl group, R) led Garrett and Goto (7) to propose a mechanism yielding a carbonium ion, R^+, at physiological pH. However, systematic kinetic studies on 1-(2-chloroethyl)nitrosoureas, which exhibit even greater antileukemic activity, have not been reported.

BACKGROUND

Previous kinetic studies on the hydrolysis of halogenated nitrosoureas II–IV (Table I) were contradictory (1–4). They primarily emphasized the analysis of hydrolytic products around physiological pH or pH 4. In the hydrolysis of nonhalogenated nitrosoureas, the major product arising from the N-1 alkyl substituent is the corresponding alcohol (1–6). However, with 1-(2-chloroethyl)nitrosoureas, the major products at pH 4 are acetaldehyde and the chloride ion instead of 2-chloroethanol. As the pH is increased, however, more 2-chloroethanol is produced at the expense of acetaldehyde.

These results indicate that additional hydrolytic mechanisms may exist for 1-(2-chloroethyl)nitrosoureas that are not available to nonhalogenated nitrosoureas (1, 3), and two mechanisms have been proposed (1–3) to account for the hydrolysis products. Neither mechanism, however, adequately explains the changes in product distribution observed at various pH values. Since the 1-(2-chloroethyl) substituent may partly determine the chemical and biological activity of these compounds, a systematic kinetic study of a 1-(2-chloroethyl)nitrosourea was warranted.

Since the rate and extent of chloride-ion production would be expected to yield valuable information on the role of the N-1 halogen substituent, chlorozotocin (I), a monochlorinated nitrosourea (*i.e.*, having a β-chloro substituent on N-1 only), was chosen as the major compound. By studying this monochlorinated compound, ambiguities about the source of the chloride ion produced could be eliminated. Furthermore, chlorozotocin has greater aqueous solubility than other monochlorinated nitrosoureas (III and IV), which allows quantitation in totally aqueous solvents. Also, a detailed kinetic study on streptozotocin (V) (a nonchlorinated analog of I) was available (5), and possible analysis of comparative kinetic values of I and V could be useful in understanding the role of the 1-(2-chloroethyl) substituent. Finally, it was necessary to conduct limited kinetic studies on II–IV and VI (Table I) to clarify some ambiguous results reported in the literature and to support the proposed hydrolytic pathways for other halogenated nitrosoureas.

EXPERIMENTAL

Materials—The nitrosoureas and their analogs[1], I[2], II[3], III[4], IV[5], V[6], VI[7], and 1-(2-chloroethyl)-3-(2-deoxy-D-glucopyranosyl)urea[8] (VII), were used as received. All other chemicals were reagent grade.

Buffers—Citrate and phosphate buffers (pH 2–8) were prepared from 0.25 *M* stock solutions of citric acid and monobasic sodium phosphate, respectively, by the addition of sodium hydroxide to the desired pH.

Tris(hydroxymethyl)aminomethane buffer (pH 6–8.5) was prepared by dissolving the appropriate amount of base in distilled water and adjusting the pH with hydrochloric acid. When chloride-ion determinations were to be made, nitric acid was used for pH adjustment.

No attempts were made to maintain a constant ionic strength because preliminary studies revealed that ionic strength did not appreciably affect the hydrolysis rate.

Equipment—Absorbance readings were determined using a recording spectrophotometer[9] equipped with a programmable thermostated cell holder[10]. Temperature in the cell compartment was regulated within ±0.1° using a circulating water bath. Chloride-ion determinations were made with an automatic chloride titrator[11]. Fluoride-ion determinations were made with a combination fluoride electrode[12] using the expanded scale of a pH meter[13]. All pH measurements were conducted at room temperature.

Kinetic Studies—*Spectrophotometric Determination*—The first-order rate constants for the hydrolysis of nitrosoureas were determined as reported (6, 7) by following the loss of UV absorption at 231 nm with time. The general procedure consisted of making a stock solution of nitrosourea (~1×10^{-2} *M*) in methanol, diluting the solution with thermally preequilibriated buffers in a 1-cm UV cell to an initial concentration of ~1×10^{-4} *M*, and recording the absorbance at 231 nm *versus* time. The solution pH remained constant (within 0.05 unit) throughout the kinetic runs.

Chloride-Ion Measurements—The decomposition rate of I also was determined by following the production of free chloride ion. A weighed amount of nitrosourea was dissolved in thermally equilibrated buffer (~1×10^{-3} *M*), and the chloride-ion concentration was determined by titrating an aliquot out of the reaction mixture at specified intervals. For this purpose, 2.0 ml of the reaction mixture was placed in a 6-ml beaker and 2.0 ml of titration reagent [consisting of 0.2 *M* HNO_3 and 20% (v/v) acetic acid] was added.

Titration was performed by an electrolysis of a silver wire to produce free silver ions at a constant rate, the end-point of titration being determined by an excess of free silver ions. The time of electrolysis (which is displayed by the titrator), after appropriate correction for the reagent blank, was proportional to the amount of free chloride ion. The titrator was calibrated with standard sodium chloride solution, and the medium range of the titrator was used. During the titration procedure, the mixture

[1] Obtained through the National Cancer Institute.
[2] Synthesized by Stark Associates, lot PH 37-43-1.
[3] Synthesized by Merck & Co., lot L-596025-0-7.
[4] Synthesized by Stark Associates, lot CC 52-21-1.
[5] Synthesized by Ash-Steven, lot JOS-01-282-1.
[6] Synthesized by Pfanstehl, lot 10337-B.
[7] Synthesized by Southern Research Institute, lot 4689-23-1.
[8] Synthesized by Stark Associates, lot PH 28-98-1.
[9] Cary-17, Varian Instruments, Palo Alto, Calif.
[10] Cary-1729 programmer, Varian Instruments, Palo Alto, Calif.
[11] Model 4-4433, American Instrument Co., Silver Spring, Md.
[12] Model 96-09, Orion Research, Cambridge, Mass.
[13] Model 76, Beckman Instruments, Fullerton, Calif.

