Determination of Ethinyl Estradiol in Dissolution Study—The release rate of ethinyl estradiol was determined at 37° using the USP rotating-basket apparatus in a dissolution medium containing 900 ml of distilled water. The basket was rotated at 100 rpm. At each sampling interval, 6 ml of the medium was withdrawn and extracted with 5 ml of chloroform. Then 4 ml of the chloroform layer was evaporated to dryness under a nitrogen stream. Ethinyl estradiol was analyzed as described.

RESULTS AND DISCUSSION

Figure 1 shows the excitation and emission spectra of ethinyl estradiol. Ethinyl estradiol exhibited strong fluorescence spectra at excitation and emission wavelengths of 460 and 490 nm, respectively. However, norethindrone did not exhibit fluorescence, even at the very high concentration of 2000 ng/ml (Fig. 1).

Figure 2 shows fluorescence stability for 1 hr after sulfuric acid addition. As can be seen in Fig. 2, the fluorescence of ethinyl estradiol was relatively stable; moreover, an excellent linearity between the fluorescence intensity and the ethinyl estradiol concentration over 17.5–280 ng/ml was observed; the minimum detection limit for the drug was ~10 ng/ml using this procedure.

The results of this study also indicate that the reproducibility of the method is excellent and that common tablet excipients do not interfere with the determination of ethinyl estradiol (Table II).

Finally, the method was valuable for the determination of small amounts of the drug in the dissolution study. Figure 3 shows the release rate of ethinyl estradiol from the sustained-release tablet (Vial 2) and the rapid-release tablet (Vial 4).

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COMMUNICATIONS

Potential Errors in Kinetic Studies of Hydrolysis of Nitrogen Mustards Based on Chloride-Ion Determination

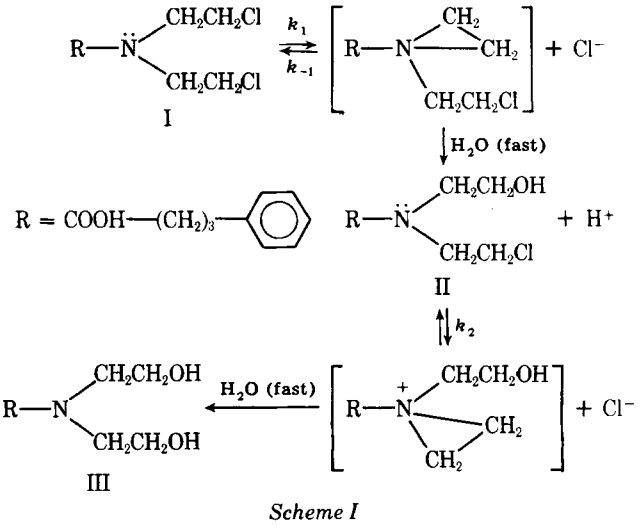
Keyphrases □ Chlorambucil—hydrolysis kinetics, potential errors based on chloride-ion determination □ Hydrolysis—chlorambucil, potential errors in kinetic studies based on chloride-ion determination □ Nitrogen mustards—chlorambucil, hydrolysis, potential errors in kinetic studies based on chloride-ion determination

To the Editor:

Chlorambucil (I), an aromatic nitrogen mustard, has been used clinically in the treatment of chronic lymphocytic leukemia and primary microglobulinemia and in the management of ovarian and testicular carcinomas (1). In aqueous solutions, I and other nitrogen mustards undergo hydrolysis, with the release of chloride ion and the formation of the cyclic ethyleneimmonium ion (1–3). This unstable cyclic intermediate then is attacked by water and other nucleophiles to yield II and other products. The same sequence then is repeated for the hydrolysis of the chloroethyl group in II (Scheme I).

Owen and Stewart recently reported (3) the kinetics of I hydrolysis using the chloride-ion measurement as a parameter for the concentration of intact I remaining. They plotted log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time and calculated k_1, the degradation rate of I, from the apparent linear plots. In using chloride-ion measurements to obtain k_1, Owen and Stewart (3) made two important assumptions: (*a*) that the loss of I is always accompanied by the loss of two chloride ions, which is a valid assumption in the case of nitrogen mustards; and (*b*) that both chloride ions must be released simultaneously, *i.e.*, $k_2 \gg k_1$. However, there is no theoretical basis for assuming that $k_2 \gg k_1$.

