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Kinetics of Chlorambucil Hydrolysis Using High-Pressure Liquid Chromatography

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Abstract □ A stability-specific high-pressure liquid chromatographic (HPLC) method was developed to assay intact chlorambucil (I) in the presence of its hydrolytic decomposition products. The HPLC method was used to follow the degradation kinetics of I over pH 1.0–10.0 in the presence of various buffers with and without added chloride ion. In the absence of chloride ion, the hydrolysis of I followed first-order kinetics and the pH rate profile showed a sharp inflection around pH 2.5 attributable to the ionization of the nitrogen mustard and a shallower inflection around pH 5.0 attributable to the ionization of the carboxylic group. The rate was pH independent over pH 6.0–10.0 and independent of buffer species in the absence of chloride ion. In the presence of chloride ion, the kinetics of I hydrolysis was still first order. However, the degradation half-life at a particular pH and buffer concentration increased linearly with chloride concentration. Kinetic evidence is presented to show that the mechanism of chloride stabilization involves the attack of chloride ion on the unstable cyclic ethyleneimmonium intermediate to give back I. Implications of the kinetic data obtained on the fate of orally administered I are discussed.

Keyphrases □ Chlorambucil—kinetics of hydrolysis, high-pressure liquid chromatography □ Hydrolysis—chlorambucil, analysis of kinetics using high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis of chlorambucil hydrolysis kinetics □ Kinetics—chlorambucil hydrolysis, high-pressure liquid chromatography

Nitrogen mustards were one of the first classes of cancer chemotherapeutic agents systematically studied, and they still are used clinically (1). Although the general mechanisms of hydrolysis and alkylation for nitrogen mustards are well known (1–6), little detailed kinetic data are available on their hydrolysis. One reason for the lack of comprehensive kinetic work on the mustards has been the lack of suitable stability-specific analytical methods.

BACKGROUND

Most early work on the kinetics of mustards was performed by estimating the amount of free chloride ion liberated. However, this indirect method of determining intact nitrogen mustards and subsequent kinetic analysis can lead to incorrect stability estimates (3). Furthermore, the stabilizing effect of chloride on mustard hydrolysis cannot be quantitatively evaluated with this method. More recent reports (4, 5) on the hydrolysis of melphalan using stability-specific high-pressure liquid chromatography (HPLC) produced some useful information on the kinetics of melphalen hydrolysis and on the effect of chloride ion on its stability.

Chlorambucil (I), an aromatic nitrogen mustard, is considered stable enough to be administered primarily as an oral dosage form. However, there are no reported data on the stability of I over the GI pH range at 37°. It was recently shown (3), using a stability-specific HPLC method for I, that the half-life of I degradation was only 25 min at pH 3.0 and 37°. Since pH 3.0 is not unrealistic for the stomach, these data showed potential stability problems for orally administered I. Thus, a kinetic study of I hydrolysis over the pH range of the GI system seemed warranted.

EXPERIMENTAL

Materials—Chlorambucil USP reference standard was used for all kinetic studies. Water was double distilled in an all-glass apparatus, and glass-distilled solvents[1] were used for HPLC. All other chemicals were reagent grade.

Buffers—Citrate buffers (pH 2.0–7.0), acetate buffers (pH 3.5–5.5), phosphate buffers (pH 6.0–9.0), and borate buffers (pH 8.5–10.0) were prepared from the 0.25 *M* stock solutions of citric acid, acetic acid, monobasic sodium phosphate, and boric acid, respectively, by the addition of sodium hydroxide solution to the desired pH. Addition of chloride ion was avoided except when specified. Ionic strength was maintained con-

[1] Burdick & Jackson Laboratories, Muskegon, Mich.