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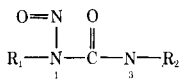
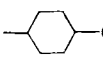
Table I—Structures of Various Nitrosoureas



Drug	Chemical Name	R_1	R_2	Mol. Wt.
Chlorozotocin (I)	1-(2-Chloroethyl)-3-(2-deoxy-D-glucopyranosyl)-1-nitrosourea	CH_2CH_2Cl	*O*-Glucopyranosyl	313.7
II	1,3-Bis(2-chloroethyl)-1-nitrosourea	CH_2CH_2Cl	CH_2CH_2Cl	213.9
III	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea	CH_2CH_2Cl		233.5
IV	1-(2-Chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea	CH_2CH_2Cl		247.5
Streptozotocin (V)	1-Methyl-3-(2-deoxy-D-glucopyranosyl)-1-nitrosourea	CH_3	*O*-Glucopyranosyl	265.3
VI	1,3-Bis(2-fluoroethyl)-1-nitrosourea	CH_2CH_2F	CH_2CH_2F	181.0

pH was decreased to about 2. The short titration time (less than 1 min) along with the long half-life of I at pH 2 and room temperature (approximately 40 hr) assured that no significant nitrosourea degradation took place during the analytical procedure. The extent of chloride-ion liberation (or percentage chloride ion) was calculated as the percentage of the theoretical maximum (1 mole of drug liberating 1 mole of chloride ion). For this purpose, the drug powder was assumed to be pure; the weight of drug taken was converted to a molar basis directly to calculate the theoretical maximum of chloride liberation.

In the kinetic runs from chloride-ion measurements, particularly above pH 6.50, the end-points were slightly inaccurate, because other slow reactions probably were yielding some free chloride. Furthermore, a finite time period was needed to pipet accurately an aliquot out of the reaction mixture and then to add the titration reagent. Because of the gas bubbles (nitrogen, carbon dioxide, *etc.*) being generated during the reaction, there were some inaccuracies in pipetting. Therefore, the rate constants calculated by chloride-ion measurements have a higher uncertainty than those calculated from UV measurements.

Fluoride-Ion Measurements—A weighed amount of VI was dissolved in the buffer ($\sim 1 \times 10^{-3}$ *M*), and the fluoride-ion electrode was dipped into the reaction mixture. The millivolt reading on the pH meter was noted at different times, and the fluoride-ion concentration was determined from a standard calibration plot of millivolts *versus* sodium fluoride concentration prepared in the identical buffer. The limitation of the electrode allowed fluoride-ion measurements only between pH 5.0 and 7.0. These measurements were carried out at room temperature.

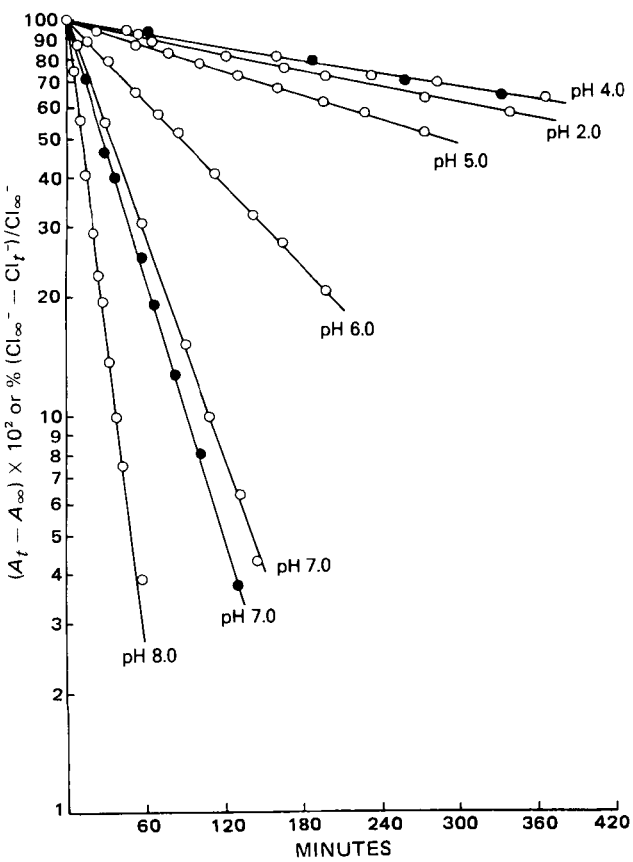


Figure 1—*Apparent first-order plots for the hydrolysis of I. Key:* ○, *from UV absorbance; and* ●, *from chloride-ion determination in 0.01* M *citrate buffer (pH 2.0, 4.0, and 5.0) and 0.01* M *phosphate buffer (pH 6.0, 7.0, and 8.0) at 37°. The* A_∞, A_t, *and* A_0 *are the absorbances at time infinity, time* t, *and time zero, respectively;* Cl_∞^- *and* Cl_t^- *are the amounts of the chloride ion produced at time infinity and time* t, *respectively. Infinity readings were taken after approximately seven half-lives.*

RESULTS AND DISCUSSION

Chlorozotocin (I) hydrolysis followed first-order kinetics between pH 1 and 9.5 as measured by either the rate of loss of UV absorbance at 231 nm or the rate of free chloride-ion production (Fig. 1). The observed rate constants for I hydrolysis as a function of pH are presented in Table II. The extent of free chloride-ion production changed with pH and buffer composition (Table III). These results suggest that three different mechanisms (Schemes I–III) exist for the hydrolysis of I and other 1-(2-haloethyl)nitrosoureas and that the relative contribution of each depends on the pH of the reaction mixture. The unstable intermediates formed during these first steps in Schemes II and III further decompose to yield the final products (Schemes IV and V).

