resonance absorption at δ 92.80 was subsequently shown to arise from the reaction of acrolein with Tris. The aforementioned experiment with *cis*-12 and Tris was used to record the ^1H NMR spectrum for a reaction mixture that contained ~70% **22–24**; no signals were observed at δ >5 for C*H*=N.

Reaction of 2/3 with Sulfur-Containing Compounds. The following procedures were adapted from synthetic methods reported by Peter and Hohorst.[66] A solution of *cis*-12 (100 mg, 0.34 mmol) and Ph_3P (89 mg, 0.34 mmol) in CH_2Cl_2 (0.5 mL) was stirred for 15 min at ambient temperature and then concentrated on a rotary evaporator without heating. The resultant oil was dissolved in a minimal volume of Et_2O, a seed crystal of Ph_3PO was then added, and the solution was stored at −20 °C overnight. The ether solution was removed and concentrated first on a rotary evaporator and then under high vacuum to afford crude **2/3** as a white semisolid.

A solution of crude **2/3** (~0.34 mmol) in CH_2Cl_2 (0.2 mL) was added to a mixture of **2-mercaptoethanol** (48 μL, 0.68 mmol) and trichloroacetic acid (0.5 mg, 3 μmol) in CH_2Cl_2 (0.1 mL) at ~5 °C, and the resultant solution was stirred for 1 h at ~5 °C before removal of volatile material in vacuo. The residual oil was dissolved in lutidine buffer (1.35 mL of 1 M, pH 7.4) and D_2O (0.15 mL) for ^{31}P NMR analysis, which showed the presence of two major peaks (ca. 1:1 ratio) at δ 11.35 and 12.59 that were ascribed to the expected[66] products, viz., *cis*- and *trans*-**7** (R = CH_2CH_2OH); a trace amount of the acyclic hemithioacetal conjugates was seen at δ 20.36. Attempts to isolate these products by either low-temperature crystallization or chromatography on silica gel were unsuccessful.

A solution of crude **2/3** (~0.34 mmol) in CH_2Cl_2 (1 mL) was added to a mixture of ***N*-acetyl-L-cysteine** (55 mg, 0.34 mmol) and trichloroacetic acid (0.5 mg, 3 μmol) in acetone (2 mL) at ~5 °C, and the resultant solution was stirred for 1.5 h at ~5 °C before removal of volatile material in vacuo. ^{31}P NMR analysis of the residual solid, as described above, showed the presence of major peaks at δ 11.25 and 11.35 for *cis*-**27** and at δ 12.29 and 12.56 for *trans*-**27** in approximately equimolar amounts, as well as less intense signals at δ 20.36 and δ 13.8–13.9 for **26** and 4/alkylation products, respectively. Repetition of this procedure with crude **2/3**, derived by ozonation (vide supra) of (*S*)-**1**, led to ^{31}P NMR signals for *cis*- and *trans*-**27** at δ 11.25 and 12.29, respectively.

To a solution of crude **2/3** (~0.34 mmol) in acetone (2 mL) at ~5 °C was added $Na_2S_2O_3 \cdot 5H_2O$ (84 mg, 0.34 mmol) and trichloroacetic acid (0.5 mg, 3 μmol). The resultant suspension was allowed to warm to room temperature, more acetone (1 mL) was added, and the reaction mixture was then stirred for 8 h at room temperature. Removal of solvent in vacuo afforded a semisolid, which was dissolved in lutidine buffer (1.35 mL of 1 M, pH 7.4) and D_2O (0.15 mL) for ^{13}C and ^{31}P NMR analyses, which failed to show the presence of signals indicative of either a cyclic (δ_P 11–13) or an acyclic (δ_P 20–21) conjugate; signals ascribed to **17** (δ_C 69.78 and δ_P 10.01) were also not evident.

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Registry No. 1-5,5-d_2, 59720-09-1; *cis*-**2**, 88852-77-1; *trans*-**2**, 88852-78-2; **3**, 35144-64-0; **4**, 10159-53-2; **5**, 88685-78-3; *cis*-**7**, 61903-94-4; *trans*-**7**, 61903-98-8; **10**-*14*-^{13}C, 27046-19-1; (±)-*cis*-**12**, 62435-42-1; *cis*-**12**-5,5-d_2, 88803-00-3; *cis*-**12**-*4*-^{13}C, 88803-05-8; **13**, 88803-04-7; **13** (hydroxy derivative), 88803-03-6; **16**, 88802-97-5; **17**, 88825-35-8; (*E*)-**21**, 88803-06-9; (*Z*)-**21**, 88803-07-0; L-(*S*)-**26**, 88802-98-6; L-(*R*)-**26**, 88852-79-3; *cis*-**27** (isomer 1), 88802-99-7; *cis*-**27** (isomer 2), 88852-80-6; *trans*-**27** (isomer 1), 88852-81-7; *trans*-**27** (isomer 2), 88852-82-8; Tris, 77-86-1; L-R*SH, 616-91-1; $Cl_2P(O)M$, 127-88-8; $MeO^{13}CH_2OMe$, 88803-02-5; $^{13}CH_2O$, 3228-27-1; MeOH, 67-56-1; $HS(CH_2)_3SH$, 109-80-8; $MeONH_2 \cdot HCl$, 593-56-6; acrolein, 107-02-8; 3-buten-1-ol, 627-27-0; 3-butenyl *N*,*N*-bis-(2-chloroethyl)phosphorodiamidate, 39800-29-8; 2,2-dideuterio-3-deuteroxypropionitrile, 88803-01-4; 1-amino-2,2-dideuteriopropan-3-ol, 59720-08-0; 1,3-dithiane-2-^{13}C, 88157-07-7; 2-mercaptoethanol, 60-24-2.

