REPORTS

Proton Magnetic Resonance Studies of the Decomposition of 4-Hydroxy-cyclophosphamide, a Microsomal Metabolite of Cyclophosphamide[1]

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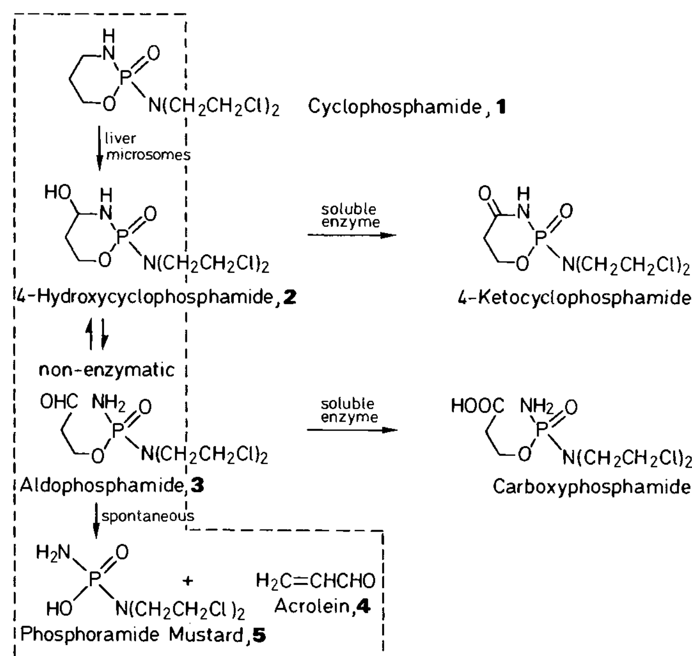
Abstract: The proposed tautomeric equilibrium between the microsomal metabolite of cyclophosphamide, 4-hydroxycyclophosphamide, and the open chain aldophosphamide, and the subsequent facile β-elimination to generate acrolein and phosphoramide mustard have been confirmed by proton magnetic resonance studies. When 4-hydroxycyclophosphamide, initially maintained in $CDCl_3$ at −20°C, was allowed to equilibrate at 15°C, a singlet at 9.76 δ and a triplet at 2.88 δ appeared concomitantly which were assigned to the aldehydic proton and the protons α to the carbonyl of aldophosphamide, respectively. Further reaction led to the appearance of several NMR signals that indicated the irreversible formation of acrolein (multiplet at 9.55 δ) and phosphoramide mustard. Polymerization occurred approximately 2 hours after the initiation of the reaction. The kinetic data of the reaction sequence are discussed.

The requirement of metabolic activation for the cytotoxic activity of cyclophosphamide (**1**) has long been recognized (1). Recent investigations from several laboratories have contributed to the understanding of the metabolic fate of cyclophosphamide as represented in Scheme 1 (2–8). Formation of 4-hydroxylcyclophosphamide (**2**) via the microsomal mixed function oxidases system is the first step necessary for all subsequent metabolic pathways. The identity of **2** has been elucidated by isolation, chemical derivatization, mass spectrometric and other spectroscopic analysis, and chemical synthesis. The hydroxylated metabolite **2** has been postulated to be in tautomeric equilibrium with the open chain isomer, aldophosphamide (**3**) (9), which then undergoes β-cleavage to form phosphoramide mustard (3–8). The last compound is believed to be the ultimate alkylating metabolite responsible for the cytotoxicity of cyclophosphamide (6, 8). The identity of **3**, however, has been difficult to establish (7–15) because of its instability (6, 8, 10) and thus remained elusive. Several attempts to synthesize aldophosphamide (**3**) failed to yield significant amounts of isolable material (10, 11, 12). Several investigators have succeeded in trapping **3** as adduct derivatives (1, 8, 13), and some have claimed to have isolated it within chromatographic systems (10, 14, 15). Direct physical evidence of the formation of **3** from **2** and the existence of a tautomeric equilibrium between them is still lacking. Our experiment was aimed at providing this link. Using fourier transformed NMR, we have established the tautomeric equilibrium of **2** and **3**, and additionally, demonstrated the subsequent β-elimination to generate acrolein (**4**) and phosphoramide mustard (**5**); the latter has been suggested as the key alkylating species rather than intact cyclophosphamide (6).