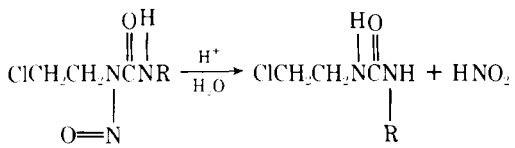
Acid-Catalyzed Hydrolysis—In the acidic region (pH < 2) the hydrolytic rate of I increased sharply with decreasing pH, indicating hydrogen-ion catalysis. Scheme I presents the classical hydrogen-ion-catalyzed hydrolysis of the nitroso group to yield the nitrite ion but no chloride ion. In contrast, the water- and hydroxide-ion-catalyzed reac-

Table II—Observed First-Order Rate Constants, K_{obs}, for Chlorozotocin (I) Decomposition in Aqueous Solution at Various pH Values and Buffer Concentrations at 37°

Buffer Concentration, *M*	pH	Observed Rate Constants, min^{-1}: From Absorbance Measurements	From Chloride-Ion Determinations
Hydrochloric acid			
0.50	0.42	0.0289	—
0.01	2.00	0.00159	—
Citrate			
0.01	2.01	0.00145	—
0.01	2.81	0.00140	—
0.01	3.28	0.00138	—
0.01	4.00	0.00140	0.0017
0.01	5.00	0.00245	—
0.01	6.00	0.00785	0.0061
Phosphate			
0.01	6.00	0.0078	—
0.10	6.50	0.0139	—
0.01	7.00	0.024	0.021
0.10	7.00	0.025	0.022
0.20	7.00	0.025	0.024
0.01	8.00	0.062	—
0.10	8.00	0.068	—
Tris(hydroxymethyl)-aminomethane			
0.01	8.00	0.0552	—

Table III—Production of Chloride Ion from the Decomposition of Chlorozotocin (I) in Various Buffers at 37°

Buffer Concentration, *M*	pH	Total Percent Chloride Ion
Nitric acid		
0.5	0.40	18.7
0.10	1.10	57.1
0.03	1.50	72.0
Citrate		
0.10	2.00	81.4
0.10	2.63	84.8
0.10	3.00	87.8
0.10	3.13	90.5
0.10	3.50	91.6
0.10	4.00	90.5
0.10	5.02	67.9
0.10	5.55	47.0
0.10	6.00	41.6
0.10	6.50	35.1
0.10	7.00	30.0
0.02	7.00	29.6
Phosphate		
0.01	6.00	41.6
0.10	6.00	41.0
0.01	7.00	33.6
0.10	7.00	51.4
0.20	7.00	59.8
0.40	7.00	68.6
0.01	7.50	32.8
0.10	7.50	52.8
Tris(hydroxymethyl)aminomethane		
0.02	7.00	30.8
0.10	7.00	31.2
0.10	8.45	25.7
0.10	9.26	27.2



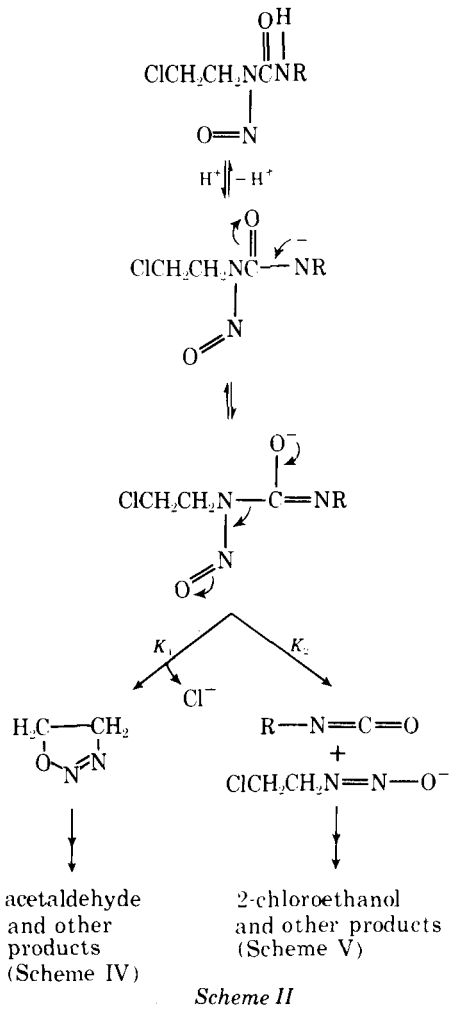
Scheme I

tions (Schemes II and III, respectively) yield chloride ion. Therefore, as the pH is lowered, the hydrogen-ion-catalyzed reaction becomes increasingly important, and there should be a decrease in chloride-ion production. As seen in Table III, the yield of chloride ion decreased as the pH was decreased below pH 3, thus supporting increasing hydrogen-ion catalysis at lower pH values.

Hydroxide-Ion-Catalyzed Reaction—Two separate mechanisms were proposed (2, 3) for hydroxide-ion-catalyzed hydrolysis of nitrosoureas, both involving prior ionization of the N-3 proton. These mechanisms were based on the product and kinetic analysis at various pH values. The major product arising from the hydrolysis of nonhalogenated nitrosoureas is the corresponding alcohol, and the rate of hydrolysis increases with increasing pH above about pH 4. This result led Garrett and Goto (7) to propose a mechanism yielding the corresponding carbonium ion (same as step K_2 in Scheme II, which yields the carbonium ion as shown in Scheme V) and Reed *et al.* (2) to propose the same mechanism for 1-(2-chloroethyl)nitrosoureas.