Base-Catalyzed Hydrolysis of 4-Hydroperoxycyclophosphamide: Evidence for Iminocyclophosphamide as an Intermediate

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cis-4-Hydroperoxycyclophosphamide (**5**) undergoes facile reaction with aqueous phosphate or Tris buffers at pH 7–8 and 30 °C. The kinetics of **5** are complex, and the *trans*-4-hydroperoxy isomer **6** is produced and subsequently disappears over the course of the reaction. Addition of hydrogen peroxide to the reaction mixture retards the disappearance rate of **5** and increases the amount of **6** generated. Rate constants for the reversible disappearance of **5** and appearance of **6** and 4-hydroxycyclophosphamide (**2**) have been determined by nonlinear least-squares methods. The reaction is catalyzed by hydroxide ion, Tris free base, and HPO_4^{2-}, with catalytic constants of 0.032 min^{-1} (pH 8.0), 0.052, and 0.115 M^{-1} min^{-1}, respectively. The major product in the presence of Tris is the oxazolidine **8b** arising from the addition of Tris to aldophosphamide, not **2** as assumed previously. These results are consistent with a mechanism involving general-base-catalyzed elimination to produce iminocyclophosphamide **7** as a transient intermediate; the imine can react with the hydrogen peroxide evolved in the reaction to give **5** and **6**, with water to give **2**, or, in general, by addition of a nucleophile to C-4. The significance of these findings with respect to other 4-substituted cyclophosphamides is discussed.

Cyclophosphamide (**1**) continues to be one of the most widely used and intensively studied agents available for the treatment of malignancy. The chemistry, pharmacology, and metabolism of **1** have been reviewed;[1-3] the activation process appears to involve initial hydroxylation by the hepatic cytochrome P450 system to produce 4-hydroxycyclophosphamide (**2**) of unknown stereochemistry. Intermediate **2** undergoes ring opening to aldophosphamide **3**, which in turn suffers β-elimination to produce acrolein and phosphoramide mustard **4**, generally

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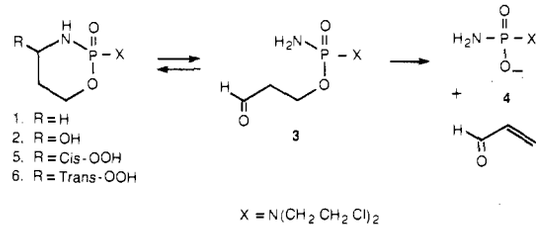
Scheme I



believed to be the ultimate cytotoxic agent. It is apparent that **2** occupies a critical position as an intermediate in a complex array of both activation and deactivation processes, and recent evidence suggests that it may represent the important circulating metabolite therapeutically.[4] Products arising from substitution at the C-4 position have been described;[5-9] those derivatives possessing 4-mercapto groups are especially interesting because of the controversy over whether these compounds are deactivated[6] or, in fact, represent "activated" analogues.[7,8] Several mechanisms have been suggested for C-4 substitution, including direct displacement,[5] a sequence involving ring opening to **3**, nucleophilic addition to the aldehyde, and ring closure of the adduct,[6] and formation of and subsequent addition to the intermediate imine via a sequence of elimination–addition.[9] Fenselau et al. have provided persuasive evidence for the transient existence of iminocyclophosphamide **7** both from the decomposition of 4-hydroperoxycyclophosphamide (**5**) and from the enzymatic activation of **1**.[10] We describe here our studies of the hydrolysis of **5** as a mechanistic model for exchange reactions at the 4-position of activated cyclophosphamides. The results confirm the existence of **7** as a transient intermediate produced from **5** via base-catalyzed elimination, although its concentration and/or lifetime is insufficient to permit detection by either ^1H or ^{31}P NMR spectroscopy.