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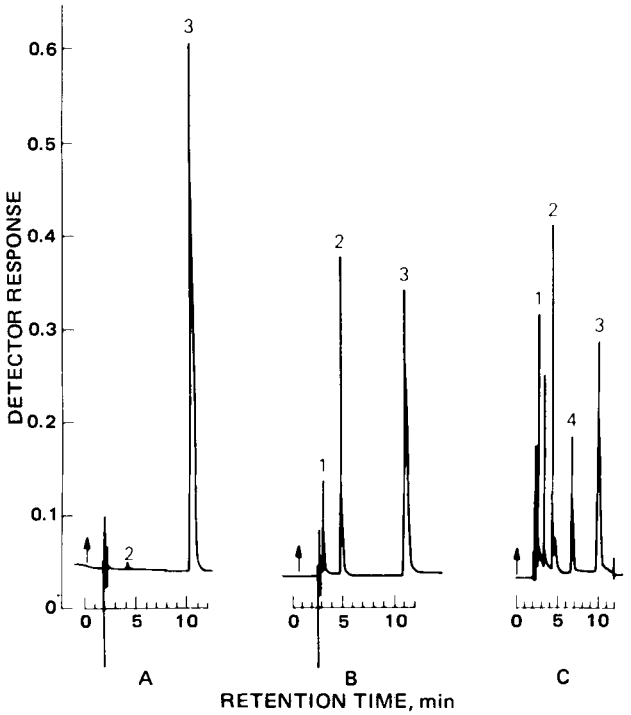


Figure 1—*Chromatogram obtained by the described HPLC method. Key: A, freshly prepared solution of I in water, 200 μg/ml; B, solution of I degraded ~50% in water; and C, solution of I degraded ~50% in 0.05* M *acetate buffer, pH 3.5.*

stant where noted with potassium nitrate. Other ingredients, when added, were weighed into the buffers before adjustment of pH.

Equipment—The high-pressure liquid chromatograph[2] was equipped with a fixed-volume loop injector[3] and a fixed-wavelength detector[4]. A 250- × 4.6-mm i.d. reversed-phase column[5] was used. All pH measurements[6] were taken at room temperature. All kinetic runs were carried out in a constant-temperature water bath[7] capable of maintaining the temperature within ±0.1°. Chloride-ion determinations were made with an automatic chloride titrator[8].

Kinetic Studies—Solutions of I were prepared by adding stock solution of I in acetone to 25.0 ml of buffer preequilibrated at the desired temperature for at least 30 min. The concentration of I in the final solution was ~0.04 mg/ml, and acetone concentration in the final reaction mixture was <1%. The solutions were kept in the constant-temperature water bath and were assayed for intact I at various time intervals. In several cases, the solutions were also assayed for chloride-ion production as described previously (3).

HPLC Assay Method—Separation was performed on a reversed-phase column[5] and methanol–acetonitrile–0.01 *M* acetate buffer, pH 4.5 (65:5:30), at a flow rate of 1.6 ml/min was used as the mobile phase. A 10-μl full loop volume was quantitatively injected, and the recorder was set at 0.32 aufs (254-nm detector). The peak due to I had a retention time of ~10.6 min and was the last to elute in the chromatogram. The peak height of I was used to calculate the amount of intact I present in the sample.

RESULTS AND DISCUSSION

Chromatography—The reversed-phase liquid chromatography permitted the analysis of intact I in the presence of its major hydrolytic degradation products in various buffer systems. Figure 1A shows the chromatogram of a freshly prepared solution of I in water. Peak 3, with a retention time of 10.6 min, was due to I; the small peak (peak 2), with a retention time of 4.0 min, was attributed to the monohydroxy product (II). When completely degraded solutions of I, both in water alone and in various buffer systems, were chromatographed, no peak corresponding to the retention time of I was observed. Therefore, none of the final degradation products interfered with the assay for intact I. During the degradation of I in water, peak 2 was the first to appear and increased through approximately one half-life of I, after which time it began decreasing.

Peak 1, which eluted close to the solvent front, increased steadily throughout the degradation; Fig. 1B shows a typical chromatogram when ~50% of I had degraded in water. Based on the mechanism of I hydrolysis, peak 2 was attributed to II and peak 1 was attributed to the dihydroxy product (III). This is also consistent with the order of elution of these peaks, where the dihydroxy compound, III, eluted faster than the monohydroxy compound, II, which, in turn, eluted faster than the relatively nonpolar I. The order of elution was similar to that reported for the reversed-phase HPLC of melphalan (4). Therefore, major intermediate degradation products in water alone do not interfere with the assay.