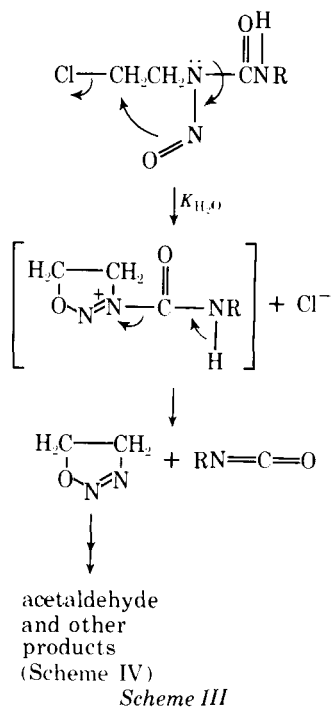
However, the major product arising from 1-(2-chloroethyl)nitrosoureas at pH 4 is not 2-chloroethanol but acetaldehyde (1, 3). Observation of this unusual product led Montgomery *et al.* (3) to propose a cyclic mechanism involving the attack of negatively charged carbonyl oxygen to form an unstable oxazolidine intermediate, which then decomposes to acetaldehyde (similar to step K_1 in Scheme II, except that the carbonyl oxygen instead of the nitroso oxygen displaces the chloride). Both mechanisms, however, do not explain fully the experimental findings. Thus, the cyclic mechanism, if it is valid over the pH 3–8 range, should yield similar amounts of the chloride ion irrespective of pH and should not yield any 2-chloroethanol. At pH 4, the chloride-ion yield was about 90% of the theoretical value, and no 2-chloroethanol formed. However, at pH 7, the chloride-ion yield was only about 30%, and a significant amount (40% of the theoretical) of 2-chloroethanol formed at the expense of acetaldehyde (1, 3). The cyclic mechanism of Montgomery *et al.* (3) alone fails to explain this change in product distribution.

Similarly, the 2-chloroethylcarbonium-ion intermediate proposed by Reed *et al.* (2) does not account for the acetaldehyde product. Reed's

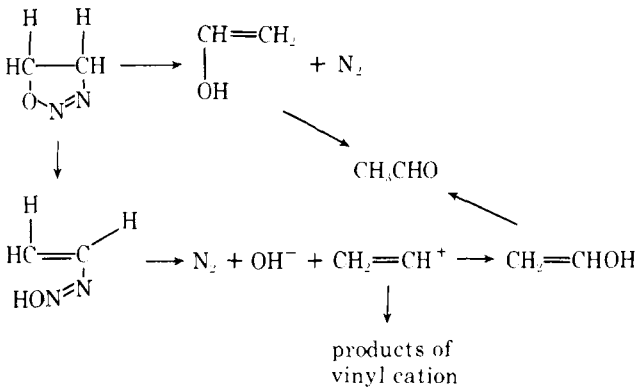


Scheme II

mechanism (2) postulates that dehydrohalogenation of the 2-chloroethylcarbonium ion yields vinyl cation, which subsequently forms vinyl alcohol and rearranges to acetaldehyde. It is difficult to explain why the



Scheme III

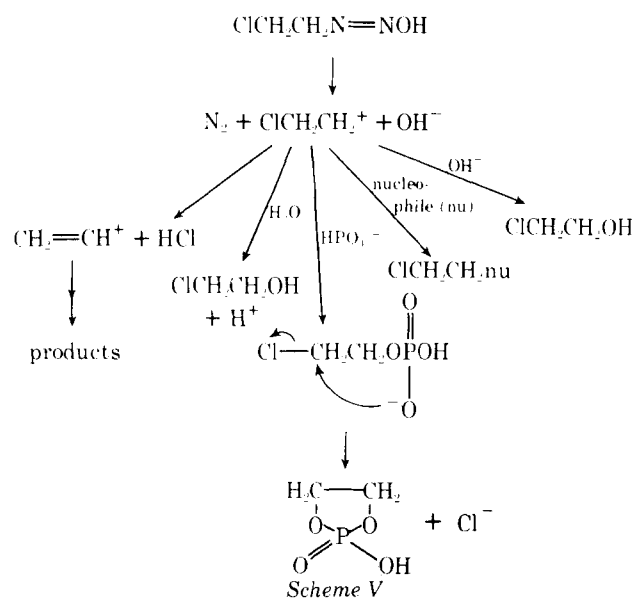


Scheme IV

yield of acetaldehyde should decrease with increasing pH. Moreover, 3-(2-chloroethyl)-1-triazinoimidazole-4-carboxamide, an excellent precursor of the 2-chloroethylcarbonium ion (9), was reported to yield mostly 2-chloroethanol in water (1). Therefore, the 2-chloroethylcarbonium ion does not appear to be the source of acetaldehyde; the cyclic structure, either the oxazolidine intermediate proposed by Montgomery or the oxadiazole intermediate proposed in Scheme II, is more likely the source. Furthermore, as will be discussed later, in unbuffered aqueous solution (pH ~4) where maximal acetaldehyde production is observed (1), ionization of the N-3 proton is not important and an alternative mechanism, other than the 2-chloroethylcarbonium ion, must yield the acetaldehyde.

Studies on I hydrolysis (Tables II and III) showed that, although the total amount of the chloride ion produced generally decreased with increasing pH, the hydrolytic rates determined by either the loss of UV absorbance or chloride-ion production were the same at each pH value. This result indicated that the reactions responsible for the loss of chloride ion and the loss of nitroso moiety (the chromophore at 231 nm) were not always the same. This observation provided the basis for proposing a parallel pathway for alkaline hydrolysis of I in which involvement of the chloroethyl moiety is the differentiating factor (Scheme II).

