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Communications to the Editor

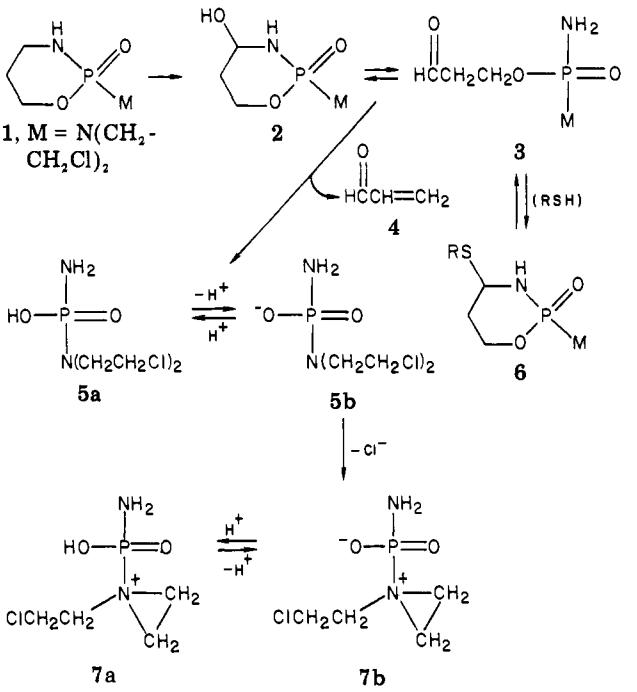
^{31}P NMR Investigations of Phosphoramide Mustard: Evaluation of pH Control over the Rate of Intramolecular Cyclization to an Aziridinium Ion and the Hydrolysis of This Reactive Alkylator

Sir:

The metabolic transformations shown in Scheme I for the anticancer drug cyclophosphamide (**1**)[1] are generally viewed as an initial "activation step" (**1** → **2/3**) which is followed by partitioning of the primary metabolites among competing toxification (**2/3** → **4/5**) and reversible deactivation[2] (**2/3** → **6**) pathways. Causal relationships between these events and the oncostatic specificity of **1** have not as yet been clearly established; however, the toxicity of phosphoramide mustard (**5**)[3] is reasonably associated with DNA cross-linking.[4,5] Friedman[6] originally rationalized the high alkylating reactivity of **5** in terms of an electronically enhanced rate of intramolecular cyclization of conjugate base **5b** to form aziridinium ion **7** (Scheme I), the trapping of which has been recently reported by Colvin et al.[7] The influence of pH on the alkylating activity of **5** has been recognized as an important reactivity feature;[6-9] however, details in this connection have been heretofore unavailable. This lack of fundamental information concerning cyclophosphamide's ultimate alkylating metabolites prompted our investigation of **5** by high-resolution NMR, and we now report preliminary ^{31}P NMR measurements which accurately define the pH–rate profile for **5** → **7** and also reveal a striking pH dependency for subsequent hydrolytic reactions of **7**. These findings bear upon numerous recent studies involving **5**[2,5,7,10-14] and provide the basis for a new perspective from which the oncostatic specificity of **1** may be considered.

The purity of a recrystallized (absolute EtOH–Et_2O) sample of the cyclohexylammonium salt of **5** (mp 109 °C, lit.[15] mp 107–108 °C) was confirmed by ^{31}P NMR analysis,[16] which showed only one absorption signal at 26.3 ppm in anhydrous Me_2SO/Me_2SO-d_6. Kinetic studies with this material were conducted at 37 °C using 50 mM initial concentrations in 1.0 M buffers, which provided acceptably small pH decreases (<0.2 unit) during the eventual formation of 2 equiv of HCl. Bistris was used for pH 5.7 and 6.5, while pH 7.0, 7.4, 8.2, and 9.0 were maintained with Tris buffering.[17] A 300-s thermal equilibration period was employed in each kinetic run, and the accumulation of a free-induction decay signal over 200 s was repeated every 600 s. Fourier transformation gave frequency-domain spectra in which resulting peak heights were used to measure the relative concentration of all detectable phosphorus-containing solution components, and such data were used to plot ln ($[5]_{rel}$) vs. time. A straight line (correlation coefficient >0.996) was obtained at each pH

