

Studies on the Hydrolysis of Cyclophosphamide II.  
Isolation and Characterization of Intermediate Hydrolytic Products (I)

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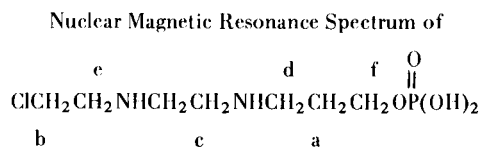
Two new products of hydrolysis of cyclophosphamide in water at 100°, *N*-(2-chloroethyl)-*N'*-(3-phosphatopropyl)ethylenediamine and *N*-(2-hydroxyethyl)-*N'*-(3-phosphatopropyl)ethylenediamine, have been isolated after 30 minutes, and 6 hours of reaction times, respectively. These products have been shown to be intermediates leading to the formation of *N*-(2-hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine, the principal ultimate product of cyclophosphamide hydrolysis. The nature of these new products supports the previously postulated mechanism involving an intramolecular alkylation as the initial step in the hydrolytic process although the pathway appears to be an unlikely model for the metabolic transformations of cyclophosphamide *in vivo*.

In a previous communication (3), we reported the isolation and identification of *N*-(2-hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine (V) and 1-(3-hydroxypropyl)piperazine (VI) as the ultimate products from hydrolysis of cyclophosphamide (I), *N,N*-bis(2-chloroethyl)-*N',O*-propylenephosphorodiamidate, in water. The present paper reports the isolation and characterization of two hydrolytic intermediates leading to the formation of these products.

The hydrolysis of cyclophosphamide (I) was followed by the liberation of ionic chlorine determined in samples drawn intermittently from the hydrolysis mixture (4). After 30 minutes of boiling in water about half of the total chlorine and after about 6 hours essentially all of the chlorine was liberated in ionic form. When the reaction was stopped at the end of 30 minutes, the extremely hygroscopic product characterized as *N*-(2-chloroethyl)-*N'*-(3-phosphatopropyl)ethylenediamine (III) as the hydrochloride was isolated in 51% yield. This product was transformed into a crystalline oxalate in about 60% yield.

The structure III has been assigned on the basis of elemental analysis, as well as physical and chemical evidence. The infrared absorption in potassium bromide pellet gave bands at 3435 cm<sup>-1</sup> (NH-stretch), 1210 cm<sup>-1</sup> (P=O, hydrogen bonded), and 1058 cm<sup>-1</sup> (P-O-C aliphatic vibration). The band appearing in the spectrum of cyclophosphamide (I) at 750 cm<sup>-1</sup>, due to a P-N stretching vibration, is totally lost in the spectrum of III. The lack of any absorption in the region 820-780 cm<sup>-1</sup> for III indicates the absence of either the P-N <math>\overset{\curvearrowright}{\text{C}}</math> structure or P-C bond. The proton magnetic resonance spectrum in

deuterium oxide reveals one singlet at  $\delta$  3.65 whose area integrated to four hydrogens, which are assigned for methylene hydrogens (e). The center of a multiplet at  $\delta$  = 2.2, equivalent to two hydrogens, is attributed to the methylene hydrogens (a). The rest of the methylene hydrogens appear in the region  $\delta$  = 3.8-4.3 as would be expected in these types of compounds (5-7).



	b	c	a	
	$\delta$ ppm		Multiplicity	Integration
a	2.2		m	2
b	3.4		t	2
c	3.65		s	4
d,e,f	3.8-4.3		m	6

On acetylation with acetic anhydride in pyridine the chlorodiaminophosphate (III) gave a water-soluble product which could not be isolated in a crystalline state. The infrared spectrum of this product in chloroform solution shows strong absorption at 1658 cm<sup>-1</sup>, indicating the presence of one or more *N*-acetyl groups.

On hydrolysis in water for 5 hours, III gave *N*-(2-hydroxyethyl)-*N'*-(3-phosphatopropyl)ethylenediamine (IV), isolated as its oxalate, in about 70% yield. This hydroxydiaminophosphate (IV) on further hydrolysis for

a period of 40 hours produced *N*-(2-hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine (V) (isolated as the oxalate) in 85% yield. The dioldiamine V was obtained directly in 65% yield when a solution of cyclophosphamide in water was kept under reflux for 48 hours. When a solution of I was kept at reflux for 6 hours IV and V, separated by paper chromatography and isolated as their oxalates, were obtained in about 60% and 10% yield, respectively.

