^{31}P NMR Kinetic Studies of the Intra- and Intermolecular Alkylation Chemistry of Phosphoramide Mustard and Cognate N-Phosphorylated Derivatives of *N*,*N*-Bis(2-chloroethyl)amine[1,2]

Thomas W. Engle,[†] Gerald Zon,*[,†] and William Egan[‡]

Department of Chemistry, The Catholic University of America, Washington, DC 20064, and Division of Biochemistry and Biophysics, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205. *Received April 6, 1982*

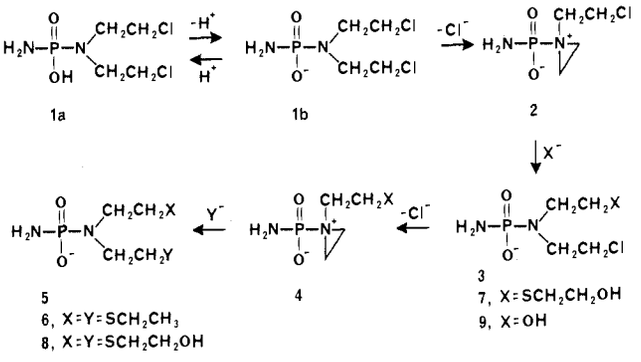
^{31}P Fourier-transform NMR spectroscopy at 40.25 MHz was used to measure the pK_a (4.75 ± 0.03) of the cyclohexylammonium salt of phosphoramide mustard (1·CHA) at 20 °C and to study the kinetics and products of the decomposition of 1·CHA at solution pH values between 5.7 and 9.0, at 37 °C, and at pH 7.4 in the presence of either metal ions or nucleophilic trapping agents. The half-life ($\tau_{1/2}$) of 1 was approximately constant (18 ± 3 min) between pH 9.0 and 7.0 and then increased sharply with lowered pH (~100 min at pH 5.7); the rate deceleration caused by metal ions was less pronounced (e.g., $\tau_{1/2} \simeq 52$ min for 1 M $MgCl_2$, pH 7.4). Hydrolysis of P–N bonds was dominant at pH ≤ 6.5, whereas at pH 7.4–9.0 the *individual rate constants* for intramolecular nucleophilic displacement of chloride ion to give aziridinium ion **2** (Scheme I) and ring opening of **2** by hydroxide ion were measurable, giving $\tau_{1/2}$ (average) ≃ 18 min for 1, and $\tau_{1/2}$ = 30, 18, and 15 min for **2** at pH 7.4, 8.2, and 9.0, respectively. At pH 7.4, 37 °C, aziridinium ions **2** and **4** were intercepted relatively rapidly by an excess of 2-mercaptoethanol and afforded separate ^{31}P NMR signals for the resulting monosubstituted (**7**) and disubstituted (**8**) products. The signal intensities for **7** and **8** were fitted to the integrated rate equations for, in effect, two consecutive "first-order" reactions (1 → **7** → **8**), thus allowing quantification of the reactivity of intermediate **7**, $\tau_{1/2} \simeq 16$ min. Similar results were obtained for the reaction of 1·CHA with sodium 2-mercaptoethylsulfonate ("mesnum"), whereas thiourea gave no evidence (^{31}P NMR) for the accumulation of *S*-alkyl trapping products. The decomposition kinetics for nine analogues of 1 (Scheme II: **13**, **14**, and **25**–**31**) were also studied by ^{31}P NMR, and their relative reactivities were interpreted in terms of resonance and inductive effects, which alter the electron density at the nitrogen position in the PN-$(CH_2CH_2Cl)_2$ moiety. The three *N'*-alkyl derivatives of 1·CHA (**27**–**29**) provided evidence for a considerable amount (30–50%) of intramolecular O-alkylation leading to 2-(alkylamino)-3-(2-chloroethyl)-1,3,2-oxazaphospholidine 2-oxides (e.g., **34**, Scheme III). The above data are briefly discussed with regard to the role of 1 as a cytotoxic metabolite of the anticancer drug cyclophosphamide, and the data are also considered in connection with previously reported studies dealing with 1 and other nitrogen mustard alkylating agents.

Almost 2 decades after Friedman and Seligman's[3] synthesis of phosphoramide mustard (**1**, Scheme I) as a candidate for tumor-specific release of *N*,*N*-bis(2-chloroethyl)amine, Colvin, Padgett, and Fenselau[4] established that **1** was produced by murine microsomal metabolism of the anticancer drug cyclophosphamide.[5] Shortly thereafter, **1** was detected in the plasma and urine of patients receiving cyclophosphamide therapy.[6,7] The importance of **1** in the mechanism of action of cyclophosphamide is related to its high cytotoxicity.[8] In this connection, it has been shown that **1** is a potent alkylating agent at physiological pH: **1** reacts with sulfhydryl[9] groups, guanosine,[10] guanosine 5′-monophosphate,[11] deoxyguanosine,[10] and phosphodiester groups in DNA;[12] **1** also produces both DNA–protein and intrastrand DNA cross-links.[13] Theories regarding cyclophosphamide's oncostatic selectivity have prompted numerous investigations concerning the metabolic *precursors* of **1**;[14–18] however, the fundamental alkylation chemistry of **1** is, by comparison, a poorly understood subject.

We now report ^{31}P NMR spectroscopic studies of media and structural factors that influence the dynamical and chemical properties of **1**, transient species derived from **1**, and allied alkylating agents. The twofold goal of this work was the elucidation of possible chemical reasons for selective alkylation by **1** and the refinement of structure–reactivity concepts that extend to diverse phosphoramide mustards.[14,19–21]

The greater alkylating reactivity of phosphorodiamidic acid **1**, relative to its ester derivatives, was attributed by Friedman[19] to the electronically enhanced rate of intramolecular cyclization of conjugate base **1b** to form aziridinium ion **2** (Scheme I). Intermolecular reaction of **2** with a biological nucleophile (X^-) gives a monosubstituted intermediate (**3**), which can in turn form a second aziridinium ion (**4**) and subsequently afford a disubstituted (bisalkylated) product (**5**), such as the recently found[11]

Scheme I



[†] The Catholic University of America.
[‡] FDA.

(1) A preliminary communication regarding this work has been published: T. W. Engle, G. Zon, and W. Egan, *J. Med. Chem.*, **22**, 897 (1979).
(2) *N*,*N*-Bis(2-chloroethyl)phosphorodiamidic acid has been generally referred to as "phosphoramide mustard". For the sake of simplicity, this trivial name has been adopted herein for the naming of analogous structures; systematic nomenclature for all compounds is given under Experimental Section.
(3) O. M. Friedman and A. M. Seligman, *J. Am. Chem. Soc.*, **76**, 655 (1954).
(4) M. Colvin, C. A. Padgett, and C. Fenselau, *Cancer Res.*, **33**, 915 (1973).
(5) D. L. Hill, "A Review of Cyclophosphamide", Charles C. Thomas, Springfield, IL, 1975.
(6) C. Fenselau, M.-N. N. Kan, S. Billets, and M. Colvin, *Cancer Res.*, **35**, 1453 (1975).
(7) I. Jardine, C. Fenselau, M. Appler, M.-N. Kan, R. B. Brundrett, and M. Colvin, *Cander Res.*, **38**, 408 (1978).
(8) C. L. Maddock, A. H. Handler, O. M. Friedman, G. E. Foley, and S. Farber, *Cancer Chemother. Rep.*, **50**, 629 (1966).
(9) M. Colvin, R. B. Brundrett, M.-N. N. Kan, I. Jardine, and C. Fenselau, *Cancer Res.*, **36**, 1121 (1976).
(10) J. R. Mehta, M. Przybylski, and D. B. Ludlum, *Cancer Res.*, **40**, 4183 (1980).

0022-2623/82/1825-1347$01.25/0

Table I. ^{31}P NMR Derived Kinetic Data for the Disappearance of Phosphoramide Mustard (1) Under Various Reaction Conditions at 37 ± 2 °C

run no.	conditions[a]	k,[b] min^{-1}	$\tau_{1/2}$, min
1	pH 5.7, Bistris	0.0072	96
2	pH 6.5, Bistris	0.014	51
3	pH 7.0, Tris	0.051	14
4	pH 7.4, Tris	0.038 (±0.006)	18 (±3)
5	pH 7.4, Tris (HNO_3)	0.038	18
6	pH 8.2, Tris	0.036	19
7	pH 9.0, Tris	0.032	20
8	pH 7.4, Tris, 1 M LiCl	0.020	35
9	pH 7.4, Tris, 1 M NaCl	0.018	38
10	pH 7.4, Tris, 1 M $CaCl_2$	0.017	40
11	pH 7.4, Tris, 1 M $MgCl_2$	0.013	52
12	pH 7.4, Tris, $HSCH_2CH_2OH$[c]	0.036	19
13	pH 7.4, Tris, $HSCH_2CH_2SO_3^-Na^+$[c]	0.038	18
14	pH 7.4, Tris, $H_2NC(S)NH_2$[c]	0.035	19
15	pH 7.4, Tris, BSA[d]	0.040	17

[a] In each run, the initial concentration of 1·CHA was approximately 50 mM; Bistris = $(HOCH_2CH_2)_2NC(CH_2OH)_3$, Tris = $H_2NC(CH_2OH)_3$; the 1 M buffer pH values were adjusted with concentrated HCl, except for run 5, wherein concentrated HNO_3 was used. In each run, ΔpH ≤ 0.2 after complete reaction. [b] First-order rate constants obtained by least-squares fits ($r > 0.996$) of ln (% P) vs. time, where the quantity "% P" refers to the ^{31}P NMR signal intensity of 1, relative to all observable signals, which were recorded with NOE. The error limits between runs were estimated to be ca. 10–20%; the error limits indicated for run 4 refer to five independent experiments (cf. footnote 23). [c] Tenfold molar excess, relative to the initial concentration of 1·CHA. [d] Twofold weight excess of BSA (bovine serum albumin), relative to 1·CHA.

dimer adduct in which each "arm" of 1 has N-alkylated the 7-position in guanosine 5′-monophosphate. That this stepwise pathway, rather than direct S_N2 displacements of Cl^- by X^- and Y^-, is mechanistically competent was unabiguously established by Colvin et al.,[9] who reacted specifically deuterated 1 [*N*,*N*-bis(2-chloro-2,2-dideuterioethyl)phosphorodiamidic acid] with an excess of ethanethiol at 37 °C, pH 7.0–7.5, and found by mass spectrometry that the bisalkylated product (6) contained equal amounts of the deuterium labels at all of the ethylene positions, which required symmetrization via aziridinium ions 2 and 4. On the other hand, *kinetic* data[14] pertaining to the reaction sequence in Scheme I was scanty, and *direct observation* of aziridinium ions 2 and 4 was desirable. We have addressed these points by using ^{31}P Fourier-transform (FT) NMR spectroscopy. The combined advantages of ^{31}P NMR as opposed to either ^1H, ^{13}C, or ^{15}N NMR are the 100% natural abundance of ^{31}P, the favorable gyromagnetic ratio and relaxation time of this nucleus, the sensitivity of ^{31}P chemical shifts to molecular structure, and the spectral simplicity under conditions of ^1H decoupling.