Scheme I



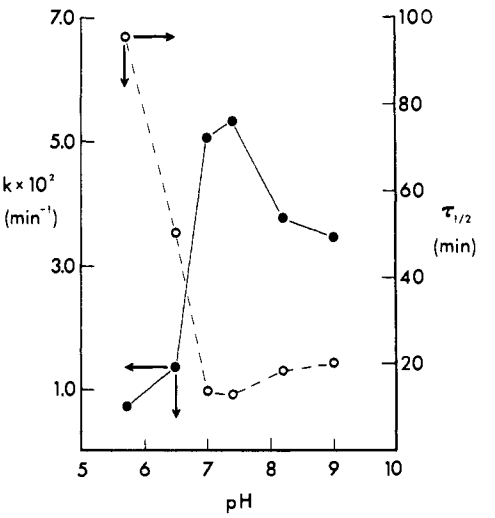


Figure 1. Combination plot showing the pH dependence of the rate of disappearance of **5** expressed as either a first-order rate constant (k, solid curve) or half-life ($\tau_{1/2}$, dashed curve); temperature = 37 °C.

and the slope was equated with the first-order rate constant (k) for disappearance of **5**: (pH, $k \times 10^2$ min^{-1}) 5.7, 0.72; 6.5, 1.37; 7.0, 5.04; 7.4, 5.33; 8.2, 3.75; 9.0, 3.43. These

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rate data are displayed in Figure 1 as a pH–rate profile and reveal that a maximum disappearance rate for **5** occurs in the vicinity of pH 7.4. The reactivity of **5** is further seen to sharply decrease as the pH is lowered, while comparable shifts in the alkaline direction have much less of an effect on **5**. The inverse relationship between k and the half-life ($\tau_{1/2}$) of **5** results in the "L-shaped" profile for $\tau_{1/2}$ vs. pH, which is also shown in Figure 1. In particular, it is noteworthy that the half-life of **5** at pH 7.4 is 13 min and that it increases to nearly 100 min at pH ~6 vs. an increase to only 20 min at pH 9.0.

The decelerating effect of acidity on the disappearance rate of **5** leads to the expectation that acid production in the absence of buffer will lead to curved plots of ln ($[5]_{rel}$) vs. time which show a continual diminution in reaction velocity. While these predictions were verified by ^{31}P NMR measurements in both unbuffered D_2O (36 °C) and 0.9% NaCl–D_2O (25 °C), they conflict with earlier reported[18] first-order kinetics for the disappearance of **5** which had been studied by ^1H NMR (60 MHz) under identical reaction conditions. The apparent discrepancy may be rationalized, however, by noting that for each reaction a straight line with the reported[18] slope was found to pass through the ^{31}P NMR derived data points in such a manner as to give a "reasonable" correlation coefficient but unacceptably large positive and negative deviations of the ln ($[5]_{rel}$) values. *The pH and buffering capacity of a medium should now be recognized as critical factors in studies with 5 which intend to either measure a half-life or assume that a reported half-life is operative.* In this connection, it is worthwhile to note that our value of $\tau_{1/2}$ = 50 min at pH 7.0 in 1.0 M Bistris agrees quite well with the half-life for **5** of 48 min at the same pH and temperature (37 °C) in 0.07 M phosphate buffer, which has been indirectly determined[19] by measurements of the "alkylating capacity" of **5** toward 4-(*p*-nitrobenzyl)pyridine (NBP).

Reversible dissociation of acid **5a** into conjugate base **5b** is relatively rapid on the NMR time scale and the ^{31}P signal for **5**, which is the weighted average of signals for **5a** and **5b**, was expected and found to be strongly pH dependent. From simultaneous measurement of the chemical shift of **5** (δ_{av}) and the ambient pH in unbuffered water at 20 °C, a titration curve was generated by plotting δ_{av} vs. pH over the range of pH 4.0–6.5. Chemical-shift measurements at lower pH were foiled by exceedingly *fast* hydrolysis rates, presumably due to proton-catalyzed P–N bond cleavage; however, a least-squares fit of the data to the Henderson–Hasselbalch equation gave a pK_a of 4.75 ± 0.03, which is close to the reported[2] value of 4.8 at 4 °C.