Materials and Methods

All deuterated and common solvents and chemicals are reagent grade and were used without further purification. 4-Hydroxycyclophosphamide (**2**) was synthesized by the method of Takamizawa et al. (10) using 1-buten-3-ol with N,N-bis-(2-chloroethyl)-phosphoramidic dichloride followed by ozonization to form 4-hydroperoxycyclophosphamide. Reduction of the hydroperoxy compound with triphenylphosphine afforded **2** in good yield (ca. 50%).



Scheme 1. Major metabolic pathways of cyclophosphamide.

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NMR was measured on a Varian XL-100 instrument using either CW or FT mode. For FT NMR, a typical spectrum was run with an acquisition time of 2.67 sec, sweep width of 1500 Hz, repetition time 2.67 secs, pulse angle 45°, pulse width 12 µsec 4 uses and 8 K data points. For area integration, both FT and CW modes were used. The concentration of **2** ranged from 0.3 to 1M in $CDCl_3$. Because of the instability of **2**, excess manipulation was avoided, and high purity of **2** was not obtained. Therefore, **2** contained small amounts of added triphenylphosphine and its oxide, the latter derived from the reaction, and CH_2Cl_2. Concentrations of **2** and other species were estimated by integration of appropriate proton signals of the species against those of known quantity of added solvents. For **2** the signal at 1.8 ppm (C_5-H_2) was integrated against added known quantity of cyclohexane (1.43 ppm). For aldophosphamide (**3**) the aldehydic proton (9.76 ppm) was integrated against bromoform (6.85 ppm) or benzene (7.37 ppm). The latter internal standard (benzene) was less satisfactory because of the occasional interference by triphenylphosphine. For the assay of acrolein (**4**) the aldehydic proton (9.5 ppm) was integrated also against bromoform.

For the NMR experiment, solid synthetic **2** initially kept at −78° C in vacuo prior to the experiment was quickly transferred into an NMR tube containing 1 ml $CDCl_3$ immersed in an ice bath. Appropriate amounts of internal standards, usually 1 µl each, and TMS were added with the use of a Hamilton syringe. The tube was inverted several times to effect mixing and dissolution and was again immersed in a bath of dry ice-acetone. Temperatures were measured by replacing the sample tube with an NMR tube containing a thermocouple immersed in the solvent. The accuracy was ±1°C. The NMR experiment was begun by warming the sample to 0°C, and the CW or FT spectrum was accumulated for 20 transients. The following signals (in ppm downfield from tetramethylsilane (TMS)) were obtained: 1.43 (s, cyclohexane), 1.8 (m, C_5-H_2), 3.0–3.87 (m, α and β protons of the mustard side chain), 4.2 (m, C_6-H_2), 4.9 (m,C_4-H) 4.9–5.19 (m, NH and OH), 5.3 (s, residual CH_2Cl_2), 6.85 (s, bromoform), 7.37 (s, benzene), and 7.4 to 8 (m, residual Ph_3P and Ph_3PO) (Fig. 1). These assignments are based on the published data of **2** in DMSO (10).

In order to test for temperature effects on the postulated equilibrium $\mathbf{2} \rightleftharpoons \mathbf{3}$, the sample tube containing approximately 0.4 M of **2** in $CDCl_3$ was inserted into the NMR probe which was initially maintained at −4°C. FT-NMR data were acquired for 1 minute intervals and the region between 7–10 ppm was plotted (duration 4 minutes). Then the temperature was quickly raised to +10° C and the data were acquired and displayed as before. The procedure was repeated at +15°C, 0°C, −4°C, +20°C, −4°C, +1°C, and +20°C with an average cycle time of approximately 10 minutes.

