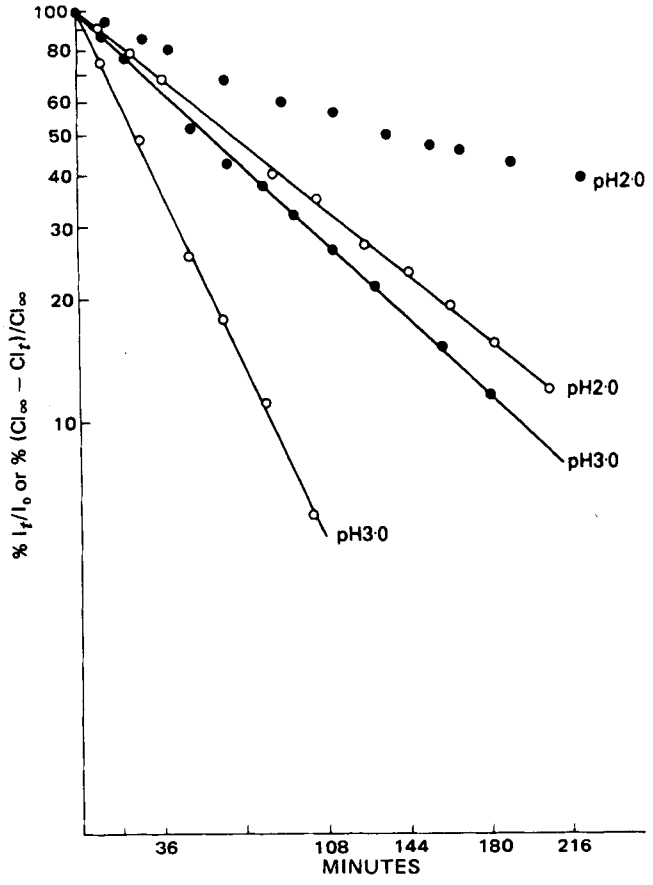

Figure 2—*Plots for the hydrolysis of I. Key:* ○, *data from HPLC runs (% I_t/I_0); and* ●, *data from the chloride-ion determination [% $(Cl_\infty - Cl_t)/Cl_\infty$]; in 0.05* M *acetate buffer (pH 3.0) and in dilute nitric acid (pH 2.0).*

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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Table II—Analysis of Variance for the Orthogonal Polynomial Analysis of the Data for Hydrolysis of Chlorambucil at 25.0°

Order of Fit	Regression Sum of Squares	Remainder Sum of Squares	Degrees of Freedom	Remainder Mean Square	Variance Ratio	p
0	0.306E + 01	0.110E + 01	27	0.406E − 01	0.754E + 02	<0.001
1	0.110E + 01	0.538E − 04	26	0.207E − 05	0.529E + 06	<0.001
2	0.101E − 07	0.528E − 04	25	0.215E − 05	0.468E − 02	0.94
3	0.203E − 04	0.335E − 04	24	0.140E − 05	0.145E + 02	0.001
4	0.425E − 06	0.331E − 04	23	0.144E − 05	0.295E + 00	0.60

The use of correlation coefficients to test the goodness of fit of the data to this model is inadequate. A statistical technique capable of testing for curvature of the regression is required. Linear regression with appropriate analysis of variance can be used but requires replicate data that are either extremely difficult or not possible to obtain. Orthogonal polynomial analysis, with the successive fitting of higher order polynomials and subsequent statistical testing to determine whether these higher order polynomials have significantly improved the fit, is the method of choice in these circumstances. The principal orthogonal polynomial algorithms were reviewed (3), and the Gram–Schmidt algorithm (4) was chosen for the original study.

Chatterji's theoretical data were regenerated with a random error variance typical of that obtained in the original study. The log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time regressions were subjected to orthogonal polynomial analysis, and a summary of the analyses of variance is provided (Table I). Curvature was detected in all regressions, except in the limiting situations when $k_2 \gg k_1$ and $k_2 = 0.5k_1$. While there may be some doubt as to the linearity of the regression when $k_2/k_1 = 20$, the regression clearly is linear when $k_2/k_1 = 30$ and 50 and when $k_2/k_1 = 0.5$. These results demonstrate clearly the sensitivity of this numerical technique to detect curvature in regressions.

In the original study of chlorambucil hydrolysis (2), typical first-order plots (Fig. 2 in Ref. 2) showed no significant curvature when subjected to orthogonal polynomial analysis (Table II). The literature (5, 6) suggested that the chlorohydrin hydrolyzed much faster than the original dichloroethyl compound, and a rate-determining step involving the hydrolysis of the first chlorine thus was proposed.

Furthermore, Chatterji (1) plotted log $(Cl_\infty - Cl_t)/Cl_\infty$ *versus* time. While this method is satisfactory in the theoretical plots, the Cl_0 term should be added in the chlorambucil hydrolysis, *i.e.*, log $(Cl_\infty - Cl_t)/(Cl_\infty - Cl_0)$ *versus* time. For the true first-order model:

$$Cl_\infty - Cl_t = (Cl_\infty - Cl_0)e^{-kt} \qquad \text{(Eq. 2)}$$

and therefore:

$$\ln (Cl_\infty - Cl_t)/Cl_\infty = -kt + \ln (1 - Cl_0/Cl_\infty) \qquad \text{(Eq. 3)}$$

The absence of a Cl_0 term will force the regression through the zero intercept and cause an error in estimation of the rate constant.

Chatterji (1) also stated that Owen and Stewart assumed that the loss of chlorambucil always is accompanied by the loss of two chloride ions. This hypothesis was clearly tested in the original study by the chloride concentrations at time infinity (2), which demonstrated the loss of two chloride ions.

The present investigator agrees that analytical methods that determine original reactants are preferred over those measuring reaction products, particularly in complex reactions. The original research was initiated over 10 years ago, and methods to measure reactant concentrations, including high-pressure liquid chromatographic (HPLC) methods, were not available. The measurement of chloride ion by specially prepared selective-ion electrodes (3) was the most suitable method to follow the reaction at low concentrations and was amenable to continuous monitoring and automatic data collection. While HPLC methods possess a considerable advantage in the measurement and separation of products and reactants, such methods do have some limitations in fast reactions, *e.g.*, consideration of the extent of decomposition on the column during analysis, difficulties in the calibration of unstable compounds, long-term instability with subsequent changes in retention times, and reduction in the amount of data collected, causing a limitation in the use of numerical techniques.

In addition, the measurement of chloride ion in Chatterji's work may be subject to some error. In a previous study, Chatterji *et al.* (7) pointed out the inaccuracies of the use of the automatic chloride titration in faster reactions due to the production of chloride ion during the titration, the finite time to prepare samples and perform the analysis, and the inaccuracies in pipetting. The use of solid-state chloride-selective electrodes would overcome these difficulties.

Nevertheless, it is difficult to disagree with the results of the current kinetic study, where the k_2 value seems to be about half of the k_1 value. The conclusions in the original study were based on sound numerical techniques that tested for a limiting form of a more complex model ($k_2 \gg k_1$). Consideration was not given to a second limiting form ($k_2 \rightarrow 0.5k_1$) because of the literature indications to the contrary at that time. However, the current work does show that the second limiting form may be a realistic alternative, and further research is warranted.

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