Characterization of the Major Sheep Urinary Metabolites of

Cyclophosphamide, a Defleecing Chemical

Two metabolites of cyclophosphamide (I) in the urine from sheep orally dosed with I were characterized as O-(2-carboxyethyl) N,N-bis(2-chloroethyl)-phosphorodiamidate (IV) and 2-[bis(2-chloroethyl)-amino]tetrahydro-2H-1,3,2-oxazophosphorine 2,4-dioxide (4-ketocyclophosphamide) (II). Compounds IV and II represented approximately 45 and 5%, respectively, of the urinary radioactivity.

Homan et al. (1968) induced wool loss in sheep by intravenous doses of the antitumor agent, cyclophosphamide (I). Dolnick et al. (1969) demonstrated that oral drenching of sheep with 10 to 30 mg per kg of I would also induce wool loss. Hill et al. (1970) identified 4-ketocyclophosphamide (II) as a metabolite in the urine of dogs given I intravenously. This report concerns the isolation of II and the ring-opened analog of compound II—compound IV—from the urine of sheep orally dosed with I.

EXPERIMENTAL

Wether sheep were given, by gelatin capsule, 30 mg per kg of either ring-labeled (0.180 μCi per mg) or side-chain-labeled (0.040 μCi per mg) ^{14}C-cyclophosphamide (I). The urine was collected at 4-hr intervals in an iced container. After assay for radioactivity, the urine was frozen.

The gel filtration column was a 180 $\times$ 2 cm column of water-equilibrated Sephadex LH-20. The column was eluted with water at a flow rate of 1.5 ml per min.

The alumina column was prepared with 20 g of basic alumina (Wolem) poured in ethanol–ammonia–water (80 : 15:5; v/v/v). The samples were eluted with the above solvent pumped at 0.5 ml per min.

The Bio-Rad AG-50X8 [H]$^+$ was packed in a 1 $\times$ 20-cm column and washed with water prior to addition of the samples.

The elution of radioactivity from the columns was monitored using a Packard model 320E liquid scintillation flow system. Radioactive components on paper chromatograms were located using a Packard model 7200 chromatogram scanner. All quantitation of radioactivity was done by liquid scintillation in counting solution A (Bakke et al., 1967). Elution of radioactivity from the gas chromatograph was monitored by trapping each peak in a glass tube and assaying each tube for radioactivity in counting solution A.

All paper chromatography was performed with Whatman No. 1 using butanol–acetic acid–water (6:2:2, v/v/v). Gas chromatography utilized a 6 ft, $^1/_8$ in. i.d. glass column packed with either 3% SE-30 (gc column A) or 3% OV-225 (gc column B) on 60/80 mesh Chromosorb W in a Perkin-Elmer 801 gas chromatograph fitted with an effluent splitter (10% to the flame ionization detector). The carrier gas was helium at 30 ml per min. The temperature was programmed at 10° per min from 100° to 150° C. The injector was maintained at 200° C. The detector was located within the column oven.

Mass spectra were obtained with a Varian M-66 mass spectrometer equipped with a V-5500 control console, using the solid sample inlet system. The nmr spectra were taken with a Varian A-60A nmr spectrometer equipped with a Varian V-6058A spin decoupler and a Fabri-Tek 1062/SW-3 time averager. All spectra were determined in pyridine-d_5.

Methyl β - (Dimethylphosphoryl)propionate Synthesis. Methyl 3-hydroxypropionate, 8.9 g (35 mmol), was added dropwise with stirring to 13.1 g (85 mmol) of phosphorous oxychloride at room temperature. After the addition was complete, the reaction mixture was heated on a steam bath for 30 min, then cooled to room temperature, and 30 ml of anhydrous methanol was added. The reaction was stirred for 5 hr at room temperature. Removal of the solvent yielded a clear oil which appeared to be quite pure on the basis of tlc and glc, but only moderately pure on the basis of nmr. Distillation through a micromolecular distillation apparatus effected substantial purification; ir (KBr) 1750 cm^{-1} (CH$_3$-OCO); nmr (CDCl$_3$) δ 2.70 (doublet of triplets, 2, J = 6.3 Hz and $J_{P,H}$ = 1.0 Hz, COCH$_2$), 3.70 (s, 3, CH$_3$OCO), 3.75 [d, 6, $J_{P,H}$ = 11.0 Hz, PO(OCH$_3$)$_2$], 4.30 (d of t, 2, J = 6.3 Hz and $J_{P,H}$ = 7.5 Hz, CH$_2$OPO).