When I degrades in the presence of various buffers, other peaks, in addition to those due to II and III, also appear. Thus, Fig. 1C shows the chromatogram of a solution of I degraded in 0.05 *M* acetate buffer, pH 3.0. The peaks, in addition to those due to I–III, were probably due to the degradation products resulting from the attack of nucleophilic buffer species on reactive cyclic ethyleneimmonium intermediates. Thus, based on the time course of appearance and disappearance and also the retention time of peaks, peak 4 in Fig. 1C was tentatively attributed to the

Table I—Effect of pH and Buffer Concentration on Rate and Half-Life of I Hydrolysis at 37°

pH	Buffer[a]	$t_{1/2}$, min	K_{obs} (±*SD*)[b] × 10^2 min^{-1}
1.20	0.1 *M* nitric acid	378.0	0.18
2.00	0.01 *M* nitric acid	95.1	0.73
2.50	0.01 *M* citrate, 0.1 *M* ionic strength	34.9	1.99 (±0.06)
2.50	0.05 *M* citrate	38.4	1.81
3.00	0.01 *M* citrate, 0.1 *M* ionic strength	26.0	2.67 (±0.08)
3.00	0.05 *M* citrate	27.6	2.51
3.50	0.01 *M* citrate	21.4	3.24 (±0.11)
3.50	0.05 *M* citrate	21.7	3.19
3.60	0.01 *M* citrate, 0.1 *M* ionic strength	21.4	3.23 (±0.18)
3.85	0.01 *M* citrate, 0.1 *M* ionic strength	21.3	3.26 (±0.17)
4.00	0.01 *M* acetate	19.6	3.54
4.00	0.05 *M* citrate	19.6	3.54
4.10	0.01 *M* citrate, 0.1 *M* ionic strength	21.0	3.32 (±0.40)
4.35	0.01 *M* citrate, 0.1 *M* ionic strength	18.5	3.74 (±0.25)
4.50	0.05 *M* citrate	19.4	3.58
4.60	0.01 *M* citrate, 0.1 *M* ionic strength	19.2	3.61(±0.07)
4.85	0.01 *M* citrate, 0.1 *M* ionic strength	18.7	3.70 (±0.15)
5.00	0.01 *M* acetate	16.8	4.14
5.00	0.05 *M* acetate	16.9	4.13
5.00	0.05 *M* citrate	18.3	3.79
5.10	0.01 *M* citrate, 0.1 *M* ionic strength	16.8	4.13 (±0.06)
5.35	0.01 *M* citrate, 0.1 *M* ionic strength	16.5	4.20 (±0.06)
5.60	0.01 *M* citrate, 0.1 *M* ionic strength	16.4	4.23 (±0.14)
5.85	0.01 *M* citrate, 0.1 *M* ionic strength	16.4	4.23 (±0.03)
6.00	0.05 *M* citrate	15.4	4.51
6.10	0.01 *M* citrate, 0.1 *M* ionic strength	15.6	4.44 (±0.27)
6.50	0.01 *M* citrate, 0.1 *M* ionic strength	15.7	4.43 (±0.25)
7.00	0.01 *M* citrate, 0.1 *M* ionic strength	15.7	4.42 (±0.02)
7.00	0.02 *M* phosphate	16.1	4.31
7.00	0.05 *M* phosphate	16.1	4.32
8.00	0.05 *M* phosphate	16.3	4.26
9.00	0.05 *M* phosphate	15.5	4.47
10.00	0.05 *M* borate	15.4	4.50

[a] Buffers contained no chloride ion, and ionic strength was maintained only where noted. [b] Standard deviations, where noted in parentheses, are for three runs.

[2] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[3] Velco-type valve, Spectra-Physics, Santa Clara, Calif.
[4] Model 225, Spectra-Physics, Santa Clara, Calif.
[5] Zorbax C-8 column, 6 μm average particle size, Dupont Instruments, Wilmington, Del.
[6] Expandomatic SS-2 pH meter, Beckman Instruments, Fullerton, Calif.
[7] Electronic relay system, Precision Scientific Co., Chicago, Ill.
[8] Model 4-4433, American Instrument Co., Silver Spring, Md.

