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# Alkylating Properties of Phosphoramide Mustard[1]

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## SUMMARY

The relative alkylating activities of two of the cytotoxic metabolites of cyclophosphamide, phosphoramide mustard and nornitrogen mustard, have been studied at pH 4.6 and 7.4. The products formed on alkylation of ethanethiol by these metabolites have been identified, confirming that phosphoramide mustard undergoes alkylation reactions as an intact molecule. Deuterated analogs of the two metabolites have been synthesized, namely *N,N*-bis(2,2-dideutero-2-chloroethyl)-phosphorodiamidic acid and *N,N*-bis(2,2-dideutero-2-chloroethyl)amine, and used to determine that alkylation proceeds directly via an aziridinium intermediate rather than a direct $S_N2$ displacement of the chlorine atom.

## INTRODUCTION

In recent years the metabolic degradation pathway of the antitumor agent cyclophosphamide has been elucidated (4, 6, 17, 19) and is shown in Chart 1. Of the compounds shown, carboxyphosphamide (Chart 1, *VII*) and 4-ketocyclophosphamide (Chart 1, *VI*) do not possess significant *in vivo* or *in vitro* cytotoxic activity (17) and must be regarded as inactivated metabolites of cyclophosphamide. The compounds, 4-hydroxycyclophosphamide (Chart 1, *IIa*), phosphoramide mustard (Chart 1, *III*), and nornitrogen mustard (Chart 1, *V*), are all cytotoxic both *in vivo* and *in vitro* and thus, presumably, one of these compounds, or some combination thereof, is responsible for the cytotoxic properties of the parent compound. Since the spontaneous decomposition of 4-hydroxycyclophosphamide leads to the formation of phosphoramide mustard, which decomposes to form nornitrogen mustard, it will be difficult to establish the relative role of each of these metabolites in the biological activities of cyclophosphamide. An understanding of the chemical properties of these compounds should help in the elucidation of their relative roles.

In the work reported here we have studied the alkylating properties of phosphoramide mustard and nornitrogen mustard and offer evidence that the pharmacologically significant alkylating agent produced by the metabolism of cyclophosphamide is phosphoramide mustard.

[1] This research was supported by USPHS, NIH Grants CA-16783 and GM-21248, and by Grants from the Andrew Mellon Fund and the American Cancer Society, Maryland Division. The synthesis of *N,N*-bis(2,2-dideutero-2-chloroethyl)phosphorodiamidic acid was reported at the Symposium on Metabolism and Mechanism of Action of Cyclophosphamide, London, U. K., July 10-12, 1975.

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## MATERIALS AND METHODS

**NBP[4] Assay.** A modification of the NBP assay described by Friedman and Boger (11) was used. One-tenth ml of aqueous sample containing 100 nmoles of the alkylating agent, 0.1 ml of 0.1 M buffer (either sodium acetate, pH 4.6, or potassium phosphate, pH 7.4), and 0.5 ml of 5% NBP in acetone were incubated at 37° for 2 hr. At the end of the incubation, 1.0 ml of 25% 3-amino-1-propanol in tertiary butyl alcohol was added to develop a blue color that was quantitated at 540 mμ in a Gilford spectrophotometer. In the published procedure (11), the blue color is extracted into ethyl acetate for determination of the absorbance. Using this procedure we observed that much of the color generated by alkylation of NBP with phosphoramide mustard remained in the aqueous layer. The modification described above provided a 1-phase system with color stability at least as good as the extraction technique.

**Incubation with Ethanethiol.** The phosphoramide mustard incubation was done by mixing 50 mg of the cyclohexylamine salt of phosphoramide mustard or $d_4$-phosphoramide mustard and 6 ml saturated ethanethiol aqueous solution at 37° for 1.5 hr. During the incubation, the pH of the solution was monitored and adjusted by adding $NH_4OH$ to keep the pH between 7 and 7.5. The nornitrogen mustard and $d_4$-nornitrogen mustard incubations were done in the same way, using 30 mg of the hydrochloride salts.

