# Kinetics and Mechanism of Chlorambucil Hydrolysis

**W. R. OWEN and P. J. STEWART** ˣ

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**Abstract** □ The kinetics and mechanism of hydrolysis of the cytotoxic drug chlorambucil were investigated. A study of the reaction order and of the pH, dielectric constant, and ionic strength effects on the reaction rate revealed that the hydrolysis was a limiting unimolecular nucleophilic substitution, the rate-determining step being the ionization of the first 2-chloroethylamine chlorine. The hydrolysis rate was markedly dependent on the protonation degree of the chlorambucil basic group, indicating that a cyclic ethyleneimmonium ion may be involved in the rate-determining step.

**Keyphrases** □ Chlorambucil—hydrolysis, kinetics and mechanism, reaction order, pH, dielectric constant, ionic strength □ Antineoplastic agents—chlorambucil, hydrolysis, kinetics and mechanism, reaction order, pH, dielectric constant, ionic strength □ Hydrolysis—chlorambucil, kinetics and mechanism

The cytotoxic drug chlorambucil has been used clinically in the treatment of malignant conditions associated with white blood cell proliferation (follicular lymphoma, Hodgkin's disease, *etc.*) and ovarian carcinoma (1–3). Dosage forms include intravenous injections and perfusion fluids. In these aqueous media, chlorambucil, an aromatic nitrogen mustard, undergoes hydrolysis by nucleophilic substitution. The hydrolytic pathway involves several consecutive steps. Previous stability studies (4–8) were semiquantitative or revealed little information regarding the kinetics and mechanism of hydrolysis in conditions appropriate to formulation.

The formation of a cyclic ethyleneimmonium ion has been proposed as an intermediate in aliphatic nitrogen mustard hydrolysis (7). A cyclic intermediate has not been suggested for aromatic nitrogen mustards but would seem highly probable by analogy with the aliphatic series. However, in aromatic compounds, such an intermediate would probably be less favored since the availability of the lone electron pair on the nitrogen would be reduced somewhat by resonance with the $\pi$-electrons of the aromatic ring. Therefore, this investigation was undertaken to elucidate the reaction mechanism under conditions appropriate to formulation.

## EXPERIMENTAL

**Materials**—Chlorambucil BP (mp 65.8–66.0°) was recrystallized from a light petroleum (bp 40–60°, dried over sodium)–benzene (fractionated and dried over sodium) mixture. Silver chloride was prepared by potentiometrically titrating silver nitrate (25 ml, 0.1 *M*) and nitric acid (1 ml) with sodium chloride (0.1 *M*) to the exact end-point using an automatic titrimeter. The supernatant liquid was decanted, and the silver chloride precipitate was washed six times with oxygen-free distilled water. The following buffers were used: boric acid (0.1 *M*) in potassium nitrate (0.1 *M*)–sodium hydroxide (0.1 *M*) for pH 10.1–7.8, and sodium acetate (0.1 *M*)–nitric acid (0.1 *M*) for pH 5.5–1.63. Other chemicals were reagent grade.

The silver–silver chloride electrodes were prepared electrolytically (9, 10) and possessed asymmetric potentials of $< \pm 0.02$ mv.

**Kinetic Methods**—*Potentiometric*—An alcoholic solution of chlorambucil (1 ml, 10 mg/ml) was added to the buffer (500 ml) in the reaction half-cell of the silver–silver chloride concentration cell (Fig. 1). The concentration cell was equilibrated previously in a water bath (25.00 ± 0.01°). The pH was measured[1] (±0.002 pH unit) initially and on completion of the kinetic run (pH difference was within ±0.05 pH unit in all experiments). The temperature was monitored throughout the experiment. Throughout the hydrolysis, the electromotive force (emf) and time were monitored on a data logging unit[2]. Selection of the appropriate range and sensitivity multiplier ensured maximum accuracy in the electromotive force measurement.

The silver–silver chloride concentration cell allowed continuous monitoring of the liberated chloride. At low chloride-ion concentrations and constant ionic strength, the electromotive force of the concentration cell was related to the liberated chloride by the following equation (10):

$$E = E' + 2.303RT/nF \log [c_A/2 + \sqrt{K_S + (c_A/2)^2}] \quad \text{(Eq. 1)}$$

where $E$ is the electromotive force of the concentration cell; $E'$ is the standard potential of the concentration cell, dependent on the reference half-cell potential and the activity coefficient correction term; $R$ is the gas constant (8.314 J mole$^{-1}$ deg$^{-1}$); $T$ is the absolute temperature (°K), $F$ is the Faraday constant (96,487 coulomb equivalents$^{-1}$); $n$ is the number of electrons transferred per ion; $c_A$ is the liberated chloride; and $K_S$ is the silver chloride solubility product.