Table II—Effect of Chloride Ion and Other Buffers on the Rate and Half-Life of I Hydrolysis in 0.02 *M* Phosphate Buffer, pH 7.0, at 37°

Run	Additive	Chloride-Ion Concentration, moles/liter	$t_{1/2}$, min	$K_{obs} \times 10^2$ min^{-1}
1	—	—	16.6	4.18
2	0.08 *M* potassium chloride	0.08	27.9	2.48
3	0.10 *M* potassium chloride	0.10	29.2	2.38
4	0.20 *M* potassium chloride	0.20	41.6	1.66
5	0.24 *M* potassium chloride	0.24	50.3	1.38
6	0.40 *M* potassium chloride	0.40	67.8	1.19
7	0.08 *M* potassium nitrate	—	16.0	4.34
8	0.24 *M* potassium nitrate	—	15.0	4.63
9	0.40 *M* potassium nitrate	—	15.2	4.56
10	0.08 *M* imidazole	—	18.8	3.69
11	0.40 *M* potassium nitrate + 0.08 *M* imidazole	—	15.8	4.38
12	0.08 *M* potassium chloride + 0.08 *M* imidazole	0.08	21.8	3.18
13	0.10 *M* potassium chloride + 0.30 *M* imidazole	0.10	19.1	3.63
14	0.40 *M* potassium chloride + 0.08 *M* imidazole	0.40	34.1	2.04

monoacetyl derivative of I. Similarly, when the solvents contained substantial amounts of methanol or ethanol, other peaks that probably were due to the methoxy- or ethoxy-substituted compounds appeared. No further attempts were made to isolate and identify these peaks, and the major concern was to make sure that these peaks did not interfere with the assay of intact I.

The peak height due to I was linearly related with its concentration over 10–200 μg/ml. Therefore, peak heights were used to calculate the amount of intact I present in the sample. Acetone was used as the solvent for the stock solution, because I degrades slowly in hydroxylic solvents such as methanol or ethanol.

Kinetic Studies—Hydrolysis of I followed first-order kinetics over pH 1.0–10.0 as determined from linear semilogarithmic plots of the amount of intact I remaining *versus* time. Table I summarizes the rates of I hydrolysis in various buffers and pH values in the absence of added chloride ion. Table II shows the rates in the presence of added chloride ion. The rate of I hydrolysis was relatively independent of buffer species and concentration but was strongly influenced by pH and chloride-ion concentration. Therefore, the effects of pH and chloride-ion concentration are discussed separately.

Effect of pH—Figure 2 shows the pH rate profile for I hydrolysis at 37° in buffered solutions with 0.1 *M* ionic strength where no chloride ion was added. The profile shows a sharp inflection around pH 2.5 and a shallower one around pH 5.0, followed by a plateau region at pH 6.0–10.0

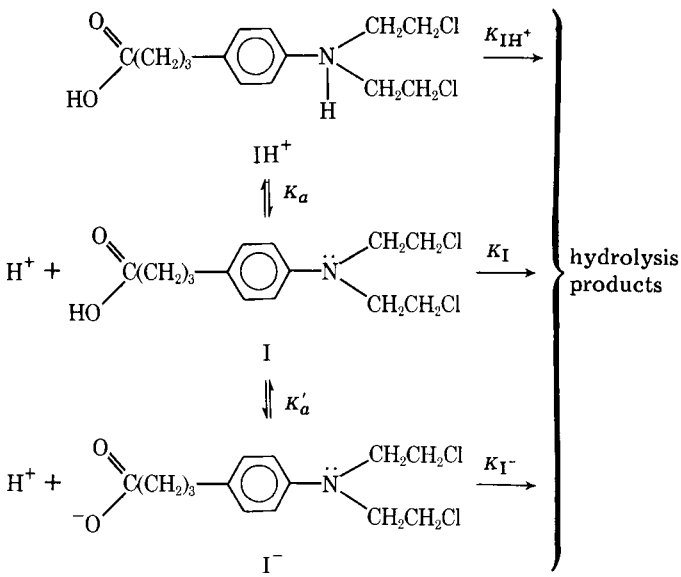
The pH-independent rate of I hydrolysis at pH 6.0–10.0 excludes the possibility of a significant hydroxide-ion catalysis for I hydrolysis over pH 1.0–10.0. In addition, the decreasing rate of I hydrolysis as the pH was lowered suggests that hydrogen-ion catalysis is also not important in this range. Thus, change in the rate of I hydrolysis with pH in this range must be attributable to the ionization of reactive functional groups in I.