Results and Discussion

cis-4-Hydroperoxycyclophosphamide (**5**)[11] was synthesized in 10–15% yields by ozonolysis of cyclophosphamide by a modification of the published procedure.[6] The cis 1,3-diaxial relationship between the 4-OOH and P=O groups was confirmed by the large H_4–P coupling (26.9 Hz in $CDCl_3$, 25.8 Hz in D_2O) indicative of an equatorial C-4 proton[12] and the downfield position of the C-6 axial proton (4.65 ppm) compared to that (4.25 ppm) of the trans isomer, which has the P=O group in the equatorial position (vide infra).[12] These data are in accord with that reported by Takamizawa et al. for **5**[13] and confirms that the preferred conformation in solution is the same as that of the crystal.[14] In some experiments a small amount (~1%) of another product was obtained, which chemical-ionization mass spectrometry confirmed was an isomer of **5**. The ^1H NMR spectrum showed a H_4–P coupling of 17.5 Hz, and the chemical shifts of the C-6 axial and C-4 protons were 0.4-ppm upfield and 0.17-ppm downfield, respectively, compared to those of **5**. These data confirm[12] that the minor product is the trans isomer **6**, with the major solution conformer possessing axial OOH and NR_2 groups. X-ray analysis has shown that the OOH group is axial in both isomers of 4-hydroperoxyisophosphamide;[15] the downfield shift of the OOH proton in **5** and the multiplicity of the CH_2Cl protons in **6** (suggesting magnetic nonequivalence due to restricted rotation about the P–N bond) are consistent with stabilization of these conformers via hydrogen bonding between OOH and the axial phosphorus substituent, as noted in the crystal structure.[14,15]

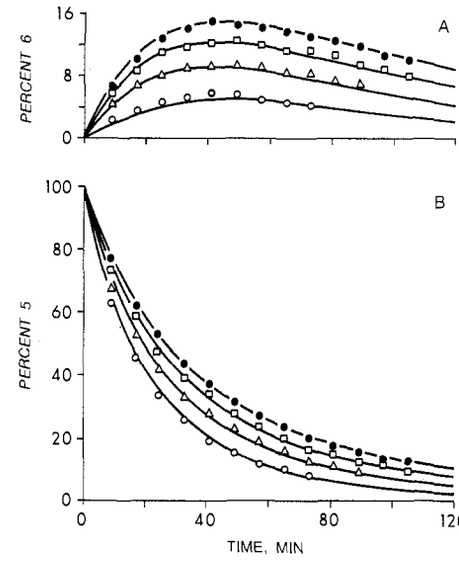


Figure 1. Reaction of *cis*-4-hydroperoxycyclophosphamide (**5**) with Tris buffer (1 M), pH 8.0, 30 °C, μ = 1.0, in the presence of the following initial hydrogen peroxide concentrations: (○) 0 mM; (△) 10 mM; (□) 20 mM; (●) 30 mM. Symbols represent individual data points. The lines represent calculated curves using the rate constants in Scheme II and eq 4 and 5 (see text for details). (A) Appearance and disappearance of **6**; (B) disappearance of **5**.

The hydrolysis of **5** was examined at 30 °C as a function of pH and buffer concentration in Tris and phosphate buffers; disappearance of **5** was monitored by either reverse-phase HPLC or ^{31}P NMR. Although good first-order kinetics were observed over approximately the first 25% of the reaction, the first-order plots showed increasing upward curvature with increasing reaction time. This nonlinearity was tentatively attributed to the generation of hydrogen peroxide over the course of the reaction, which underwent addition to a reactive intermediate to regenerate **5**, consequently slowing its apparent disappearance. This hypothesis was tested by running the reaction (1 M Tris, pH 8.0) in the presence of added hydrogen peroxide (Figure 1B); increasing concentrations of H_2O_2 clearly

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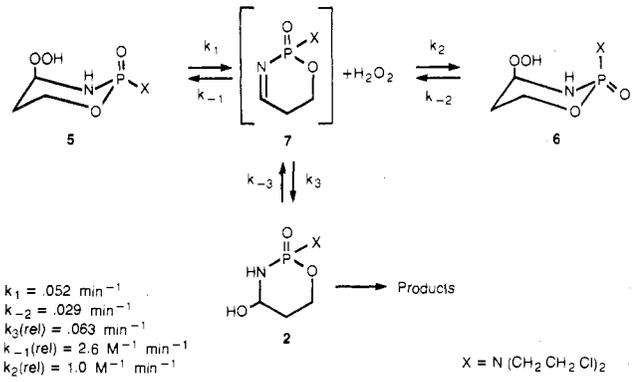
Scheme II



retard the reaction rate of **5**. The appearance and disappearance of a minor product was also noted by HPLC (Figure 1A), the kinetics for which clearly depend upon hydrogen peroxide concentration. A sample of this minor component was isolated from the reaction of **5** by HPLC; ^1H and ^{31}P NMR and mass spectrometry confirmed that it was the trans compound **6**. The fact that larger quantities of **6** are produced at higher H_2O_2 concentrations indicates that **6** is formed as a result of trapping, rather than direct isomerization or stereomutation of **5**. These data suggest that **5** in aqueous buffer initially produces a reactive intermediate that can undergo attack by hydrogen peroxide to regenerate **5** or to produce **6**, or react with water to give the hydroxy compound **8**. Additional evidence, to be presented below, indicates that the rate-limiting step involves general-base-catalyzed elimination to give imine **7**; we have been unable to detect **7** under a wide variety of conditions by both ^1H and ^{31}P NMR, suggesting that it reacts rapidly with water or other nucleophiles present.

