Clearly, a great deal of work yet remains in order to clarify the biochemical lesion caused by *o,p'*-DDT or *p,p'*-DDT which results in the blockage of the known biological functions of cholecalciferol, since these compounds do not have a deleterious effect on the metabolism of the vitamin to its more active forms. Also, the extrapolation of our data to the bird in the field is difficult. We are presently carrying out further experiments in an attempt to define the relationship of the known biological activities of vitamin D to the thin eggshell syndrome.

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# Metabolism of Cyclophosphamide by Sheep

Jerome E. Bakke,* Vernon J. Feil, Connie E. Fjelstul, and Edward J. Thacker

Eight sheep urinary metabolites from a single oral dose of cyclophosphamide-$^{14}C$ (I) have been either identified or characterized. These metabolites represented approximately 95% of the urinary radioactivity from ring-$^{14}$C-labeled I and 88% of the urinary radioactivity from side chain-$^{14}$C-labeled I. The major route of excretion was in the urine (70–90% of the dose) and the major route of metabolism was oxidation of the ring followed by hydrolysis.

Cyclophosphamide (I, Figure 1) is a nitrogen mustard derivative which has been used in the treatment of many types of cancer (Nissen-Meyer and Host, 1960; Sullivan, 1967; Haggard, 1967; Sutow, 1967). It also causes a temporary loss of hair. Homan *et al.* (1968) and Dolnick *et al.* (1969) have reported the application of this latter property to the defleecing of sheep.

The purpose of the present study was to determine the chemistry of the residues that would result from the use of cyclophosphamide as a chemical defleecing agent.

We have previously reported (Bakke *et al.*, 1971) the identification of two of the major urinary metabolites (Compounds II and VI, Figure 1). These resulted from oxidation of I at the 4 position of the tetrahydro-2*H*-1,3,2-oxazaphosphorine ring to yield 4-ketocyclophosphamide (II), and hydrolysis of II to yield the phosphorodiamidate (VI). These have been identified by Hill *et al.* (1970) and Struck (1971) in the urine from dogs given cyclophosphamide by intravenous injection.

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EXPERIMENTAL

**Purification of Cyclophosphamide.** A solution of 0.242 g (1.5 μCi) of side chain-labeled cyclophosphamide (New England Nuclear Corp., Boston, Mass.) and 3.758 g of unlabeled cyclophosphamide monohydrate in 6 ml of acetone was placed on a 2.9 × 17 cm column of acid-washed alumina (Merck 71695) packed in acetone. The column was eluted slowly with acetone–ethanol (9:1), and the eluents were monitored with a radioactive flow monitor equipped with a cerium-activated glass bead cell. The large peak that eluted was collected, except for its leading and trailing edges, and the solvents were evaporated with a flash evaporator at water aspirator vacuum. A small amount of 2-methyl-4-keto-2-pentanol (acetone dimer) which

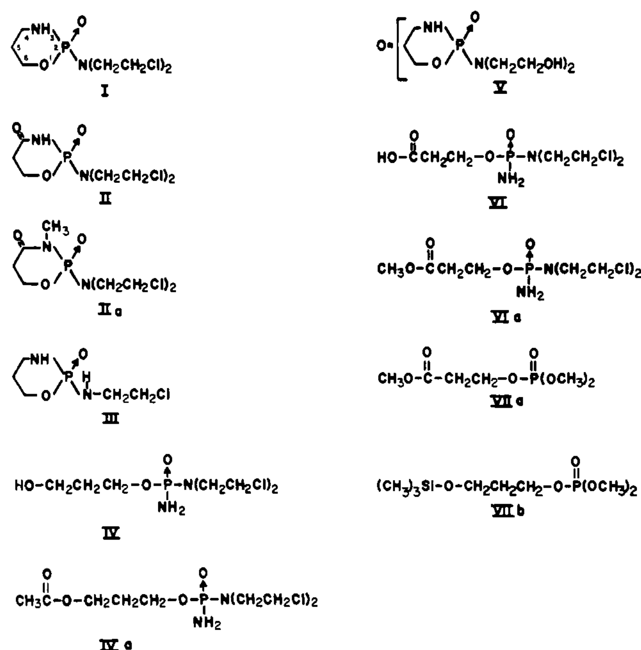

Figure 1. Structures of the metabolites and/or derivatives of the metabolites referred to in the text, Tables, and Figures