The sharp inflection around pH 2.5 is attributable to the ionization of the nitrogen mustard of I. The mechanism of nitrogen mustard hydrolysis is known to involve the attack of unprotonated nitrogen mustard to expel chloride, forming an unstable cyclic ethyleneimmonium intermediate. This is followed by the attack of water and other nucleophiles to give subsequent products (1–6). The availability of a free electron pair on the nitrogen mustard is essential for its reactivity (1), and protonation of this nitrogen makes the compound much less reactive. The pKa of the nitrogen mustard of I was reported (2) to be on the order of 2.5. Therefore, as pH increases from 1.0 to 4.0, more of I would be in the unprotonated form and the rate of I hydrolysis should increase. The sharp inflection (Fig. 2) near the pKa of I strongly supports the mechanism that the unprotonated nitrogen mustard is the reactive species.

This mechanism alone, however, does not explain the shallow inflection around pH 5.0. If the ionization of the nitrogen mustard was the only determinant in the rate of I hydrolysis, a plateau above pH 4.5 (*i.e.*, 2 pH units above the pKa) would be expected. However, the difference in rate constants in the final plateau region (pH >6.0) and that of the plateau range of pH 4.0–4.5 is real. Experiments in this pH region (pH 3.0–5.5) were done at very frequent pH intervals (Fig. 2 and Table I), often repeated three times each, and ionic strength was strictly maintained, thus leaving little doubt that there is an inflection between pH 4.5 and 6.0.

Since the rate of I hydrolysis in the pH 4–6-range is totally independent of hydrogen ion, hydroxide ion, or buffer catalysis (acetate, citrate, and phosphate buffer concentration had no effect on the rate, Table I), the increase in rate with increasing pH in this range must be attributed to ionization of another group in I that would affect the rate of I hydrolysis. The only ionizable group in I (except the nitrogen mustard) is the carboxylic group, and thus the inflection must be attributed to this group. The inflection region around pH 5.0 is also consistent with the ionization constant of an aliphatic carboxylic group.

A slightly higher rate of hydrolysis of I^- over I is also not unreasonable. The ionization of the carboxylic group and resultant formal negative charge on the alkyl substituent is expected to have some long-range electron-donating inductive effect on the nitrogen mustard, making it slightly more reactive than I where the carboxylic group is unionized. Therefore it is suggested that the second inflection point is due to the ionization of the carboxylic group and that the plateau area, in the 6.0–10.0-range, is the limiting rate of ionized I^- (Scheme I).



Scheme I

This mechanism is supported by calculation of rate at any particular pH. Thus, for the reaction shown in Scheme I, it can be shown that:

$$K_{obs} = (K_{IH^+}[H^+]^2 + K_I[H^+]K_a + K_{I^-}K_aK'_a)\frac{I_t}{D} \quad \text{(Eq. 1)}$$

where K_{obs} is the observed rate constant; K_a and K'_a are the ionization constants of the species IH^+ and I, respectively; and:

$$I_t = [IH^+] + [I] + [I^-] \quad \text{(Eq. 2)}$$

$$D = [H^+]^2 + K_a[H^+] + K_aK'_a \quad \text{(Eq. 3)}$$

The value of K_{IH^+} can be estimated from pH 1 data and thus shown to be negligible. The value of K_{I^-} can be obtained from the limiting rate at pH >6, which was experimentally found to be 0.0440 min^{-1} (Table I). The pKa value was reported (2) to be on the order of 2.5; that of pKa′, the ionization constant of an aliphatic carboxylic group, should be on the order of 5.0. The value of K_I should be close to the rate between pH 3.5 and 4.0, which was experimentally found to be between 0.032 and 0.035 min^{-1}. The solid line in Fig. 2, obtained using Eq. 1 and $K_{IH^+} = 0.0$ min^{-1}, $K_I = 0.0335$ min^{-1}, $K_{I^-} = 0.0440$ min^{-1}, pKa = 2.4, and pKa′ = 4.9 (all realistic values), represented the best fit of the theoretical line to the