The kinetics were treated by using the steady-state approximation for **7** as an intermediate and the following set of differential equations (see Scheme II):

$$dC/dt = -k_1[C] + k_{-1}[I][H] \quad (1)$$

$$dT/dt = k_2[I][H] - k_{-2}[T] \quad (2)$$

$$dI/dt = k_1[C] + k_{-2}[T] - [H][I](k_{-1} + k_2) - k_3[I] \quad (3)$$

where C = **5**, T = **6**, I = **7**, and H = H_2O_2. Assuming $dI/dt = 0$ at steady state, solving eq 3 for [I], and substituting into eq 1 and 2, we have eq 4 and 5.

$$\frac{dC}{dt} = \frac{-k_1[C](k_3 + k_2[H]) + k_{-1}k_2[H][T]}{k_3 + [H](k_{-1} + k_2)} \quad (4)$$

$$\frac{dT}{dt} = \frac{k_1k_2[H][C] - k_{-2}[T](k_3 + k_{-1}[H])}{k_3 + [H](k_{-1} + k_2)} \quad (5)$$

These equations assume that the contribution from k_{-3} is negligible under the reaction conditions (see Scheme II)—a point to be discussed in detail below. Inspection of eq 4 reveals that, at early time points in the absence of added H_2O_2, [T] ~ [H] ~ 0, and the rate expression simplifies to $dC/dt = -k_1[C]$ as expected for a first-order reaction. Thus, k_1 was obtained under a variety of different conditions by first-order analysis of the first 25% of the reaction. A computer program using eq 4 and 5 was written to calculate the theoretical curves for the kinetics of **5** and **6** at four initial concentrations of added H_2O_2 (0–30 mM); using the value of k_1 determined from the initial reaction period, we determined the remaining rate constants by simultaneously minimizing the sum of least-squares errors for all four experiments. The calculated best fit values are

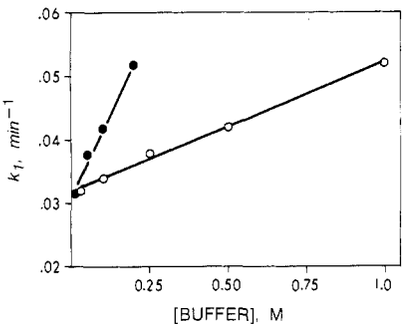


Figure 2. Effect of buffer concentration on the rate constant for the disappearance of **5**, pH 8.0, 30 °C, μ = 1.0: (○) Tris buffer; (●) phosphate buffer. Lines represent best-fit straight lines determined by linear regression ($r^2 > 0.995$).

shown in Scheme II; although it is not possible to calculate exact errors for these rate constants, changes of 5% in any one value caused a significant increase in least-squares error at one or more added H_2O_2 concentrations. Because of the high degree of dependency of k_{-1}, k_2, and k_3, the values determined for these rate constants are relative. Based upon our inability to detect **7**, we can place an upper limit of ~0.02 on the equilibrium constant K_1, so the value of these three rate constants represents a reasonable lower limit to their real values ($K_1 = k_1/k_{-1} < 0.02$). The data points are shown with the calculated curves in Figure 1; reasonable agreement is observed for the concentrations of **5** and **6** as a function of time at all four initial H_2O_2 concentrations. As a further check on the rate constants, the first 25% of the reaction was also examined in the presence of added H_2O_2. Under these conditions [T] ~ 0, and eq 4 simplifies to eq 6. In eq 6, the calculated values

$$\frac{dC}{dt} = \left(\frac{-k_1(k_3 + k_2[H])}{k_3 + [H](k_{-1} + k_2)}\right)[C] = k_{obsd}[C] \quad (6)$$

for k_{obsd} were identical (within 0.001 min^{-1}) with those obtained experimentally: 0.038, 0.032, and 0.028 min^{-1} at added H_2O_2 concentrations of 10, 20, and 30 mM, respectively.

The effect of buffer concentration on k_1 was determined at pH 8.0 for Tris and phosphate buffers by examination of the initial disappearance of **5** in the absence of added H_2O_2. The results (Figure 2) indicate that, under these conditions, phosphate is approximately 5-fold more effective as a catalyst. The catalytic contribution of hydroxide ion was determined by extrapolation to zero buffer concentration and was found to be 0.032 min^{-1} at pH 8.0. The effect of pH was studied over the pH range of 7–8 for both buffers; as expected, the reaction proceeded faster at higher pH (Figure 3). The buffer contribution to the rate constant was determined by subtraction of the hydroxide contribution calculated for each pH, and after correction for total buffer concentration it was plotted against the fraction of buffer in its basic form (HPO_4^{2-} in the case of phosphate). The results indicate that the basic component of the buffer is the catalytically active species, that the acid form of Tris is catalytically inactive, and that $H_2PO_4^-$ possesses very little catalytic activity. The k_{cat} for HPO_4^{2-} is approximately twice that for Tris (0.115 vs. 0.052 M^{-1} min^{-1}). The temperature dependence of k_1 gave an Arrhenius plot over the range 10–40 °C, from which the activation energy of the reaction was determined (16.2 kcal/mol in 1 M Tris, pH 8.0). Finally, definitive evidence for the elimination–addition mechanism was obtained by examining the initial disappearance rates of **5** in H_2O vs. D_2O (1 M Tris, pH 7.4, 37 °C). The observed rate constants under these conditions were 0.031 and 0.008 min^{-1},