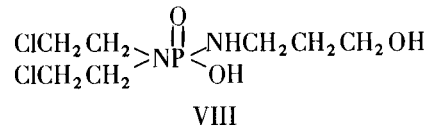
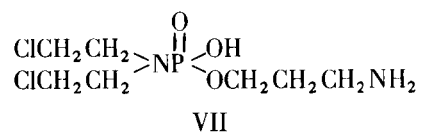
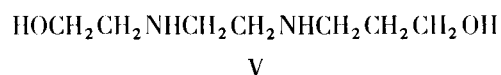
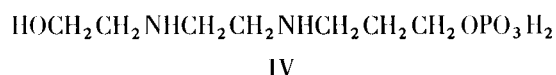
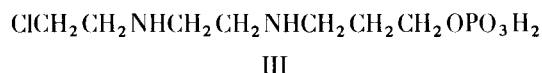
Attempts to isolate any phosphorylated products as cyclohexylammonium salts from the hydrolysis mixture of I in water after 48 hours gave only phosphoric acid as its bis-cyclohexylammonium salt.

The isolation and characterization of these hydrolytic intermediates support the initial intramolecular alkylation mechanism suggested earlier (3). However, hydrolysis of cyclophosphamide (I) in water under various mild conditions, 38°, 50° and 60° or in the presence of 1 mole of sodium bicarbonate at 100° in an attempt to isolate the postulated bicyclic intermediate II were unsuccessful. Paper or thin layer chromatographic examination of the hydrolysis mixtures failed to indicate the presence of a product of this type.

#### Discussion.

Our earlier study (3) on the spontaneous hydrolysis of cyclophosphamide revealed that it gives rise to some rather unusual products unpredictable on the basis of a simple hydrolytic mechanism. When cyclophosphamide was hydrolyzed in distilled water, two ultimate products of decomposition were isolated: one a major product accounting for approximately 60 per cent of the original cyclophosphamide, and the other a minor product in trace amounts. These products have been identified as *N*-(2-hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine (V) and *N*-(3-hydroxypropyl)piperazine (VI), respectively.

We have now found that when the reaction is stopped at the end of 30 minutes and 6 hours, respectively, we are able to isolate in substantial yield two intermediate products III and IV. On further hydrolysis the former III was converted into the latter IV and IV was transformed into the ultimate product, the dioldiamine V, both in substantial yields. These products have been identified as the chlorodiaminophosphate III, and the dihydroxy-diaminophosphate IV, respectively.