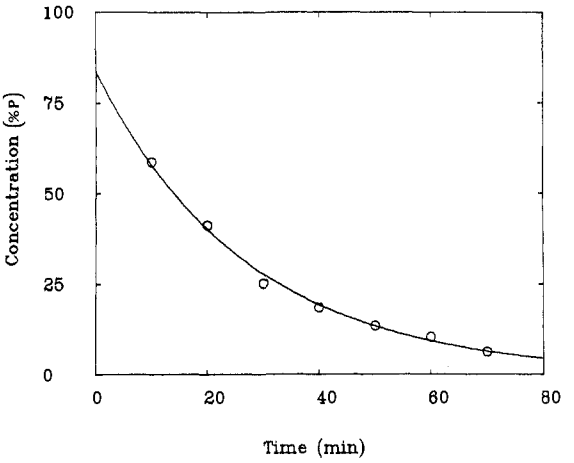


Figure 1. A representative time course for the disappearance of 1·CHA (50 mM initial concentration) in 1 M Tris[22] buffer at pH 8.2, 37 °C. The concentration term "% P", which was derived from time-averaged (200 s) ^{31}P NMR spectra (with NOE), refers to the signal intensity of 1·CHA, relative to the total signal intensity of all of the detectable reaction components. The smooth curve was obtained by fitting the experimental data points to a first-order rate equation (k = 0.036 min^{-1}, $\tau_{1/2}$ = 19 min). The indicated times refer to the start of data acquisition; the spectrum corresponding to "zero" time (data point not shown) was acquired after a 5-min delay for thermal equilibration of the NMR sample.

Results and Discussion

Phosphoramide Mustard. Decomposition Kinetics. ^{31}P NMR kinetic measurements with the cyclohexylammonium salt of 1 (1·CHA) were carried out at 37 °C with 50 mM initial concentrations of 1·CHA in 1 M Bistris[22] and Tris[22] buffers, which covered a pH range of 5.7–9.0 and provided acceptably small pH decreases (Δ pH < 0.2) during the eventual release of 2 equiv of HCl. In each kinetic run, a series of time-averaged spectra provided signal intensities that were equated to the relative concentrations ("% P") of all of the detectable reaction components. Linear least-squares analysis ($r > 0.99$) of plots of ln (% P) vs. time gave the first-order rate constants (k) for disappearance of 1 listed in Table I (runs 1–7). The concentrations for 1 were also fitted to the exponential rate expression for first-order decay (e.g., Figure 1), which assured equal weighting of data points, and the resultant values of k were essentially identical with those presented in Table I. The reactivity of 1, as measured by k, was, within experimental error,[23] constant between pH 7.0 and

(11) V. T. Vu, C. C. Fenselau, and O. M. Colvin, *J. Am. Chem. Soc.*, **103**, 7362 (1981).
(12) H. Lindemann and E. Harbers, *Arzneim.-Forsch.*, **30**, 2075 (1980).
(13) L. C. Erickson, L. M. Ramonas, D. S. Zaharko, and K. W. Kohn, *Cancer Res.*, **40**, 4216 (1980).
(14) O. M. Friedman, A. Myles, and M. Colvin, *Adv. Cancer Chemother.*, 1, 143–204 (1979).
(15) G. Zon, *Progr. Med. Chem.*, in press.
(16) E. Wrabetz, G. Peter, and H. J. Hohorst, *J. Cancer Res. Clin. Oncol.*, **98**, 119 (1980).
(17) B. E. Domeyer and N. E. Sladek, *Biochem. Pharmacol.*, **29**, 2903 (1980).
(18) J. H. Hipkens, R. F. Struck, and H. L. Gurtoo, *Cancer Res.*, **41**, 3571 (1981).
(19) O. M. Friedman, *Cancer Treat. Rep.*, **51**, 347 (1967).
(20) F.-T. Chiu, F.-P. Tsui, and G. Zon, *J. Med. Chem.*, **22** 802 (1979).
(21) H. Arnold, F. Bourseaux, and N. Brock, *Arzniem.-Forsch.*, 11, 143 (1961).

(22) Bistris refers to 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris refers to tris(hydroxymethyl)-aminomethane.
(23) Five independently performed ^{31}P NMR measurements using different synthetic samples of 1·CHA and freshly prepared buffer gave an average $\tau_{1/2}$ value of 18 ± 3 min at pH 7.4, 37 °C. The 13 min half-life reported in our preliminary communication[1] was anomalously low. Solution temperature control, which usually constitutes the major source of inaccuracy in kinetic measurements by NMR, was especially problematic in these studies, due to the high ionic strength of the buffer (dielectric heating) and the relatively large sample volumes (2 mL in 10-mm tubes). Consequently, the estimated error limits (ca. ±10–20%) for the rate data in Table I are rather high by comparison with other kinetic methods.

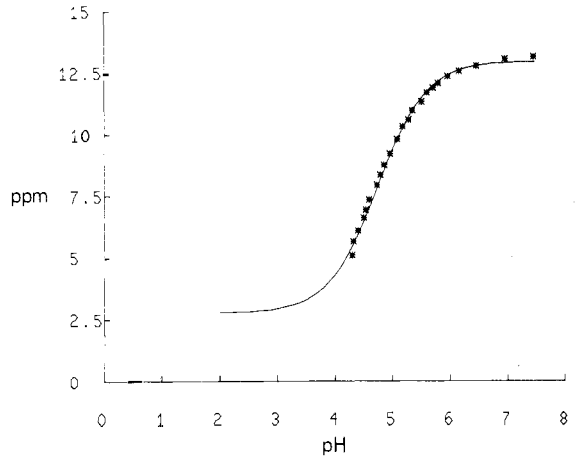


Figure 2. ^{31}P NMR chemical shifts (δ, ppm) of 1·CHA (50 mM initial concentration), as a function of pH, during its spontaneous decomposition in unbuffered water at 20 °C. The smooth curve was generated by a computerized least-squares fit of the data to the Henderson–Hasselbalch equation, which gave $pK_a = 4.75 \pm 0.03$.

9.0 but decreased sharply as the pH was lowered: e.g., lowering the pH from 7.4 to 5.7 caused an approximately 5- to 6-fold decrease in reactivity. For comparative purposes, it is worthwhile to note that the 51 min half-life ($\tau_{1/2}$) of 1 in 1 M Bistris buffer at pH 6.5 agreed quite well with the value of 48 min previously measured[24] for 1 in 0.07 M phosphate buffer, at the same pH and temperature, and with 4-(*p*-nitrobenzyl)pyridine (NBP) as a colorimetric reagent.[25]

Proton exchange between acid **1a** and conjugate base **1b** is fast on the NMR time scale, and the ^{31}P signal observed for 1, which is the weighted average of the signals for **1a** and **1b**, was therefore strongly pH dependent. These circumstances proved to be useful, since the spontaneous decomposition of 1·CHA in *unbuffered* water at 20 °C was slow enough to allow alternating measurements of the chemical shift (δ) of 1 and the corresponding pH of the solution, which had an initial value of 7.5 but steadily decreased due to the generation of HCl. In accord with the aforementioned data in Table I, the rate of disappearance of 1 gradually decreased during these measurements; however, when the pH fell below ca. 4.5, the decomposition of 1 was markedly *accelerated* and thus precluded reliable determinations of δ vs. pH beyond this point in the reaction.[26] These ^{31}P NMR chemical-shift "titration"[27,28] data (δ vs. pH) were fitted to the Henderson–Hasselbalch equation (Figure 2) and gave a pK_a value of 4.75 ± 0.03, which was in excellent agreeement with the values of 4.8[29] and 4.7[30] previously determined by other experimental techniques; theoretical calculations[31] regarding 1 have indicated a pK_a value of 4.58. The relatively rapid disappearance of 1 at pH <4.5 was indicative of the expected[32] hydrogen ion catalyzed hydrolysis of P–N bonds (vide infra), while the measured pK_a value for 1 and the response of k to pH values between 5.7 and 9.0 were consistent with Scheme I; i.e., the unimolecular conversion of conjugate base **1b** into aziridinium ion **2** was suppressed when the initial preequilibrium step (**1a** ⇄ **1b**) was disfavored by lowering the pH of the reaction medium.

Further mechanistic insight was obtained from several additonal kinetic results: (1) the presence of 1 M LiCl, NaCl, $CaCl_2$, or $MgCl_2$ in solutions of 1 in 1 M Tris buffer led to ca. 2- to 3-fold decreases in the reactivity of 1 (runs 8–11 vs. run 4); (2) use of HNO_3 in place in HCl for the preparation of the 1 M Tris buffer did not alter the reactivity of 1 (run 5 vs. run 4); (3) the rate of disappearance of 1 at pH 7.4 was unaffected by addition of 10-fold molar excesses of either $HSCH_2CH_2OH$, $HSCH_2CH_2SO_3^-Na^+$, or thiourea (runs 12–14 vs. run 4); and (4) a 2-fold weight excess of bovine serum albumin (BSA) had no signficant influence on the value of k (run 15 vs. run 4). These data were consistent with a rate-limiting ionization step (**1b** → |**2** + Cl^-|) and kinetically negligible internal return by the subsequently formed solvent-separated ion pair. A mechanistically analogous situation obtains for the hydrolysis of cyclophosphamide,[33] whereas chloride ion *is* a significant competitor for both the aziridinium ion derived from methylbis(2-chloroethyl)amine[34] and the thiiranium ion produced by bis(2-chloroethyl) sulfide.[35] The apparently greater stabilizing influence of Mg^{2+} (run 11), as opposed to Ca^{2+} (run 10), suggested that Mg^{2+} was superior to Ca^{2+} in removing negative charge density from anion **1b**; however, other explanations for these relatively small effects are feasible, such as those discussed[36] for the role metal ions in organophosphate hydrolyses.