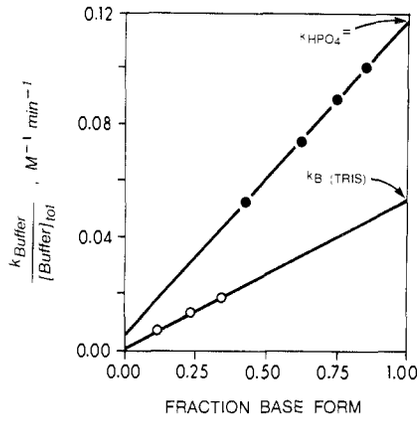
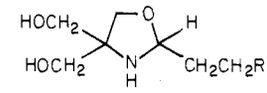


Figure 3. Effect of pH on buffer catalysis for the disappearance of **5**, 30 °C, μ = 1.0: (○) Tris buffer (1.0 M), k_B = 0.052 min^{-1}; (●) phosphate buffer (0.1 M), $k_{HPO_4^{2-}}$ = 0.115 min^{-1}. Lines represent best fit straight line determined by linear regression (r^2 > 0.995).

respectively, corresponding to an isotope effect of approximately 4. An isotope effect of this magnitude can only be explained by assuming rapid exchange of the N–H proton for deuterium, followed by rate-limiting cleavage of the N–D bond with concomitant expulsion of hydroperoxide. In effect, this is a primary rather than a solvent deuterium isotope effect, and its large value reflects considerable N–H bond breaking in the transition state. These data argue convincingly for a mechanism involving rate-limiting general-base-catalyzed elimination of hydrogen peroxide from **5** to form **7** as a transient intermediate, which reacts with available nucleophiles to produce **5**, **6**, or **2**.

In our preliminary report on the reaction of **5** we assumed that the product observed in the Tris buffer reaction was **2** and suggested that the unusual stability of **2** in Tris as compared to phosphate and bicarbonate buffers may be a consequence of accelerated ring opening of **2** via bifunctional catalysis for the latter.[16] These assumptions were incorrect; the ^{31}P NMR signal (−5.65 ppm) observed as the major product in Tris occurs in the region characteristic of the open-chain metabolites [e.g., aldophosphamide (**3**) and its hydrate[17] and *O*-methyloximes[24]]. We have also shown that the conversion of **2** to **4** and acrolein occurs via general-base catalysis and that carbonate is the catalytically significant species when the reaction is run in bicarbonate buffer.[18] This suggests that Tris may be forming a stable adduct with a ring-opened intermediate, presumably **3**, and that this adduct may be susceptible to reversal by bifunctional catalysts. The reaction of propionaldehyde with Tris (pH 8.0, D_2O) was studied with ^1H NMR as a model system. Within minutes after the addition of Tris (4 equiv) to the aldehyde, the resonances at 9.68 and 4.94 ppm (C-1 protons of the aldehyde and hydrate, respectively) were replaced by a new triplet at 4.42 ppm (J = 5.7 Hz). A new AB quartet [3.54 and 3.63 ppm (J = 11.8 Hz)] appeared in the region of the Tris resonance, suggesting the presence of nonequivalent alkoxymethylene protons. These results suggest that Tris reacts rapidly with the aldehyde to produce the oxazolidine **8a**, with the



8a, R = H
b, R = $OP(=O)(NH_2)N(CH_2CH_2Cl)_2$

quartet arising from the geminal ring protons. The reaction of **5** in Tris/D_2O was similarly monitored by ^1H NMR. The C-4 proton resonance centered at 5.17 ppm gradually disappeared, but no evidence of signals attributable to *cis*- or *trans*-**2** was seen.[17] Instead, a new triplet appeared at 4.63 ppm (J = 5.6 Hz), and the complex resonances for the nonequivalent C-6 axial and equatorial protons were replaced by a simple doublet of triplets centered at 4.11 ppm (J_{H-P} = 7.0 Hz, J_{H-H} = 5.5 Hz). The AB quartet seen in the model reaction was also evident at 3.6 ppm. Thus, the product of the "hydrolysis" reaction in Tris is presumably **8b**, and the trapping of **3** by Tris is presumably rapid compared with cyclization of **3** to **2**. In contrast to Tris, the phosphate-catalyzed reaction gives a mixture of *cis*- and *trans*-**2**, **3**, and its hydrate.[17]

Examination of the rate constants in Scheme II indicate that the cis isomer **5** undergoes elimination approximately twice as fast as the trans isomer **6**. The basis for this difference is not obvious, inasmuch as the OOH group is axial in the predominant solution conformer of both isomers. Significant differences in isomer stability have been noted for the 4-mercapto-[7] and 4-cyanocyclophosphamide[10] derivatives, although the stereochemistry at C-4 has not been established for these compounds. Addition of hydrogen peroxide to **7** favors the cis over the trans product by a factor of 2.6; again, the basis for this difference is not known. One would expect that addition of water to **7** would show stereoselectivity similar to that observed for H_2O_2; unfortunately, the cis and trans isomers of **2** undergo facile interconversion via **3**,[17] effectively precluding the examination of stereoselectivity in the water addition reaction.