Variations in absorption frequency with pH were also observed for products derived from **5**. This complication prevented reliable deciphering of all of the ^{31}P spectral information; however, the following salient generalizations were evident.

Under acid conditions, the decrease in signal intensity for **5** (13.2 ppm) over 2 half-lives was primarily accompanied by the growth of absorption signals which were within a few parts per million of the external H_3PO_4 and were subsequently identified (by addition of authentic compound) as the expected P–N hydrolysis products, viz., phosphoric acid and phosphoramidic acid, $H_2NP(O)(OH)_2$. Spectra obtained at pH 7.0–9.0 were quite different in that they showed the initially rapid growth of a transient absorption (13.9 ppm) which then began to diminish in intensity during concurrent evolution of two permanent absorptions grouped near **5** and a relatively weak inorganic phosphate signal. Figure 2 presents the time course of the

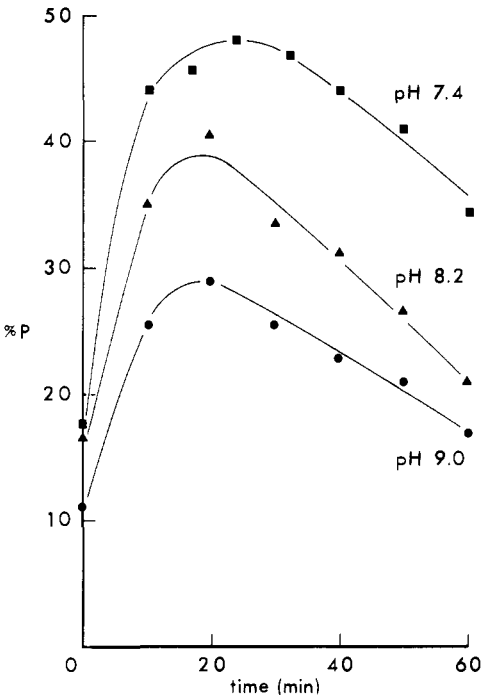

Figure 2. Effect of pH on the time course of the intensity of the signal at ca. 13.9 ppm for aziridinium ion **7**, with %P = 100[(^{31}P NMR peak height of **7**)/(sum of all ^{31}P NMR peak heights)]; temperature = 37 °C.

intensity of the transient absorption, relative to all ^{31}P signals, and the effect of pH on this dynamical concentration. It is readily apparent that the maximum intensity drops from ca. 50% at pH 7.4 to only ca. 30% at pH 9.0. While elucidation of the products associated with the resonances close to **5** is currently under investigation, persuasive assignment of the transient absorption at 13.9 ppm to azridinium ion **7** was achieved by directly monitoring its alkylation of 2-mercaptoethanol ($HSCH_2CH_2OH$). This approach was based on the previously demonstrated[7] use of mercaptoethane as a trapping agent for **7** via sequential alkylation reactions. The essence of our findings is illustrated by considering the reaction of **5** in the presence of a tenfold excess of 2-mercaptoethanol in 1 M Tris buffer at pH 7.4, which had no effect on the half-life of **5** and, moreover, prevented the accumulation of a detectable concentration of **7** during the entire course of reaction. Instead, the near-exclusive final product grew in at 13.3 ppm and was subsequently characterized as $H_2NP(O)(OH)N(CH_2CH_2SCH_2CH_2OH)_2$ by ^1H NMR (200 MHz).