**Identification of Alkylation Products.** The reaction mixture of phosphoramide mustard was lyophilized after incubation and then treated with diazomethane. The reaction mixture of nornitrogen mustard was extracted with chloroform at pH 8. All reaction products were then analyzed and identified on a Dupont 491 mass spectrometer coupled through a jet separator to a Varian 2700 gas chromatograph. A 6-ft glass column packed with 3% Dexsil 300 on Supelcoport was used. The oven temperature was programmed from 130° at 6°/min. The injection port was maintained at 250° and the ionization source at 200°. Chemical ionization spectra were obtained using isobutane reagent gas.

### Synthesis of Deuterium-substituted Derivatives

**Bis(2,2-dideutero-2-hydroxyethyl)amine.** To a stirred, ice-cooled mixture of lithium aluminum deuteride (10 g, 0.24 mole) and tetrahydrofuran (180 ml, freshly distilled from $LiAlH_4$) we added a solution of diethyl iminodiacetate (30 g, 0.16 mole) over a period of 1 hr. The mixture was then

[4] The abbreviation used is: NBP, nitrobenzylpyridine.

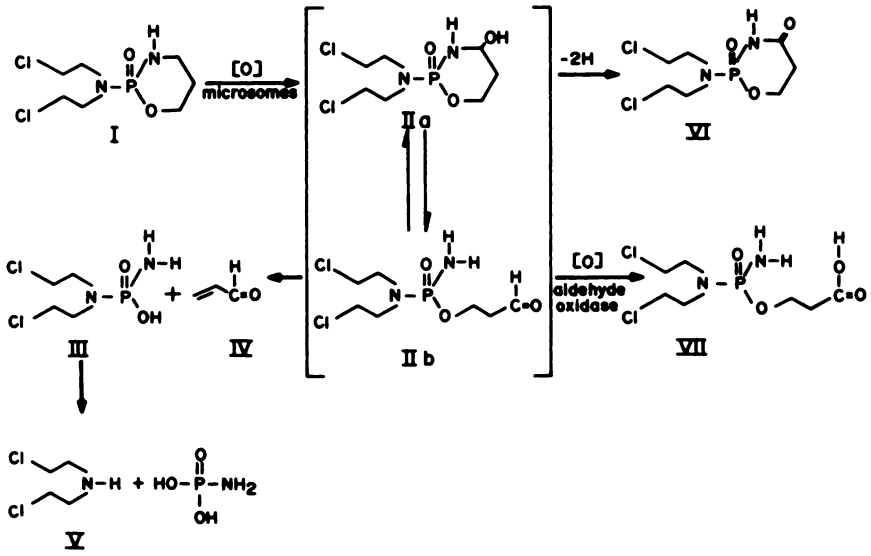


Chart 1. The metabolism of cyclophosphamide.

heated overnight under reflux. It was cooled to 0° and water (20 ml) was added dropwise. Solid material was collected by filtration and extracted in a Soxhlet extractor with tetrahydrofuran overnight. The tetrahydrofuran solutions were combined, the solvent was removed under vacuum, and the residue was distilled under vacuum (150°, ~10 mm) to give a colorless liquid (12 g, 0.11 mole, 70%): nuclear magnetic resonance ($d_6$-dimethyl sulfoxide) δ 3.7 (3 H, s), 2.6 (4 H, s).

**Bis(2,2-dideutero-2-chloroethyl)amine hydrochloride.** This was prepared from the above alcohol and thionyl chloride following the procedure of Mann (16): nuclear magnetic resonance ($d_6$-dimethyl sulfoxide) δ 9.8 (2 H, s), 3.4 (4 H, s).

**N,N-bis(2,2-dideutero-2-chloroethyl)phosphoramidic dichloride.** This was prepared from the above amine and phosphorus oxychloride following the procedure of Friedman and Seligman (13): nuclear magnetic resonance ($CDCl_3$) δ 3.7 (d, $J_{HP}$ = 17 Hz).

