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Urinary Metabolites of the Antitumor Agent Cyclophosphamide

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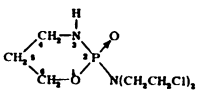
SUMMARY

2-Carboxyethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate and 4-ketocyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-one 2-oxide) have been isolated and identified as urinary metabolites of dogs treated with the anticancer agent cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide). 2-Carboxyethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate is the major urinary metabolite, whereas 4-ketocyclophosphamide is a minor metabolite and represents the first known product of cyclophosphamide which retains the ring structure. Another urinary metabolite yields β-hydroxypropionamide on hydrolysis, a further indication that the primary oxidative step in the metabolism of cyclophosphamide occurs on carbon 4 of the ring. Neither of the two isolated metabolites is highly cytotoxic to human epidermoid cancer cells *in vitro* or to Leukemia L1210 cells *in vivo*. Neither compound is on a pathway leading to an active form of the drug.

INTRODUCTION

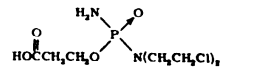
Cyclophosphamide (2-[bis(2-chloroethyl)-amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (compound I) is widely used in the treatment of many types of cancer (1–5). This agent has little cytotoxic or alkylating activity until it is acted upon by a mixed-function oxidase of liver microsomes, which requires TPNH and oxygen (6, 7). Following activation of cyclophosphamide by the livers of animals treated with this drug, cytotoxic, alkylating materials are present in the serum, urine, and bile (6, 8).

Study of metabolites of cyclophosphamide has revealed that nornitrogen mustard (bis-2-chloroethylamine), 2-chloroethylaziridine, and hydracrylic acid are formed in small amounts (6, 9, 10). These three compounds represent small portions of the original molecule. We report here the isolation



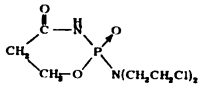
Cyclophosphamide

I



2-Carboxyethyl N,N-bis(2-chloroethyl)phosphorodiamidate

II



4-Ketocyclophosphamide

III

and identification of the major urinary metabolite [2-carboxyethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate, II] and a minor urinary metabolite (4-ketocyclophosphamide, III) which retain all the carbon and

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nitrogen atoms of cyclophosphamide. A preliminary report of the isolation of 4-ketocyclophosphamide (III) has been published (11).

MATERIALS AND METHODS

Ring-labeled [6-^{14}C]cyclophosphamide was a gift from the Mead-Johnson Research Center, Evansville, Ind., and was supplied to us by Dr. G. P. Wheeler. It was further purified by column chromatography on DEAE-Sephadex A-25. Side chain-labeled ^{14}C-cyclophosphamide was purchased from New England Nuclear Corporation. Neither compound, as used, contained detectable impurities on column or paper chromatography or on mass spectrometry. 6-Methylcyclophosphamide was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Md., and β-hydroxypropionamide was a product of K & K Laboratories, Inc.

Beagle dogs were given an intravenous injection of 20 mg/kg of either ring- or side chain-labeled ^{14}C-cyclophosphamide (20–30 nCi/mg), and urine was collected for periods up to 24 hr. Approximately 65% of the administered radioactivity was recovered in 24 hr. Urine was either pooled from several animals or collected individually.

^{14}C-Cyclophosphamide (either ring- or side chain-labeled) was administered intravenously to humans in doses of 10–30 mg/kg (20–30 nCi/mg), and urine was collected for at least 24 hr as a solution buffered at pH 7.0. Approximately 60% of the administered radioactivity was recovered in 24 hr. In some cases the urine from several patients was pooled.

The urine samples were treated in one of three ways. The first method involved addition of several volumes of ethanol and removal of the insoluble materials by filtration or centrifugation. At this stage, 75% of the radioactivity originally present in the urine samples was present in the aqueous ethanol solution. Ethanol was evaporated at 30° under reduced pressure. The second procedure involved lyophilization of urine, trituration with ethanol–water (11:2, v/v; 12 ml/g of urine residue), and filtration; the filtrate contained 90–92% of the radioactivity originally present in the urine. The third procedure, which was employed for the isolation of 4-ketocyclophosphamide, involved prolonged extraction of urine with ether; after removal of the solvent under reduced pressure at room temperature, the ether-soluble materials were suspended in 0.02 M NH_4HCO_3. The ethanol-soluble or ether-soluble fractions were then applied to a column (2.5 × 100 cm) of DEAE-Sephadex A-25 equilibrated with 0.02 M NH_4HCO_3. The anionic character of the urinary metabolites has been noted (12). The columns were washed with 1 column volume of 0.02 M NH_4HCO_3, and a linear gradient of 0.02–0.2 M NH_4HCO_3 was applied; 95% of the radioactivity applied to the columns was recovered.