Determination of Rate Constants for Consecutive Reactions. ^{31}P NMR spectra obtained during the reaction of 1 with 10 mol equiv of 2-mercaptoethanol are displayed in Figure 3. This stack plot showed that the gradual disappearance of the signal (δ 13.2, "A") due to 1 was accompanied by the steady formation of a new signal (δ 14.0, "C") atrributable to the expected[9] bisalkylated product, **8**, which was subsequently identified by ^1H NMR

(24) G. Voelcker, T. Wagner, and H. J. Hohorst, *Cancer Treat. Rep.*, **60**, 415 (1976).
(25) O. M. Friedman and E. Boger, *Anal. Chem.*, **33**, 906 (1961).
(26) The decelerating effect of hydrogen ions on the rate of disappearance of 1 (cf. Table I, runs 1–4) requires that the decomposition of 1, *in the absence of a buffering agent*, afford curved plots of ln ($[1]_{rel}$) vs. time, showing a continual diminution in reaction velocity due to HCl formation. This was verified by obtaining ^{31}P NMR data (not reported) for solutions of 1·CHA in both D_2O (36 °C) and 0.9% NaCl–D_2O (25 °C); however, other investigators [P. L. Levins and W. I. Rogers, *Cancer Chemother. Rep.*, **44**, 15 (1965)] using ^1H NMR data have claimed "linear" first-order kinetic plots for 1 under these reaction conditions. The apparent discrepancy was resolved by finding that, for each reaction, a straight line with the slope reported by Levins and Rogers could by "fitted" to our ^{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 ($[1]_{rel}$) values from this line.
(27) I. K. O'Neill and C. P. Richards, *Annu. Rep. NMR Spectrosc.*, **10A**, 141–142 (1980).
(28) For a recent study of the effect of ionic strength on pH measurements by ^{31}P NMR, see J. K. M. Roberts, N. Wade-Jardetzky, and O. Jardetzky, *Biochemistry*, **20**, 5389 (1980).
(29) G. Voelcker, H. P. Giera, L. Jäger, and H. J. Hohorst, *Z. Krebsforsch.*, **91**, 127 (1978).
(30) N. Brock and H. J. Hohorst, *Z. Krebsforsch.*, **88**, 185 (1977).
(31) W. Ulmer, *Int. J. Quantum Chem.*, **19**, 337 (1981).
(32) J. Rahil and P. Haake, *J. Am. Chem. Soc.*, **103**, 1723 (1981); T. A. Modro and D. H. Graham, *J. Org. Chem.*, **46**, 1923 (1981); for related information, see M. E. Phelps, P. W. Woodman, and P. V. Danenberg, *J. Med. Chem.*, **23**, 1229 (1980).
(33) G. Zon, S. M. Ludeman, and W. Egan, *J. Am. Chem. Soc.*, **99**, 5785 (1977).
(34) P. D. Bartlett, S. D. Ross, and C. G. Swain, *J. Am. Chem. Soc.*, **69**, 2971 (1947).
(35) P. D. Bartlett and C. G. Swain, *J. Am. Chem. Soc.*, **71**, 1406 (1949).
(36) F. Ramirez, J. F. Marecek, and J. Szamosi, *J. Org. Chem.*, **45**, 4748 (1980); J. MacB. Harrowfield, D. R. Jones, L. F. Lindoy, and A. M. Sargeson, *J. Am. Chem. Soc.*, **102**, 7733 (1980), and pertinent references in these articles.

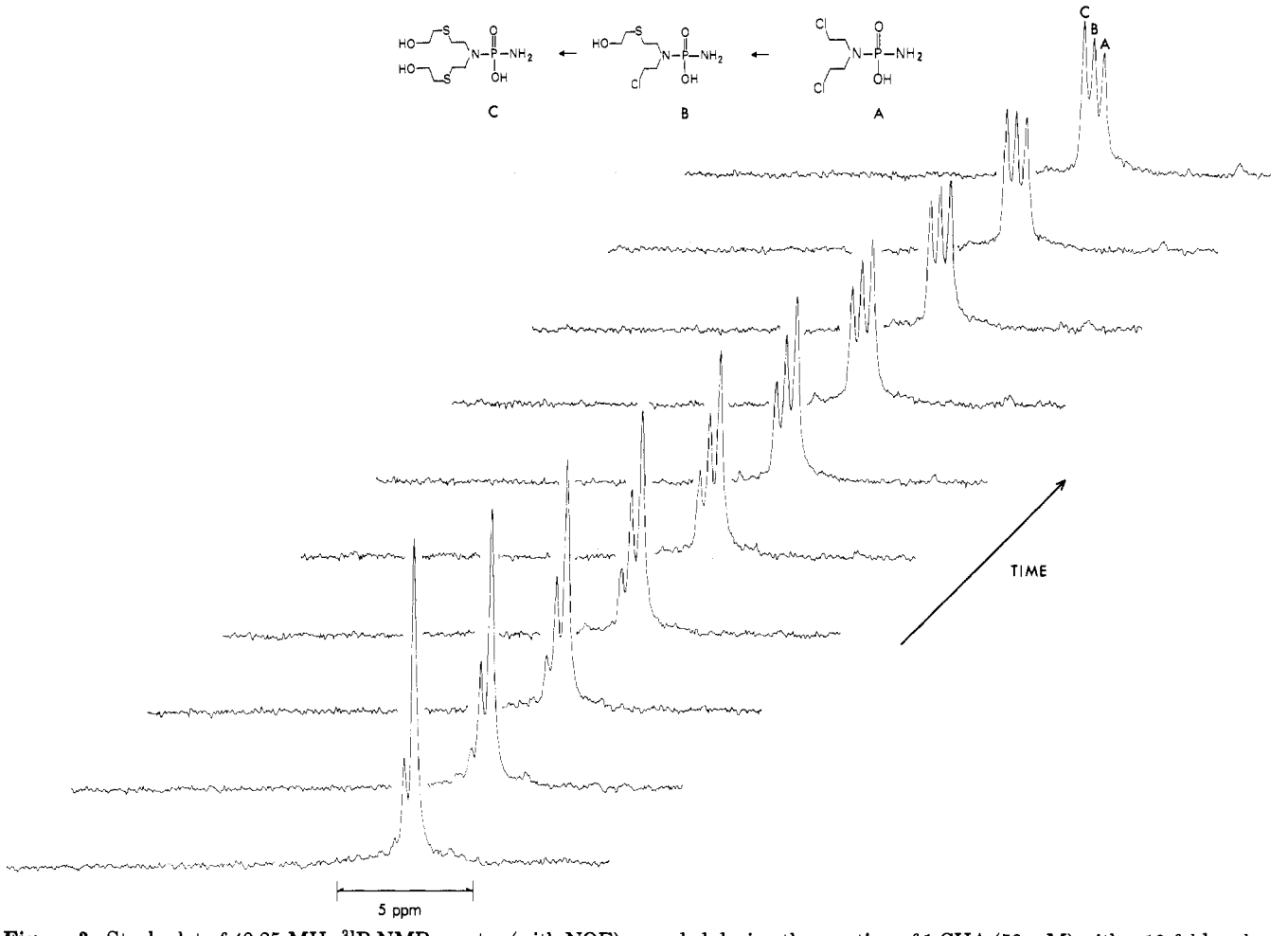


Figure 3. Stack plot of 40.25-MHz ^{31}P NMR spectra (with NOE) recorded during the reaction of 1·CHA (50 mM) with a 10-fold molar excess of 2-mercaptoethanol in 1 M Tris[22] buffer at pH 7.4, 37 °C. The starting material and product structures and their corresponding signals are designated by the letters "A–C". The time interval between each time-averaged spectrum (40 pulses, 4.5-s pulse repetion time) was 3 min (see Figure 4 for kinetic plot).

analysis. A plot of relative concentrations (% *P*) vs. time (Figure 4) indicated that the centrally located resonance absorption (δ 13.6, "B") in Figure 3 arose from a *transient precursor* of **8**. Since the lifetimes for aziridinium ions **2** and **4** should be very short, relative to **1** and **7**,[34] the assignment of the δ 13.6 signal to **7** was supported by finding that a computerized analysis of the data in Figure 4 as consecutive "first-order" reactions ($1 \xrightarrow{k'} 7 \xrightarrow{k''} 8$) gave $k' = 0.036$ min^{-1} and $k'' = 0.043$ min^{-1}, which were virtually identical (standard error limits ±0.002) and thereby consistent with the similar reactivity expected for **1** and **7**.

Analogous trapping experiments were carried out with 1·CHA and 10-fold molar excesses of either sodium 2-mercaptoethylsulfonate ($HSCH_2CH_2SO_3^-Na^+$, "mesnum") or thiourea. The former compound has been reported[37] as an antidote against the urotoxicity accompanying cyclophosphamide therapy, and the latter material has been proposed[38] as a nucleophilic interceptor of aziridinium ions. The reaction of 1·CHA with mesnum at pH 7.4, 37 °C gave ^{31}P NMR spectra (not shown), which were essentially identical with those presented in Figure 3, whereas 1·CHA and thiourea gave the same spectra as those obtained with a solution of 1·CHA in the pH 7.4 Tris buffer (vide infra);

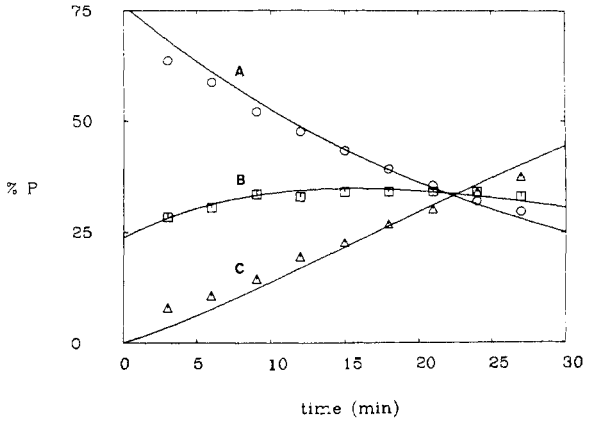


Figure 4. ^{31}P NMR derived time course for the reaction of 1·CHA ("A") with a 10-fold molar excess of 2-mercaptoethanol in 1 M Tris[22] buffer at pH 7.4, 37 °C, to give intermediate "B" (**7**) and final product "C" (**8**); see Figure 3 for spectra and structures. The concentration term "% *P*" refers to relative signal intensities (peak heights), and the indicated times are relative to the start of data acquisition. As detailed under Experimental Section, the smooth curves were obtained by simultaneously fitting the experimental data for reaction components A–C to the integrated kinetic expressions for $A \xrightarrow{k'} B \xrightarrow{k''} C$.

i.e., there was no evidence for *accumulation* of S-alkylated thiourea interception products.

At pH values ≤6.5, in the *absence* of an added trapping agent, the decrease in the ^{31}P NMR signal intensity of **1**

(37) W. Scheef, H. O. Klein, N. Brock, H. Burkert, U. Günther, H. Hoefer-Janker, D. Mitrenga, J. Schnitker, and R. Voigtmann, *Cancer Treat. Rep.*, **63**, 501 (1979).
(38) L. A. Zwelling, J. Filipski, and K. W. Kohn, *Cancer Res.*, **39**, 4989 (1979).