**Cyclohexylammonium hydrogen-N,N-bis(2,2-dideutero-2-chloroethyl)phosphorodiamidate (Phosphoramide Mustard).** This was prepared from the above phosphoramidic dichloride by reaction with sodium benzylate, ammonia, and hydrogenolysis, following the procedures of Friedman *et al.* (12): m.p. 112–114°.

**Structure Confirmation.** The isotope purity of each of the deuterated compounds was confirmed by both electron impact and chemical ionization mass spectrometry to be greater than 99% $d_4$ species. The 2,2-position of the deuterium atoms was confirmed by examining the fragment ions recorded in the mass spectra. Loss of $CH_2Cl$ (49 mass units) from the unlabeled compound (6) is replaced by loss of $CD_2Cl$ (51 mass units) from the labeled analog.

## RESULTS

The alkylation of NBP by nornitrogen mustard and phosphoramide mustard after incubation for 2 hr at 37° was measured at the usual pH of the NBP test, 4.6, and at physiological pH, 7.4. For comparison, the alkylating activity of nitrogen mustard [methylbis(2-chloroethyl)amine] was also measured under these conditions. As can be seen from the results shown in Table 1, all 3 agents alkylate NBP at the lower pH, but at pH 7.4 only phosphoramide mustard and nitrogen mustard retain alkylating activity. Under all conditions, nitrogen mustard appears to be a more reactive molecule than the other 2. The disparity in alkylating activity between nornitrogen mustard and phosphoramide mustard is further illustrated in Chart 2, in which increasing alkylation of NBP with increasing amounts of phosphoramide mustard is shown, whereas increasing the concentration of nornitrogen mustard incubated at physiological pH with NBP did not result in alkylation of the nitrobenzylpyridine.

To ascertain whether phosphoramide mustard reacts as

Table 1

*Reaction of alkylating agents with NBP*

The experimental conditions are described in the text.

| | Net absorbance at 540 mμ | |
|---|---|---|
| | pH 4.6 | pH 7.4 |
| Phosphoramide mustard | 0.479 | 0.291 |
| Nornitrogen mustard | 0.562 | 0.000 |
| Nitrogen mustard | 2.400 | 2.030 |

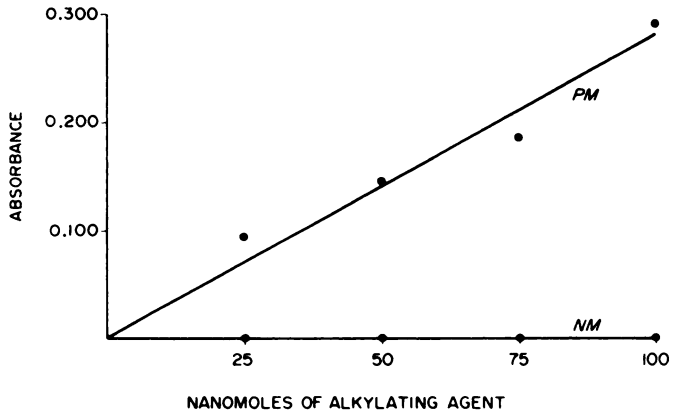


Chart 2. Extent of alkylation of NBP at physiological pH at various concentrations of nornitrogen mustard (*NM*) and of phosphoramide mustard (*PM*).