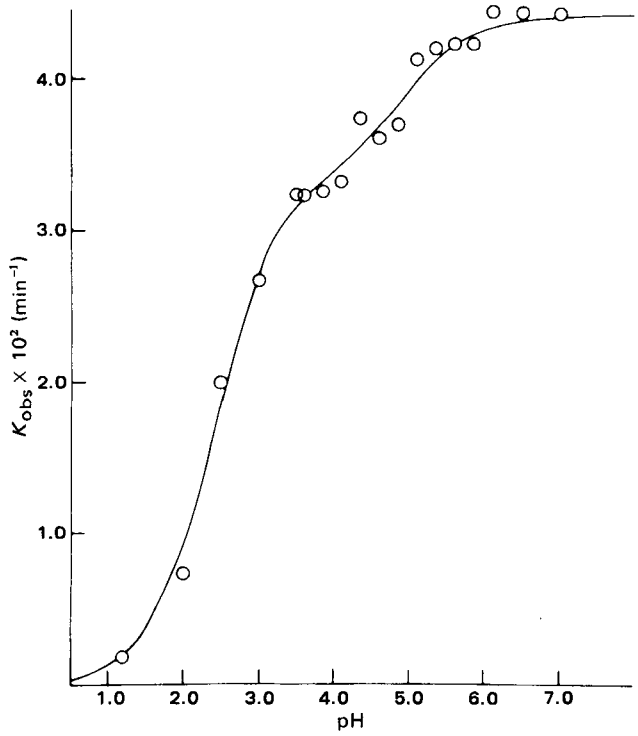


Figure 2—*The pH rate profile for degradation of I in the absence of added chloride ion at 37°. The solid line is the best fit theoretical line using Eq. 1 and values mentioned in the text. Circles are experimental points. All points above pH 2.0 were determined in 0.01* M *citrate buffer, 0.1* M *ionic strength.*

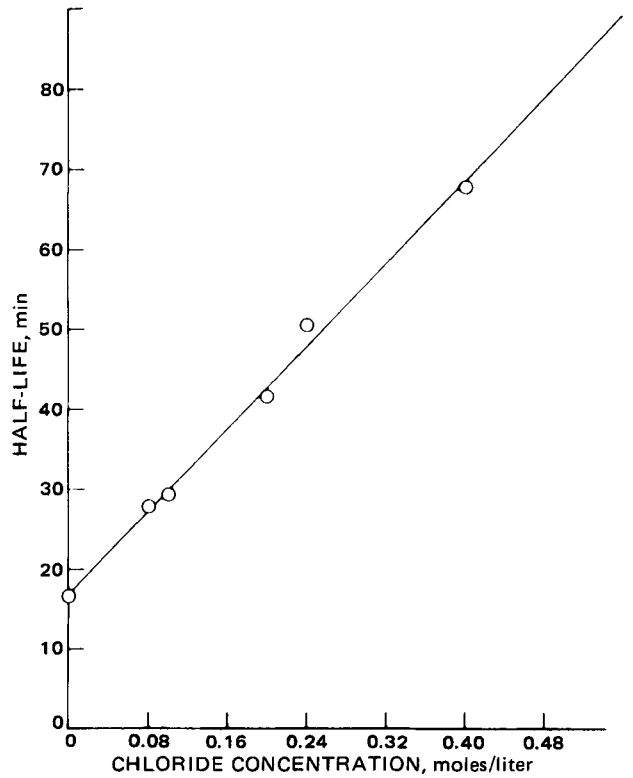
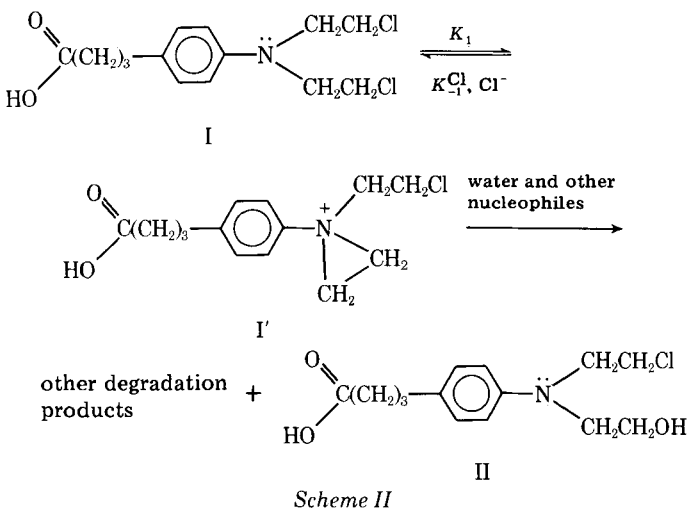


Figure 3—*Plot of adjusted half-life (see text) for I hydrolysis* versus *the concentration of added chloride ion.*

experimental points. This result supports the proposed kinetic analysis (Scheme I) for the hydrolysis of I.