2-Carboxyethyl *N*,*N*-bis(2-chloroethyl)-phosphorodiamidate (II) was synthesized in accordance with the reported procedure (13).

N^3-Methylcyclophosphamide was prepared by a reported method (14).

Mass spectra were obtained with a Hitachi high-resolution, double-focusing mass spectrometer (RMU-6-D-3), and infrared spectra, in pressed KBr discs with Perkin-Elmer infrared spectrophotometers (models 521 and 621). Nuclear magnetic resonance spectra were obtained with a Varian A-60A spectrometer in [6-^{2}H]dimethyl sulfoxide with tetramethylsilane as internal reference.

Human epidermoid Carcinoma 2 (H.Ep.2) cells were maintained in culture and grown as clones as described previously (15). Procedures for cytotoxicity tests with H.Ep. cells in monolayer cultures (16), with L1210 leukemia cells in swirl cultures (17), and with L1210 cells implanted in mice (18) have been described previously.

RESULTS AND DISCUSSION

Major metabolite. Urine from dogs given ^{14}C-cyclophosphamide labeled at C-6 was lyophilized. The residue was triturated with ethanol–water (11:2, v/v) and filtered. Evaporation of the aqueous, ethanolic solution gave a residue, which was separated by DEAE-Sephadex A-25 column chromatography and gave three radioactive peaks [cyclophosphamide, 4-ketocyclophosphamide

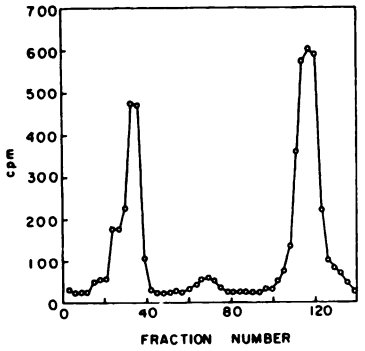


FIG. 1. *Elution pattern of cyclophosphamide metabolites from DEAE-Sephadex A-25*

The column was equilibrated with 0.02 M NH_4HCO_3 and eluted with a linear gradient of 0.01–0.2 M NH_4HCO_3.

(III), and ionic metabolites, respectively] as previously described (11) in the ratio of approximately 20:1:60 (Fig. 1). Independent analysis of the pooled or individual urine samples according to the method of Mellett *et al.* (19) gave similar results with respect to the ratio of unchanged drug to metabolites.

The third radioactive fraction from the Sephadex column was lyophilized, and the residue was again triturated with ethanol–water (11:2, v/v) and filtered. Evaporation of the filtrate, which contained 90% of the radioactivity present in the column fraction residue, gave a syrup which was purified by thin-layer chromatography; separation on preparative silica gel plates[2] in chloroform–methanol (3:1) gave a major, strongly positive, 4-(*p*-nitrobenzyl)pyridine-reactive (20) band at R_F 0.24 along with a second, weaker NBP[3]-reactive band at the origin. Liquid scintillation counting indicated a radioactivity ratio of 3.6:1 for the two bands (R_F 0.24:0.0). These data indicated that the band at R_F 0.24 contained the major C-6-containing metabolite, which appeared in the third fraction from DEAE-Sephadex A-25 chromatography of urine. The band at R_F 0.24 was eluted with ethanol and filtered.

[2] Merck 2-mm-layer coated plates obtained from Brinkmann Instruments, Inc., Westbury, N. Y.

[3] The abbreviation used is: NBP, 4-(*p*-nitrobenzyl)pyridine.

Evaporation of the ethanol left a syrup, which upon mass spectral analysis gave no definitive results; the syrup was then treated with diazomethane in ether. After removal of excess diazomethane and ether, the sample was analyzed by infrared and mass spectrometry. The infrared spectrum showed