LiAlH$_4$ Reduction. The reduction of the major urinary metabolite off LH 20 (Fraction 4) was accomplished by refluxing ring labeled Fraction 4 with excess LiAlH$_4$ in anhydrous tetrahydrofuran for 18 hr. The excess LiAlH$_4$ was destroyed by the addition of ethylacetate, followed by the addition of water. The resulting aqueous phase was separated and freeze-dried. The radioactivity was extracted from the freeze-dried residue with methanol, and the methanol extract paper chromatographed in the butanol–acetic acid–water system.

The three radioactive fractions separated on the paper were extracted from the chromatogram with methanol and the fractions taken to dryness. Separate aliquots of these fractions were treated with either benzoyl chloride in pyridine or acetylchloride in pyridine. The reacted samples were taken to dryness on a steam bath under a stream of nitrogen. The dried residues were extracted with diethyl ether and the ether extracts gas chromatographed on gc column B. Samples of known 3-aminopropanol and 1,3-propanediol were acylated by the same method.

RESULTS AND DISCUSSION

The urine samples collected to 8 hr, which contained from 25.4 to 56.2% of the administered radioactivity from either side-chain or ring-labeled I, were used for isolation of the metabolites.

Seven radioactive components were separated on the Sephadex LH-20 column from sheep urine containing either ring-labeled or side-chain-labeled cyclophosphamide metabolites. The fractions eluting between 380 and 400 ml (Fraction 4) and 570 and 600 ml (Fraction 7) contained radioactivity from both ring- (4-R and 7-R) and side-chain-labeled (4-S and 7-S) compound I. Fractions 4-R, 4-S, 7-R, and 7-S contained, respectively, 51, 42, 4.5, and 6.4% of the radioactivity applied to the column. All four fractions had the same R_f (0.81) in the paper chromatographic system.

Mass spectral analysis of the freeze-dried residues from 7-R and 7-S gave peaks at m/e 238, 225, and 189. The 238 and 225 (base peak) fragments each contained one chlorine

atom. These high mass fragments from the metabolite spectra exhibited a pattern similar to the high mass fragments from I (m/e 224, 211, 175, and 120), but at positions 14 mass units higher. In the mass spectra from I, m/e 211 was the base peak and both m/e 224 and 211 contained one chlorine atom. The molecular ion (M^+) for I, with two chlorine atoms, appeared at m/e 260; no corresponding M^+ was present at m/e 274 in the 7-R or 7-S spectra.

Assuming that these metabolite fragments arose by the same mechanism as the high mass fragments from I, the addition of 14 mass units suggested the metabolite to be an oxidation product of I. Since the high mass fragments from I can be accounted for by fragmentations in the side chains [m/e 224, M^+-HCl; m/e 211, M^+-CH_2Cl; m/e 175, M^+-(CH_2Cl + HCl); and m/e 120, M^+-$N(CH_2CH_2Cl)_2$], the oxidation was assumed to have taken place on the ring portion.

Fractions 4-R and 4-S were further purified by either paper chromatography alone or on the alumina column followed by paper chromatography.

Mass spectral analysis of both 4-R and 4-S that had been through alumina gave the same high mass fragments as 7-R and 7-S. Fractions 4-R and 4-S that had been subjected to only paper chromatography did not give these high mass fragments until after they have been treated with trifluoroacetic anhydride. This indicated that 4-R and 4-S were ring-opened forms of fractions 7-R and 7-S, and that 4-R and 4-S could be cyclized either on the alumina or with a dehydrating agent. Fractions 4-R and 4-S eluted from the alumina column in a broad band after approximately three column volumes of solvent (Fractions 7-R and 7-S eluted from the alumina column with one column volume of solvent).