The mechanism of cyclic ethyleneimmonium-ion formation should be the same for both the k_1 and k_2 steps, and there is no reason to expect that the presence of a hydroxyl function in II would make II much more unstable



Scheme I

relative to I. From a statistical consideration alone, the k_1 step probably should be faster, because there are two hydrolyzable chloro groups. Furthermore, if k_1 is not the rate-determining step, the plot of log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time would be dependent on the relative magnitude of k_2/k_1. This relation can be shown theoretically from the classical kinetic treatment of consecutive reactions (4). Thus, for the reaction shown in Scheme II:

$$I \xrightarrow{k_1} II + Cl^- \xrightarrow{k_2} III + Cl^-$$

Scheme II

it follows that:

$$[I]_t = I_0 e^{-k_1 t} \quad \text{(Eq. 1)}$$

$$[II]_t = \frac{I_0 k_1}{(k_2 - k_1)}(e^{-k_1 t} - e^{-k_2 t}) \quad \text{(Eq. 2)}$$

$$[III]_t = \frac{I_0}{(k_2 - k_1)}[k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})] \quad \text{(Eq. 3)}$$

$$[Cl]_t = [II]_t + 2[III]_t \quad \text{(Eq. 4)}$$

where $[I]_t$, $[II]_t$, $[III]_t$, and $[Cl]_t$ are the concentrations of

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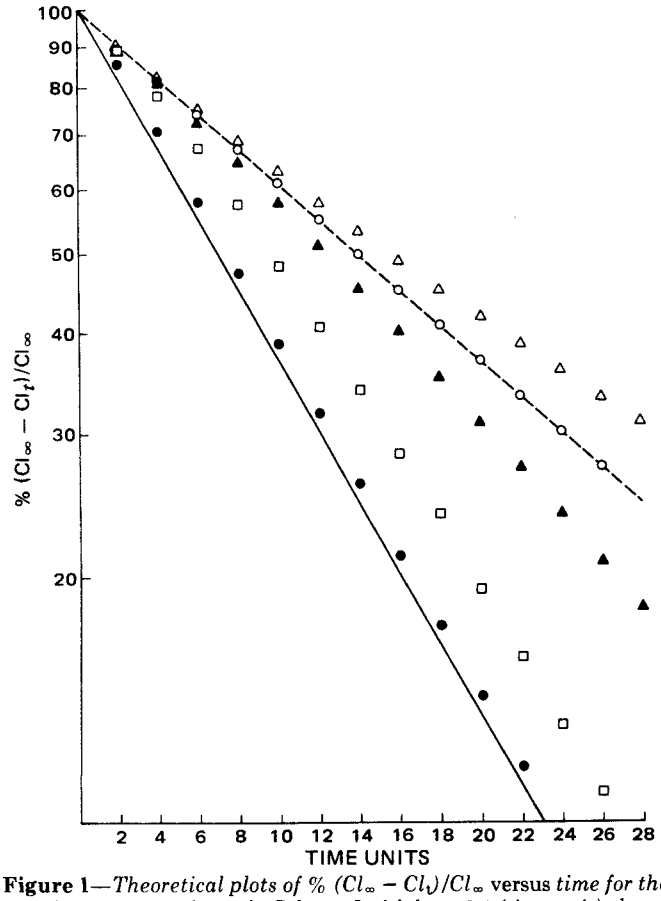

Figure 1—*Theoretical plots of % $(Cl_\infty - Cl_t)/Cl_\infty$* versus *time for the reaction sequence shown in Scheme I with* $k_1 = 0.1$ *(time unit)*$^{-1}$ *and* $I_0 = 1.0$ *and with various values of* k_2/k_1. *Key:* ●, *10.00;* □, *2.00;* ▲, *0.75;* ○, *0.50; and* △, *0.33. The solid line represents the theoretical plot of the percent of I remaining, thus representing the true* k_1 *value; the broken line has a slope equal to* $0.50k_1$.

I, II, III, and chloride ion, respectively, at time t, and I_0 is the initial concentration of I. Since each molecule of I eventually yields two molecules of chloride ion, it follows that the chloride-ion concentration at infinite time is given by:

$$Cl_\infty = 2I_0 \qquad \text{(Eq. 5)}$$

Figure 1 shows theoretical plots obtained by plotting log I or log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time for various values of k_2/k_1 (the solid line represents the actual rate of loss of I). As seen in Fig. 1, in addition to the expected linearity of the chloride plot when $k_2 \geq 10k_1$, a perfect linear chloride plot also is obtained when $k_2 = 0.5k_1$[1]. However, the slopes of the two lines are different; only in the case where $k_2 \geq 10k_1$ does the slope yield a value close to k_1. The slope of the straight line plot when $k_2 = 0.5k_1$ is equal to $0.5k_1$.

Therefore, it is apparent that a linear first-order plot of the chloride ion produced does not necessarily establish k_1 as the rate-determining step. In fact, as seen in Table I, an apparent linear relationship of the chloride ion produced can be observed over a wide range of k_2/k_1 values, within experimental errors, particularly if the data are subjected to a computer-assisted linear regression without actually plotting the points. However, the apparent rate constants depend on the value of k_2/k_1 and are smaller than k_1 (Table I). As explained earlier, k_1 probably is not the rate-determining step for the hydrolysis of I, and k_2 probably is of the same magnitude as k_1. Therefore, it is possible that the apparent linear plots of chloride-ion concentrations obtained during I hydrolysis (3) do not yield the true degradation rate of I. To clarify this ambiguity, it is necessary to follow the hydrolysis of I by a method that actually determines the concentration of intact I and to compare the kinetic data obtained with those obtained from chloride titration.