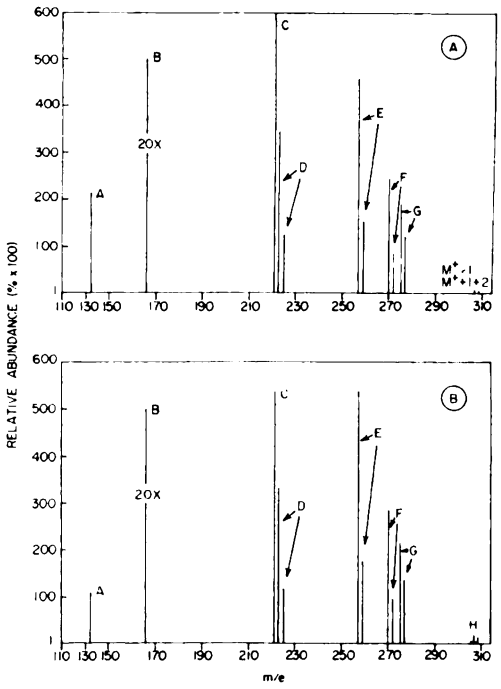
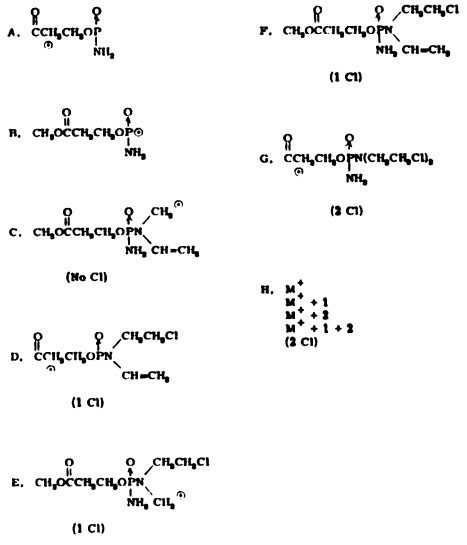
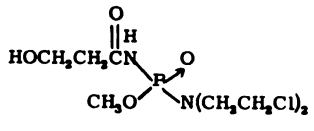
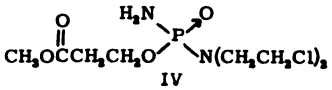


FIG. 2. *Mass spectra of a cyclophosphamide metabolite (A) and synthetic 2-carbomethoxyethyl N,N-bis(2-chloroethyl)phosphorodiamidate (IV) (B)*

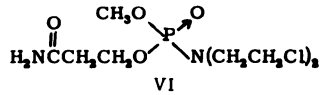




V



IV



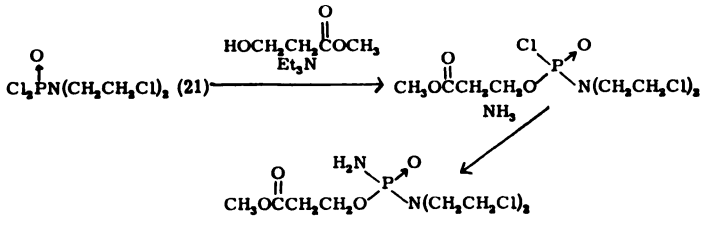
VI

an intense band at 1730 cm^{-1}, and the mass spectrum (Fig. 2) showed strong peaks of *m/e* 275 (M^+—OCH_3, 2 Cl), *m/e* 270 (M^+—HCl, 1 Cl), *m/e* 257 (M^+—CH_2Cl, 1 Cl), *m/e* 221 (*m/e* 257—HCl, no Cl), *m/e* 166 [M^+—$N(CH_2CH_2Cl)_2$, base peak, no Cl], and *m/e* 135 (*m/e* 166—OCH_3, no Cl). The mass fragmentation was reasonably derivable from a molecular ion of *m/e* 306, although a molecular ion peak was not observed. Since the three isomers IV, V, and VI could all give this fragmentation, the identity of the metabolite was not immediately apparent.

Further purification of the methylated metabolite fraction by thin-layer chromatography on silica gel in acetone–chloroform (3:1) gave one strong, NBP-reactive band at R_F 0.55 along with a weak, NBP-reactive band at the origin. The infrared spectrum of the residue, obtained by ethanol elution of the band at R_F 0.55 and subsequent evaporation, was essentially identical with that of the material before chromatography; the strongest band appeared at 1730 cm^{-1} and suggested structure IV for the major component.

Proof was obtained by synthesis of compound IV by the sequence shown in Scheme 1.