Benckhuysen et al. observed a "lag phase" in the release of acrolein from **5** and from the anhydro dimer of **2** in aqueous buffer and concluded from this observation that radical intermediates were involved in this conversion.[23] We also observed this "lag phase" in the reaction of **5** with phosphate buffer but found that the duration of the lag decreased with increasing buffer concentration and pH (see Charts 3–5 in ref 16). This dependence on pH and buffer concentration would be expected for a general-base-catalyzed reaction but is not consistent with the intermediacy of free radical species. Thus, the lag phase presumably arises not as a consequence of radical intermediates but rather from the relative rates of the stepwise sequence as we suggested previously.[16] Zon and co-workers also observed 4-ketocyclophosphamide as a product from the anhydro dimer in aqueous buffer and attributed its formation to free radical cage reactions.[24] We did not observe this product from the reaction of **5**, nor was it found by Zon in the reaction of 4-(*tert*-butylperoxy)cyclophosph-

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amide.[24] Conceivably, 4-ketocyclophosphamide might be produced by competitive attack of base at the C-4 proton and concomitant cleavage of the O–O bond to produce **2** and the 4-keto derivative. It is not clear why this would occur only with the anhydro derivative; perhaps steric factors make the N–H proton abstraction and elimination of **5** less favorable in this case. In any case, mechanisms involving general-base catalysis can be invoked to explain all of these results; there is neither evidence nor the need to postulate free radical intermediates.

Elimination–addition via the imine represents a probable common mechanism that explains a number of reported observations: (a) the "equivalence" of 4-hydroperoxy- and 4-hydroxycyclophosphamide in aqueous buffer systems, albeit with a "latent period";[19] (b) conversion of the 4-peroxy dimer to **5** by reaction with hydrogen peroxide;[22] (c) facile interconversion of the less stable to the more stable isomer of 4-cyanocyclophosphamide;[10] and (d) rapid conversion of 4-mercapto derivatives to **4** and acrolein, presumably via elimination–addition to give **2**.[7,8] This mechanism requires that the C-4 substituent be a satisfactory leaving group with respect to elimination, and this in turn should be a function of the pK_a of the substituent conjugate acid. Thus, the stability of 4-substituted derivatives in aqueous buffers should decrease with respect to elimination in the order OEt > OH > OOH > SR ≈ CN. It is interesting to note that rate constants reported for the hydrolysis of several 4-mercapto derivatives (70 mM phosphate, pH 7.0, 37 °C) are in the range 0.04–0.17 min^{-1} with thiol pK_a's of 9.5–10.5.[7,8] This compares with the somewhat slower rate (0.016 min^{-1}) that we observe for **5** under these conditions, where the leaving group pK_a is 11.6. This dependence of rate on leaving group pK_a would be characteristic of elimination reactions that involve expulsion of strongly basic leaving groups (pK_a > 10).[20]

Conclusion

The kinetic and mechanistic data presented, in particular the conversion of *cis*- to *trans*-4-hydroperoxycyclophosphamide in the presence of an agent that traps the 4-hydroxy compound as an aldehyde derivative, argue for the existence of a β-elimination mechanism leading to the formation of iminocyclophosphamide **7**. Our inability to detect **7** spectroscopically suggests that it is present as a transient intermediate and is rapidly intercepted by water or other nucleophiles. The extent of β-elimination in the reaction of 4-substituted cyclophosphamide derivatives will depend upon the basicity of the 4-substituent, and attempts to design "stabilized" analogues should consider this an important factor for determination of stability. Finally, our initial assumption[16] that 4-hydroxycyclophosphamide is stable to nonbifunctional base catalysts has been proved incorrect, and the conclusions drawn regarding the role of bifunctional catalysis in selective activation apply to the oxazolidine derived from Tris and aldophosphamide, not to the hydroxy compound itself. These concepts are currently being applied to the synthesis and study of activated cyclophosphamide analogues in our laboratory.