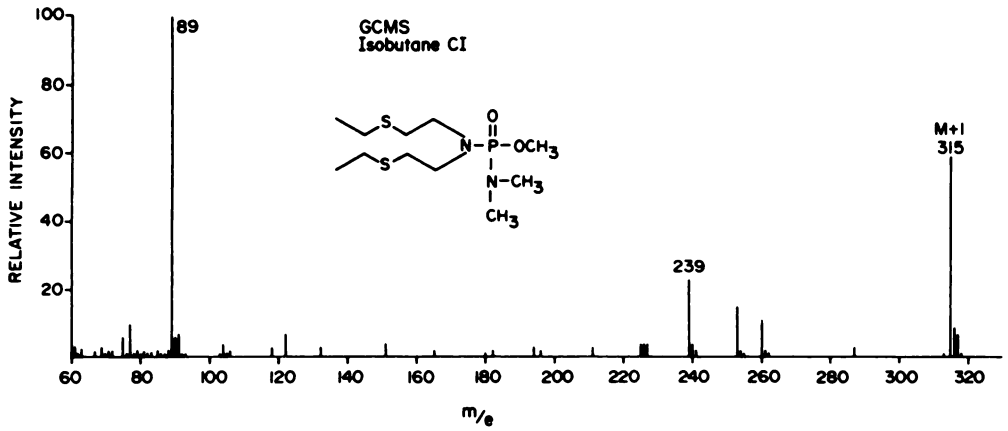


Chart 3. Chemical ionization mass spectrum of *N,N*-bis[2-(ethylthio)-ethyl]phosphorodiamidic acid formed in the reaction of phosphoramide mustard with ethanethiol (trimethyl derivative).

an intact molecule, phosphoramide mustard was allowed to react with ethanethiol at 37° at pH 7.4. After 2 hr of incubation, the reaction mixture was lyophilized and treated with diazomethane. The chloroform-soluble components were then analyzed by gas chromatography-mass spectrometry. Two sulfur-containing products were found. The chemical ionization mass spectrum and structure of the trimethyl derivative of 1 product, *N,N*-bis[2-(ethylthio)ethyl]phosphorodiamidic acid, are shown in Chart 3. The chemical ionization spectrum is shown because it characterizes the molecular weight more emphatically. Fragmentation seen in this spectrum, and more definitively in the electron impact spectrum (6, 9), confirms the disubstituted structure shown. Mono- and dimethyl derivatives of this product were also formed by the diazomethane reaction (6) and identified by gas chromatography mass spectrometry. Thus, intact phosphoramide mustard is seen to be an alkylating agent. Cleavage to nornitrogen mustard is not a prerequisite for its reaction with the model nucleophile ethanethiol.

The other alkylation product of the incubation of phosphoramide mustard and ethanethiol is 2,2'-bis(ethylthio)-diethylamine, whose structure and chemical ionization mass spectrum are shown in Chart 4. This compound could arise either by scission of the N—P bond in phosphoramide mustard and subsequent alkylation of ethanethiol by nornitrogen mustard, or by alkylation of ethanethiol by phosphoramide mustard and subsequent scission of the N—P bond. The superior alkylating activity of phosphoramide mustard at pH 7.4 and data presented below suggest that most of the 2,2'-bis(ethylthio)diethylamine was generated by the latter pathway.

The incubation of nornitrogen mustard with ethanethiol led to the formation of a small amount of monosubstituted product, 2-chloro-2'-(ethylthio)diethylamine. Unreacted nornitrogen mustard and 3-(2-chloroethyl)oxazolidone formed by reaction with atmospheric carbon dioxide were also identified in the product mixture. No disubstituted product and no hydrolysis products were detected in incubations maintained at pH 7.4, although hydrolysis of the chloride groups (alkylation of water) occurs at acidic pH.

In an effort to understand the mechanism of alkylation of phosphoramide mustard and nornitrogen mustard and their different behavior at basic pH values, an experiment was

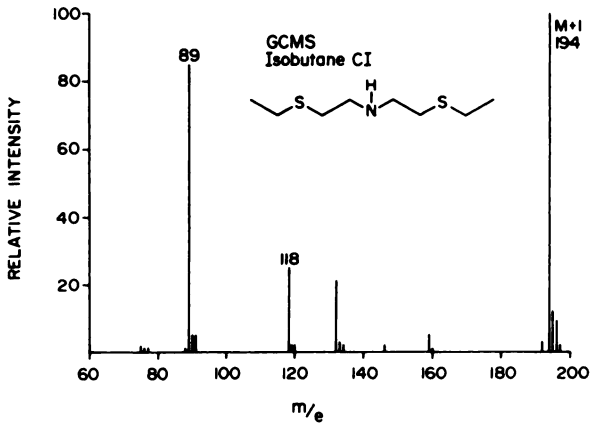


Chart 4. Chemical ionization mass spectrum of 2,2'-bis(ethylthio)diethylamine formed in the reaction of phosphoramide mustard with ethanethiol.