A solution of methyl β-hydroxypropionate (4.5 g, prepared by addition of β-hydroxypropionic acid to diazomethane in ether) in triethylamine (6 ml) was added dropwise during 30 min at 0° with stirring to bis(2-chloroethyl)phosphoramidic dichloride (21) (11.2 g, 0.043 mole) in *N*,*N*-dimethylacetamide (25 ml). The ice bath was removed, and the mixture was stirred at room temperature for 1 hr and filtered to remove triethylamine hydrochloride. The filtrate was treated with ammonia at a moderate rate at room temperature until precipitation of ammonium chloride was complete. After filtration, the solution was evaporated under vacuum, and the resulting syrup was passed through a silica gel column (prepared from 200 g of silica gel) in acetone–chloroform (3:1); 25 fractions of 25 ml each were collected and analyzed by thin-layer chromatography. Two strongly positive, NBP-reactive components were detected; mass spectral analysis of the two components showed that one was the expected product, whereas the other was 2-carbomethoxyethyl *N*,*N*-bis(2-chloroethyl)-*N'*,*N'*-dimethylphosphorodiamidate (M^+ 334 observed along with expected fragments). The fractions containing compound IV were combined and purified by thin-layer chromatography on preparative silica gel plates[2] in acetone–chloroform (1:1). The NBP-reactive band at R_F 0.25 was collected, eluted with acetone, and filtered, and the filtrate was evaporated, giving chromatographically pure compound IV.



IV

SCHEME 1

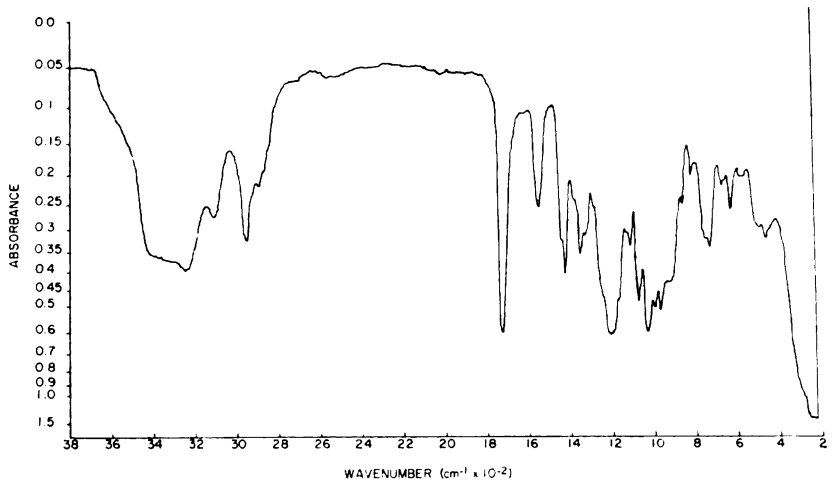


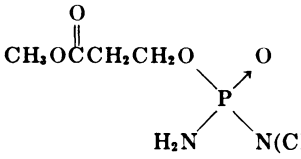
FIG. 3 *Infrared spectrum of synthetic 2-carbomethoxyethyl N,N-bis(2-chloroethyl)phosphorodiamidate (IV)*

Mass (Fig. 2) and infrared (Fig. 3) spectra of the synthetic ester (IV) confirmed structure II for the major urinary metabolite. A weak molecular ion (M^+ 306) was observed for the synthetic material along with a more intense, protonated molecular ion ($M^+ + 1 = 307$). The relative abundances of the mass fragments listed above for the methylated metabolite were identical for the synthetically and metabolically derived specimens. All bands in the infrared spectrum of the synthetic material (IV) were present in the same relative intensity in the spectrum of the chromatographically purified sample of the methylated metabolite. The presence of several bands in the latter spectrum that were absent from the spectrum of the synthetic material showed that the methylated metabolite fraction was not completely pure. However, thin-layer chromatography of the methylated metabolite and detection by NBP showed only one alkylating active component, with an R_F value identical with that of the synthetic ester in seven systems (Table 1).

Our results were not in agreement with a recent report by Norpoth *et al.* (22), who electrophoretically compared a synthetic specimen of compound II with the major metabolite from rat urine and concluded that the two were not identical. To investigate the discrepancy, we electrophoretically separated the total, anionic, DEAE-Sephadex A-25 column fraction described above under conditions reported by Rauen and Norpoth (10) for the metabolite from rat serum. The NBP-reactive pattern we observed for the electrophoretogram matched that reported (10); four NBP-positive, anionic components were detected which were separated by the same relative distances. Scanning of the electrophoretogram indicated that the second band (Rauen and

TABLE 1

Thin-layer chromatographic comparison of methylated metabolite with synthetic



Solvent system	R_F	
	Methylated metabolite	Synthetic terminal acid methyl ester
Acetone	0.8	0.8
Acetone–benzene (1:1)	0.3	0.3
Acetone–chloroform (1:1)	0.25	0.25
Acetone–chloroform (3:1)	0.55	0.55
Benzene–methanol (1:1)	0.85	0.85
Benzene–methanol (9:1)	0.4	0.4
Methanol–toluene (1:5)	0.4	0.4