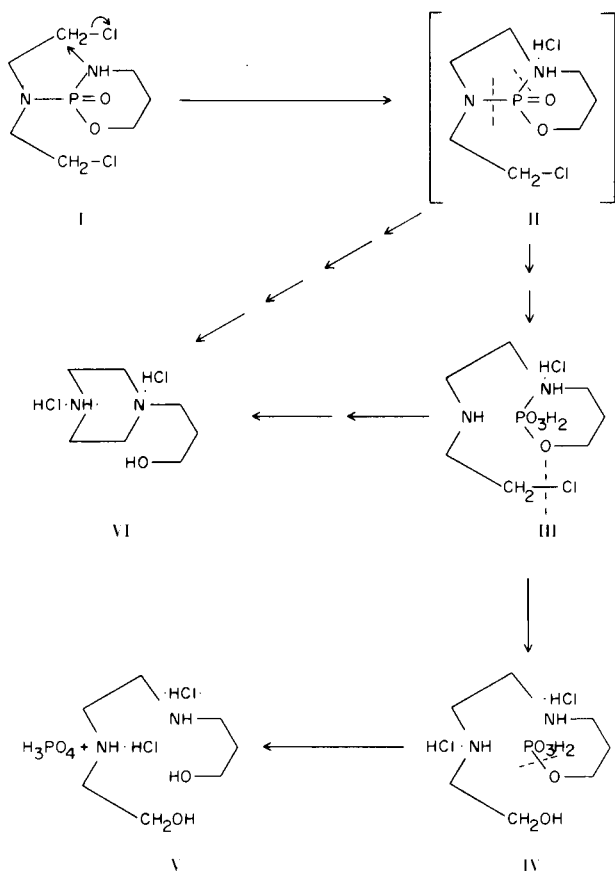


From the nature of these hydrolytic products, it seems unlikely that the spontaneous hydrolysis of cyclophosphamide simply involves a sequential hydrolysis of the *P-N* and *P-O* bonds with release of free bis-( $\beta$ -chloroethyl)-amine (nor HN2) or propanolamine. This view is supported by the fact that analysis of the reaction mixture at any stage by assay (for alkylating activity) with *p*-(4-nitrobenzoyl)pyridine (NBP) (8) or gas chromatography gave no evidence for the presence of free nor HN2, diethanolamine or propanolamine, and by the fact that an equimolar mixture of nor HN2 and propanolamine did not give a detectable amount of the dioldiamine V when heated to reflux under conditions used for the hydrolysis of (I). Moreover, as no alkylating activity could be detected by NBP assay, it seems unlikely that the course of hydrolysis involves an initial cleavage of either the *P-N* bond or the *P-O* bond in the ring. Such cleavage would give rise to the known products, cytoxyl amine (VII) and cytoxyl alcohol (VIII), respectively, both of which are active alkylating agents.

The most likely explanation of these results is that an intramolecular *N*-alkylation of the type I  $\rightarrow$  II occurs prior to *P-N* or *P-O* bond cleavage as the initial event in the course of spontaneous hydrolysis. The resultant bicyclic product II then undergoes a sequence of hydrolytic cleavages of *P-N* and *P-O* bonds to give rise to the various products that are produced. In the case of production of the piperazine derivative VI, a second alkylation of the ring nitrogen apparently intervenes at some early stage in the hydrolytic process. The overall reaction scheme that we postulate is illustrated diagrammatically below.

Cyclophosphamide is a potent antitumor agent and is one of the most effective compounds now in use in clinical cancer chemotherapy. The compound is biologically inactive *per se* and becomes biologically active only upon metabolic transformation *in vivo* by a mechanism as yet not fully understood. The pathway of spontaneous hydrolysis that has been described here is an unlikely model for what may occur *in vivo*. It does not, for example, account for the appearance of the alkylating activity in the tissue of animals injected with cyclophosphamide (9) or for the alkylating products isolated from the urine of animals and man after administration of cyclophosphamide (10). Also in preliminary testing at the

Children's Cancer Research Foundation, Boston, the various products thus far isolated [the dioldiamine (V), the piperazine (VI), the hydroxyphosphate ester (IV) and the chlorophosphate ester (VII)] have shown little evidence of cytotoxicity in cell culture systems. These latter results, however, will have to be confirmed by testing for antitumor activity in animals when these products are available in sufficient quantity.



#### EXPERIMENTAL

\* Melting points were determined using a Koffler block apparatus and were not corrected. Infrared spectra were taken with a Perkin-Elmer 137 spectrophotometer. Ultraviolet absorption spectra were measured on a Perkin-Elmer 202 spectrophotometer. Nmr spectra were recorded with a Varian Associates Model A-60 spectrometer with TMS as external standard (in a capillary tube). Vapor phase chromatographic analyses were done on an Aerograph Autoprep A-700 (Wilkins) with an E. H. Sargent recorder and a disc integrator attachment.

#### *N*-(2-Hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine (V).

Cyclophosphamide (I) (0.5 g.) was dissolved in 25 ml. of water and the solution kept under mild reflux for 48 hours. On evaporation under reduced pressure, the solution gave a colorless gum which was taken up in methanol and a few drops of acetone added to it. The mixture, on standing in the refrigerator, afforded

crystalline hygroscopic material (0.25 g.), m.p. 97-101°, which on recrystallization (ethanol-acetone) gave an analytically pure product, 0.17 g., yield 41%, m.p. 106-108°; ir:  $\gamma$  max (potassium bromide) 3447  $\text{cm}^{-1}$ , 2981-2796  $\text{cm}^{-1}$  (broad), 1625  $\text{cm}^{-1}$ , 1445  $\text{cm}^{-1}$ , 1064-1025  $\text{cm}^{-1}$  (broad).