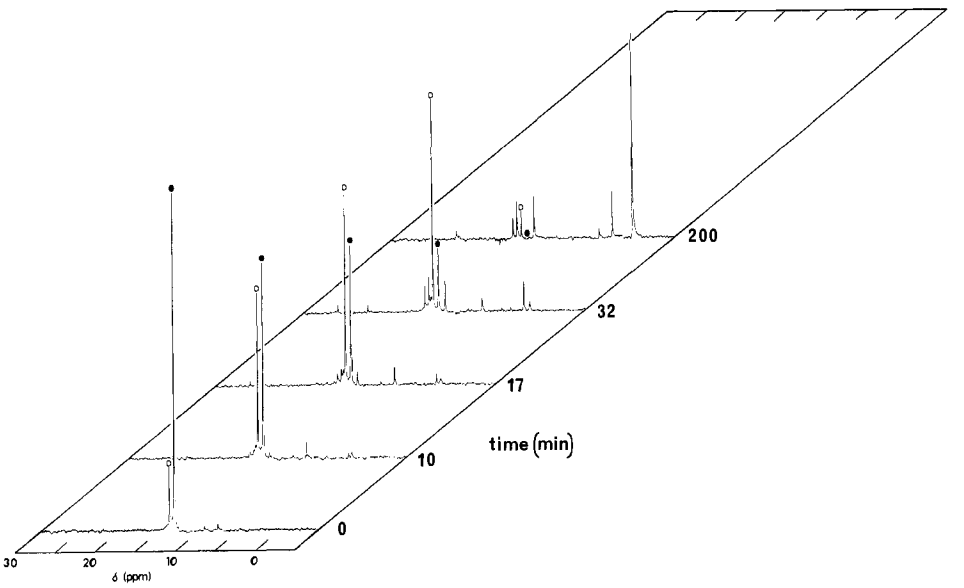
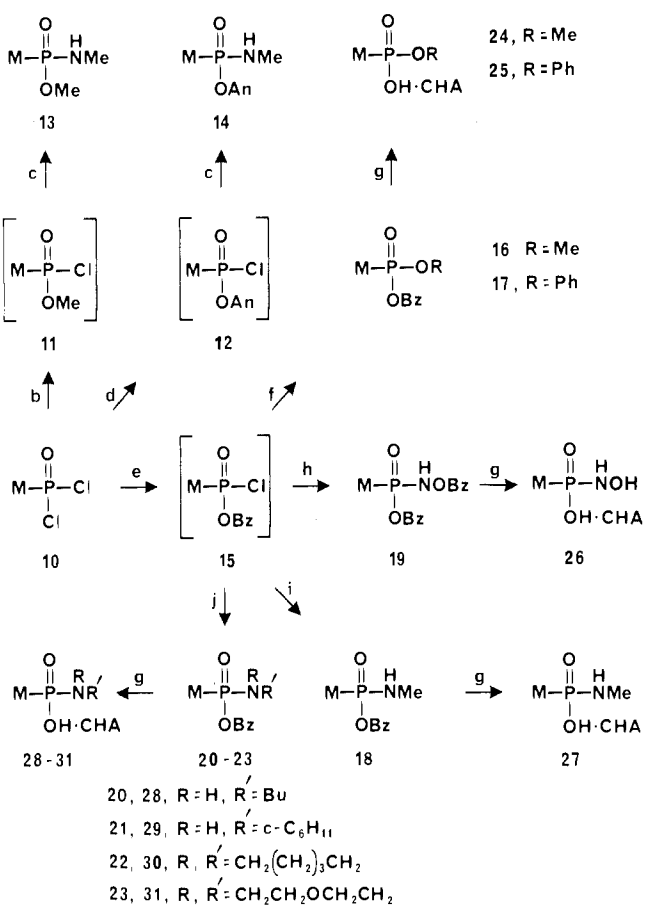


Figure 5. Stack plot of 40.25-MHz ^{31}P NMR spectra (with NOE) recorded for 1·CHA (50 mM initial concentration) in 1 M Tris[22] buffer at pH 7.4, 37 °C. The symbol ● indicates the signal arising from 1·CHA (δ 13.2), and the symbol O indicates the signal (δ 13.9) assigned to aziridinium ion **2**. The signals seen in the 200-min spectrum at approximately δ 0 and 5 are due to H_3PO_4 and $H_2NPO_3H_2$, respectively. The designated times refer to the start of data acquisition (52 pulses, 5-s pulse repetition time), with "zero" time corresponding to 5 min after placement of the sample in the spectrometer probe.

over 2 half-lives was primarily accompanied by the concomitant appearance of upfield-shifted resonance absorptions for H_3PO_4 and $H_2NPO_3H_2$. These signals were identified by addition of authentic materials, and their detection provided direct evidence for the hydrogen ion catalyzed hydrolysis of P–N bonds. By contrast, ^{31}P NMR spectra recorded for 1 at pH 7.4–9.0 showed (e.g., Figure 5) that the disappearance of 1 (δ 13.2) was primarily accompanied by formation of a transient intermediate (δ 13.9) that was converted into a number of secondary products (δ 12.5–14.5) and ultimately gave rise to the aforementioned inorganic phosphates. The relative concentrations measured for 1, the transient intermediate seen at δ 13.9, and the mixture of secondary products were simultaneously fitted to the rate equations for consecutive first-order reactions, with spectral data obtained at pH 7.4, 8.2, and 9.0. Over this pH range, the calculated average half-life for 1 was 17.5 ± 3.2 min, which agreed with the 19 ± 1 min average half-life for 1 computed from the corresponding values listed in Table I (runs 4, 6, and 7). At each pH, there was good agreement between the calculated and measured kinetic profiles (Figure 6) for the transient intermediate. Moreover, the intermediate's half-life gradually decreased with increasing hydroxide ion concentration: 30 ± 1.6 min at pH 7.4, 18 ± 0.7 min at pH 8.2, and 15 ± 0.4 min at pH 9.0. This pH dependency was consistent with the behavior expected for aziridinium ion **2** and was contrary to the reactivity pattern anticipated for monohydroxy compound **9**, the half-life of which should be pH independent, as was established for 1 in this pH range. Attempts to use ^{13}C NMR as an adjunct spectroscopic method for further characterization of **2** were foiled by chemical-shift changes resulting from HCl liberation in unbuffered ("^{13}C-transparent") solutions of 1·CHA during the early stages of its decomposition.

Analogues of Phosphoramide Mustard. Synthesis. Scheme II summarizes the synthetic routes to phosphoramide mustard analogues **13**, **14**, and **24–31**. The preparative strategies involved relatively routine organophosphorus chemistry, and the acidic phosphorus products (**25–31**) were generated by catalytic hydrogenolysis (Pd/C, 1 atm of H_2) of PO–Bz bonds, which was the same methodology previously employed by Friedman and co-workers[3,39] in their original synthesis of several of these target compounds. For unknown reasons, the debenzylation of diester **16** was exceptionally slow under 1 atm of H_2; the

Scheme II



(39) O. M. Friedman, E. Boger, V. Grubliauskas, and H. Sommer, *J. Med. Chem.*, **6**, 50 (1963).

Table II. ^{31}P NMR Derived Kinetic and Product Data for the Reaction of Phosphoramide Mustard (1) Analogues in 1 M Tris[a] Buffer at pH 7.4, 37 ± 2 °C

compd	k,[b] min^{-1}	$\tau_{1/2}$, min	$\sim k_{rel}$ ($\equiv k/k_1$)	O-alkylation,[c] %
1	0.038 (±0.006)	18 (±3)	1.0	⩽2
13	<0.00053[d]	>1300	<0.014	
14	<0.00053[d]	>1300	<0.014	
25	0.00038[e]	1800	0.010	
26	0.00063	1100	0.016	
27	0.063	11	1.6	31 (δ 22.4)
28	0.046	15	1.2	50 (δ 21.7)
29	0.046	15	1.2	30 (δ 20.5)
30	0.53	1.3	14	6 (δ 23.1)
31	0.027	26	0.7	3 (δ 29.0)

[a] Tris = $H_2NC(CH_2OH)_3$. [b] First-order rate constants obtained by least-squares fits ($r > 0.96$) of ln (% P) vs. time, where the quantity "% P" refers to the ^{31}P NMR signal intensity of the starting compound, relative to all observable signals, which were recorded with NOE. The error limits were estimated to be ca. 10–20%; the error limits indicated for 1 refer to five independent experiments (cf. footnote 23). [c] See text for details. The values in parentheses are the ^{31}P NMR chemical shifts of the O-alkylated products; a blank indicates not applicable. [d] Estimated upper limit; see footnote 49 for details. [e] At 80 °C, $k = 0.070\ min^{-1}$ and $\tau_{1/2} = 10$ min.

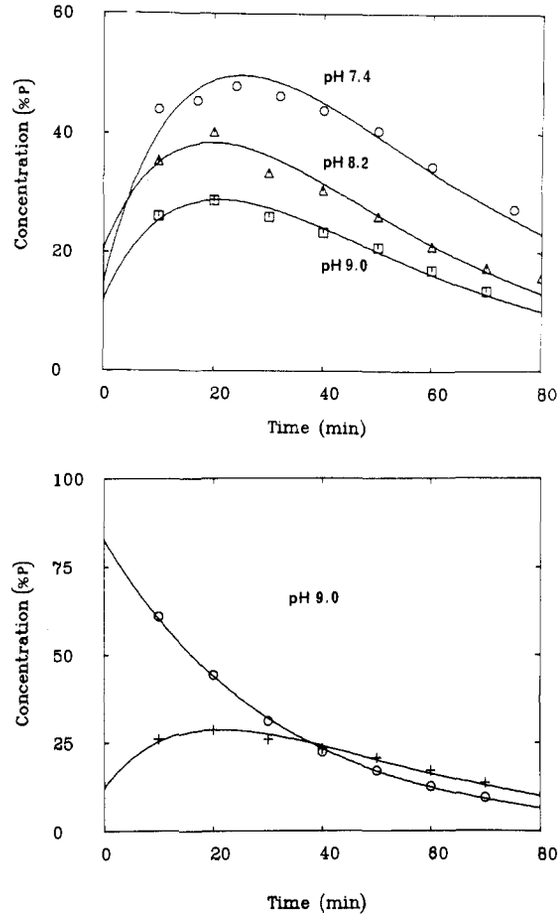
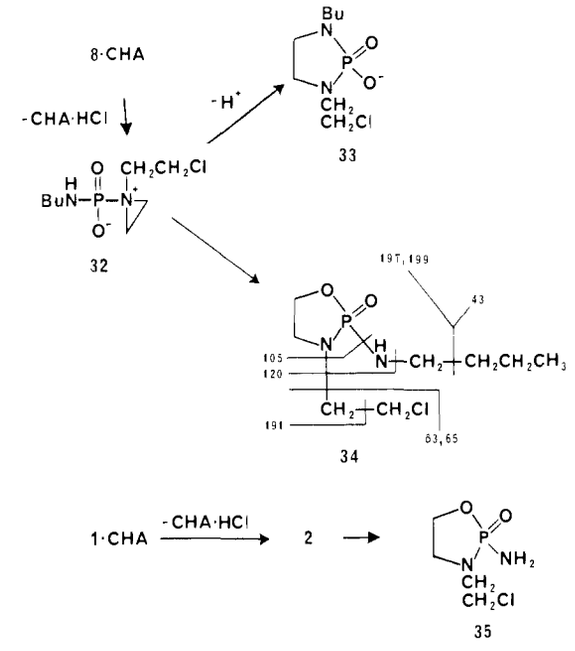


Figure 6. The upper portion of the figure shows the effect of pH on the time course of the ^{31}P NMR signal (δ 13.9) assigned to aziridinium ion **2**, which was produced by the 37 °C reaction of 1·CHA in 1 M buffers having the indicated pH values. The concentration term "% P" refers to the signal intensity of **2**, relative to the total signal intensity of all of the detectable reaction components. As detailed under Experimental Section, the smooth curves were obtained by simultaneously fitting the experimental data for **1**, **2**, and secondary products to the integrated rate expressions for consecutive first-order reactions; for clarity, the corresponding fits for **1** and the secondary products are not shown. The lower portion of the figure presents the data points and calculated curves for **1** and **2** at pH 9.0; the corresponding plots for reaction at pH 7.4 and 8.2 were qualitatively similar with regard to the degree of fit for **1**.

use of 40 psi of H_2 led to the disappearance of **16** and the isolation of CHA·HCl, rather than **24**. We were also unsuccessful in repeated attempts[40] to prepare *N'*-phenylphosphoramide mustard [$PhNHP(O)(OH)N(CH_2CH_2Cl)_2$] by either Pt-catalyzed hydrogenolysis[41] of its phenyl ester or demethylation of its methyl ester with either PhSH[42] or *t*-$BuMe_2SiI$.[43]

Scheme III



Decomposition Kinetics and Products. The phosphoramide mustard analogues were monitored by ^{31}P NMR under a standard set of reaction conditions (1 M Tris buffer, pH 7.4, 37 °C), and in those cases (**25–31**) where the reaction proceeded at a measurable rate, the disappearance of starting material was found to obey a first-order rate law ($r > 0.96$), giving the rate parameters listed in Table II.