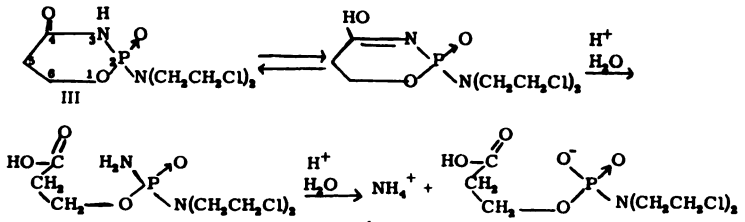
Norpoth's band 2) contained about 95% of the radioactivity. The radioactive band was eluted with water; the eluate, after lyophilization, was methylated with diazomethane. Thin-layer separation of the methylated material on silica gel in acetone:benzene (1:1) showed three NBP-positive spots, at R_f 0.0, 0.3, and 0.8, the strongest of which was at R_f 0.3, with a radioactivity ratio of 1.8:3.4:1, respectively. The mass spectrum (M^+ 306 fragmentation observed) and thin-layer chromatographic behavior of the component at R_f 0.3 (R_f identical with that of co-chromatographed, synthetic compound IV) indicated that the major metabolite observed in this electrophoresis experiment was the same as that observed in our chromatographic experiments, namely, structure II.

In order to eliminate the possibility of modification of the metabolites during thin-layer chromatography or electrophoresis, the DEAE-Sephadex A-25 anionic column fraction was lyophilized, and the total residue was treated with diazomethane. A mass spectrum of the total sample gave chlorine-containing fragments derivable only from compound IV, as shown by the relative abundances of the fragments. Thin-layer separation of the total sample on silica gel in acetone–benzene (1:1) gave a pattern identical with that observed earlier (R_f 0.0, 0.3, and 0.8), with the strongest NBP-reactive spot again having the same R_f value as that of co-chromatographed, synthetic compound IV. Mass spectra of the three components, isolated by elution with ethanol, revealed that only the component at R_f 0.3 gave recognizable chlorine-containing peaks and that its spectrum matched that given by synthetic compound IV. The data again implicate structure II as that of the major urinary metabolite.

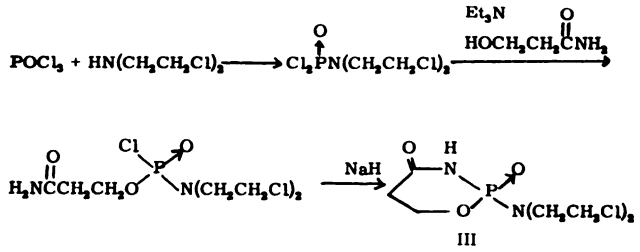
Thin-layer chromatographic comparison of the major metabolite with synthetic compound II (13) in chloroform–methanol (3:1) on silica gel showed that both samples behaved identically. Thin-layer chromatographic and infrared and mass spectrometric comparison of the methyl ester of the major metabolite with compound IV, prepared from synthetic II by treatment with diazomethane, indicated the complete identity of the methyl esters.

The major urinary metabolite (II) represents at least 25% of the administered dose in 6-hr urine samples of dogs and 15% of the dose in human urine after 4 hr. From 40 to 50% of the total radioactivity in the urine of dogs and 25–30% in the urine of humans can be attributed to compound II. Unchanged cyclophosphamide in the urine of dogs and humans amounts to approximately 7% and 16%, respectively, of the administered dose and approximately 11% and 27% of the urinary radioactivity. If the criterion of alkylating activity (as judged by reaction with NBP) is added to that of radioactivity, structure II accounts for even higher percentages of urinary metabolites which are potential alkylating agents.