The site of oxidation of the cyclophosphamide ring can be inferred from the nmr spectra (Table I) of I and metabolite 4-S that had been through the alumina column to form 7-S. Double resonance experiments with the dimethylamino analog (III) and I demonstrated that the protons of the position 5 could be observed as a triplet by irradiating position 4 protons or position 6 protons. The signal from position 4 or position 6 was not completely collapsed to a singlet by the reverse experiment, indicating the influence of the phosphor-amide and phosphate bonds. The spectrum of the metabolite had two peaks due to ring protons, one of which occurred at δ 2.95 as a triplet. This triplet and the multiplet at δ 4.47 could be decoupled, indicating that the two methylenes are adjacent. The carbonyl must then be on position 4 or 6, and the resonance at δ 4.47 must be due to the protons of the remaining methylene group (*i.e.*, position 6 or 4). Assignment of the carbonyl to position 4 would result in a change in the chemical shift of the protons on position 6 of 0.2 ppm, a realistic amount for the introduction of a β carbonyl. Assignment of the carbonyl to position 6 would result in a change in chemical shift of the protons on position 4 of 1.3 ppm, a shift as great as for the methylene protons at position 5
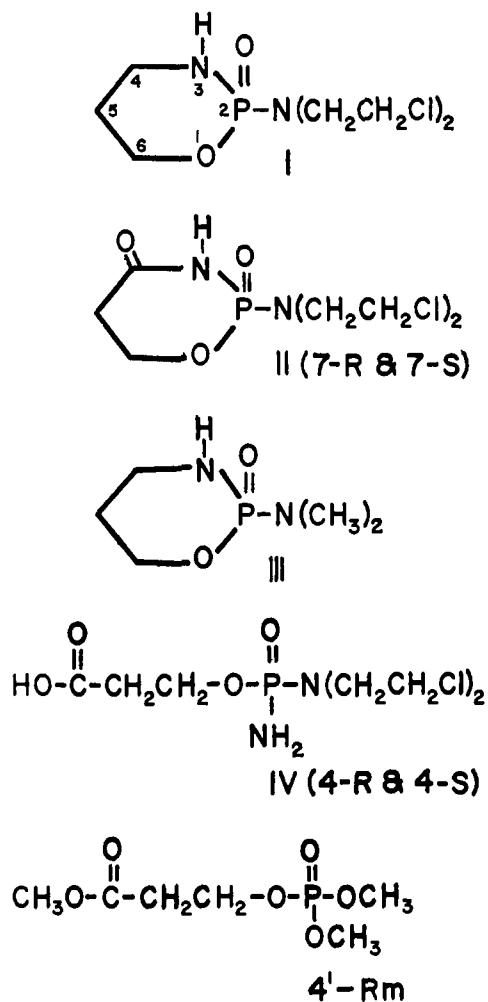
Figure 1. Structures of the compounds referred to in the text and Table I

(α to the carbonyl), clearly an unrealistic amount. The difference in chemical shift (approximately 1.3 ppm) is about that expected for introduction of an adjacent carbonyl. Double resonance experiments on the metabolite (7-S) demonstrated that the signals at δ 2.5 and δ 4.47 were coupled. The resonance from position 5 could be reduced to a singlet, verifying that the methylene at either position 4 or position 6 is missing. Again the reverse experiment did not yield a singlet. If the resonance was from the methylene at position 4, the change in chemical shift required is about 1.3 ppm; if the resonance was from position 6, the expected change would be about 0.2 ppm. Since the change in shift for the resonance of the protons on position 5 was 1.3 ppm, with a carbonyl introduced adjacent to it, a change in shift for a proton β to the carbonyl would be much less. The carbonyl, therefore, is on position 4.

Mass spectral analysis of methylated 4-R and 4-S (diazomethane) gave fragments at m/e 275 with two chlorine atoms, m/e 270 and 257 with one chlorine atom, and m/e 221, 166 (base peak) and 135 without chlorine. The fragments at m/e 270, 257, and 221 were considered analogous to the side-chain fragmentations found with 7-R, 7-S, and I. These fragments appeared at positions 32 mass units higher than those found with the proposed ring-closed metabolite (7-R and 7-S). This would correspond to the addition of water to fraction 7 (compound II, Figure 1), followed by replacement of one proton with a methyl group. No molecular ion

for the proposed compound was present at m/e 306. The fragment at m/e 166 would correspond to the loss of M^+-$N(CH_2CH_2Cl)_2$ analogous to the fragment at m/e 120 from compound I. The fragment with two chlorine atoms at m/e 275 most probably represents the loss of a methoxyl group (M^+-31) from the proposed methyl ester of 4-R and 4-S.