designed to distinguish between alkylation by direct ($S_N2$) displacement of the chloride ion and alkylation by a previously formed aziridinium ion. Such structures are illustrated in Chart 5, and it can be seen that the 2 carbon atoms in an *N*-2-chloroethyl chain become equivalent when chloride is displaced by nitrogen to form the aziridinium ion. Phosphoramide mustard was synthesized carrying 2 deuterium atoms in place of hydrogen on the $\beta$ carbon in each chloroethyl chain and incubated with ethanethiol as before. These isotope labels, in conjunction with mass spectral analysis, permit the positions in the alkylation products to be distinguished of the carbon atoms, which carried chlorine in the starting material. *N,N*-bis[2-(ethylthio)ethyl-$d_2$]phosphorodiamidic acid was identified, as before, by gas chromatography mass spectrometry of its trimethyl derivative, M.W. 318. The electron impact mass spectrum of this derivative is shown in Chart 6. The base peak at *m/e* 91 confirms the presence of 2 deuterium atoms in each ethylthioethyl chain. Cleavage occurs between the $\alpha$ and $\beta$ carbons of 1 chain in the formation of ions of mass 241 and 243. These ions are formed with equal abundance by loss of $CH_3CH_2SCD_2$ and of $CH_3CH_2SCH_2$ (Chart 6). The formation of alkylation products with deuterium labels in either of the 2 ethylene positions indicates that ethanethiol has reacted by opening the cyclic aziridinium ion and not by direct dis-

placement of chloride ion. The fact that equal amounts of these deuterated products are formed indicates there is no secondary deuterium isotope effect, an effect that would favor opening the aziridinium ring at the $CH_2$ position.

Mass spectral analysis of the 2nd alkylation product isolated from this reaction confirmed that 2,2′-bis(ethylthio)diethylamine contained 4 deuterium atoms and that here too the deuterium-bearing carbon occurred in equal amounts in the $\alpha$- and $\beta$-ethylene positions.

Bis(2,2-dideutero-2-chloroethyl)amine ($d_4$-nornitrogen mustard) was reacted with ethanethiol. The electron impact mass spectrum of the $d_4$-mono adduct is shown in Chart 7. Here again the key fragmentation process cleaves the $\alpha$- and $\beta$-ethylene carbons, allowing them to be distinguished. And here again equal amounts of adduct are detected that carry the deuterium atoms on the $\alpha$ carbon and on the $\beta$ carbon. [Peaks occur at *m/e* 94, 96, and 98, reflecting the presence of 2 isotopes of chlorine in each of the variously deuterated ions (6, 14).] Thus, alkylation of ethanethiol by nornitrogen mustard also proceeds through an aziridinium intermediate.

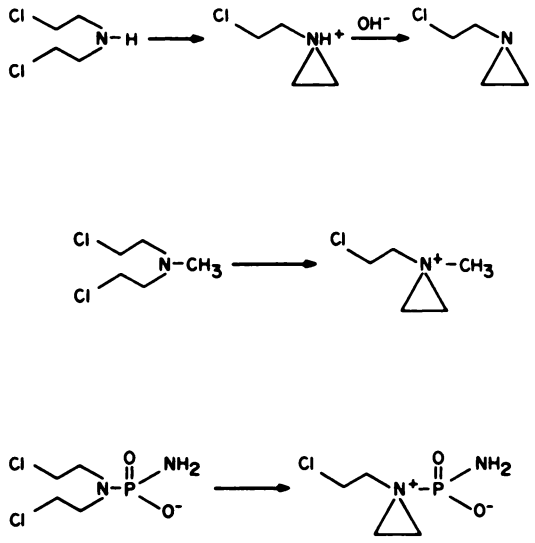

Chart 5. Aziridine intermediates formed from nornitrogen mustard, nitrogen mustard, and phosphoramide mustard.