Minor metabolite. The second DEAE-Sephadex fraction (Fig. 1) was further purified by paper chromatography with 1-butanol–acetic acid–water (6:2:2 by volume) and by subsequent passage over a Sephadex G-10 column (1 × 100 cm). Mass spectral analysis of this fraction showed the presence of a molecular ion (M^+) at *m/e* 274, which contained 2 chlorine atoms, as indicated by the M^+:$M^+ + 2$ intensity ratio. The *m/e* value of 274 for M^+ was consistent with either a monomethylated or a keto derivative of cyclophosphamide. Mass spectra of 6-methylcylophosphamide (14) and N^3-methylcyclophosphamide (14) were clearly different from that of the metabolite, although all three contained molecular ions at *m/e* 274. The infrared spectrum of the unknown product contained a strong band at 1695 cm^{-1} and suggested a keto derivative. The metabolite fraction, which at this stage amounted to only approximately 100 μg, was soluble in ether or ethanol. A solution of the fraction in 1 ml of ethanol, concentrated in a stream of nitrogen to about 0.1 ml, yielded a few crystals (square plates). A single-crystal melting point of 148–149° was observed. The mass spectrum of the crystallized specimen again gave a molecular ion at *m/e* 274 (Fig. 4) with a base peak at *m/e* 225 (1 Cl), corresponding to the loss of $—CH_2Cl$. Other prominent peaks in the spectrum were the following: M^+—Cl (*m/e*



SCHEME 2



SCHEME 3

239, 1 Cl); *m/e* 225—HCl (*m/e* 189, no Cl); *m/e* 225—CH_2CH_2Cl + H (*m/e* 163, no Cl); and M^+—$N(CH_2CH_2Cl)_2$ (*m/e* 134, no Cl).

Infrared spectral studies provided the information necessary to locate the position of the carbonyl function. A semimicro infrared spectrum of the crystals showed a carbonyl band (1695 cm^{-1}) of reduced intensity in comparison with the earlier spectrum of the total fraction. Two bands of medium intensity appeared at 1630 and 1615 cm^{-1}, suggesting the presence of a —C=C— or —C=N— group and indicating possible keto-enol or lactam-lactim tautomerism of the metabolite. When the sample was dissolved in water and immediately frozen and lyophilized, the infrared spectrum of the residue showed a decrease in intensity of the bands at 1630 and 1615 cm^{-1} and an increase in the carbonyl band (1695 cm^{-1}), further supporting the idea of tautomerism for the metabolite. When the sample, which had been reclaimed from the infrared spectral disc, was allowed to remain in distilled water (about pH 6) at room temperature for 1 hr, the spectrum of the residue showed strong bands attributable to NH_4^+, an indication that both P—N and C—N bond rupture had occurred. Such facile liberation of NH_4^+ can best be explained by initial hydrolysis of a lactim to generate a P—NH_2 function which readily undergoes acid-catalyzed hydrolysis to produce NH_4^+. The infrared spectral behavior of the metabolite can be explained by the transformations shown in Scheme 2.

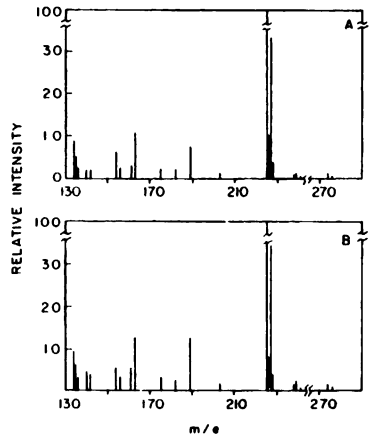


FIG. 4. *Mass spectra of a cyclophosphamide metabolite (A) and synthetic 4-ketocyclophosphamide (B)*

4-Ketocyclophosphamide (III)[4] was synthesized according to Scheme 3. A solution of β-hydroxypropionamide (8.6 g, 96.7 mmoles) and triethylamine (9.76 g, 96.7 mmoles)

[4] Oxo-Endoxan, the name given to 4-ketocyclophosphamide (III) by ASTA-Werke, Brackwede, West Germany, was synthesized prior to our report (11) by Arnold (23).

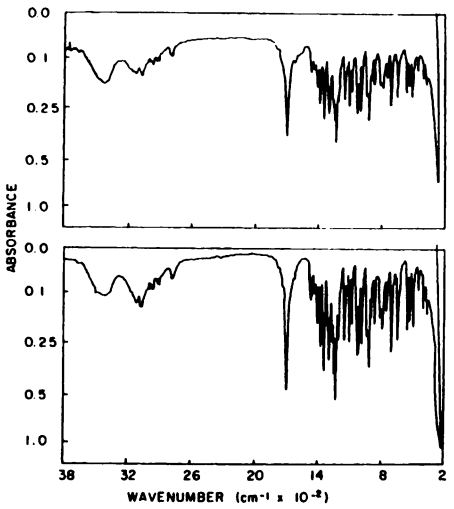


FIG. 5. *Infrared spectra of a cyclophosphamide metabolite (A) and synthetic 4-ketocyclophosphamide (B)*