Experimental Section

Cyclophosphamide monohydrate and Tris were obtained from Sigma Chemical Co. HPLC analyses were carried out on a Supelco C8 (15 or 25 cm × 4.6 mm) reverse-phase column on a Beckman HPLC system consisting of a Model 110A pump, Model 210 injector equipped with a 10-μL injection loop, and a Model 155 detector set at 205 nm; acetonitrile/water (1:3) was used as the elution solvent. Preparative liquid chromatography was carried out on a system consisting of a Fluid Metering, Inc., solvent pump, a Waters Model R401 refractive index detector, a Rheodyne six-port injection valve, and a Merck LiChroprep RP8 (size B) column. Proton NMR spectra were obtained on a Nicolet NT300 or IBM WP-270-SY instrument using 5-mm sample tubes, a 3000-Hz spectral width, pulse widths of 2 or 4 μs with pulse repetition times of 2 or 8 s, respectively. For spectra run in D_2O, the residual HOD signal was suppressed by either gated homonuclear decoupling or application of a 180°–τ–90° pulse sequence. Chemical shifts are reported in parts per million from internal references of Me_4Si ($CDCl_3$) or 1-(trimethylsilyl)propanesulfonate (D_2O). ^{31}P NMR spectra were recorded on an XL-100, Nicolet NT300, or IBM WP-270-SY instrument using 10-mm sample tubes, a 5000-Hz spectral width, a 15-μs pulse width, and a pulse repetition time of 0.6 s. Chemical shifts are reported in parts per million from 5% hexamethylphosphoric triamide (HMPA) in $CHCl_3$ in a coaxial tube. Low-power, broad-band decoupling was used. Chemical-ionization mass spectra were recorded on a Finnigan 4000 instrument at 0.3 torr with ammonia as reagent gas.

***cis*- and *trans*-4-Hydroperoxycyclophosphamide (5 and 6).** A solution of cyclophosphamide (1.0 g, 3.8 mmol) in 7 mL of acetone, 14 mL of water, and 1.4 mL of 30% hydrogen peroxide was ozonized at 0 °C, with the reaction course monitored twice hourly by HPLC. When the peak arising from **5** reached a maximum value (typically ~4 h), the 4-ketocyclophosphamide byproduct that had precipitated was removed by filtration, and the resulting solution was extracted with methylene chloride (3 × 50 mL). The combined extracts were dried ($MgSO_4$) and filtered, and the filtrate was evaporated to give an oily residue. This material was dissolved in 2 mL of methanol and introduced into the injection loop of the preparative LC system. Elution was carried out with methanol/water (3:7) at a flow rate of 15 mL/min. The retention time of **5** was approximately 50 min, and 300 mL of eluent containing the desired product was collected. This was divided into two portions, and each was extracted with methylene chloride (3 × 75 mL). The combined extracts were dried ($MgSO_4$) and filtered, and the filtrate was evaporated; the residue was dissolved in 2 mL of chloroform, ether was added to the cloud point, and the product was crystallized by cooling at −20 °C. The product was collected and washed with cold 3:1 ether/chloroform to give 150 mg (14%) of **5**: mp 105–107 °C (lit.[21] 107–110 °C); HPLC single peak of retention time 5.1 min; ^1H NMR ($CDCl_3$) δ 5.11 (1 H, dddd, J_{HP} = 26.9 Hz, H_4), 4.65 (1 H, dddd, H_{6a}), 4.15 (1 H, dddd, H_{6e}), 3.62 (4 H, t, CH_2Cl), 3.42 (4 H, d of t, J_{HP} = 12 Hz, NCH_2), 2.12 (1 H, dddd, H_{5a}), 1.93 (1 H, dddd, H_{5e}); ^{31}P NMR ($CDCl_3$) δ −15.44 (s); mass spectrum, *m/e* 292 (M^+).

In some experiments a minor peak was also collected (retention time 40 min on preparative LC) and isolated as described above to give **6** (10 mg, 1%) as a waxy solid: HPLC single peak of retention time 4.0 min; ^1H NMR ($CDCl_3$) δ 5.28 (1 H, dddd, J_{HP} = 17.5 Hz, H_4), 4.25 (2 H, m, H_{6a} and H_{6e}), 3.64 (4 H, m, CH_2Cl), 3.47 (4 H, m, NCH_2), 2.22 (1 H, m, H_{5a}), 1.90 (1 H, m, H_{5e}); ^{31}P NMR ($CDCl_3$) δ −16.77 (s); mass spectrum, *m/e* 292 (M^+).

Kinetics. Reactions were followed by the following two methods: (a) ^{31}P NMR with 5% HMPA/$CDCl_3$ in a coaxial tube used as internal standard for integration of the peak area corresponding to **5**; (b) HPLC at a flow rate of 2.0 mL/min with or without acetophenone as internal standard, with detection at 205 nm set at 0.1 AUFS. Reproducibility was generally better with the HPLC method because of superior control of reaction temperature (±0.2 °C with a thermostatted water bath for HPLC measurements vs. ±2 °C with temperature control of the NMR sample). The ionic strength was maintained at unity with sodium chloride. The pH was measured before and after the kinetic runs and was generally within ±0.02 pH unit over the course of the reaction.

Long-Term (3–4 Half-Lives) Kinetics. A stock solution of **5** was prepared in methanol (29.5 mg/250 μL; 0.4 M), and the appropriate buffer solution was prepared at a concentration 5% higher than desired for the reaction. Hydrogen peroxide was added when needed for the kinetic determination. The reaction was initiated by the addition of 5 μL of the stock solution to 95 μL of buffer at 30 °C; the resulting concentration of **5** was 20 mM. An aliquot of 15 μL was removed at 8-min intervals and analyzed for **5** and **6** by HPLC using peak-height measurements. The initial peak height was determined in triplicate for each run by carrying out the addition at 0 °C and then vortexing the mixture briefly

and immediately analyzing it by HPLC. Each experiment was carried out in duplicate.