Preliminary electrode response calibrations showed that the concentration cell operated in a theoretical and reproducible manner. Use of $E'$, slope ($2.303RT/nF$), and $K_S$, derived by a nonlinear parameter estimation (11) for each ionic strength, allowed calculation of the liberated chloride concentration. The first-order rate constants were determined by a linear least-squares regression–orthogonal polynomial analysis (12), using the $c_\infty$ derived from all of the data by a nonlinear parameter estimation (10).

*Conductimetric*—An alcoholic chlorambucil solution was added to

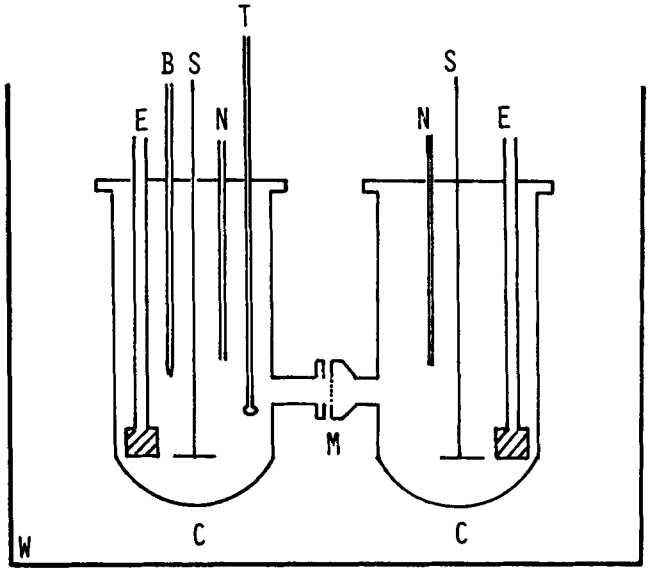


**Figure 1**—*Silver–silver chloride concentration cell for chloride determination. Key: W, thermostated water bath (±0.01°) containing the concentration cell (C); M, Millipore sintered-glass filter separating the half-cells of the concentration cell; E, silver–silver chloride electrodes; B, buret tip; S, mechanical stirrer; N, nitrogen inlet/outlet; and T, thermometer (0–50°).*

[1] Radiometer (pHM26).

[2] Solartron command range unit (EC 1475), digital voltmeter (LM 1420.2), punch drive (LU 1967), digital clock (LU 1963), scanner (LU 1461), and Facit paper tape punch (model 4070).



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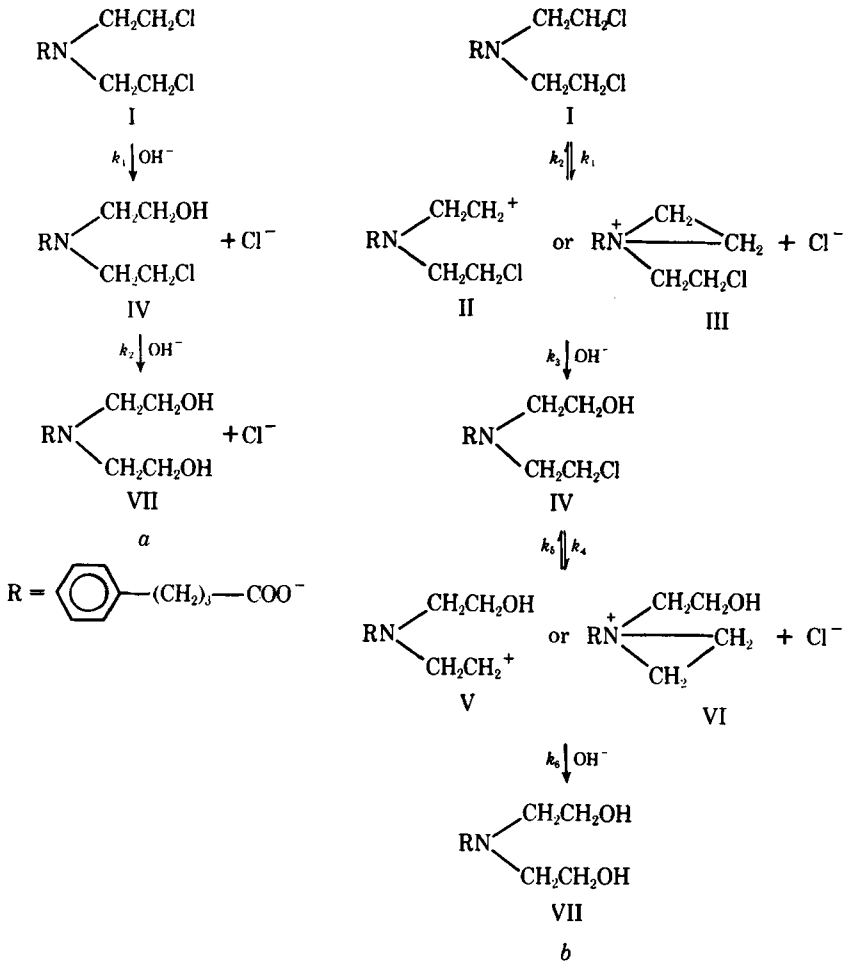