The scheme essentially integrates the two reported mechanisms (2, 3). Pathway K_1 involves a cyclic mechanism similar to that proposed by Montgomery *et al.* (3) for halogenated nitrosoureas, except that the nitroso oxygen rather than the carbonyl oxygen is considered the cyclizing nucleophile. The reasoning for a cyclic mechanism *via* the nitroso oxygen will be discussed later. Pathway K_2 is the same as that proposed by Reed *et al.* (2). The occurrence of both mechanisms around physiological pH was suggested, but no evidence was provided (10). The kinetic data reported in this paper support the parallel mechanism of Scheme II in which the change in product distribution with changing pH from 4 to 7 can be accounted for by assuming that increasing pH specifically favors pathway K_2 over K_1, thus yielding more 2-chloroethanol and smaller amounts of the chloride ion and acetaldehyde.



Scheme V

Table IV—Observed First-Order Rate Constants and Chloride-Ion Production from the Decomposition of the Urea Analogs of Chlorozotocin (VII) at pH 4.0–7.0 at Room Temperature

Buffer, 0.05 *M*	pH	Observed Rate Constant, min^{-1}	Total Percent Chloride Ion
Citrate	4.0	0.00085	36.8
Citrate	6.0	0.00064	39.4
Phosphate	6.0	0.00067	36.6
Phosphate	7.0	0.00073	36.9

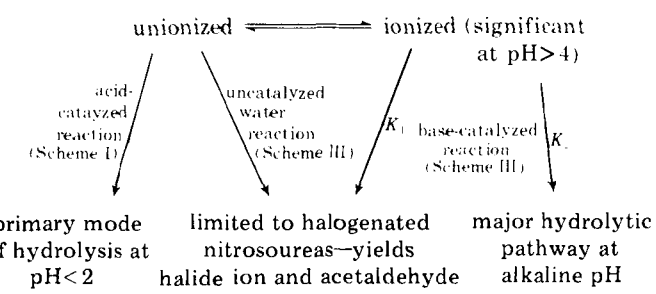
Water Reaction—Careful analysis of the studies at pH 2–4 revealed that some results cannot be explained solely by the reactions presented in Schemes I and II. Thus, a plateau region was observed between pH 3.0 and 4.0, where observed rates of hydrolysis of I and extents of chloride-ion production were similar (Tables II and III). Based on Schemes I and II, similar yields of the chloride ion would be difficult to justify if this plateau region is due only to a competition between base- (Scheme II) and acid- (Scheme I) catalyzed reactions because acid-catalyzed hydrolysis does not yield any free chloride ion. Therefore, another reaction yielding free chloride ion must be considered.

This reasoning formed the basis for proposing a spontaneous water reaction in Scheme III. Evidence for the proposed water reaction on the unionized I was obtained by conducting kinetic studies on its urea analog, VII. Table IV shows the extent and the rate of chloride production from VII at pH 4–7. Between pH 4.0 and 7.0, the rate and extent of chloride-ion production were independent of pH and buffer species. Furthermore, the chloride-ion production rate was too fast (compared to hydrolysis of simple alkyl halides) to be explained by simple nucleophilic displacement by water and other buffer species. At pH 4–7, VII, a substituted urea, is not expected to ionize appreciably; therefore, the chloride ion must come from the cyclization involving the carbonyl oxygen of unionized VII.

The pKa of the N-3 proton of nitrosoureas is estimated to be 8–9 (5, 6); therefore, it is reasonable to conclude that no appreciable ionization of I would take place at pH 3–4. Thus, the major reaction at pH 3–4 is attributed to the water reaction involving the unionized drug (Scheme III) where hydroxide- and hydrogen-ion catalyses are least significant. On the acid side, the hydrogen-ion-catalyzed reaction becomes more important and, at pH > 4, hydroxide-ion catalysis is increasingly the dominant reaction. Scheme VI summarizes the pathways for the decomposition of nitrosoureas.

Further Evidence for Schemes II and III—The cyclic mechanisms K_{H_2O} (Scheme III) and K_1 (Scheme II), which are only available to 1-(2-haloethyl)nitrosoureas, are more evident from the kinetic studies of V (nonchlorinated analog of I). As presented in Table V, the hydrolysis rate of I at pH 4.0 was about seven times faster than that of V. As the pH became increasingly alkaline, the hydrolytic rates of I and V became closer (Table V), and the amount of chloride liberated from the hydrolysis of I decreased (Table III). This result indicates that, at higher pH (*i.e.*, ~pH 7–8), both I and V must be primarily hydrolyzing by a similar mechanism and that the increased reactivity of I at lower pH (~pH 3–4) may be related to a specific mechanism involving the 2-chloroethyl moiety available only to I.

The proposed mechanisms of Schemes II and III are supported also by kinetic studies conducted with VI, where the β-fluoro substituent, a poor leaving group, was shown to be resistant to decomposition by pathway K_{H_2O} (Scheme III). As seen in Table V, the decomposition rate of VI at pH 4.0 was about 10 times slower than that of its chlorinated analog, II, and was similar to that of the nonhalogenated nitrosourea, V. This result indicates that VI is relatively resistant to halogen-assisted hydrolysis as presented in Scheme III. However, at pH 7.0, the hydrolysis



Scheme VI

Table V—Comparison of Observed Hydrolytic Rate Constants at pH 4.0 and 7.0 for Various Nitrosoureas at 37°