The presently reported ^{31}P NMR kinetic data for **5** have demonstrated that the half-life of this metabolite of **1** exhibits appreciable variation over the physiologically relevant pH range of 6–8 and that conjugate base **5b** is the required precursor to aziridinium ion **7**. The marked sensitivity of the pH–rate profile and hydrolytic product distribution represent a caveat with regard to studies dealing with **5**, as they underscore a complexity in both reaction dynamics and products which has been largely ignored. From a more speculative viewpoint, we suggest that pH control over the reactivity of **5** and **7** provides a chemical basis for rationalizing at least part of the oncostatic specificity of **1** and as such is formally analogous to the previously noted deactivation[2] concepts. For example, reference to Figures 1 and 2 suggests that the rate for cyclization of **5** into **7** would be roughly 50% slower in tumor cells that may be more acidic than normal cells (pH 6.9 vs. 7.4)[20] and that once the active alkylator (**7**) is

generated it will have a longer lifetime under relatively acidic conditions, due to less frequent interception by hydroxide ion. Whether alone or in concert, these circumstances with regard to **5** and **7** provide for greater probability of encountering and alkylating DNA and, in effect, represent a form of selective cross-linking.

There are significant unanswered questions which must be addressed in connection with the biologically pertinent chemistry of **5**, and our continued work in this area will employ, among other methods, multinuclear NMR techniques.

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References and Notes

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(16) ^{31}P NMR spectra were recorded at 40.25 MHz on a JEOL FX-100 spectrometer equipped with a 10-mm variable-temperature probe. Normal operating conditions utilized continuous broad-band ^1H decoupling, a 26-μs ($\pi/2$) pulse, 5-kHz spectral window and filter, and 8192 data points. Prior to Fourier transformation, the free-induction decay signal was zero filled with 8192 points and exponentially multiplied so as to result in an additional 1-Hz line broadening in the frequency-domain spectrum. A 2-s pulse delay time was used throughout these studies, as longer waiting periods were shown not to have a measurable effect upon relative absorption intensities. The lock signal was provided by 20% (v/v) D_2O, and chemical shifts were measured relative to an external solution of 25% (v/v) H_3PO_4 in water that was contained in a 1-mm coaxial capillary tube. A sample temperature of 37 °C was directly measured under normal operating conditions by immersion of a calibrated thermometer.
(17) Abbreviations used: Bistris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane. The specified pH values refer to buffer solutions before the addition of 20% (v/v) D_2O and are not corrected for isotope effects [cf. R. Lumry, E. L. Smith, and R. R. Glantz, *J. Am. Chem. Soc.*, **73**, 4330 (1951)].
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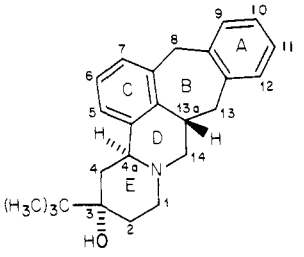
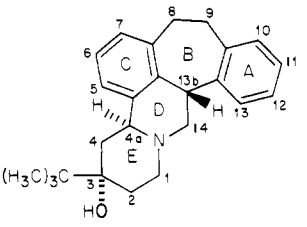
(+)-Isobutaclamol: A Crystallographic, Pharmacological, and Biochemical Study

Sir:

We have recently described[1] the synthesis and some pharmacological properties of (±)-isobutaclamol (I), a



I (isobutaclamol)



II (butaclamol)

compound having a neuroleptic profile virtually identical with that of (±)-butaclamol (II).

The activity of (±)-butaclamol, both in vivo and in vitro, has been shown to reside exclusively in the (+)-3*S*,4a*S*,-13b*S* enantiomer.[2-4] Based on a proposed model of the central dopamine receptor,[1,5] we had predicted that (±)-isobutaclamol's neuroleptic activity resides solely in its 3*S*,4a*S*,13a*S* enantiomer.

With respect to the stereochemistry of isobutaclamol at positions 3, 4a, and 13a, no chemical evidence was available to aid in the assignment of relative configurations. However, assuming a requirement for identical molecular topographies in the rings B, C, D, and E regions of both butaclamol and isobutaclamol, the latter was assigned[1] the 4a,13a-trans and 3(OH),13a(H)-trans relative configurations, shown in I, analogous to those found in butaclamol by crystallographic analysis.[6]

The purpose of the present investigation was to assess the validity of these predictions by resolving (±)-isobutaclamol, examining some aspects of the neuroleptic profile of the enantiomers, and determining relative and absolute configurations by a crystallographic analysis.

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