*Scheme I—Alternative hydrolytic pathways for chlorambucil. Key:* a, *bimolecular nucleophilic substitution; and* b, *unimolecular nucleophilic substitution.*

the reaction medium (500 ml) in a stoppered reaction vessel previously equilibrated in a water bath (25.00 ± 0.01°). The conductance was measured[3] at predetermined times with a platinized conductance cell (13). The temperature and pH were monitored throughout the experiment.

The ionized chlorambucil decomposition products (mainly chloride) were monitored conductimetrically. Preliminary experiments showed that the conductance–chloride-ion response calibrations were linear over the chloride concentration range required for the kinetic study. Rate constants were determined as in the potentiometric method, using conductance instead of concentration terms.

## RESULTS AND DISCUSSION

**Order of Reaction**—The general shape of the chloride concentration–time curve (Fig. 2) typified first-order behavior. Fitting the data to the first-order model:

$$\log [(c_\infty - c_0)/(c_\infty - c)] = kt/2.303 \qquad \text{(Eq. 2)}$$

where $c$ is the concentration of chloride at time $t$, $c_0$ is the initial concentration of chloride at time $t = 0$, $c_\infty$ is the concentration of chloride at infinity, and $k$ is the first-order rate constant (seconds$^{-1}$), and testing the linearity of this model (12) confirmed first-order behavior (Fig. 2). Attempts to fit more complex models were unrewarding and did not improve the fit.

The infinite chloride concentration in Fig. 2 was indicative of the hydrolysis of both chloroethyl chains (*i.e.*, $0.114 \times 10^{-3}$ *M* compared to $0.120 \times 10^{-3}$ *M*). This situation was typical of all kinetic runs. Therefore, the rate-determining step must be the hydrolysis of the first chlorine, because a consecutive pathway involving the hydrolysis of the chlorohydrin as the rate-determining step would result in a nonlinear first-order plot. This result was consistent with previous reports (5). For a first-order reaction, the hydrolysis mechanism of the first chlorine must be either limiting unimolecular ($k_1 \gg k_2$ and $k_3 \gg k_1$), with the rate-determining step being the ionization of the first chlorine (Scheme I*b*), or limiting bimolecular ($k_1 \ll k_2$), with the rate-determining step being the reaction of chlorambucil with $H_2O$ or $OH^-$ (Scheme I*a*).

**pH Effect**—Duplicate rate determinations for chlorambucil hydrolysis were made over pH 1.63–10.10 using the potentiometric method (Fig. 3).

Two discrete sections of the profile were obvious. First, at pH 5–10, the chlorambucil reaction rate with the amino group unionized [*i.e.*, pKa = 2.499 (10)] was independent of hydroxide ion, and a bimolecular substitution with the poorly nucleophilic water molecule seemed unlikely (14). Therefore, a unimolecular hydrolytic mechanism is suggested. Second, below pH 5, a decrease in the reaction rate occurred with a decrease in pH, *i.e.*, with increased protonation of the chlorambucil amino group. The decreased availability of the lone pair of nitrogen electrons should have little effect on conventional $S_N1$ or $S_N2$ reaction rates owing to the two-carbon separation from the chlorine. If any influence did occur in a bimolecular solvolysis, the decreased inductive effect should promote the reaction rate.