Nitrosourea	Buffer	pH	Observed Rate Constant, min^{-1} [a]	Total Percent Chloride Ion
Chloro-zotocin (I)	0.01 *M* Citrate	4.00	0.00136	90.5
	0.01 *M* Phosphate	7.00	0.0248	33.6
	—	9.25	0.693[b]	—
II	0.01 *M* Citrate	4.00	0.0013	94
	0.01 *M* Tris(hydroxy-methyl)aminomethane	7.00	0.0086	—
	0.01 *M* Phosphate	7.00	0.0109	41.3
	0.10 *M* Phosphate	7.00	0.0112	—
	0.20 *M* Phosphate	7.00	—	55.8
III	0.01 *M* Tris(hydroxy-methyl)aminomethane	7.00	0.00635	—
	0.10 *M* Phosphate	7.00	0.0089	—
IV	0.01 *M* Tris(hydroxy-methyl)aminomethane	7.00	0.0064	—
	0.10 *M* Phosphate	7.00	0.0091	—
Strepto-zotocin (V)	0.17 *M* Acetate	3.98	0.00020[c]	—
	0.125 *M* Phosphate	6.89	0.0054[c]	—
	—	9.25	0.594[b]	—
VI	0.10 *M* Citrate	4.00	0.00015	—
	0.01 *M* Tris(hydroxy-methyl)aminomethane	7.00	0.0072	—
	0.05 *M* Phosphate	7.00	0.011	—

[a] From absorbance measurements. [b] Room temperature. [c] Estimated by doubling the rate constant at 30.2° from Tables I and II, Ref. 5.

rates of II and VI were similar, indicating that the 2-chloroethyl-moiety-dependent water reaction is not important and that the reactions presented in Scheme II are dominant. Step K_2 is not expected to be affected by the fluoro group; in step K_1, the negatively charged nitroso group is expected to displace some fluoride (Table VI). These rate constants at pH 7.0 agreed well with those reported at pH 7.2 in phosphate buffer (1, 3).

Further evidence supporting the mixed water- and hydroxide-ion-catalyzed reaction from pH 3.0 to 8.0 comes from the calculation of values of the chloride ion produced at various pH values. At pH > 8, the major reaction is hydroxide-ion catalysis where the extent of the chloride ion produced plateaus at about 26% of the maximal theoretical value (Table III). Thus, at pH > 8, a ratio of K_1/K_2 of 26/74 is expected. If one assumes that this ratio does not change from pH 3 to 8, it can be concluded that, for each mole of ionized I degrading by Scheme II, 0.26 mole of chloride ion is produced. However, unionized chlorozotocin undergoing hydrolysis through Scheme III should yield ionic chloride on an equimolar basis. Thus, the theoretical amount of the chloride ion can be calculated from Eq. 1 if the fraction of chlorozotocin undergoing degradation by Schemes II and III is known:

Table VI—Effect of Various Nucleophiles on Observed Rate Constants and Fluoride-Ion Production from VI at pH 7.0 and Room Temperature

Buffer	Nucleophile	Observed Rate Constant[a], min^{-1}	Total Percent Fluoride Ion
0.10 *M* Tris(hydroxymethyl)-aminomethane	—	—	20.5
0.01 *M* Phosphate	—	0.00271	26.5
0.01 *M* Phosphate	0.05 *M* $Na_2S_2O_3$	0.00233	21.7
0.40 *M* Phosphate	—	0.00265	75.7
0.40 *M* Phosphate	0.05 *M* $Na_2S_2O_3$	0.00280	65.8
0.20 *M* Phosphate	—	0.00281	59.5
0.20 *M* Phosphate	0.20 *M* KNO_3	0.00299	59.0
0.20 *M* Phosphate	0.20 *M* KCl	0.00299	59.4
0.20 *M* Phosphate	0.20 *M* NaI	0.00305	50.0

[a] From fluoride-ion measurements.

Table VII—Comparison of Chloride-Ion Production Observed Experimentally and That Calculated from Eq. 1 for Chlorozotocin (I) Decomposition at 37°

pH	K_{obs}, min^{-1}	K_{H_2O}/K_{obs}	Total Percent Chloride Ion	
			Theoretical	Experimental
3.0	0.00140	0.8643	90.0	90.5
4.0	0.00140	0.8643	90.0	90.5
5.0	0.00245	0.4939	62.5	67.9
6.0	0.00785	0.1541	37.4	41.6
6.5	0.0139	0.0871	32.4	35.1
7.0	0.025	0.048	29.6	30.0
8.0	0.0610	0.0198	27.5	26.0

$$\text{total \% chloride} = 100\,(X) + 26\,(1 - X) \qquad \text{(Eq. 1)}$$

where X is the fraction of chlorozotocin undergoing hydrolysis by Scheme I and can be calculated from:

$$X = \frac{K_{H_2O}}{K_{obs}} \qquad \text{(Eq. 2)}$$

where K_{obs} is the observed first-order rate constant. Hydrogen-ion catalysis is assumed to be negligible above pH 3.0.

At pH 3.0–4.0, maximal chloride-ion formation is observed equivalent to 90% of the chlorozotocin decomposition. By substituting this value in Eq. 1, X at this pH is found to be 64/74 (0.865). By substituting this value of X and K_{obs} at pH 4.0 in Eq. 2:

$$0.865 = K_{H_2O}/(0.0014\ min^{-1}) \qquad \text{(Eq. 3)}$$

$$K_{H_2O} = (0.865)\,(0.0014) = 0.0012\ min^{-1} \qquad \text{(Eq. 4)}$$

The value of X at any pH can be calculated from Eq. 2 if K_{obs} at that pH is known. By substituting this value of X in Eq. 1, theoretical chloride values can be calculated. Table VII presents the theoretically calculated values of chloride ion along with the observed values at pH 3.0–8.0. The close agreement between theoretical and experimental values lends support to the proposed mechanisms and to the assumption that K_{H_2O} (Scheme III) and the ratio K_1/K_2 (Scheme II) are relatively unaffected by pH.