For kinetic studies, a typical data point was acquired for 10 transients of 2.66 sec duration followed by 4.1 min pulse delays constituting a 5 min cycle. FT spectra were organized and stored in the automatic mode. No intervention was attempted nor found necessary to start the decomposition of **2**. The process ensues spontaneously after 2 hours at 15°C during which time the colorless solution undergoes no visible change. Decomposition is accompanied by marked spectral changes, yellowing of the solution and, finally, formation of an orange-brown residue. The obtained NMR spectra were analysed by plotting and integration. The kinetic data were obtained by estimation of the ratio of the integrated signal area over the appropriate signals of the known added internal standards.

Results and Discussion

When approximately 1M of synthetic 4-hydroxycyclophosphamide (**2**) originally kept at −20°C in $CDCl_3$ was subjected to both CW and FT-NMR measurements at 0°C, we obtained the spectrum shown in Fig. 1 a. The resonances were assigned to **2** by analogy to the cyclic structure in cyclophosphamide (see Experimental). Characteristic signals were multiplets at 1.8 ppm (C_5-H_2) and at 4.9 ppm downfield (C_4-H) from TMS which are consistent with the reported resonances in **1** (10). When the probe temperature was raised to 15°C, a singlet appeared at 9.76 ppm over a short time with a concomitant appearance of a broad triplet at 2.8 ppm (Fig. 1 b). The intensity of these peaks after their abrupt rise remained relatively constant for about 2 hours, and no appreciable spectral change was seen during this period. Further reaction at 15°C in the NMR tube then led to the

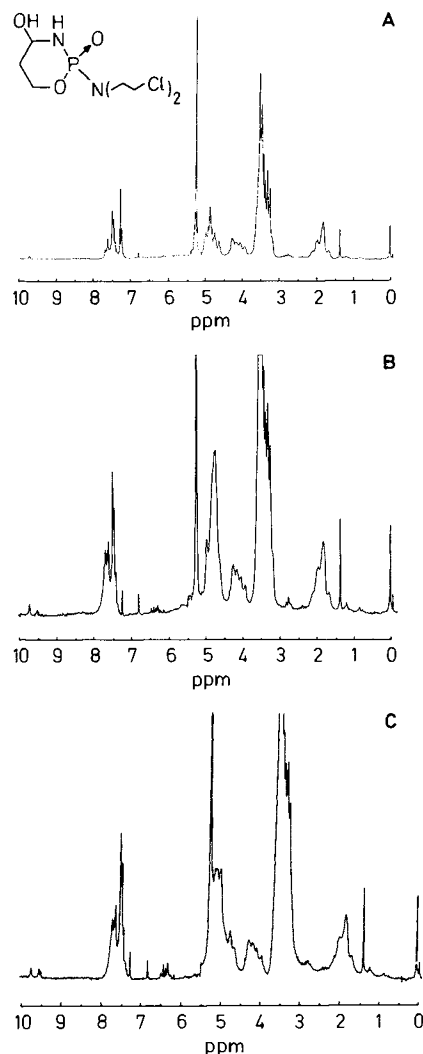

Fig. 1. (a) FT proton NMR spectrum of 4-hydroxycyclophosphamide (**2**) in $CDCl_3$ at 0°C. Small known amounts of cyclohexane, bromoform, and benzene were added to facilitate integration at different spectral regions. See experimental for signal assignments. (b) FT proton NMR spectra of **2** in $CDCl_3$ at 15°C recorded at 120 minutes. (c) Same as that in b recorded at 150 minutes.

appearance of a multiplet at 9.55 and 6.4 ppm (Fig. 1 c). Additionally, significant spectral changes in the entire spectrum, particularly in the region between 4–5 ppm, were seen and were accompanied by yellowing and an increase in viscosity (Fig. 1 c). In less than three hours from the beginning of the experiment, the sample became an orange-brown residue. Similar results were observed in $(CD_3)_2C{=}O$ at 15°C and $(CD_3)_2S{=}0$ at 5°C. The singlet at 9.76 ppm was assigned to the aldehydic proton and the triplet at 2.8 ppm to the protons α to the carbonyl in aldophos-

phamide (**3**). The changes of the signals with time in the spectral region between 9–10 ppm downfield form TMS are shown in Fig. 2.