The lone pair, however, is essential for the formation of the cyclic ethyleneimmonium ion as the intermediate in a unimolecular pathway (Scheme I). Amino group protonation will greatly affect the reaction rate by decreasing the "effective" species concentration. The formation of a cyclic ethyleneimmonium ion would adequately explain the rate dependence on the availability of a free lone pair on the nitrogen atom.

**Proposed Kinetic Models**—While the primary chlorambucil decomposition pathway was the limiting unimolecular hydrolysis of the unprotonated basic species, the possibility of a secondary parallel reaction mechanism, which could become dominant as the ratio of the protonated to unprotonated species increased, must be considered. Two possible secondary mechanisms emerge.

[3] Cambridge conductance bridge L-380287.

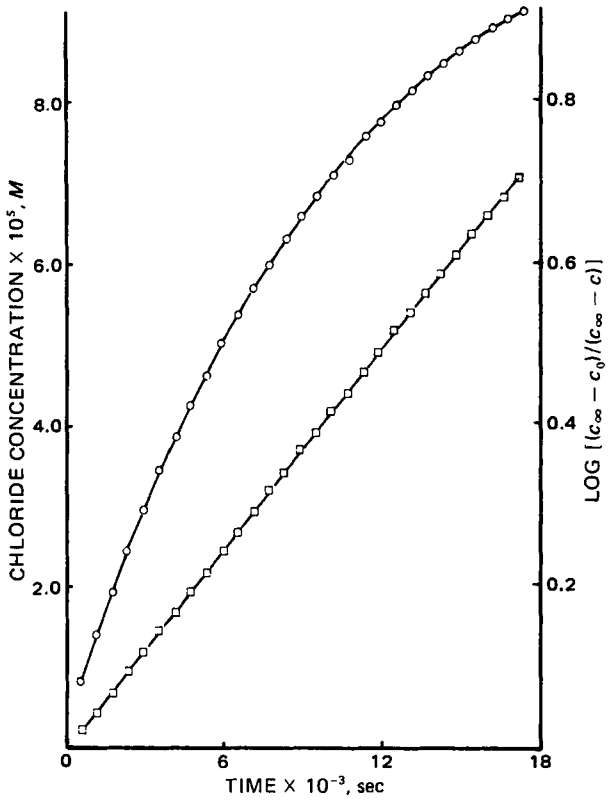


**Figure 2**—*Chlorambucil hydrolysis in borate buffer (pH 9.0, $\mu$ = 0.05) at 25.0° showing first-order behavior. Key:* ○, *chloride concentration;* □, *log* $[(c_\infty - c_0)/(c_\infty - c)]$; —, *linear least-squares regression;* $c_0$, *0.214* × $10^{-5}$ M; *and* $c_\infty$, *0.114* × $10^{-5}$ M.

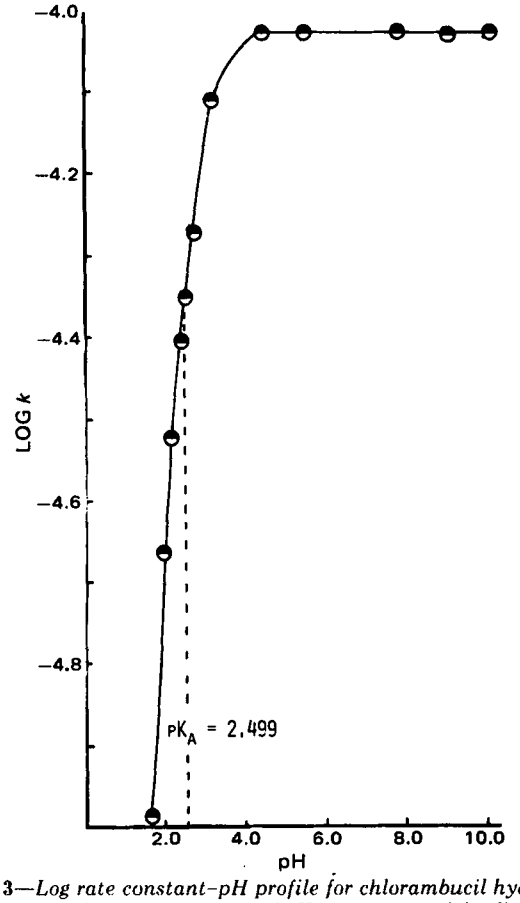


**Figure 3**—*Log rate constant–pH profile for chlorambucil hydrolysis in aqueous solution ($\mu$ = 0.1) at 25.0°. Key:* ⊖, *mean of duplicate rate determinations.*