*Anal.* Calcd. for  $\text{C}_7\text{H}_{18}\text{N}_2\text{O}_2 \cdot 2\text{HCl}$ : C, 35.7; H, 8.6; N, 11.9; Cl, 30.1. Found: C, 35.6; H, 8.6; N, 11.8; Cl, 30.0 (ionic).

The mother liquor from the above, when treated with an excess of a 10% solution of cyclohexylamine in ethanol, produced a precipitate, which on crystallization from methanol afforded a shiny flaky product, 0.1 g., m.p. 204-206°.

*Anal.* Calcd. for  $(\text{C}_6\text{H}_{13}\text{N})_2 \cdot \text{H}_3\text{PO}_4$ : C, 48.7; H, 9.9; N, 9.4; P, 10.4. Found: C, 49.0; H, 10.0; N, 9.6; P, 10.6.

Cyclophosphamide (0.5 g.), when hydrolyzed under the same conditions as described above, and the product treated with an excess of a saturated solution of oxalic acid in water, afforded the dioxalate directly, which was crystallized in water-methanol, 0.4 g., yield 64%, m.p. 197-199° dec., ir:  $\gamma$  max (potassium bromide) 3503  $\text{cm}^{-1}$ , 3130  $\text{cm}^{-1}$ , 2895  $\text{cm}^{-1}$ , 1728  $\text{cm}^{-1}$  (broad), 1518-1483  $\text{cm}^{-1}$  (broad).

*Anal.* Calcd. for  $\text{C}_7\text{H}_{18}\text{N}_2\text{O}_2 \cdot [\text{C}_2\text{H}_2\text{O}_4]_2 \cdot \text{H}_2\text{O}$ : C, 36.7; H, 6.6; N, 7.7. Found: C, 37.0; H, 6.2; N, 7.7.

The mixture m.p. and ir spectra were found identical with an authentic synthetic sample. An oxalate derivative prepared from a synthetic sample of (V) hydrochloride melted at 199-200° dec. *N*-(2-Chloroethyl)-*N'*-(3-phosphatopropyl)ethylenediamine (III).

A solution of 0.5 g. of cyclophosphamide in 25 ml. of water was refluxed for 30 minutes. The solution was chilled in ice and evaporated to dryness under reduced pressure at room temperature. To the gummy residue was added a few drops of absolute ethanol and acetone and the mixture was again evaporated. The solution of this residue in absolute methanol, containing a few drops of ethanol, when kept in the refrigerator gave 0.32 g. of a hygroscopic solid, which was recrystallized from a mixture of absolute methanol-ethanol and dried under vacuum over phosphorus pentoxide, 0.27 g., yield 51%, extremely hygroscopic, m.p. 101-105°; ir  $\gamma$  max (potassium bromide) 3435  $\text{cm}^{-1}$ , 2972-2702  $\text{cm}^{-1}$  (broad), 1613  $\text{cm}^{-1}$ , 1471-1429  $\text{cm}^{-1}$  (broad), 1211  $\text{cm}^{-1}$  (broad), 1053  $\text{cm}^{-1}$  (broad).

*Anal.* Calcd. for  $\text{C}_7\text{H}_{18}\text{ClN}_2\text{PO}_4 \cdot \text{HCl}$ : N, 9.4; P, 10.2; Cl, 23.9. Found: N, 9.7; P, 10.7; Cl, 25.2.

Cyclophosphamide (0.5 g.) in 25 ml. of water was heated under reflux for 30 minutes, and the solution chilled and concentrated in a rotary evaporator at room temperature. An excess saturated solution of oxalic acid in water was added. The resulting precipitate was filtered and the residue dissolved in a mixture of water and methanol. On concentration of this solution and cooling a crystalline solid appeared which was filtered and dried under vacuum, 0.45 g., yield 64%, m.p. 109-191° dec.; ir:  $\gamma$  max (potassium bromide) 3385  $\text{cm}^{-1}$  (sh), 3042  $\text{cm}^{-1}$  (broad), 2856  $\text{cm}^{-1}$  (broad), 1725  $\text{cm}^{-1}$ , 1515  $\text{cm}^{-1}$ , 1475  $\text{cm}^{-1}$ , 1205  $\text{cm}^{-1}$  (broad), 1060  $\text{cm}^{-1}$  (broad).