With regard to products, the *n*-Bu derivative (**28**), which gave rise to a signal at δ 9.8, simultaneously afforded two

(40) T. W. Engle and G. Zon, unpublished observations.
(41) R. F. Struck, M. W. Trader, D. J. Dykes, T. H. Corbett, and W. R. Laster, Jr., *Current Chemotherapy and Infectious Disease*, Proceedings of the International Congress of Chemotherapy, 11th, Boston, Oct 1–5, 1979, American Society for Microbiology, Washington, DC, 1980, p 1520.
(42) C. B. Reese, R. C. Titmas, and L. Valente, *J. Chem. Soc., Perkin Trans. 1*, 2451 (1981).
(43) T. Morita, Y. Okamoto, and H. Sakurai, *Tetrahedron Lett.*, 2523 (1978).

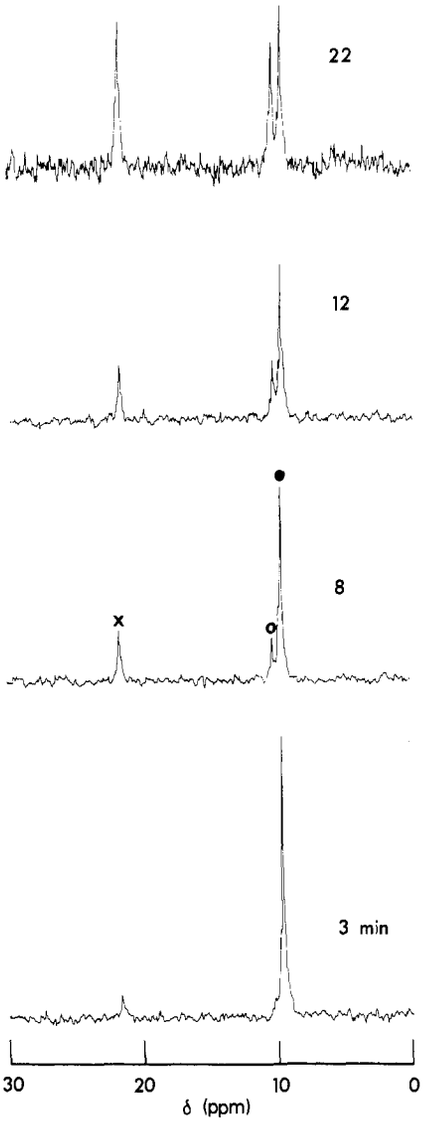
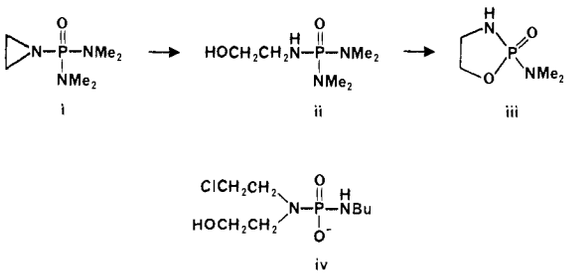


Figure 7. Partial displays of 40.25-MHz ^{31}P NMR time-averaged spectra (with NOE) recorded for the decomposition of the cyclohexylammonium salt of *N'*-*n*-butylphosphoramide mustard (**28**·CHA, 50 mM initial concentration) in 1 M Tris[22] buffer at pH 7.4, 37 °C. The symbols ●, ○ and x indicate the signals for **28**·CHA (δ 9.8), aziridinium ion **32** (δ 10.4), and 1,3,2-oxazaphospholidine derivative **34** (δ 21.7), respectively. The designated times refer to the start of data acquisition (52 pulses, 2-s pulse repetition time), with "zero" time corresponding to 2 min after placement of the sample in the spectrometer probe.

major product absorptions seen (Figure 7) at δ 10.4 and 21.7. Assignment of the former product signal to aziridinium ion **32** (Scheme III) was based on its 0.6-ppm downfield chemical shift, relative to **28**, which was very close to the 0.7 ppm Δδ value found for **2** (δ 13.9), relative to **1** (δ 13.2). The much larger downfield chemical shift of the product signal at δ 21.7 was indicative of a five-membered ring *P*(V)-oxide,[44,45] such as **33** or **34** (Scheme III), resulting from intramolecular N- and O-alkylation of aziridinium ion **32**, respectively. Chloroform extraction of the final reaction mixture gave a single substance (^{31}P NMR) that was identified as 1,3,2-oxazaphospholidine derivative **34**, based on its MS fragmentation pattern (Scheme III) and molecular ions (*m/e* 240, 242). The conversion of **32** into **34** (50% yield, ^{31}P NMR) represented an intramolecular version of the reported[12] O-alkylation of DNA phosphodiester linkages by aziridinium ion **2**; moreover, **32** → **34** has precedence in chemistry previously reported[46] for **1**·CHA, which produced **35** (Scheme III) in *acetone* solvent at room temperature. In the present studies with *aqueous* solutions of **1**·CHA at pH 7.4, several relatively weak ^{31}P NMR signals were observed (cf. Figure 5) in the range of δ 20–30, and one of these downfield-shifted signals could possibly have arisen from **35**. After complete disappearance of aziridinium ion **2**, only the signal at δ 22.0 remained, having a relative intensity of 2%. If the δ 22.0 signal was due to **35**, then the ratio of rate constants (k_{inter}/k_{intra}) for hydroxide ion interception of **2** to give **9**, and intramolecular O-alkylation of **2** to give **35**, must be ca. 25-times greater than k_{inter}/k_{intra} for **32**. Since the signal at δ 22.0 may have arisen from some other minor reaction product, this partitioning factor[34] represented a lower limit.[47] In either case, it was evident that substitution of an *n*-Bu group for hydrogen in aziridinium ion **2** dramatically altered the fate of this transient alkylating agent.[48] Downfield-shifted ^{31}P NMR product signals in the range of δ 20–30 were also observed during the decomposition of other *N'*-alkylphosphoramide mustards at pH 7.4, and, by extension, these signals were *tentatively* assigned to the corresponding 2-(alkylamino)-3-(2-chloroethyl)-1,3,2-oxazaphospholidine 2-oxides. The yields of these products, as determined from ^{31}P NMR signal intensities after complete reaction, are listed in Table II as "% O-alkylation". The extent of O-alkylation appeared to decrease in the order RHN > RR'N > H_2N.

The values of k/k_1 (k_{rel}, Table II) for *N'*-alkyl derivatives **27–29** were 1.6, 1.2, and 1.2, respectively, while the magnitude of k_{rel} for *N',N'*-dialkyl compound **30** indicated a rate-enhancement factor of 14. Oxygen-containing substituents had a rate-retarding influence upon the reactivity: the *N'*-hydroxy group in **26** and the more "remote" oxygen moiety in **31** led to k_{rel} values of 0.016 and 0.69, respectively. Replacement of the NH_2 group in **1** with OPh (**25**) had an even greater rate-retarding effect, viz., a 100-fold decrease in *k*. The k_{rel} value for phenyl ester **25** was considered to be an upper limit, since this analogue may have reacted by direct nucleophilic substitution pathways

(44) W. Egan, R. Schneerson, K. E. Werner, and G. Zon, *J. Am. Chem. Soc.*, **104**, 2898 (1982).
(45) L. D. Quin in "The Heterocyclic Chemistry of Phosphorus", Wiley-Interscience, New York, 1981, pp 126–271.
(46) J. A. Montgomery and R. F. Struck, *Cancer Treat. Rep.*, **60**, 381 (1976).
(47) Our attempts[40] to obtain authentic **35** by decomposition of **1**·CHA in acetone were not successful.
(48) In *aqueous acetic acid*, 1-aziridinylbis(dimethylamino)phosphine oxide (i) reportedly [J. B. Stokes, C. W. Woods, and A.



B. Borkovec, *Phosphorus Sulfur*, **10**, 139 (1981)] undergoes ring opening to give 2-hydroxyethylphosphorotriamide ii, which then cyclizes to 1,3,2-oxazaphospholidine iii. Extension of this mechanism to the reaction of **32** *at pH 7.4* would require that product **34** arise from cyclocondensation of intermediate iv, which is possible, in principle, but would most likely be too slow[44] to accommodate the relatively rapid rate of formation of **34**.

[S_N2(P) or S_N2(C)], which bypass aziridinium ion formation. The methyl (**13**) and 4-methoxyphenyl (**14**) esters of *N'*-methylphosphoramide mustard, which lack a formal negative charge but have a potentially "activating", electron-donating, *N'*-methyl group, where unreactive at 37 °C and were estimated[49] to be >70 times more stable than **1** and >100 times more stable than their parent acid, **26**.

Conclusions

The measurement of chloride ion release from N-phosphorylated nitrogen mustards has been used[50] to study the initial "activation" of these compounds by aziridinium ion formation; however, as previously noted by Friedman et al.,[14] this kinetic method fails to provide information regarding subsequent intermolecular alkylation reactions having biological (medicinal) significance. While the NBP colorimetric procedure developed by Friedman and Boger[25] has been widely employed to quantify alkylating agents, to our knowledge this technique has not been used to distinguish between kinetically similar alkylators. Moreover, both of these analytical methods are incapable of providing reliable bases for structure elucidation. By contrast, the presently reported studies have demonstrated that ^{31}P NMR can provide previously inaccessible information concerning **1**, cognate phosphoramidic mustards, their transient aziridinium ion intermediates, their transient monoalkylated products, and the final product distributions. Our salient findings and inferences are summarized in the following paragraphs.

(1) The cascade of reactions leading from **1a**/**1b** to **5** (Scheme I) and the extent of competing P–N bond hydrolyses to afford $H_2NPO_3H_2$ and H_3PO_4 are markedly pH dependent. The rate-limiting intramolecular chemistry of **1** (pK_a = 4.75) is controlled, in effect, by the concentration of conjugate base **1b**, and the dynamical concentration of aziridinium ion **2** is also responsive to pH changes. In view of the general trends exhibited by **1** and **2**, it is conceivable that the "activation" rate for **1** (i.e., **1b** → **2**) could be slower in those tumor cells that are more acidic than normal cells, and, in addition, once an active alkylator (**2** or **4**) is generated, it can have a longer lifetime under the more acidic conditions. These pH-modulated, intracellular, kinetic factors would thus result in greater probabilities for encountering and alkylating key biological macromolecules and could account for part of the oncostatic specificity elicited by cyclophosphamide, following its metabolic conversion into **1**. Unfortunately, while it has been widely assumed[51] that the intracellular pH (pH_i) of tumor cells is lower than that of normal cells, the magnitude and significance of such differences are not clearly defined at this time. In studies of glycolyzing Ehrlich ascites tumor cells, radiolabeling methods[52] and ^{31}P NMR[53] techniques have indicated ≤0.2 pH-unit lower values for pH_i, relative to the extracellular pH. Phosphorus spectra[54] of Walker 256 carcinosarcomas and normal rat muscle similarly indicate pH_i = 7.1 and pH_i = 7.2, respectively; however, other NMR results[55] have conclusively demonstrated the existence of intracellular pH "domains" due to cellular compartmentalization.[54,56] Consequently, the aforementioned role of pH_i in selectively altering the cytotoxicity of **1** is offered as a heuristic suggestion. Hamacher[57] has recently reported that acid-stimulating compounds can be used to decrease the pH_i of tumor cells to enhance the effectiveness of those oncostatic agents that are active at lower pH, and it thus appears that this type of experimental approach could be applied to test the proposals for **1**.