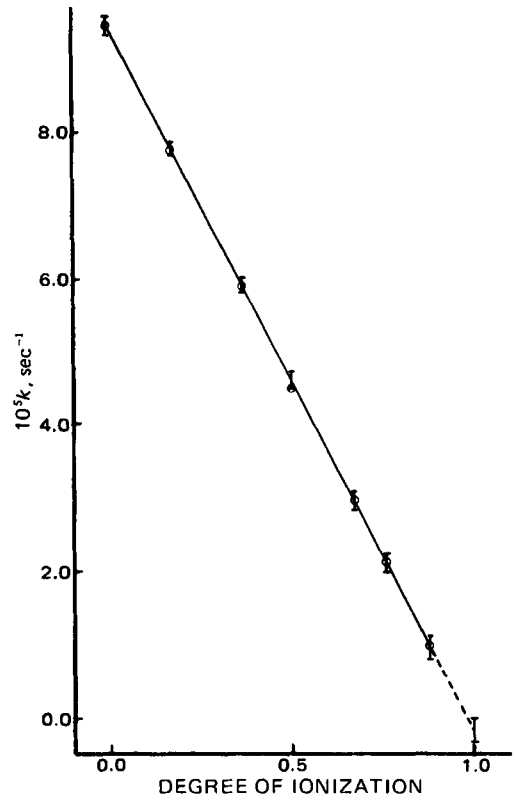


**Figure 4**—*Regression of rate constant* versus *the degree of ionization (basic group) for chlorambucil hydrolysis in an aqueous solution at 25.0°. Key:* ○, *mean of replicate rate determinations;* —, *fitted linear least-squares regression with the confidence limits of the calculated rate constants* (p = 0.95); *and* ---, *extrapolated regression to* $\alpha$ = 1.

*Unimolecular Ionized Base Hydrolysis Involving Noncyclic Carbonium Ion*—

$$dc_T/dt = k_A c_A + k_{AH} c_{AH} \quad \text{(Eq. 3)}$$

where $c_T$ is the total chlorambucil concentration equal to the sum of the unionized concentration ($c_A$) and the ionized concentration ($c_{AH}$), $k_A$ is the unimolecular rate constant for free base decomposition, and $k_{AH}$ is the unimolecular rate constant for ionized base decomposition.

$$\therefore dc_T/dt = k_A(1 - \alpha)c_T + k_{AH}\alpha c_T$$

$$= kc_T \quad \text{(Eq. 4)}$$

where $k$ is the observed first-order rate constant and equals $k_A(1 - \alpha) + k_{AH}\alpha$ and $\alpha$ is the degree of ionization.

Therefore, the model (Model A) to be tested is:

$$k = k_A + (k_{AH} - k_A)\alpha \quad \text{(Eq. 5)}$$

*Bimolecular Hydrolysis for Ionized Base Involving Noncyclic Intermediate*—

$$dc_T/dt = k_A c_A + k_{AH} c_{AH} c_{OH} \quad \text{(Eq. 6)}$$

where $k_{AH}$ is the bimolecular rate constant for decomposition of the ionized base and $c_{OH}$ is the concentration of hydroxyl ions.

$$\therefore dc_T/dt = [k_A(1 - \alpha) + k_{AH}\alpha c_{OH}]c_T \quad \text{(Eq. 7)}$$

For pseudo-first-order kinetics at constant pH:

$$k = k_A(1 - \alpha) + k_{AH}\alpha c_{OH} \quad \text{(Eq. 8)}$$

where $k$ = observed first-order rate constant (seconds$^{-1}$).

$$\therefore k = (k_A K_a + k_{AH} K_w)/(K_a + c_H) \quad \text{(Eq. 9)}$$

where $c_H$ is the hydrogen-ion concentration, $K_a$ is the dissociation constant for the basic group of chlorambucil, and $K_w$ is the water dissociation constant.