Cyclic Oxadiazole Intermediate—In Schemes II and III, formation of a cyclic oxadiazole intermediate involving the nitroso oxygen as the attacking nucleophile is proposed. Montgomery *et al.* (1, 3), however, proposed an oxazolidine intermediate involving the carbonyl oxygen as the attacking nucleophile. This proposal was not supported by any direct evidence but was based on the fact that acetaldehyde and products derived from vinyl cation were formed. However, the oxadiazole intermediate as proposed in Schemes II and III also could account for the production of acetaldehyde and products of vinyl cation as shown in Scheme IV. Therefore, based on product analysis alone, either of the two oxygens could act as the nucleophile in displacing the chloride ion.

Evidence supporting the cyclic oxazolidine intermediate comes from the fact that VII (urea analog of I), which does not contain the nitroso moiety, liberates the chloride ion at a rate comparable to I in aqueous buffers at pH 4.0 (Tables II and IV). The absence of the nitroso group in VII allows unequivocal assignment of the carbonyl oxygen as the attacking nucleophile. At this pH, the urea would be unionized and thus it must be the polarized carbonyl group that displaces the chloride group. However, the yield of the chloride ion from the urea compound was only 40%, whereas in I the yield was 90% at pH 4; the kinetic schemes presented in this paper dictate that the cyclic mechanism yield nearly 100% free chloride ion. Since the yield of chloride ion was only 40% in the absence of nitroso group, it is more reasonable to assume that nitroso oxygen is the primary cyclizing nucleophile in I.

Furthermore, although the carbonyl oxygen will be highly polarized in alkyl-substituted analogs (VII), this polarity will be less pronounced in nitrosoureas where the N-1 nitrogen lone pair of electrons will be strongly resonating with the nitroso group and thus be unavailable for amide resonance with the carbonyl group. This assumption is supported by the IR spectra[14] of I and VII where the carbonyl peak for VII appears as a doublet at 1575 and 1620 cm^{-1} (typical of ureas, showing considerable resonance), but that for I appears upfield at 1690 cm^{-1}, or roughly the same as that of an ordinary carbonyl group. This relative lack of polarity of the carbonyl in nitrosoureas (upfield shift of carbonyl peak indicating less single bond character) makes it an unlikely candidate to act as a

[14] In potassium bromide pellet.

nucleophile in displacing the β-chloro group of the N-1 substituent and favors the highly polarized *N*-nitroso group as the nucleophile giving a cyclic oxadiazole intermediate.

Also, if the carbonyl oxygen is the attacking species, then the cyclic oxazolidine intermediate formed will still have the nitroso group (the chromophore at 231 nm) and, therefore, must decompose instantly with the loss of the nitroso chromophore to account for the observed first-order hydrolysis kinetics as determined by the UV method. There is no reason to expect such instability of 2-oxazolidines. In contrast, the oxadiazole intermediate proposed in Schemes II and III has no nitroso group, so first-order kinetic behavior does not require its instant decomposition. Other evidence against the carbonyl oxygen acting as the cyclizing nucleophile (and thus favoring the nitroso oxygen as the primary nucleophile) is the similarity of rates observed for various nitrosoureas at pH 4.0 and 7.0 (Table V). Nucleophilic attack of the carbonyl oxygen on the β-chloro group should be influenced by steric and electronic effects of various N-3 substituents, whereas those effects would not be as significant for the attack by nitroso oxygen. Therefore, the cyclic structure presented in Schemes II and III is the more likely intermediate. However, because of the lack of strong direct evidence, this assignment in favor of the oxadiazole intermediate is tentative; the oxazolidine intermediate as proposed by Montgomery *et al.* (1, 3) cannot be ruled out.

Role of Phosphate in Hydrolysis—The role of the phosphate dianion (HPO_4^{2-}) in the catalysis of the overall hydrolysis rate of nitrosoureas has been controversial. Garrett (5) suggested a catalytic effect of HPO_4^{2-} on V hydrolysis. Loo *et al.* (4) demonstrated that phosphate buffer had no appreciable effect on the hydrolysis rate of II. Montgomery *et al.* (3) reported significant rate increases in phosphate buffer compared to tris(hydroxymethyl)aminomethane and acetate buffers at pH 7.2, specifically with III, where the rate in phosphate buffer was reported to be more than threefold faster than in acetate buffer. However, their reports (1, 3) did not mention the molarity of the acetate buffer used for comparison at pH 7.2 and its buffer capacity could not be ascertained. Therefore, it is difficult to compare data when the maintenance of pH is not demonstrated.

The present data (Table V) showed that the hydrolysis rates of nitrosoureas at pH 7.0 were about 25% faster in phosphate buffer than in tris(hydroxymethyl)aminomethane buffer. The present values at pH 7.0 in phosphate buffer agreed well with those reported (1, 3, 8).

Increased Chloride Production by Phosphate Dianion—The observation that the phosphate dianion increases the yield of chloride has not been reported. As can be observed from Table III, the yield of chloride increased substantially at pH 7.0 with an increase in the phosphate dianion concentration. However, at pH 6.0, where most of the phosphate would be monobasic, there was very little change in chloride production with increasing phosphate concentration. The yield of chloride was independent of buffer species and buffer concentration at pH 7.0 for other buffers tested [tris(hydroxymethyl)aminomethane and citrate (Table III)]. Although phosphate dianion appeared to have a specific effect in increasing the chloride-ion production, the overall decomposition rate (or the production rate of chloride) was unaffected by phosphate buffer concentration (Table II).