## DISCUSSION

Recently, Struck *et al.* (18) reported that both phosphoramide mustard and nornitrogen mustard reacted with NBP at 37° and pH 4.6, but 4-hydroxycyclophosphamide did not. While phosphoramide mustard retained significant alkylating activity at pH 7.4, nornitrogen mustard did not.

In our studies we have confirmed the findings of Struck *et al.*, that at physiological pH, phosphoramide mustard is a more reactive alkylating agent than nornitrogen mustard. Furthermore, our model alkylation experiments with ethanethiol demonstrate that phosphoramide mustard can alkylate as an intact molecule, without prior or concomitant scission of the N—P bond.

The experiments with deuterium-labeled phosphoramide mustard and nornitrogen mustard indicate that these molecules alkylate through an aziridinium intermediate. The absence of a deuterium isotope effect suggests that the ring opening is a concerted reaction in which the carbon atoms never bear significant positive charge. The intermediacy of the aziridinium ion in the alkylation reactions suggests an explanation for the superior alkylating activity of phosphoramide mustard at pH 7.4 (Chart 5). Friedman and Boger (11) have pointed out that the formation of the aziridinium ion from nornitrogen mustard generates a compound that can easily be converted to chloroethylaziridine. The neutral

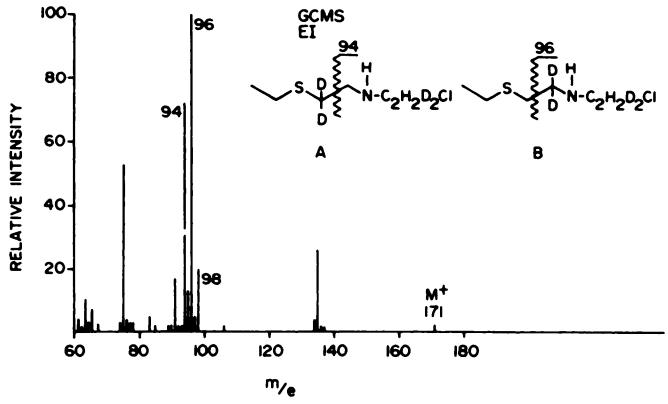


Chart 7. Electron impact mass spectrum of *N*-(2-chloroethyl)-*N*-(ethylthioethylene)amine in the reaction of $\beta$-$d_4$-nornitrogen mustard with ethanethiol.

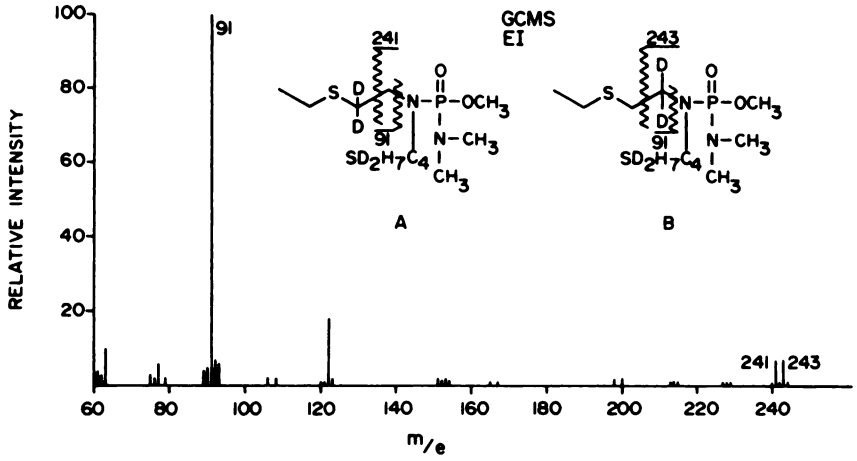


Chart 6. Electron impact mass spectrum of *N,N*-bis[2-(ethylthio)-$d_2$-ethyl]-phosphorodiamidic acid formed in the reaction of $\beta$-$d_4$-phosphoramide mustard with ethanethiol (trimethyl derivative).

chloroethylaziridine would be a more stable molecule and a less active alkylating agent (7) than the tetrasubstituted aziridinium cations that are formed from nitrogen mustard or phosphoramide mustard. The $pK_a$ of ethanolaziridine, which should be close to that of chloroethylaziridine, has been reported to be 7.12 (8), but it was determined in our laboratory to be 6.85. We suggest that at physiological pH, most of the molecules of the chloroethylaziridine are uncharged and are poor alkylating agents.