Therefore, the model (Model B) becomes:

$$\log k = \log (k_A K_a + k_{AH} K_w) - \log (K_a + c_H) \quad \text{(Eq. 10)}$$

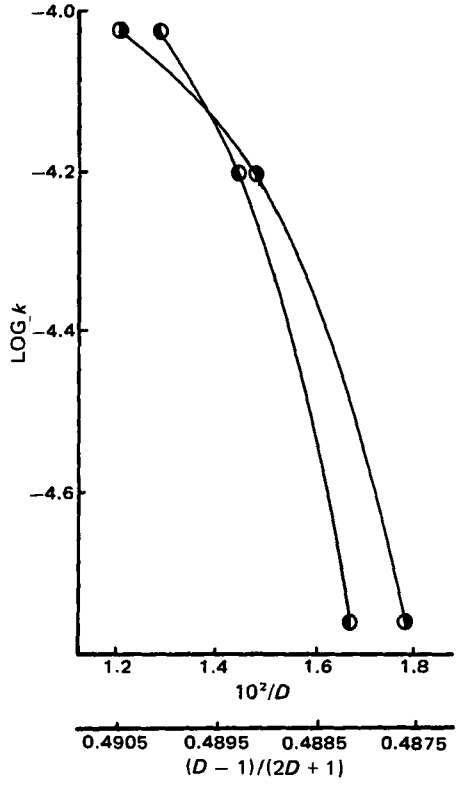


**Figure 5**—*Solvent effect on the chlorambucil hydrolysis rate in aqueous-alcohol solutions at 25.0°. Key:* ◑, *dielectric constant function* = (D − 1)/(2D + 1); *and* ◐, *dielectric constant function* = 1/D. *Each point is the mean of three replicates.*

Model A was tested by regressing the observed rate constant ($k$) against the degree of ionization ($\alpha$) (Fig. 4). An analysis of variance showed no significant deviations from linearity ($p > 0.2$). Thus, the data can be adequately interpreted by this model; *i.e.*, the reaction proceeds by a unimolecular hydrolysis of the free base *via* a cyclic intermediate in parallel with a similar unimolecular hydrolysis of the ionized base.

The magnitude of the two parallel mechanisms can be determined by calculation of the rate constants $k_A$ and $k_{AH}$. Linear regression analysis provided the following results:

$$k_A = 9.436 \times 10^{-5} \pm 0.988 \times 10^{-6} \quad (p = 0.95) \qquad \text{(Eq. 11)}$$

$$k_{AH} = -0.210 \times 10^{-5} \pm 0.225 \times 10^{-5} \quad (p = 0.95) \qquad \text{(Eq. 12)}$$

The hypothesis that $k_{AH}$ does not differ significantly from zero cannot be rejected [$t$ test: $p(t = 1.93, 22\ df) = 0.1$–$0.05$]. Under these circumstances, the rate constant $k_{AH}$ must be extremely small or zero. Hence, the decomposition occurred substantially by unimolecular hydrolysis of the free base. Hydrolysis of the ionized base was insignificant. Therefore, the data can be adequately represented by the model:

$$k = k_A(1 - \alpha) \qquad \text{(Eq. 13)}$$

In addition, since there was no significant departure from linearity shown by the analysis of variance, nonlinear terms such as $k_{AH}\alpha c_{OH}$ in the bimolecular Model B were unnecessary. Consequently, testing of Model B was irrelevant.

**Solvent Effects**—Replicate rate determinations were made in 0.0, 20.0, and 40.0% (v/v) ethanol in water at zero ionic strength by the conductimetric method. The effect of the dielectric constant on the hydrolysis rate is shown in Fig. 5, where log $k$ is plotted as a function of the dielectric constant (15), *i.e.*, log $k$ *versus* $1/D$ (Amis theory) or log $k$ *versus* $(D - 1)/(2D + 1)$ (Laidler and Eyring theory). The chlorambucil reaction rate increased markedly with the solvent dielectric constant. For a unimolecular mechanism, this finding reflected the increased solvation (and, therefore, stabilization) of the transition state (*i.e.*, ethyleneimmonium ion) with respect to the uncharged reactant (14). In contrast, for a bimolecular reaction, an increase or decrease could be expected depending on whether the attacking nucleophile is uncharged or charged and, therefore, on the degree of charge dispersion in the transition state (14). The results obtained are thus consistent with the proposed unimolecular model but do not provide absolute proof of the reaction pathway.