Indirect evidence for higher chloride production in phosphate buffer around pH 7 was presented when Montgomery *et al.* (1) reported a lower yield of 2-chloroethanol in phosphate buffer than in acetate buffer and Reed *et al.* (2) reported lower yields of 2-chloroethanol in phosphate buffer compared to the yield in tris(hydroxymethyl)aminomethane buffer. To determine if the reaction involved a simple nucleophilic displacement of chloride by phosphate dianion, studies were conducted with VII. As can be seen from Table IV, both the rate and extent of chloride-ion production were independent of pH and buffer species over pH 4–7. Therefore, simple nucleophilic substitution by phosphate dianion was ruled out. As the overall decomposition rate of I did not change with phosphate buffer strength (Table II), an almost twofold increase in the amount of chloride produced (by increasing the phosphate concentration from 0.01 to 0.2 *M*; Table III) must be explained by some other mechanism.

At pH 7.0, only the pathways presented in Scheme II are important. Therefore, higher production of chloride could come from either (*a*) phosphate dianion catalysis of the cyclic mechanism K_1, or (*b*) phosphate dianion reaction with 2-chloroethylcarbonium ion, which then cyclizes to yield free chloride (Scheme V). Route *a* could be ruled out on the basis that it would have to increase the overall rate. Pathway *b*, involving the intramolecular nucleophilic substitution, appears more likely because this reaction would not change the overall decomposition rate.

If pathway *b* is correct, then addition of strong nucleophiles, which would compete for 2-chloroethyldiazonium hydroxide (Scheme V), should decrease the yield of free chloride ion (*i.e.*, less 2-chloroethyl phosphate would form). Therefore, an attempt was made to determine the yield of chloride in phosphate buffer in the presence of strong nucleophiles such as sulfide, thiocyanate, thiosulfate, or iodide. However, the chloride ion could not be assayed in the presence of these nucleophiles by the method used in this study because the titration is based on precipitating chloride by silver cation, and the strong nucleophiles interfere with the assay of chloride.

An alternative compound, VI, was chosen because it liberates the fluoride ion, which could be assayed without being affected by the presence of strong nucleophiles. Also, if one assumes that both VI and other 2-(chloroethyl)nitrosoureas hydrolyze by similar mechanisms (Schemes I–V), the conclusion drawn in experiments with VI could be directly related to 1-(2-chloroethyl)nitrosoureas. As expected, increasing the phosphate concentration at pH 7.0 increased the yield of the fluoride ion (Table VI). However, addition of strong nucleophiles, thiosulfate or iodide ion, significantly decreased the yield of the fluoride ion (Table VI). In contrast, weaker nucleophiles (*i.e.*, chloride or nitrate ion) had no effect on the yield of the fluoride ion (Table VI). Therefore, it appears that strong nucleophiles, by competing with phosphate dianion, reduce the production of 2-fluoroethyl phosphate monoanion and thus reduce the yield of free fluoride ion by making smaller amounts of 2-fluoroethyl phosphate monoanion available for cyclic displacement (Scheme V). These results support the cyclic phosphate pathway presented in Scheme V. Simple nucleophilic displacement of fluorine is extremely difficult, and liberation of the fluoride ion almost certainly points to an intramolecular substitution by a negatively charged atom, both in Scheme II (K_1) and from 2-fluoroethyldiazonium hydroxide (Scheme V).

The problem of assigning the active alkylating intermediate under physiological conditions is still unanswered. Current reports favor 2-chloroethyl cation (or the corresponding diazonium hydroxide) as the active intermediate. The increased chloride-ion production by phosphate dianion led to a proposed mechanism whereby the 2-chloroethylcarbonium ion acts as a bifunctional alkylating agent of phosphate dianion (Scheme V). Other compounds having more than one nucleophilic center might react similarly with 2-chloroethylcarbonium ion. Low nucleophilic selectivity of the nitrosoureas led Veleminsky *et al.* (11) to suggest that the groups being alkylated are nitrogens or phosphates.

It is possible, therefore, that 2-chloroethylcarbonium ions derived from 1-(2-chloroethyl)nitrosoureas act as bifunctional alkylating agents to form interstrand or intrastrand nucleic acid cross-links. The inability of nonhalogenated nitrosoureas to form bifunctional reactive intermediates may be responsible for their lower activity. The higher activity of halogenated nitrosoureas also may be due to significant production of acetaldehyde and vinyl cation products at physiological pH (Scheme VI). Further experiments are needed to isolate and identify the complexes formed and to correlate the observed reactivity of phosphate dianion to the *in vivo* effects of chlorinated nitrosoureas.

REFERENCES

(1) J. A. Montgomery, R. James, G. S. McCaleb, M. C. Kirk, and T. P. Johnston, *J. Med. Chem.*, **18**, 568 (1975).

(2) D. J. Reed, H. E. May, R. B. Boose, K. M. Gregory, and M. A. Beilstein, *Cancer Res.*, **35**, 568 (1975).

(3) J. Montgomery, R. James, G. S. McCaleb, and T. P. Johnston, *J. Med. Chem.*, **10**, 668 (1967).

(4) T. L. Loo, R. L. Dion, R. L. Dixon, and D. P. Rall, *J. Pharm. Sci.*, **55**, 492 (1966).

(5) E. R. Garrett, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 767 (1960).

(6) E. R. Garrett, S. Goto, and J. F. Stubbins, *J. Pharm. Sci.*, **54**, 119 (1965).

(7) E. R. Garrett and S. Goto, *Chem. Pharm. Bull.*, **21**, 1811 (1973).

(8) P. A. Laskar and J. W. Ayers, *J. Pharm. Sci.*, **66**, 1073 (1977).

(9) Y. F. Shealy, C. A. O'Dell, and C. A. Krauth, *ibid.*, **64**, 177 (1975).

(10) G. P. Wheeler, in "ACS Symposium Series 30, Cancer Chemotherapy," A. C. Sartorelli, Ed., American Chemical Society, Washington, D.C., 1976, p. 87.

(11) J. Veleminsky, S. Osterman-Golkar, and L. Ehrenberg, *Mutat. Res.*, **10**, 169 (1970).