(2) In principle, the Lewis-type acidity of metal ions can influence the reactivity and, hence, the selectivity of **1** in the same manner as that suggested above for hydrogen ion. While the kinetic results (Table I, runs 8–11) obtained for the reaction of **1** in the presence of Li^+, Na^+, Ca^{2+}, and Mg^{2+} were in accord with the expected decrease in reactivity due to negative-charge dispersal from **1b** to these electron acceptors,[58] other causes[34,36] may be operative. Differences in Na^+ and K^+ levels in normal vs. cancerous cells cause distinct variations in the ^{31}P NMR relaxation times measured for intracellular phosphorus compounds,[59] and it may be possible, in the future, to correlate such in vivo spectroscopic data with the relative reactivity and cytotoxicity of phosphoramidic mustards.

(3) To our knowledge, the data obtained for the reactions of **1** with 2-mercaptoethanol and sodium 2-mercaptoethylsulfonate (mesnum) represent the first spectroscopic measurements of the sequential conversion of a bisalkylating agent into its mono- and disubstituted products. These spectral and kinetic methods can be applied to phosphoramide mustard analogues having nonidentical leaving groups and would thereby provide important kinetic information concerning the relative reactivity of each "arm" in these unsymmetrical bisalkylating agents, which are metabolites of a promising class of anticancer prodrugs.[60]

(4) The relatively rapid interception of aziridinium ions **2** and **4** by mesnum provided evidence for a pathway that could, *in part*, contribute to the reported[37] antiurotoxic properties of this sulfhydryl compound. The antidotal effect of mesnum during cyclophosphamide therapy has heretofore been attributed[61] to its conjugation with the acrolein and 4-hydroxy metabolites of cyclophosphamide; however, the proposed[61] 4-thioalkyl adduct derived from

(49) As is usually the case, a signal to noise ratio (S/N) of 2 was taken to be the lower limit for NMR detection. Since product signals were *not* detected after heating **13** and **14** at 76 °C for 40 min, the starting material's S/N value and a hypothetical-product S/N value of 2 were used to calculate an upper limit for k at 76 °C, which was then divided by a temperature correction factor of 18.2 to obtain the reported upper limits for k at 37 °C. The temperature correction factor was derived from values of k at 80 and 37 °C measured for analogue **25**. The magnitude of this factor was in reasonably good agreement with the ca. 2-fold change in k per 10 °C change in temperature generally used for kinetic approximations (viz., 2^4 = 16 vs. 18.2).

(50) H. Arnold, F. Bourseaux, and N. Brock, *Arzneim.-Forsch.*, 11, 143 (1961).

(51) Z. B. Papanastassiou, R. J. Bruni, E. White, V, and P. L. Levins, *J. Med. Chem.*, **9**, 725 (1966), and pertinent references therein.

(52) D. T. Poole, *J. Biol. Chem.*, **242**, 3731 (1967).

(53) G. Navon, S. Ogawa, R. G. Shulman, and T. Yamane, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 87 (1977).

(54) J. R. Griffiths, A. N. Stevens, R. A. Iles, R. E. Gordon, and D. Shaw, *Biosci. Rep.*, 1, 319 (1981).

(55) S. M. Cohen, S. Ogawa, H. Rottenberg, P. Glynn, T. Yamane, R. Brown, and R. G. Shulman, *Nature (London)*, **273**, 554 (1978).

(56) J. S. Cohen in "Noninvasive Probes of Tissue Metabolism", J. S. Cohen, Ed., Wiley-Interscience, New York, 1982, p 19.

(57) H. Hamacher, *Pharmazie*, **36**, 391 (1981).

(58) P. Liebmann, G. Loew, A. D. McLean, and G. R. Pack, *J. Am. Chem. Soc.*, **104**, 691 (1982).

(59) T. Glonek, *Biochem. Med.*, **19**, 246 (1978).

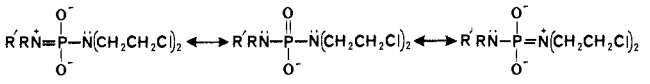
(60) A. Takamizawa, S. Matsumoto, T. Iwata, I. Makino, K. Yamaguchi, N. Uchida, H. Kasai, O. Shiratori, and S. Takase, *J. Med. Chem.*, **21**, 208 (1978). For earlier studies of potential carcinolytic agents bearing dissimilar reactive functions, see A. B. Foster, M. Jarman, W. C. J. Ross, and M. J. Tisdale, *J. Med. Chem.*, **15**, 869 (1972).

(61) N. Brock, *Beitr. Onkol.*, **5**, 1 (1980).

the latter metabolite is hydrolytically unstable[62] and leads to formation of **2**/**4**, which can be *irreversibly* trapped by mesnum. On the other hand, Cox[63] has reported that acrolein is the causative agent in cyclophosphamide cystitis.

(5) It has been recently suggested[38] that S-alkylation of thiourea by aziridinium ions provides protection of cells against the toxic actions of L-phenylalanine mustard and *N,N*-bis(2-chloroethyl)amine. The present ^{31}P NMR studies with 1·CHA and thiourea at pH 7.4 gave no evidence for the accumulation of adducts between aziridinium ions **2**/**4** and thiourea, suggesting that S-alkylated adducts, if formed, have labile $NCH_2CH_2SC(=NH)NH_2$ functionalities, relative to the $NCH_2CH_2SCH_2CH_2X$ moieties produced by ethanethiol[9] (X = H), 2-mercaptoethanol (X = OH), and mesnum (X = $SO_3^-Na^+$). The isothioureido group represents a good *nucleofuge*, which can lend itself to re-formation of **2**/**4**. It follows that thiourea may be incapable of providing cells with protection against the cytotoxicity of **1**, despite the *formal* similarities between this bisalkylating agent and the aforesaid nitrogen mustards.

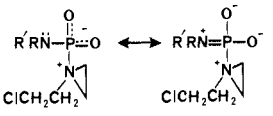
(6) The relative reactivity of the N′-substituted *N,N*-bis(2-chloroethyl)phosphorodiamidic acids studied by ^{31}P NMR can be neatly rationalized by refining the resonance model discussed by Friedman et al.[14] *Consider the electronically opposing nature of canonical forms* ***36*** *and* ***37***:



36 **37**

N′-substituents (R, R′) favoring canonical form ***36*** *increase the electron density at nitrogen in the mustard group, enhance the nucleophilicity of this nitrogen, and thus facilitate rate-limiting aziridinium ion formation, whereas N′-substituents favoring structure* ***37*** *(i.e., disfavor structure* ***36****) lead to slower rates of aziridinium ion formation.* As a first approximation, substituent-induced pK_a differences can be neglected at pH 7.4. Accordingly, the slightly increased reactivity (cf. k_{rel}, Table II) found for *N′*-alkyl compounds **27**–**29** can be attributed to inductive (+*I*) stabilization of **36**, relative to the case for **1**, wherein R = R′ = H. The roughly equal values of k_{rel} for **27**–**29** suggested that steric differences among these compounds play a comparatively minor role in controlling rate-limiting aziridinium ion formation. Inductive effects in multisubstituted systems generally operate either constructively or in opposition. The former mode was reflected in the greater reactivity (k_{rel}) of *N′,N′*-dialkyl compound **30** vs. **27**–**29**, and the latter mode was evidenced by the lower reactivity of **31** vs. **30**, due to the replacement of a methylene unit (+*I*) with an oxo moiety (−*I*). The hydroxy substituent *directly bonded* to the N′ position in **26** led to a comparatively large decrease in reactivity, consistent with −*I* destabilization of canonical form **36** (R = H, R′ = OH). Intramolecular H bonding between N–OH and P–O^- in the conjugate base of **26** could have also contributed to its sluggish reactivity; unfortunately, it was not possible to study the *N*-OBz analogue of **26** in this connection, due to the exceedingly low solubility of **26** in the 1 M Tris buffer. Synthetic difficulties prevented comparative studies with *N′*-phenylphosphoramide mustard;[40] however, the −*I and* −*R* effects of phenyl and other electron-withdrawing aryl groups should indirectly favor **37**. Hence, this class of compounds was predicted to have low reactivity, relative to **1**.

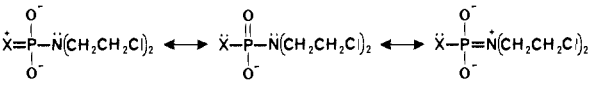
(7) The greater extent of intramolecular O-alkylation [formation of 2-(alkylamino)-1,3,2-oxazaphospholidine 2-oxide] found for phosphoramide mustard derivatives bearing an electron-donating N′ substituent (**27**–**29**, Table II) was accounted for by a combination of inductive and resonance effects in their corresponding *intermediate aziridinium ions* (**38**), leading to increased negative charge



38

density at oxygen—the locus of intramolecular electrophilic attack. However, the variable amount (30–50%) of O-alkylation in these systems and the observation of *less* O-alkylation for dialkyl derivatives (**30** and **31**, Table II) indicated that other structural effects need to be considered. Since *mono*alkylating agents are derived from intramolecular rearrangement of **38**, this competing reaction may be an important factor in determining the relative cytotoxicities of phosphorodiamidic mustards.

(8) Replacement of the NH_2 group in **1** by an OAr substituent (**25**) led to at least two orders of magnitude lower reactivity with regard to aziridinium ion formation, which was attributed to electronic effects that operate in concert: (a) less electron release by oxygen in OPh, relative to nitrogen in NH_2, thus favoring contributions by canonical form **39**, and (b) more effective "contraction" of the phosphorus d orbitals by the added oxygen, which also favors **39**. The relative reactivities of **1** and **25** in 0.025



X = NH_2, OR **39**

M acetate buffer at *pH 4.6*, 70 °C, have been previously studied by the NBP colorimetric method, which indicated roughly comparable stabilities for these two compounds.[19] From the presently reported pH studies with **1**, the hydrogen ion catalyzed P–N bond hydrolysis of **1** and **25** at pH 4.6, leading to $H_2N(CH_2CH_2Cl)_2^+$, could account for the apparent discrepancy in relative reactivities measured by the ^{31}P NMR and NBP methods.

The low reactivities of esters **13** and **14** vs. acids **27** and **1** were rationalized by the absence of a formal negative charge in the former compounds, as originally proposed by Friedman.[19] Like **25**, previous NBP comparisons[17] of compounds akin to **13** and **14** have overestimated their reactivity, relative to **1**, *at pH 4.6*. It should now be apparent that the pH and buffering capacity of a medium are critical factors in studies with N-phosphorylated nitrogen mustards that either measure alkylating reactivity or assume that a reported half-life is operative.