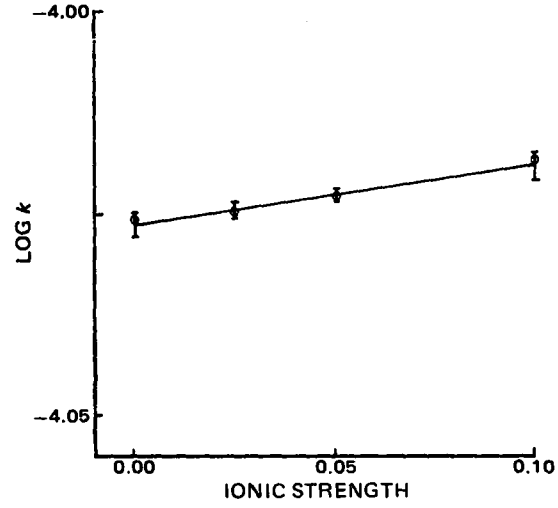


**Figure 6**—*Ionic strength effect on the chlorambucil hydrolysis rate in borate buffer (pH 9.0) at 25.0°. Key:* ○, *mean of three replicate rate determinations; and* —, *fitted linear least-squares regression with the confidence limits* (p = 0.95).

Neither kinetic model provided a linear representation of the data. However, nonelectrostatic effects in the Laidler–Eyring and Amis models were assumed to be negligible. Therefore, specific effects such as hydrogen bonding and van der Waal's forces of interaction between the solvent and reactant, which may be prevalent in chlorambucil hydrolysis in a hydroalcoholic solution, could modify the efficiency of the solvent in promoting the reaction rate. If so, linearity of the log $k$–dielectric constant function cannot be expected (16).

**Salt Effects**—The effect of common ions (chloride) on the reaction rate could not be ascertained since the required added chloride concentrations markedly reduced the sensitivity of the silver–silver chloride electrode. However, the effect of noncommon ions (*e.g.*, acetate, borate, and nitrate used in the study of pH effect) on the reaction rate was negligible, as evidenced by the pH-independent region of the log $k$–pH profile in Fig. 3. The results are indicative of a unimolecular substitution where the rate-determining step is independent of nucleophilicity. For a bimolecular mechanism, however, the reaction rate would be markedly dependent on the concentration and nucleophilic character of the attacking species (14).

The general ionic strength effect also was studied. Replicate rate determinations using the potentiometric method were performed for chlorambucil hydrolysis in borate buffer (pH 9.0, 0.01 $M$), adjusted to ionic strengths 0.100, 0.050, and 0.025 with potassium nitrate. Rate constant determinations at zero ionic strength were performed conductimetrically. Log $k$ is regressed against the ionic strength (16) in Fig. 6. An increase in ionic strength caused a small acceleration of chlorambucil hydrolysis, which reflected the greater solvation and stabilization of the transition state at higher solvent polarity. For both the unimolecular and bimolecular pathways, an increase in the reaction rate with an increase in ionic strength would be expected (14). The small degree of acceleration of the observed rate was indicative of a reaction in a high dielectric constant solvent.

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# Effects of Aspirin on $^{14}$C-Pirprofen Disposition in Rats

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Received December 18, 1978, from the *Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, NY 10502.* Accepted for publication February 8, 1979.

**Abstract** □ The effects of aspirin on $^{14}$C-pirprofen disposition in the rat were studied. An oral 60-mg/kg dose of aspirin significantly reduced plasma radioactivity during the 1–8-hr interval after an intravenous 5-mg/kg injection of $^{14}$C-pirprofen. The aspirin-treated group had only 69% as much area under the radioactivity curve as the control group. The radioactive material in plasma consisted almost entirely of $^{14}$C-pirprofen, as shown by GLC. The plasma clearance of $^{14}$C-pirprofen was 7.4 ml/hr for the aspirin-treated group and 5.1 ml/hr for the control group, while the volumes of distribution were 0.32 and 0.20 liter/kg, respectively. The apparent elimination half-life was unchanged at 5.9 hr. $^{14}$C-Pirprofen was approximately 98.6% bound to plasma proteins, and the binding decreased to an average of 97.2% in the presence of salicylate. Binding to blood cellular constituents was insignificant. Rats given $^{14}$C-pirprofen by intravenous injection without aspirin secreted 36.0–42.8% of the dose radioactivity into bile during 4 hr while a comparable group given 60 mg of aspirin/kg secreted 46.4–70.8%. TLC and GLC demonstrated that the radioactivity in rat bile was 80–90% conjugated $^{14}$C-pirprofen. The increased radioactive material secretion into bile was compensated in the intact rat by reabsorption, since the total radioactive material excreted in urine was not changed by aspirin administration.