The reported lack of alkylating activity of 4-hydroxycyclophosphamide is not surprising. In a study of a series of phosphoramide mustard derivatives of the general structure shown in Chart 8, Friedman (10) found that esterification of the oxygen ($R_1$) decreased the alkylating activity and cytotoxicity of the molecule, whereas *N*-substitution ($R_2$ or $R_3$) did not decrease the alkylating activity and in some instances seemed to enhance the cytotoxic activity. A possible explanation of the effect of esterification is that the unesterified hydroxyl group of phosphoramide mustard would be dissociated at physiological pH and thus bear a negative charge. This negative charge would be distributed over the molecule, enhancing the basicities of the phosphoramide nitrogens. Under these conditions, the tertiary (mustard) nitrogen would be nucleophilic enough to allow aziridinium ion formation and subsequent alkylation.

Thus, it would be predicted that all esters of phosphoramide mustard, including 4-hydroxycyclophosphamide and the putative aldophosphamide (Chart 1, *IIb*), would be relatively poor alkylating compounds, as is cyclophosphamide. Elimination of acrolein would generate phosphoramide mustard, an active alkylating agent. Nornitrogen mustard, a subsequent decomposition product, is also a poor alkylating agent at physiological pH.

On the basis of the evidence cited and reported here, it would appear that phosphoramide mustard is an excellent candidate for the most biologically significant alkylating agent generated by the metabolism of cyclophosphamide. The relative roles of phosphoramide mustard and its precursor, 4-hydroxycyclophosphamide (or the isomeric aldehyde), in transport into the cell remain to be determined. Since phosphoramide mustard is directly toxic to cells (15) and measurable levels of phosphoramide mustard are present in the plasma of patients after the administration of cyclophosphamide (9, 14), this extracellular material would be expected to play some role in the biological effects of cyclophosphamide. Struck *et al.* (18) were unable to detect 4-hydroxycyclophosphamide in the blood of mice after the injection of cyclophosphamide and postulated that phosphamide mustard produced outside the target cell is the significant antitumor compound generated by the metabolism of cyclophosphamide. However, we have found (5) that cyclophosphamide is more effective than phosphoramide mustard against L1210 leukemia *in vivo* and that microsomally activated solutions of cyclophosphamide are more toxic to L1210 cells *in vitro* than equally alkylating solutions of phosphoramide mustard. These facts suggest that extracellularly generated phosphoramide mustard cannot account for all of the antitumor effects of cyclophosphamide and imply either that 4-hydroxycyclophosphamide is the key metabolite to enter the target cell, or that other factors are involved in the cytotoxicity of cyclophosphamide. In this respect, it should be pointed out that the assay techniques of Struck *et al.* (18) do not preclude the existence of low but biologically significant levels of 4-hydroxycyclophosphamide.

Finally, some role may be played by the acrolein (1) released from aldophosphamide. Since the analog of cyclophosphamide, in which the bis(2-chloroethyl)amine group has been replaced by diethylamine, readily produces acrolein (2) on microsomal metabolism but lacks antitumor effect (3), it is unlikely that acrolein plays a major role in the antitumor effect of cyclophosphamide. However, the release of this toxic aldehyde may contribute to the biological effects of cyclophosphamide.

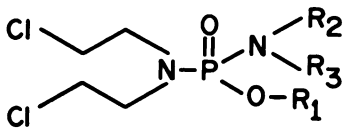


Chart 8. General structure of phosphoramide mustard derivatives.

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