in 50 ml of dimethylacetamide was added dropwise with stirring at 0° during 2 hr to 25.0 g of *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride (96.7 mmoles) (21) in 50 ml of dimethylacetamide. The mixture was stirred for 12 hr at room temperature and then filtered to remove triethylamine hydrochloride. The filtrate was treated batchwise in 30 min at 0° with 4 g of sodium hydride (57% dispersion in oil, 95 mmoles). The mixture was stirred for 4 days at room temperature and filtered through Celite. Evaporation of the filtrate *under vacuum* gave a syrup, to which 100 ml of phosphate buffer (pH 7.2, 0.05 M) were added. Aqueous potassium hydroxide (1 N) was added immediately to raise the pH back to 7.2. The solution was extracted continuously for 2 days with ether, and the extract was evaporated. The residue, in 5 ml of ethanol, was added with vigorous stirring to 200 ml of ether. Decantation of the solution from a precipitated syrup and evaporation of the ether–ethanol under vacuum left a small residue. Trituration in ether containing a drop of ethanol gave a crystalline solid: m.p. 148–149°; yield, 90 mg. Recrystallization from ether–ethanol gave pure 4-ketocyclophosphamide: m.p. 150°; yield, 67 mg.

$C_7H_{13}Cl_2N_2O_3P$

Calculated: C 30.57, H 4.76, Cl 25.78, N 10.18, P 11.26

Found: C 30.64, H 4.60, Cl 25.97, N 10.15, P 11.33

Mass spectra (Fig. 4) and infrared spectra (Fig. 5) of the metabolite and the synthetic product were identical, and a mixture melting point of the two showed no depression, thus unequivocally proving the structure of the metabolite. The NMR spectrum of the synthetic product is shown in Fig. 6.

4-Ketocyclophosphamide (III) was also identified by mass spectral analysis in ether extracts of urine from dogs given side chain-labeled ^{14}C-cyclophosphamide as well as from

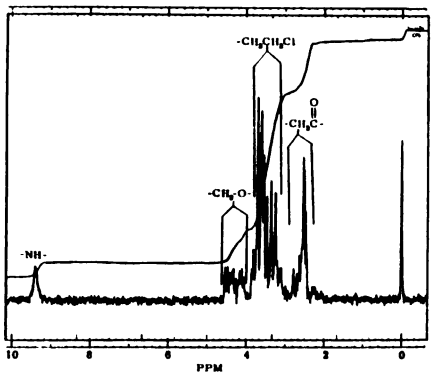


FIG. 6. *Nuclear magnetic resonance spectrum of synthetic 4-ketocyclophosphamide*

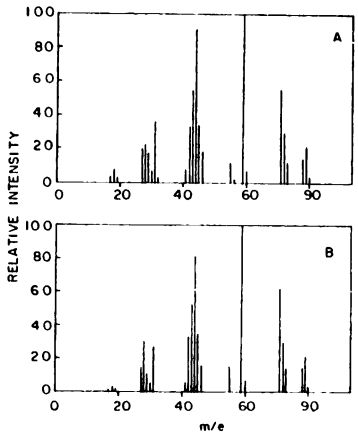
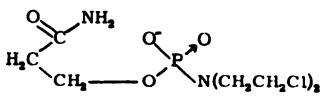


FIG. 7. *Mass spectra of the fragment from peak III (A) and authentic β-hydroxypropionamide (B)*

humans given side chain- or ring-labeled ^{14}C-cyclophosphamide.

4-Ketocyclophosphamide (III) represents 1–2% of the administered dose in the urine of dogs and humans.

Other metabolites. Further evidence that carbon 4 of the cyclophosphamide ring is oxidized by the dog came from the isolation of β-hydroxypropionamide as a fragment of a metabolite present in the third peak from DEAE-Sephadex A-25 column chromatography of ethanol-extracted urine. In this case, the urine came from a dog treated with ring-labeled cyclophosphamide. To obtain this fragment, material in the third peak was treated with 0.1 N HCl at room temperature for 24 hr. After neutralization, the preparation was again placed on a small (1 × 100 cm) DEAE-Sephadex A-25 column, and the radioactive fragment was washed from the column with 0.02 M NH_4HCO_3. It was deionized by passage through a Sephadex G-10 column (1 × 100 cm) and subjected to mass spectral analysis. The mass spectrum (Fig. 7) was nearly identical with that of an authentic sample of β-hydroxypropionamide. The minor peaks at *m/e* 32 and 56 could be attributed to trace impurities in the preparation of the fragment. A likely structure from which this fragment was derived is 2-carbamylethyl bis(2-chloroethyl)phosphoramidate (VII).