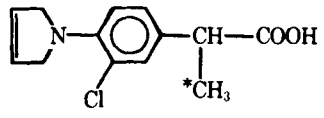
**Keyphrases** □ Aspirin—effect on pirprofen disposition, rats □ Pirprofen—disposition, effect of aspirin, rats □ Anti-inflammatory agents—pirprofen, disposition, effect of aspirin, rats

Pirprofen, 2-[3-chloro-4(3-pyrrolinyl)-phenyl] propionic acid, is a new anti-inflammatory drug of the arylalkanoic acid type (1).

Salicylate has been reported to reduce the plasma concentration of various arylalkanoic acid compounds including indomethacin (2), fenoprofen (3, 4), naproxen (5, 6), and diclofenac (7) in animals and humans. Pirprofen concentrations in human plasma as well as that bound to plasma proteins were reduced by salicylate[1], and the present study was undertaken to examine salicylate effects on the biological disposition of pirprofen in the rat.

## EXPERIMENTAL

**Radioactive Pirprofen**—$^{14}$C-Pirprofen (I), with the radioactive atom in position three of the propionic acid moiety, was used[2]. The specific

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activity was 13 μCi/mg. The labeled compound was examined by TLC using the procedures described later. The major constituent chromatographed like authentic pirprofin, and the only impurity was a substance comprising about 5% of the radioactivity.

**Animal Procedures**—Young adult male Wistar rats[3], ~200 g, were used.

$^{14}$C-Pirprofen dosage solutions were prepared in 0.2 *M* $NaHCO_3$ to provide 5 mg/kg and 3 μCi/animal in volumes of 0.3 ml for injection and 1.0 ml for intubation. Aspirin[4] was administered as a 60-mg/kg aqueous dose by intubation.

Blood samples were obtained by anesthetizing the rats with ether, opening the abdominal cavity, and exsanguinating *via* the abdominal aorta into a heparinized 10-ml syringe. Blood plasma was frozen pending analysis.

Bile duct cannulations were done under ether anesthesia for rats that were to receive intravenous $^{14}$C-pirprofen, and these rats were confined in restraining cages[5] during bile collection. Rats receiving $^{14}$C-pirprofen orally were anesthetized with urethan[4] rather than ether to preclude the necessity for restraint. A 25% solution of urethan in water was administered as a 1.25-g/kg ip dose.

**Radioactivity Measurements**—A solubilizer–phosphore solution was prepared by mixing one volume of a commercial solubilizer[6] with four volumes of toluene containing 0.5% 2,5-diphenyloxazole[7] and 0.01% *p*-bis(*o*-methylstyryl)benzene[8]. The solubilizer–phosphore solution (15 ml) was mixed with 10 μl of injection solutions, 50 μl of ultrafiltrates, or 0.1 ml of plasma, bile, or urine prior to duplicate sample counting in a liquid scintillation spectrometer[9]. Counting efficiency was measured by external standardization.

Whole blood (0.1 ml) was pipetted onto 2.5-cm circles of filter paper and dried before combustion[10]. The resulting carbon dioxide was absorbed in 4 ml of ethanolamine to which 9 ml of methanol and 6 ml of scintillator solution, containing 15.0 g of 2,5-diphenyloxazole and 1.0 g of *p*-bis(*o*-methylstyryl)benzene/liter of toluene, were added for counting.

**TLC**—Aliquots (20 μl) of bile or an equivalent amount of hydrolyzate

[1] To be published.
[2] The material was synthesized by Dr. Naba K. Chaudhuri and coworkers at Ciba-Geigy Corp., Ardsley, NY 10502.
[3] Charles River C.D. or Marland Farms.
[4] Merck and Co., West Point, PA 19486.
[5] Aerospace Industries, Garnerville, NY 10923.
[6] Bio-Solv BBS-3, Beckman Instruments, Fullerton, CA 92634.
[7] Eastman Kodak Co., Rochester, NY 14650.
[8] Packard Instrument Co., Downers Grove, IL 60515.
[9] Intertechnique SL-40, IN/US Service Corp., Fairfield, NJ 07006.
[10] Model 305 oxidizer, Packard Instrument Co., Downers Grove, IL 60515.



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