*Anal.* Calcd. for  $(\text{C}_7\text{H}_{18}\text{ClN}_2\text{PO}_4)_2 \cdot 3[\text{C}_2\text{H}_2\text{O}_4]$  or  $\text{C}_{20}\text{H}_{42}\text{Cl}_2\text{N}_4\text{P}_2\text{O}_{20}$ : C, 30.4; H, 5.4; Cl, 8.9; N, 7.1; P, 7.9; oxalic acid, 34.1. Found: C, 30.46; H, 5.24; Cl, 8.86 (organic); N, 7.07; P, 7.64; C, 30.45; H, 5.65; Cl, 8.84 (organic); N, 7.11; P, 7.63; oxalic acid (as calcium oxalate), 33.7.

#### *N*-(2-Hydroxyethyl)-*N'*-(3-phosphatopropyl)ethylenediamine (IV).

A solution of 100 mg. of cyclophosphamide in 5 ml. of water was heated under mild reflux for about 6 hours. The solution was immediately evaporated to dryness and the residue, a colorless

gum, was dissolved in a minimum amount of methanol and was applied as a streak on a sheet of Whatman chromatographic paper, 3MM, and run (descending), solvent propanol:water = 8:1 for 20 hours at room temperature. The end strip, cut out and developed with ninhydrin, revealed two spots; (A) a main very slowly moving spot, purple, just above the start line, Rf 0.01; and (B) a faster moving spot, brownish purple, Rf 0.11. The corresponding areas from the sheet were cut and eluted separated with water. The residue, in each case after concentrated at room temperature, was converted directly into oxalate derivatives, crystallized from aqueous methanol. (A) 90 mg., yield 60%, m.p. 178-180° dec.; ir:  $\gamma$  max (potassium bromide) 3410  $\text{cm}^{-1}$  (sh), 3030  $\text{cm}^{-1}$ , 2840  $\text{cm}^{-1}$ , 1716  $\text{cm}^{-1}$ , 1667-1673  $\text{cm}^{-1}$  (sh), 1512  $\text{cm}^{-1}$ , 1475  $\text{cm}^{-1}$ , 1184  $\text{cm}^{-1}$ , (broad), 1046  $\text{cm}^{-1}$ .

*Anal.* Calcd. for  $\text{C}_7\text{H}_{19}\text{N}_2\text{PO}_5 \cdot (\text{C}_2\text{H}_2\text{O}_4)_2$ : C, 31.3; H, 5.5; N, 6.6; P, 7.3. Found: C, 31.5; H, 5.7; N, 6.7; P, 7.5.

(B) 13 mg., yield 10%, m.p. 197-199° dec.; mixture m.p. and ir spectra identical with V oxalate.

To a solution of 100 mg. of III oxalate in 10 ml. of water was added a solution of 40 mg. of calcium oxalate in 2 ml. of water and the precipitated calcium oxalate was filtered. The filtrate was concentrated in a rotary evaporator at room temperature to a volume of 5 ml., which was then heated under reflux for about 5 hours. The resulting solution was chilled in an ice bath and used for paper chromatographic separation as described above. The fraction corresponding to the control sample of (IV) was converted to the oxalate: 65 mg., yield 70%, m.p. 177-179° dec.; mixture m.p. and ir spectra identical with IV oxalate.

*N*-(2-Hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylenediamine from *N*-(2-hydroxyethyl)-*N'*-(3-phosphatopropyl)ethylenediamine.

A solution of 50 mg. of IV in 2.5 ml. of water was kept under reflux temperature for 40 hours, the resulting solution on concentration under reduced pressure gave a precipitate which was

separated on a filter. The residue was dissolved in aqueous methanol which on concentration afforded crystalline material, 20 mg., m.p. 195-198° dec. Mixture m.p. and ir spectra were identical with V oxalate. A second crop of 15 mg. was obtained from the mother liquor, on further addition of oxalic acid solution; total yield 80%.

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