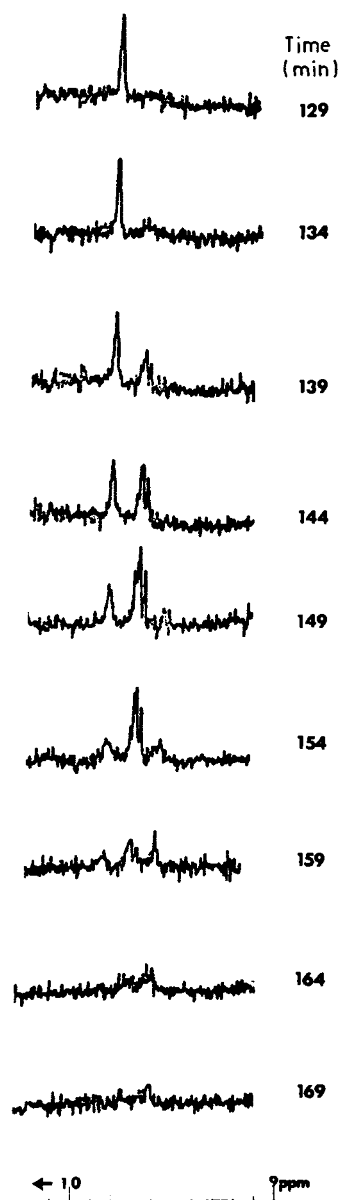

Fig. 2. FT proton NMR spectra of 4-hydroxycyclophosphamide in $CDCl_3$ between 9–10 ppm downfield from TMS at 15°C.

The NMR assignments were further supported by the spectral changes that occur at different temperatures. When sample of **2** in $CDCl_3$ or in $(CD_3)_2C{=}O$ was quickly brought to +10°C from −4°C and the FT-NMR monitored over the spectral region of 7–10 ppm, the signal at 9.76 ppm appeared as shown in Fig. 1b. The intensity of this signal increased sharply when the temperature was quickly increased to 15°C and to 20°C. When the temperature was rapidly reversed to −4°C, the intensity of the signal was found to gradually decrease. The cycle can be repeated. During this period, no new signal was detectable at 9.55 ppm or at any other regions, so that the more than two-fold decrease in **3** from 15° to −4°C cannot be explained by a reversible conversion to acrolein (**4**). If the sample is left at +20°C for approximately 40 minutes, new signals at 9.55 and at 6.4 ppm appeared as before. Using the aromatic protons of triphenylphosphine (7.43 ppm), which were present as a minor impurity of **2**, as the internal reference, the ratios of the peak intensities between the signal at 9.7 ppm and that of triphenylphosphine were estimated. The cyclic changes in these ratios corresponding to a cyclic change in temperature are consistent with the reversibility of the interconversion of **2** to **3**; moreover, the formation of **2** is favored at low temperature. Acrolein (**4**) and **5** may also be formed during this period, but the formation is probably slow at low temperature. The multiplets at 9.5 and 6.4 ppm were assigned to the acrolein aldehydic and olefinic protons, respectively, since the chemical shift and multiplicity of these signals are identical to those of acrolein measured at these solvents under similar condition.

In order to quantitate the spectral change and to measure rate constants, several separate experiments were performed with small known quantitites of benzene, bromoform, cyclohexane, and TMS as internal standards for calibration at different spectral regions. The results (Table I and Fig. 3) indicated the existence of an equilibrium between **2** and **3**, with a K_{eq} of approximately 0.10 ± .03 in favor of **2** at 15°C. This equilibrium phase persisted for approximately 2 hours followed by a rapid decline of both **2** and **3**. At approximately 2 hours acrolein (**4**) began to appear, and its concentration increased at the expense of **3**. The concentration of **4** peaked at approximately 145 minutes (Fig. 3) and declined rapidly because of polymerization until the characteristic signals of **2**, **3**, and **4** were no longer discernible. The declines of both **3** and **4** appeared to be of apparent first order (R^2 = 0.9249 and 0.9744, respectively) and essentially parallel to each other

Tab. 1 Decomposition of 4-hydroxycyclophosphamide as measured by proton NMR at 15°C.