Our use of ^{31}P NMR spectroscopy for investigating biologically related reactions between **1** and macromolecules will be reported in a future publication.

Experimental Section

C_6H_6 and Et_2O refer to anhydrous solvents, and petroleum ether refers to the hydrocarbon mixture having a 30–60 °C boiling range. Reaction mixtures that do not include H_2O were carried out with exclusion of atmospheric moisture. Elemental analyses were performed by Galbraith Laboratories, Inc., and the National Institutes of Health Microanalytical Laboratory. Melting points were obtained with a Thomas-Hoover capillary apparatus and

(62) G. Peter and H.-J. Hohorst, *Cancer Chemother. Pharmacol.*, **3**, 181 (1979).
(63) P. J. Cox, *Biochem. Pharmacol.*, **28**, 2045 (1979).

were not corrected. Flash chromatography[64] refers to a 3 × 15 cm column of EM silica gel (230–400 mesh), which was sequentially eluted with 100-mL volumes of pure $CHCl_3$, 3:1 $CHCl_3$–EtOAc, 1:1 $CHCl_3$–EtOAc, 1:3 $CHCl_3$–EtOAc, and then pure EtOAc; 15-mL fractions were collected, and the product was obtained from fractions 15–20. Analytical and preparative TLC employed 2.5 × 10 cm and 20 × 20 cm plates coated with 250-μm and 2-mm layers of silica gel GF, respectively; a 250-nm UV lamp and I_2 vapor were used for component visualization. Details regarding ^1H NMR spectroscopy at 60,[65] 100,[65] and 220[33] MHz, ^{31}P NMR spectroscopy[1,65] at 40.25 MHz, and ^{13}C NMR spectroscopy[65] at 25 MHz have been previously reported. Unless specified otherwise, ^1H NMR refers to spectra recorded at 60 MHz with $CDCl_3$ solvent and Me_4Si as an internal reference for chemical shift (δ) measurements. ^{31}P NMR δ values refer to external 25%, v/v, H_3PO_4 in D_2O, which was sealed in a capillary tube that was positioned coaxially in the NMR sample using a vortex plug; the phosphorus chemical shifts in aqueous media are pH dependent. NMR sample temperatures were measured by immersion of a precalibrated copper–constantan thermocouple attached to a digital-readout meter. Values of pH were measured with a precalibrated standard glass electrode; the "pH" values for D_2O-containing solutions correspond to the observed reading and were not corrected for deuterium isotope effects.[66] Tris and Bistris buffers[22] were prepared by use of concentrated HCl for pH adjustment; however, concentrated HNO_3 was used in one instance (cf. Table I). Details regarding electron-impact mass spectrometry have been previously reported.[67]

Methyl *N*,*N*-Bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (13). A magnetically stirred solution of **10** (2.58 g, 0.01 mol) in C_6H_6 (25 mL) was reacted at room temperature with sodium methoxide, which was added dropwise as a suspension in C_6H_6 (25 mL); the sodium methoxide had been generated in C_6H_6 by reaction of MeOH (0.32 g, 0.01 mol) with NaH (0.5 g of 57% dispersion in oil, washed with petroleum ether). After continued stirring for 1 h, 40% aqueous methylamine (3.0 g, 0.03 mol) was added, and the resultant heterogeneous mixture was stirred for 2 h. The separated organic layer was washed with H_2O (50 mL), dried with $MgSO_4$, and was then concentrated by rotary evaporation to give **13** (0.98 g, 4 mmol, 40% yield) as a colorless oil: ^{13}C NMR δ 27.00 (s, 1, NCH_3), 42.61 (s, 2, 2 CH_2Cl), 49.63 (d, J = 4.6 Hz, 2, 2 NCH_2), 52.02 (d, J = 4.9 Hz, 1, OCH_3). Anal. ($C_6H_{15}Cl_2N_2O_2P$) C, H, Cl, N.

4-Methoxyphenyl *N*,*N*-Bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (14). The procedure described above for **13** was followed using dichloride **10** (2.58 g, 0.01 mol), 4-methoxyphenol (1.24 g, 0.01 mol), NaH (0.5 g of 57% dispersion in oil), and C_6H_6 (50 mL). Workup of the organic layer, followed by flash chromatography, gave **14** (2.28 g, 6.7 mmol, 67% yield) as a colorless oil, which eventually crystallized: mp 65–67.5 °C; ^1H NMR (100 MHz) δ 2.60 (dd, J_{HP} = 12.6 Hz, J_{HH} = 5.5 Hz, 3, NCH_3, collapsed to d with J_{HP} = 12.6 Hz after shaking with D_2O), 2.80 (br m, 1, NH), 3.10–3.80 (m, 8, 2 NCH_2CH_2Cl), 3.72 (s, 3, OCH_3), 6.90 (AA'BB'q, 4, C_6H_4); ^{13}C NMR δ 27.14 (s, 1, NCH_3), 42.35 (s, 2, 2 CH_2Cl), 49.61 (d, J = 4.9 Hz, 2, 2 NCH_2), 55.60 (s, 1, OCH_3), 114.71 (s, 2, 2 C-3), 121.05 (d, J = 4.9 Hz, 2, 2 C-2), 114.24 (d, J = 6.1 Hz, 1, C-1), 156.47 (s, 1, C-4). Anal. $C_{12}H_{19}Cl_2N_2O_3P$) C, H, Cl, N.

Benzyl *N*,*N*-Bis(2-chloroethyl)phosphoramidochloridate (15). Details for the preparation of **15** (ca. 0.01 mol) by reaction of *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride (**10**; 2.58 g, 0.01 mol) with sodium benzylate (0.01 mol) in C_6H_6 (50 mL) have been previously reported by Friedman et al.[39] The resultant solution of crude **15** was used immediately, due to relatively rapid decomposition, giving, inter alia, benzyl chloride (^1H NMR).[68]

Benzyl Methyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate (16). The procedure described above for **13** was followed with a C_6H_6 (50 mL) solution of crude **15** (0.01 mol) and a C_6H_6 (30 mL) suspension of freshly prepared sodium methoxide (0.01 mol). After the solution was stirred overnight, Et_2O (30 mL) was added and the precipitated solids were removed by filtration. Flash chromatography of the concentrated filtrate gave **16** (1.63 g, 5 mmol, 50% yield) as a colorless oil: ^1H NMR δ 3.10–3.70 (m, 8, 2 NCH_2CH_2Cl), 3.75 (d, J = 11 Hz, 3, OCH_3), 5.00 (d, J = 8 Hz, 2, $CH_2C_6H_5$), 7.30 (s, 5, C_6H_6). Anal. ($C_{12}H_{18}Cl_2NO_3P$) C, H.

Benzyl Phenyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate (17). A magnetically stirred C_6H_6 (50 mL) solution of crude **15** (ca. 0.01 mol) was cooled with an ice bath and was reacted with sodium phenoxide, which was added dropwise as a suspension in C_6H_6 (30 mL); the sodium phenoxide had been generated in C_6H_6 by reaction of phenol (0.94 g, 0.01 mol) with NaH (0.5 g of 57% dispersion in oil, washed with petroleum ether). After stirring for 4 h at room temperature, the reaction mixture was filtered, and the filtrate was concentrated on a rotary evaporator to give **17** (1.98 g, 5.1 mmol, 51% yield) as a colorless oil, which was immediately used for hydrogenolysis: ^1H NMR δ 3.30–3.70 (m, 8, 2 NCH_2CH_2Cl), 5.05 (d, J = 8 Hz, 2, $CH_2C_6H_5$), 7.15–7.20 (m, 10, two C_6H_5).

Benzyl *N*,*N*-Bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (18). A magnetically stirred solution of crude **15** (ca. 0.01 mol) in C_6H_6 (50 mL) was reacted at room temperature with 40% aqueous methylamine (2.0 g), leading to the immediate formation of a gummy precipitate. After the solution was stirred overnight, H_2O (10 mL) was added and the separated organic layer was then washed with H_2O (50 mL), dried with $MgSO_4$, and concentrated on a rotary evaporator. The resultant material was purified by flash chromatography and gave **18** (6.64 g, 2 mmol, 20% yield) as a colorless oil, which was immediately used for hydrogenolysis: ^1H NMR δ 2.55 (d, J_{HP} = 12 Hz, 3, CH_3), ~3.0 (br, 1, NH), 3.05–3.80 (m, 8, 2 NCH_2CH_2Cl), 5.00 (d, J = 8 Hz, 2, $CH_2C_6H_5$), 7.30 (s, 5, C_6H_5).

Benzyl *N*-(Benzyloxy)-*N'*,*N'*-bis(2-chloroethyl)-phosphorodiamidate (19). A magnetically stirred solution of **10** (2.58 g, 0.01 mol) in freshly distilled CH_2Cl_2 (50 mL) was cooled with an ice bath and was reacted with sodium benzylate (1.30 g, 0.01 mol), which had been prepared[39] in C_6H_6 and isolated as a powder by solvent removal in vacuo. After 4 h, *O*-benzylhydroxylamine hydrochloride (1.80 g, 0.011 mol) and 0.5 M aqueous K_2CO_3 (50 mL) were added, and the two-phase reaction mixture was stirred overnight at room temperature. The separated organic layer was washed with H_2O (50 mL, 2 times), dried with $MgSO_4$, and then concentrated on a rotary evaporator to give a crude product (1.95 g), which was purified by preparative TLC (0.25 g per plate) using triple elution with $CHCl_3$. The UV-visible bands with R_f 0.36 were combined and washed with absolute MeOH to give **19** (0.97 g, 2.4 mmol, 24% yield) as a colorless oil, which eventually crystallized: mp 60–64 °C; ^1H NMR δ 3.50 (br s, 8, 2 NCH_2CH_2Cl), 4.80 (s, 2, $NOCH_2C_6H_5$), 5.05 (d, J = 8 Hz, $POCH_2C_6H_5$), 6.40 (d, J = 14 Hz, NM), 7.30 (s, 10, 2 C_6H_5). Anal. ($C_{18}H_{23}Cl_2N_2O_3P$) H, Cl, N; C: calcd, 51.81; found, 50.60.

Benzyl *N*,*N*-Bis(2-chloroethyl)-*N'*-*n*-butylphosphorodiamidate (20). A magnetically stirred solution of crude **15** (ca. 0.01 mol) in C_6H_6 (50 mL) was cooled with an ice bath, and *n*-butylamine (1.46 g, 0.02 mol) was added dropwise (15 min). After the solution was stirred overnight at room temperature, Et_2O (50 mL) was added, and the precipitate was removed by filtration. Rotary evaporation of the filtrate provided an oil, which was purified by flash chromatography and gave **20** (0.5 g, 4.3 mmol, 43% yield) as white crystals: mp 60–63 °C (Et_2O–petroleum ether); ^1H NMR δ 0.90 (t, J = 6 Hz, 3, CH_3), 1.10–1.70 (m, 4, $CH_2CH_2CH_3$), 2.70–3.10 (m, 3, $NHCH_2$), 3.25–3.80 (m, 8, 2 NCH_2CH_2Cl), 5.10 (d, J = 8 Hz, 2, $CH_2C_6H_5$), 7.35 (s, 5, C_6H_5). Anal. ($C_{15}H_{25}Cl_2N_2O_2P$) C, H, Cl, N.