Short-Term (<0.5 Half-Life) Kinetics. Because of the short time-point intervals desired (2–4 min) and the long time (8 min) required between HPLC injections, each data point required a separate experiment for determination. Thus, acetophenone was used as an internal standard and was introduced by addition to the stock solution of **5** at a concentration of 10 mM (final acetophenone concentration in the reaction solution was 0.5 mM). For each data point, 5 μL of stock solution was added to 95 μL of buffer solution at the desired temperature. HPLC analysis was carried out after the appropriate reaction time as described above. Initial peak heights were determined in triplicate for each run, and each data point was determined in duplicate. Peak heights were corrected for the reference peak height in all cases. Peak heights at time t were expressed as a fraction of initial peak height for all data calculations; rate constants were determined both by linear (short-term kinetics) and nonlinear (long-term kinetics) least-squares techniques.

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Registry No. 1, 50-18-0; **5**, 56922-83-9; **6**, 61903-31-9; **8a**, 88685-77-2; **8b**, 88685-76-1; HPO_4^{2-}, 14066-19-1; Tris, 77-86-1; hydroxide, 14280-30-9; propionaldehyde, 123-38-6.

In Situ Preparation and Fate of *cis*-4-Hydroxycyclophosphamide and Aldophosphamide: ^1H and ^{31}P NMR Evidence for Equilibration of *cis*- and *trans*-4-Hydroxycyclophosphamide with Aldophosphamide and Its Hydrate in Aqueous Solution[1]

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cis-4-Hydroxycyclophosphamide (**2**) and aldophosphamide (**4**) were generated in aqueous phosphate or cacodylate buffer by dimethyl sulfide reduction of *cis*-4-hydroperoxycyclophosphamide (**8**) and by sodium periodate cleavage of 3,4-dihydroxybutyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (**9**), respectively; the reactions of **2** and **4** were examined by ^1H and ^{31}P NMR. Within 30–60 min (pH or pD 7.0, 25 °C) the same pseudoequilibrium mixture was established in both reactions, with *cis*- and *trans*-4-hydroxycyclophosphamide (**2** and **3**), aldophosphamide (**4**), and its hydrate (**5**) present in the approximate ratio of 4:2:0.3:1. Structures of the intermediates were assigned unambiguously based upon analysis of the chemical shifts and coupling constants in the proton spectra determined in D_2O buffers, and the ^{31}P assignments followed by correlation of component ratios at equilibrium. Free energy differences of 0.4, 0.4, and 0.7 kcal/mol at 25 °C were estimated between **2**, **3**, **5**, and **4**, respectively, with **2** being the most stable. The aldehyde **4** reacted most rapidly with water to give hydrate **5**; cyclization of **4** to **3** occurred faster than to **2**, and the rate of cyclization to **2** was comparable to that for elimination to **6**. Compound **5** is formed much faster than **3** from the diol cleavage, but **5** and **3** are produced at comparable rates from **2**, suggesting that conversion of **2** to **3** can proceed by a mechanism other than ring opening. The rate of equilibration appears to be independent of buffer structure, indicating that bifunctional catalysis is not important in the ring-opening reaction. β-Elimination from **4** is rate limiting for the production of **6** and acrolein, and the rate for phosphate is 2- to 3-fold faster than for cacodylate under identical conditions. These results provide the first definitive evidence for the stability of the elusive aldehyde **4** in aqueous solution and for the existence of a preequilibrium among **2**–**5** prior to rate-limiting expulsion of phosphoramide mustard from **4**.

Cyclophosphamide (**1**) is a highly effective and extensively used agent for the treatment of many human malignancies. Its mechanism of action and the associated chemistry have been the subject of several recent reviews.[2-4] Activation of the prodrug **1** is initiated by hepatic P450 hydroxylation to produce one or both isomers[5] of 4-hydroxycyclophosphamide (**2** and/or **3**; see Scheme I). The 4-hydroxy compounds presumably undergo carbinol amide–amido aldehyde ring opening to generate aldophosphamide **4** which in turn undergoes base-catalyzed elimination to produce phosphoramide mustard (**6**) and acrolein. Intermediates **2**–**4** are susceptible to further oxidation, leading to 4-oxocyclophosphamide and carboxyphosphamide, respectively. These oxidations appear to represent the major pathway for drug detoxication in vivo. Although **4** is an obligatory intermediate in the activation sequence, efforts to isolate and characterize it have met with limited success.[6] The presence of hydrate **5** has been suggested,[6] but definitive evidence for its existence in equilibrium with **4** is lacking. Persuasive evidence has been presented for imine **7** as an intermediate in the aqueous reactions of 4-hydroperoxycyclophosphamide (**8**) and in the enzymatic oxidation of **1**;[7] recent data have confirmed

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