The radioactivity from 4-R eluted with one column volume of water from the sulfonic acid resin column. The radioactivity from 4-S could not be eluted from this resin with either water or acid. This suggested that hydrolysis of the metabolite had occurred. The freeze-dried 4-R fragment (4'-R) eluted from the AG-50 column was paper chromatographed (R_f 0.32) and either methylated (diazomethane) or silanized (Regisil) prior to gas chromatography on gc column I and and subsequent mass and infrared spectral analyses. The elution temperatures for the methylated (4'-Rm) and silanized (4'-Rs) compounds were 124° and 135° C, respectively.

The infrared and mass spectra of 4'-Rm were in agreement with the structure (4'-Rm) shown in Figure 1 and were identical with the mass and infrared spectra obtained from the synthetic methyl β-(dimethylphosphoryl)propionate (4'-Rm). A carbonyl band at 1735 cm^{-1} indicated a methyl ester of a carboxylic acid. Absorptions in the 1050 to 1030 cm^{-1} and 1190 cm^{-1} regions for alkyl phosphates and absorption in the 1300 to 1250 cm^{-1} region for the P=O group were present.

No molecular ion was present in the mass spectrum of either the isolated or synthetic 4'-Rm; however, the silyl ester (4'-Rs) gave a molecular ion at m/e 386 with a large M^+-15 fragment at m/e 371. This m/e 371 fragment had an m/e 372 isotope peak with a relative intensity compatible with the empirical formula $C_{11}H_{28}O_6PSi_3$ (calcd: 31.05%; found: 30–31%). Therefore, 4'-R would have a mol wt of 170 and its trimethylester (4'-Rm) would have a mol wt of 212. The m/e 181 fragment in the 4'-Rm spectrum would be the loss of a methoxyl group (212–31) and the m/e 153 fragment would be (212–59) the loss of COOCH$_3$. The m/e 127 and 109 fragments are the dimethyl phosphate fragments $[(CH_3O)_2P(OH)_2]^+$ and $[(CH_3O)_2PO]^+$ (Budzikiewicz et al., 1967).

The above identification of β-phosphorylpropionic acid (4'-R) as the ion-exchange hydrolysis product of 4-R established that the ring nitrogen from I could be present in 4-R and 4-S as either a phosphoramide or a carboxamide. LiAlH$_4$ reduction of 4-R established the presence of the phosphoramide structure (Compound IV, Fig. 1).

The reaction mixture from the LiAlH$_4$ reduction of 4-R contained three radioactive components by paper chromatography. The R_f's and respective amounts eluted from the paper were 0.5 (19%), 0.67 (38%), and 0.82 (42%).

Benzoylation of the R_f 0.67 fraction gave a radioactive component that gas chromatographed on gc column B at the same elution temperature (225° C) as known 1,3-propanediol dibenzoate. The mass and infrared spectra of this component were identical with those of known 1,3-propanediol dibenzoate. The identity of the other two re-

duction products has not been determined. Neither of them contained 3-aminopropane-1-ol which would have been a reduction product had the metabolite been a carboximide. Known 3-aminopropane-1-ol when acetylated gave the diacetate, which gas chromatographed on gc column B at 155° C. These components are assumed to be some reduction intermediates of 4-R.

The identification of 1,3-propanediol as a LiAlH$_4$ reduction product at Fraction 4 established that this metabolite was not a carboxamide since LiAlH$_4$ reduces carboxamides to amines (Gaylord, 1956).

From the spectral information, structure II was assigned to sheep urinary metabolite 7, which agrees with the oxidized structure identified by Hill et al. (1970) from dog urine; structure IV was assigned to metabolite 4. It has not been determined if the sheep excretes the metabolite as II, with subsequent hydrolysis to IV, or if IV is the excreted form of the metabolite.

Norpoth et al. (1970) have reported that the major rat urinary metabolite from cyclophosphamide has properties different from synthetic compound IV. The presence of IV in sheep urine indicates either a species difference in cyclophosphamide metabolism or a result of the difference in the route of administration. The latter possibility has not yet been investigated.

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