Benzyl *N*,*N*-Bis(2-chloroethyl)-*N'*-cyclohexylphosphorodiamidate (21). The procedure described above for **20** was used for reaction of a C_6H_6 solution of crude 15 (ca. 0.01 mol) with freshly distilled cyclohexylamine (1.98 g, 0.02 mol). Rotary evaporation of the filtrate gave an oil, which was dissolved in Et_2O; the resultant precipitate was removed by filtration. The volume of the filtrate was reduced by rotary evaporation, and petroleum ether was added until the solution became turbid. After

(64) W. C. Still, M. Kahn, and A. Mitra, *J. Org. Chem.*, **43**, 2923 (1978).
(65) J. A. Brandt, S. M. Lueman, G. Zon, J. A. Todhunter, W. Egan, and R. Dickerson, *J. Med. Chem.*, **24**, 1404 (1981).
(66) R. Lumry, E. L. Smith, and R. R. Glantz, *J. Am. Chem. Soc.*, **73**, 4330 (1951).
(67) F.-T. Chiu, Y. H. Chang, G. Özkan, G. Zon, K. C. Fichter, and L. R. Phillips, *J. Pharm. Sci.*, **71**, 542 (1982).
(68) Heating **15** at 140 °C in vacuo affords 1 equiv of benzyl chloride and $[(ClCH_2CH_2NPO_2]_3$.[14]

the solution was left standing overnight in a freezer, product **21** (0.82 g, 2.1 mmol, 21% yield) was collected as white crystals: mp 80–85 °C (lit.[39] mp 85–86 °C); ^{1}H NMR δ 1.10–2.20 (m, 11, c-C_6H_{11}), 3.15–3.80 (m, 9, 2 NCH_2CH_2Cl and NH), 5.00 (d, J = 8 Hz, $CH_2C_6H_5$), 7.30 (s, 5, C_6H_5). Anal. ($C_{17}H_{27}Cl_2N_2O_2P$) C, H, Cl, N.

Benzyl *N,N*-Bis(2-chloroethyl)-*N',N'*-pentamethylenephosphorodiamidate (22). The procedure described above for **20** was used for reaction of a C_6H_6 solution of crude **15** (ca. 0.01 mol) with piperidine (1.70 g, 0.02 mol). Rotary evaporation of the filtrate gave an oil, which was dissolved in Et_2O; the resultant precipitate was removed by filtration and was discarded. Flash chromatography of the concentrated filtrate afforded **22**[39] as a colorless oil (50% yield), which was then subjected to hydrogenolysis (vide infra).

Benzyl *N,N*-Bis(2-chloroethyl)-*N',N'*-(3-oxapentamethylene)phosphorodiamidate (23). The procedure described above for **20** was used for reaction of a C_6H_6 solution of crude **15** (ca. 0.01 mol) with morpholine (1.65 g, 0.02 mol). Flash chromatography gave **23** (3.14 g, 8.2 mmol, 82% yield) as a colorless oil: ^{1}H NMR δ 2.90–4.00 (m, 16, 8 CH_2), 5.00 (d, J = 8 Hz, 2, $CH_2C_6H_5$), 7.30 (s, 5, C_6H_5). Anal. ($C_{15}H_{23}Cl_2N_2O_3P$) C, H, Cl, N.

Hydrogenolysis of Benzyl Phosphoramidates and Benzyl Phosphorodiamidates. A conventional atmospheric hydrogenation apparatus was used with an absolute EtOH (20 mL) solution of the benzyl ester (0.01 mol) and 10% Pd/C catalyst (150 mg). After 1 equiv of H_2 was taken up, cyclohexylamine (1.1 mL, 0.01 mol) was added, and the catalyst was removed by filtration. The filtrate was concentrated in vacuo without heating, and the residue was triturated with Et_2O to give solid material, which was then collected and crystallized from absolute EtOH–Et_2O. Previously unreported products were identified by ^{1}H NMR comparison of the CHA and nitrogen mustard absorption intensities: *N,N*-bis(2-chloroethyl)-*N'*-*n*-butylphosphorodiamidic acid cyclohexylammonium salt (**28**, 8% yield), mp 120–122.5 °C; *N,N*-bis(2-chloroethyl)-*N'*-cyclohexylphosphorodiamidic acid cyclohexylammonium salt (**29**, 37% yield), mp 111–114 °C (lit.[39] mp 110–112 °C); *N,N*-bis(2-chloroethyl)-*N',N'*-(3-oxapentamethylene)phosphorodiamidic acid cyclohexylammonium salt (**31**, 37% yield), mp 116–118 °C (lit.[39] mp 118–119 °C); *N,N*-bis(2-chloroethyl)-*N',N'*-pentamethylenephosphorodiamidic acid cyclohexylammonium salt (**30**, 15% yield), mp 120–120.5 °C (lit.[39] mp 120–122 °C); *N,N*-bis(2-chloroethyl)-*N'*-methylphosphorodiamidic acid cyclohexylammonium salt (**27**, 21% yield), mp 155–158 °C; *N,N*-bis(2-chloroethyl)-*N'*-hydroxyphosphorodiamidic acid cyclohexylammonium salt (**26**, 50% yield), mp 109–114 °C; *N,N*-bis(2-chloroethyl)phenylphosphoramidic acid cyclohexylammonium salt (**25**, 26% yield), mp 162.5–163.5 °C (lit.[3] mp 182–184 °C).

^{31}P NMR Kinetic Studies. General Procedure. The buffer (1 M, 1.8 mL) was diluted with a relatively small amount of D_2O (0.2 mL), which provided an internal lock signal, and the resultant solution was then added to a glass vial containing the compound (ca. 30 mg) to be studied. After rapid shaking and then transfer of the solution to a 10-mm NMR tube, the solution was allowed to thermally equilibrate for 2–5 min in the spectrometer probe (37 °C) during final adjustment of the magnetic field homogeneity. At time "zero", data accumulation was initiated using a 5-kHz spectral window, 8192 data points, a $\pi/2$ pulse of 18 μs, low-power ^{1}H decoupling, and a pulse repetition time of 2 s. After 100 pulses, the free-induction decay (FID) signal was exponentially multiplied so as to result in an additional 1 Hz of line broadening in the frequency-domain spectrum obtained by Fourier transformation. The spectrum was recorded, data accumulation was again initiated, and the elapsed time relative to "zero" was noted. For compounds with relatively short half-lives, the FID for each spectrum (100 pulses), was automatically stored on the computer disk and, after a 1-s delay time, the next spectral acquisition was initiated. In each spectrum, signal intensities (peak heights) of individual components were compared with the total intensity for all observable ^{31}P signals, thus providing a measure of relative concentration, which was defined as % *P*. Reactions were generally followed for longer than 3 half-lives, and first-order kinetic plots using % *P* values were fit by linear least-squares analysis (in all cases, $r > 0.95$). Since possible nuclear Overhauser effects (NOE) were not suppressed by gated decoupling, the relative signal intensities for chemically nonequivalent species provided approximate concentration values. Other sources of error included T_1 differences and, for some reaction components, partially overlapped signals.

When the above procedure was used, reaction of 1·CHA with a 10-fold molar excess of $HSCH_2CH_2OH$ gave the spectra shown in Figure 3. Peak heights for the partially overlapped absorptions labeled as *A* (1), *B* (7), and *C* (8) provided approximations for the corresponding concentration terms, on a relative basis (% *P*), and it was assumed that $A \xrightarrow{k'} B \xrightarrow{k''} C$; i.e., the aziridinium ion intermediates (cf. Scheme I) were ignored as well as the decreasing concentration of the trapping agent (20% change after complete conversion of A into C). The values of k' and k'' were determined by a least-squares fit, using MLAB,[69] of the measured time-dependence of the concentrations of A, B, and C to the following kinetic model:

$$A = A_0 \exp(-k't) \quad (1)$$

$$B = [A_0k'/(k'' - k')][\exp(-k't) - \exp(-k''t)] + B_0 \exp(-k''t) \quad (2)$$

$$C = A_0 + B_0 + C_0 - A - B \quad (3)$$

Equations 1–3 were derived in the usual manner,[70] and the constants of integration were set equal to the % *P* values measured at "zero" time (relative). Fitting eq 1 gave $k' = 0.0377 \pm 0.0012$ min^{-1}. Simultaneous fitting of eq 1 and 2 gave $k' = 0.0327 \pm 0.0016$ min^{-1} and $k'' = 0.0410 \pm 0.0019$ min^{-1}, while simultaneous fitting of eq 1–3 gave $k' = 0.0372 \pm 0.0009$ min^{-1} and $k'' = 0.0458 \pm 0.0013$ min^{-1}. The latter fits are shown in Figure 4. Averaging all of the determinations gave $k' = 0.0359 \pm 0.0012$ min^{-1} ($\tau_{1/2} \simeq 19$ min for 1) and $k'' = 0.0434 \pm 0.0016$ min^{-1} ($\tau_{1/2} \simeq 16$ min for **7**).

A similar kinetic analysis was applied to the case represented by $1\text{·CHA} \xrightarrow{k'} \mathbf{2} \xrightarrow{k''}$ secondary products; however, in these calculations, eq 1–3 were fitted by varying k', k'', A_0, B_0, and C_0. For 37 °C reactions of 1·CHA in 1 M Tris buffer at pH 7.4, 8.2, and 9.0, the respective values for k'' were 0.0231 ± 0.0012, 0.0385 ± 0.0016, and 0.0464 ± 0.0012 min^{-1}, with the average value of k' being equal to 0.039 ± 0.007 min^{-1}.

In all of the cases involving multiparameter fitting, the dependency values[69] for the derived rate constants were relatively small.

***N,N*-Bis(5-hydroxy-3-thiapentyl)phosphorodiamidic Acid Cyclohexylammonium Salt (8).** A sample of 1·CHA (0.32 g, 1 mmol) was dissolved in H_2O (5 mL) containing 2-mercaptoethanol (1.4 mL, 20 mmol), and the solution was manually titrated with 1 N NaOH to maintain pH 7. After production of acid had ceased, lyophilization afforded material containing **8**: ^{1}H NMR (220 MHz, Me_2SO-d_6) δ 1.0–2.0 (m, 11, c-C_6H_{11}), 2.50 (6, J = 7 Hz, 4, 2 SCH_2CH_2O), 2.76 (t, J = 6.6 Hz, 4, 2 NCH_2CH_2S), 3.49 (t, J = 6.9 Hz, 4, SCH_2CH_2O), 3.59 (t, J = 6.6 Hz, 4, 2 NCH_2CH_2S).

Acknowledgment. This investigation was supported in part by research Grant CA-21345 to G.Z. from the National Institutes of Health. We thank Lawrence R. Phillips (Bureau of Biologics) for the MS measurements and his assistance in assigning the ion fragments.

(69) G. D. Knott, *Comput. Programs Biomed.*, **10**, 271 (1979).
(70) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism. A Study of Homogeneous Chemical Reactions", 2nd ed., Wiley, New York, 1961, p 166.