Table I—Correlation of Theoretical Chloride-Ion Plots Shown in Fig. 1 with Similar Calculations for Additional Values of k_2/k_1 Not Shown in Fig. 1

k_2/k_1	Correlation Coefficient[a]	Apparent Rate Constant
20.00	1.0000	0.1000
10.00	0.9999	0.0995
5.00	0.9996	0.0977
2.00	0.9983	0.0880
1.00	0.9986	0.0694
0.75	0.9994	0.0608
0.50	1.0000	0.0500
0.33	0.9991	0.0419
0.25	0.9976	0.0372

[a] Obtained for 15 points, which are two time units apart, up to 28 time units (equal to ~4 half-lives for the k_1 step). The k_1 value was 0.1 (time unit)$^{-1}$ and $I_0 = 1.0$.

A high-pressure liquid chromatographic (HPLC) method was developed to assay intact I in the presence of its major degradation products, II and III. The method involves reversed-phase chromatography[2], in which the hydroxy compounds, II and III, elute earlier than intact I since they are more polar. The details of the HPLC method and the identification of degradation peaks will be reported later.

Solutions of I (0.15 mg/ml) in 0.05 *M* acetate buffer[3] (pH 3.0) and nitric acid solution[3] (pH 2.0) were kept in a 37° water bath. At suitable time intervals, they were assayed for their intact I content by HPLC and for their chloride-ion concentration by an automatic chloride titrator[4]. Figure 2 shows the plot of the I concentration or $(Cl_\infty - Cl_t)/Cl_\infty$[5] *versus* time. The plot of I gave straight lines with half-lives of 25 and 70 min at pH values of 3.0 and 2.0, respectively. However, the points for the chloride-ion plot did not coincide with the HPLC plots (Fig. 2). At pH 3.0, the points appeared to give a straight line with a half-life of 52 min; at pH 2.0, the chloride-ion points showed distinct curvature with an apparent half-life of 165 min (r = 0.993).

Therefore, it is obvious that k_1 is not the rate-determining step in the overall hydrolysis of I. This finding was substantiated further by HPLC analysis of I hydrolysis where the peak due to II was shown to be increasing, through at least one half-life of I, followed by a decrease, as would be expected of a consecutive reaction where the intermediate is not particularly unstable compared to the initial reactants. It also is apparent that the rates calcu-

[1] The straight line nature of the chloride plot, when $k_2 = 0.5k_1$, is mathematically correct, as can be seen by substituting values of $[II]_t$ and $[III]_t$ into Eq. 4 followed by simplification to yield a monoexponential expression, $(Cl_\infty - Cl_t)/Cl_\infty = e^{-0.5k_1t}$.

[2] A Zorbax-C_8 column (DuPont) was used with a running solvent containing 60% methanol, 5% acetonitrile, and 35% acetate buffer (0.15 *M*) (pH 5.0) at a flow rate of 1.6 ml/min. The retention times of I and II were 7.5 and 3.5 min, respectively.

[3] Buffers contained 5% acetone to keep I in solution.

[4] Model 4-4433, American Instrument Co., Silver Spring, Md.

[5] The Cl_∞ and Cl_t values plotted were corrected for the blank, *i.e.*, Cl_0, which is the amount of chloride ion present at time zero.