Effect of Chloride Ion—Hydrolysis of I followed first-order kinetics in the presence of excess chloride ion. However, at a particular pH and fixed buffer concentration, the rate of I hydrolysis decreased in the presence of added chloride ion. As seen in Table II, the half-life of I hydrolysis increased with increasing chloride concentration. Although the rate of I hydrolysis was relatively independent of buffer concentrations in the absence of added chloride ion, it was strongly influenced by buffer concentration when excess chloride ion was present.

The absence of significant effects of other ions, buffer species, and specific hydrogen-ion or hydroxyl-ion catalysis (except for the pH effect on the ionization of I and consequent effects on the rate) strongly suggest that the stabilizing effect of chloride ion in I hydrolysis is a specific one. Based on these observations, the mechanism of chloride stabilization probably involves the reverse attack of chloride ion on the cyclic ethyleneimmonium intermediate (I^+) to give back parent I (Scheme II). The same mechanism was recently suggested (5) for the chloride-ion stabilization of melphalan hydrolysis.



Scheme II

The cyclic intermediate, I′, is susceptible to attack by water and other nucleophiles present in the reaction mixture. It is reasonable, therefore, to suggest that chloride ion competes with water and other nucleophiles to attack I′. The attack of water and all other nucleophiles except chloride ion yields irreversible degradation products, while the attack of chloride ion yields I. It follows that stabilization would increase as the ratio of chloride ion to other nucleophiles (including water) is increased. This relation qualitatively explains why the half-life of I hydrolysis increases with increasing concentration of added chloride ion.

The rate of I hydrolysis increases when the concentration of nucleophilic buffer species (*i.e.*, imidazole) increases in the presence of a constant excess concentration of chloride ion (compare values of Runs 2, 3, and 6 with those of Runs 12–14 in Table II). However, except for a minor ionic strength effect, buffers and other ions had no significant effect on rate when no chloride ion was added (Table I). This result can be explained by Scheme II, where increasing the concentration of other species competing to attack I′ results in a smaller share of I′ available for chloride attack and, hence, reduces stabilization. Without chloride ion, attack of I by all species leads to irreversible degradation products. Therefore, increasing the buffer concentration only changes the ratio of degradation products without affecting the rate of I hydrolysis.

This mechanism of chloride-ion stabilization is also substantiated by the kinetic analysis of the results in the presence of added chloride ion. If it is assumed that the concentration of the reactive intermediate, I′, reaches steady state, then applying classical steady-state kinetics dictates (Appendix) that, in the presence of constant excess chloride ion, the loss of I is first order with respect to I and that the half-life of I hydrolysis is related linearly to the chloride-ion concentration if all other conditions remain constant.

To check these criteria, experiments were designed to study the kinetics of I hydrolysis at a fixed pH and buffer concentration in the presence of various amounts of sodium chloride concentration. Since ionic strength would influence the rate by affecting the pKa if the pH was in the inflection region of the pH rate profile for I hydrolysis, these experiments were performed in 0.02 *M* phosphate buffer (pH 7.0), which was well within the plateau region (Fig. 2). At this pH, small changes in K_a and K'_a of I would have a minimal effect on the rate. To account for the ionic strength effect of added chloride ion on the rates, rate constants for I hydrolysis were also determined in 0.02 *M* phosphate buffers (pH 7.0) in the presence of various concentrations of potassium nitrate. The adjusted rate constants at any chloride-ion concentration were calculated by:

$$K_{adj} = K_{obs}\frac{K_0}{K'} \quad \text{(Eq. 4)}$$

where K_{adj} is the adjusted rate constant, K_{obs} is the observed rate constant at a specified sodium chloride concentration, K' is the rate constant in the absence of added chloride ion but in the presence of the same molar concentration of potassium nitrate as the concentration of sodium chloride added to determine K_{obs}, K_0 is the rate constant in the absence of any other added ion except the 0.02 M phosphate buffer (pH 7.0), and $(t_{1/2})_{adj} = 0.693/K_{adj}$.

Plotting the concentrations of I remaining against time gave first-order plots, and Fig. 3 shows the plot of adjusted half-lives against sodium chloride concentrations. The good linear plot in Fig. 3 lends strong support to Scheme II.