Time (min.)	[2][a]	[3][a]	[4][a]	K^b_{eq}
5	0.876	0.065	–[c]	0.074
10	0.696	0.055	–	0.079
20	0.844	0.071	–	0.084
25	0.658	0.070	–	0.106
30	0.723	0.079	–	0.108
45	0.697	0.107	–	0.154
55	0.866	0.081	–	0.094
60	0.658	0.089	–	0.135
80	0.688	0.072	–	0.105
85	0.633	0.063	–	0.100
100	0.562	0.064	–	0.114
105	0.836	0.066	–	0.079
120	0.734	0.064	–	0.088
125	0.841	0.056	–	0.067
130	0.679	0.074	0.018	–
135	0.689	0.097	0.049	–
140	0.613	0.073	0.081	–
145	0.603	0.059	0.108	–
150	0.471	0.037	0.081	–
155	0.294	0.030	0.071	–
160	–	–	0.041	–
165	–	–	0.030	–
Average				0.101± 0.025

[a] in M
[b] $K_{eq} = \frac{[3]}{[2]}$
[c] either not measured or not detectable

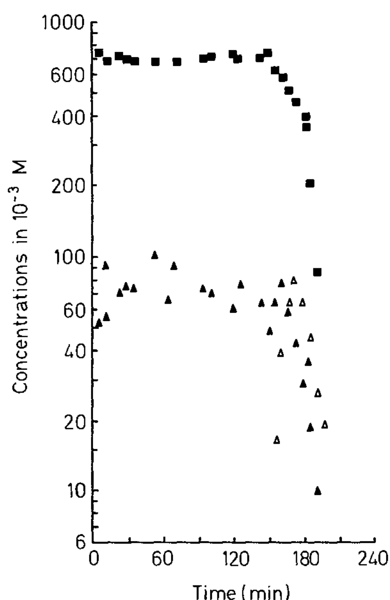

Fig. 3. Kinetic data and sequence of decomposition of 4-hydroxycyclophosphamide (**2**) in $CDCl_3$ at 15°C as estimated by proton NMR. ■, **2**; ▲, **3**;△, **4**. See experimental for the method of proton integration for each compound.

with the estimated apparent half-lives of 9.2 and 9.4 minutes, respectively. However, the decline of **2** showed two log-linear segments; one appeared immediately after the equilibrium portion of **2** and **3** and lasted for approximately 30 minutes, followed by a more rapid first order decline parallel to those of **3** and **4**.

In a duplicate experiment using 0.3M **2** in $CDCl_3$ at 15°C similar results were obtained. Following an equilibrium phase between **2** and **3** of approximately 75 minutes, **4** began to appear and peaked at approximately 85 minutes. The declines of **3** and **4** also appeared to be log linear (R^2 = 0.8654 and 0.9278 for **3** and **4**, respectively) with estimated apparent half-lives of 7.2 and 8.8 minutes, respectively. The slight difference in results between these two experiments may be due to the difference in experimental conditions, for example, the length of time for preparation and loading of NMR samples, and the lack of synchronization of the initial condition between these experiments.

Voelcker et al. (16) found a K_{eq} of 1.69 in favor of **3** in potassium phosphate buffer at pH 7 and at 37°C, using TLC separation techniques. Others (10) were not able to reproduce their results, however. The discrepancies between our results and previous reports could arise from differences in the medium and experimental conditions. All of the present experiments were carried out in organic solvents rather than aqueous solution and pH control. Since phosphoramide mustard (**5**) generated during the reaction is rather acidic, the quantitative description of the chemical kinetics of this decomposition would be difficult without a pH control. The sudden rapid disproportionation of **2** and **3** may be induced by a catalytic action of **5**. Further work with well controlled experimental conditions are necessary for a detailed investigation of the kinetics of decomposition.

The presented results unequivocally demonstrate the existence of a tautomeric equilibrium between 4-hydroxycyclophosphamide (**2**) and aldophosphamide (**3**) and give direct evidence of the sequence of the decomposition.

Acknowledgement
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