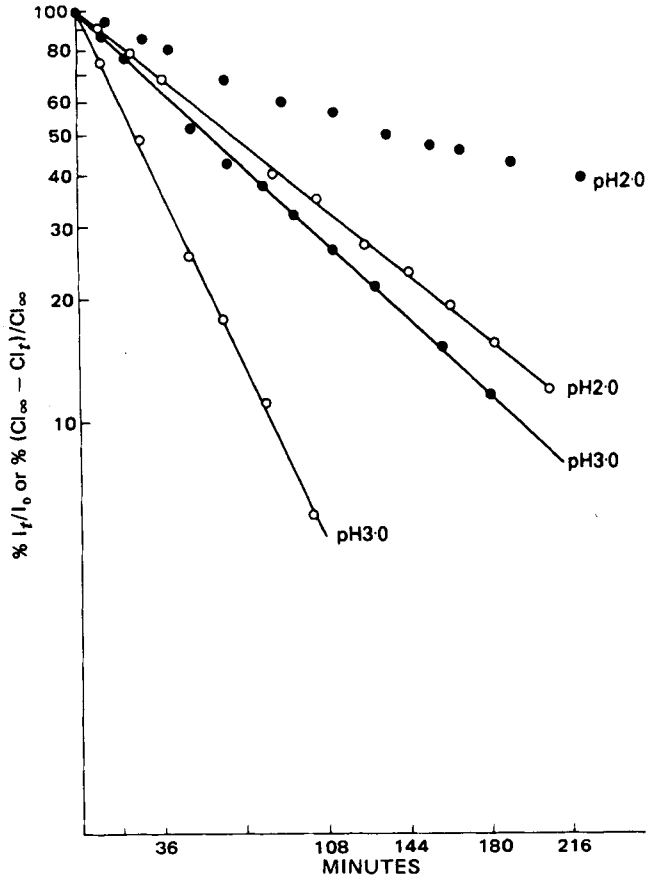

Figure 2—*Plots for the hydrolysis of I. Key:* ○, *data from HPLC runs (% I_t/I_0); and* ●, *data from the chloride-ion determination [% $(Cl_\infty - Cl_t)/Cl_\infty$]; in 0.05* M *acetate buffer (pH 3.0) and in dilute nitric acid (pH 2.0).*

lated from the chloride analyses would considerably overestimate the stability of I, at least in the two buffers tested.

These findings show the inherent potential error in obtaining kinetic parameters of a reactant by following the appearance of a degradation product, particularly when a consecutive reaction is involved as in the hydrolysis of I. The possible error in the rate constants reported for chlorambucil hydrolysis has potential practical implications. Compound I is administered routinely as oral tablets, in which case it is exposed to the pH range of the stomach (1.5–5.0). The relatively short half-lives observed for I hydrolysis at pH 2.0 and 3.0 suggest that a significant amount of I potentially may be degraded before absorption. This observation warrants a more careful study of I hydrolysis using a stability-specific assay. A more detailed kinetic study is in progress using the HPLC method for the analysis of I both to obtain practical stability data under physiological conditions and to elucidate the mechanism of I hydrolysis.

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Potential Errors in Kinetic Studies of Hydrolysis of Nitrogen Mustards Based on Chloride-Ion Determination: A Reply

Keyphrases □ Chlorambucil—hydrolysis kinetics □ Nitrogen mustards—chlorambucil, hydrolysis kinetics □ Hydrolysis—chlorambucil, kinetics

To the Editor:

The hydrolysis of chlorambucil (I) is a complex reaction, involving several reversible and consecutive steps. Chatterji (1) treated the reaction as a consecutive one involving two steps and indicated that k_1 and k_2 may be of the same magnitude. Several comments are appropriate.

The production of chloride from the consecutive model for the hydrolysis of chlorambucil is biexponential, *i.e.*:

$$Cl_t = I_0(1 - e^{-k_1 t}) + \frac{I_0}{k_2 - k_1}[k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})] \quad \text{(Eq. 1)}$$

where Cl_t is the chloride concentration at time t, I_0 is the initial concentration of chlorambucil, and k_1 and k_2 are the rate constants for the two consecutive steps.

Therefore, the theoretical plots of log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time will be nonlinear except when $k_2 \gg k_1$ and $k_2 \rightarrow 0.5k_1$. Chatterji (1) suggested that "apparent linear relationships" could be observed over a wide k_2/k_1 range by subjecting data to "computer-assisted linear regression." Apparent linearity was proposed on the basis of the correlation coefficient values. However, this approach is a gross simplification of the model and a misrepresentation of the numerical techniques used in the original work (2).

Table I—Summary of the Analyses of Variance for the Orthogonal Polynomial Analysis of the Theoretical Consecutive Model for Various k_2/k_1 Values

k_2/k_1	Probability[a] of Fitting a Polynomial of Order: Zero	1	2	3	Deviation[b] from Linearity
50	<0.001	<0.001	0.60	0.18	No
30	<0.001	<0.001	0.31	0.17	No
20	<0.001	<0.001	0.08	0.01	Yes?
10	<0.001	<0.001	0.002	<0.001	Yes
5	<0.001	<0.001	<0.001	<0.001	Yes
2	<0.001	<0.001	<0.001	<0.001	Yes
0.75	<0.001	<0.001	<0.001	<0.001	Yes
0.5	<0.001	<0.001	0.78	0.25	No
0.33	<0.001	<0.001	<0.001	0.04	Yes
0.25	<0.001	<0.001	<0.001	0.009	Yes
0.1	<0.001	<0.001	<0.001	<0.001	Yes
0.01	<0.001	<0.001	<0.001	<0.001	Yes

[a] A total of 29 data points was used (up to 28 time units); $k_1 = 0.1$ (time unit)$^{-1}$, $I_0 = 1.0$, and error variance = 0.2×10^{-5}. [b] At $p = 0.05$.

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