Physiological Implications—The preceding kinetic data suggest that I is reasonably stable at 37° to 0.1 N HCl (half-life ~12 hr). However, the stomach pH varies from 1.5 to 5.0 (7); if the pH is ~3.0, a 25-min half-life at 37° (Table I) would mean that ~10% drug would degrade in 4 min. Even in the presence of a substantial chloride-ion concentration in the stomach (*e.g.*, 0.1 M), the half-life of I would be ~50 min at pH 3.0 and 37° (still a very fast rate for an orally administered drug). Therefore, the amount of drug available for absorption apparently would be a function of stomach pH at a particular time. Thus, there is a serious potential for inconsistency in the availability of orally administered I.

The hydrolysis rate of I in human plasma at 37° was ~10–20 times slower than in the buffer of pH 7.4. This stabilization was attributed to the binding of I with plasma proteins as suggested previously (8, 9). The similar favorable effect of the proteins in the GI tract on the stability of orally administered I was not investigated in this study.

APPENDIX: EFFECT OF CHLORIDE ION ON KINETICS OF I HYDROLYSIS

For the reaction shown in Scheme II:

$$I \underset{K_{-1}^{Cl}}{\overset{K_1}{\rightleftharpoons}} I' + Cl^- \xrightarrow[H_2O,\ \text{other nucleophiles}]{K_x} \text{II + other products}$$

Scheme AI

where K_1 is the first-order rate constant of initial cyclization of I, K_{-1}^{Cl} is second-order rate constant for chloride-ion attack on I′ to yield I, and K_x is the pseudo-first-order rate constant for breakdown of I′ by all nucleophiles (including water), except chloride ion, to yield II and other degradation products:

$$-dI/dt = K_1[I] - K_{-1}^{Cl}[Cl^-][I'] \quad \text{(Eq. A1)}$$

$$dI'/dt = K_1[I] - K_{-1}^{Cl}[Cl^-][I'] - K_x[I'] \quad \text{(Eq. A2)}$$

If I′ is assumed to be very unstable and its concentration reaches steady state, then at steady state:

$$dI'/dt = 0 \quad \text{(Eq. A3)}$$

and

$$K_1[I] - K_{-1}^{Cl}[Cl^-][I'] - K_x[I'] = 0 \quad \text{(Eq. A4)}$$

Collecting terms and simplifying (Eq. A4) gives:

$$I' = \frac{K_1}{K_{-1}^{Cl}[Cl^-] + K_x}[I] \quad \text{(Eq. A5)}$$

Substituting Eq. A5 into Eq. A1 gives:

$$\frac{-dI}{dt} = K_1[I] - \frac{K_{-1}^{Cl}K_1[Cl^-]}{K_{-1}^{Cl}[Cl^-] + K_x}[I] \quad \text{(Eq. A6)}$$

Simplifying Eq. A6 gives:

$$\frac{-dI}{dt} = \frac{K_1K_x}{K_{-1}^{Cl}[Cl^-] + K_x}[I] \quad \text{(Eq. A7)}$$

If chloride ion is in excess, its concentration does not change significantly by the chloride ion liberated during the hydrolysis of I. Therefore, the term $K_1K_x/(K_{-1}^{Cl}[Cl^-] + K_x)$ in Eq. A7 is constant and designated as K_{obs}:

$$K_{obs} = \frac{K_1K_x}{K_{-1}^{Cl}[Cl^-] + K_x} \quad \text{(Eq. A8)}$$

and:

$$-dI/dt = K_{obs}[I] \quad \text{(Eq. A9)}$$

Equation A9 shows that loss of I in the presence of a fixed excess concentration of chloride ion should be the first order with respect to I with the rate constant K_{obs}. Inverting Eq. A8 yields:

$$\frac{1}{K_{obs}} = \frac{K_{-1}^{Cl}[Cl^-]}{K_1K_x} + \frac{K_x}{K_1K_x} \quad \text{(Eq. A10)}$$

Cancelling terms and multiplying Eq. A10 with 0.693 gives:

$$\frac{0.693}{K_{obs}} = \frac{0.693\,K_1^{Cl}}{K_1K_x}[Cl-] + \frac{0.693}{K_1} \quad \text{(Eq. A11)}$$

Equation A11 shows that the plot of the observed half-life ($0.693/K_{obs}$) versus the chloride-ion concentration should be a straight line, with the intercept representing the half-life when no chloride ion is present.

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