VII

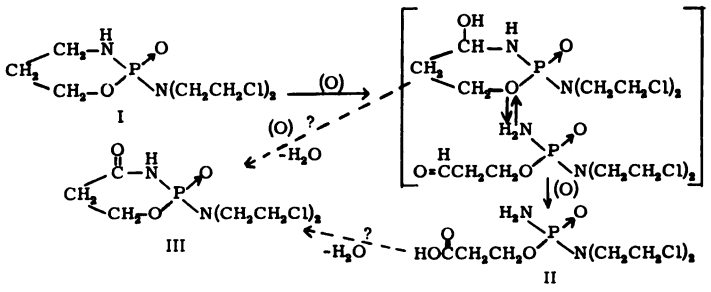
Biological evaluation. 2-Carboxyethyl *N*, *N* - bis(2 - chloroethyl)phosphorodiamidate (II), the major metabolite, inhibited clone formation of small numbers of H.Ep.2 cells in culture by 58% at 1 μg/ml (Table 2). When evaluated against L1210 leukemia in mice inoculated with 10^5 cells, the metabolite (II) gave no increase in survival time at a dose of 300 mg/kg when administered on the first day of the test. Under identical conditions, cyclophosphamide produces approximately 70% survivors and a 250% increase in survival time of the 30% nonsurvivors. At a dose of 500 mg/kg, compound II was nontoxic and gave only a 19% increase in survival time; this dose would represent approximately the LD_{90} of cyclophospha-

TABLE 2

Inhibition of clone formation of H.Ep.2 cells by 4-ketocyclophosphamide and 2-carboxyethyl N,N-bis(2-chloroethyl)phosphorodiamidate

One hundred cells were placed in 4-ounce prescription bottles containing culture fluid, and the macroscopic colonies present after 7 days were counted.

Metabolite	Concentration	Clones formed	Average	Relative values
	μg/ml			%
4-Ketocyclophosphamide	0	70, 85, 81, 66	76	100
	1	54, 57, 58	56	74
	3	56, 58, 58	57	75
	5	42, 42, 44	43	57
	7	21, 23, 25	23	30
	10	0, 0, 0	0	0
2-Carboxyethyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate	0	96, 91, 92, 86	91	100
	0.1	86, 88	77	85
	0.5	92, 47	70	77
	1	45, 30	38	42
	3	0, 0	0	0
	5	0, 0	0	0
	10	0, 0	0	0



SCHEME 4

mide. The major metabolite, then, is a radically detoxified derivative of cyclophosphamide.

Tests showed that 4-ketocyclophosphamide (III) had some biological activity. Clone formation of small numbers of H.Ep.2 cells in culture was inhibited 43% at 5 μg/ml (18 μM) of 4-ketocyclophosphamide (Table 2). Under identical conditions, 75 μg/ml of cyclophosphamide are required for comparable inhibition. However, 4-ketocyclophosphamide at 100 μg/ml inhibited the proliferation of large numbers of H.Ep.2 cells in monolayer culture by only 64%, as measured by growth after 72 hr. At 200 μg/ml no detectable activity was noted against L1210 leukemia cells in swirl culture, and at 1000 mg/kg there was no effect on implanted L1210 ascites cells in mice, as determined by the lack of increased survival. Cyclophosphamide was also not effective against L1210 cells in culture, but for the implanted L1210 cells cyclophosphamide at 180 mg/kg killed 99.99% of an administered inoculum.

The weak cytotoxic effect *in vitro* and *in vivo* of 4-ketocyclophosphamide (III) rules out the possibility that this metabolite is on the pathway leading to an active form of the drug. In an investigation, the results of which were published while our experiments were under way, Norpoth *et al.* (22) tested 4-ketocyclophosphamide (III) and 2-carbamylethyl bis(2-chloroethyl)phosphoramidate (VII) for activity against a tumor implanted on the chorioallantois of chick embryos. No activity was noted.

The metabolism of cyclophosphamide appears to proceed as shown in Scheme 4. 4-Ketocyclophosphamide could be produced either by oxidation-dehydration of the C-4-hydroxylated intermediate or by dehydration-cyclization of the major metabolite (II). Norpoth *et al.* (22) suggested such a scheme for the major metabolic pathway of cyclophosphamide leading to the acid II through the intermediacy of a ring-opened aldehyde; however, on the basis of their experimental data, they concluded that this route was unlikely and suggested C-4 hydroxylation followed by cleavage of the ester bond to generate the major metabolite.

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