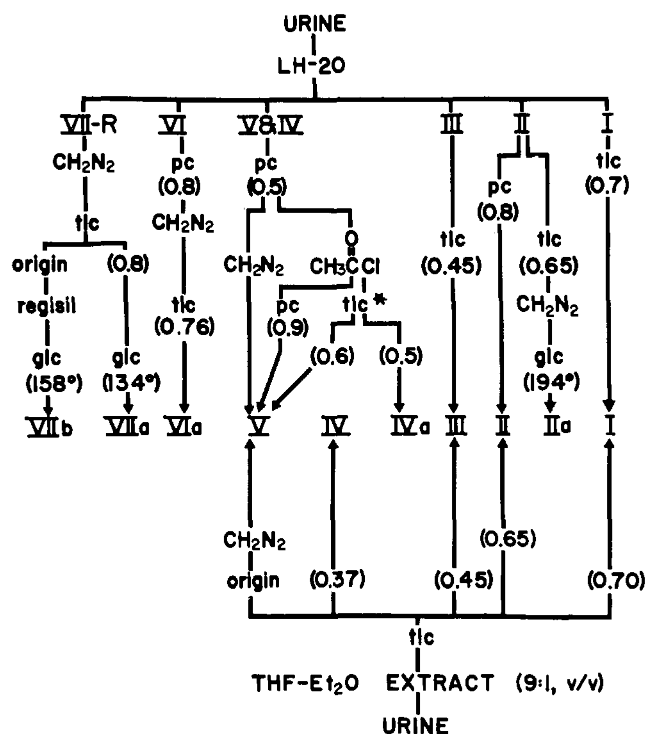


Figure 2. Flow diagram of the methods used for isolation of the sheep urinary metabolites from a single oral dose of cyclophosphamide. Values in parentheses represent either $R_f$ values or gas chromatography elution temperatures. The chromatographic methods are given in the text

was formed on the alumina column remained in the residue; this was removed by evacuating for 30 min at 0.1 Torr and 50 °C. The residue was then dissolved in ether, 120 μl of water was added, and small amounts of hexane were added while cooling on Dry Ice. After the first crop of 2.02 g of crystalline material was removed by filtration, an additional 50 μl of water was added to yield another 1.20 g of product. The combined crops of product melted at 45–48 °C. The addition of water was critical to the formation of crystalline product, since cyclophosphamide crystallizes readily only as a hydrate; in four runs with this purification method, using either ring- or side chain-labeled material, yields varied from 75 to 91%. The ring-$^{14}$C was located at the 4 position of the ring; the side chain-$^{14}$C label was located in the carbons having chlorine substituents.

**Synthesis of 2-(2-Chloroethyl)amino-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (III, Figure 1).** Reaction of propanolamine with 2-chloroethyldichlorophosphoramide (Drach and Sinitsa, 1968) under conditions similar to those of Friedman *et al.* (1963) for the preparation of cyclophosphamide (I) yielded III, mp 107–109 °C, from carbon tetrachloride.

*Anal.* Calcd for $C_5H_{12}ClN_2O_2P$: C, 30.23; H, 6.09; N, 14.11. Found: C, 30.11; H, 6.08; N, 13.94.

**Instrumentation and Quantitation of Radioactivity.** All column eluates were monitored using a Packard Model 320E liquid scintillation flow system. Radioactive components on paper and thin-layer chromatograms were located using a Packard Model 7200 chromatogram scanner. Quantitation of radioactivity was done by liquid scintillation in counting solution A (Bakke *et al.*, 1967) using a Nuclear-Chicago Mark I liquid scintillation spectrometer. Mass spectra were obtained with a Varian M-66 mass spectrometer equipped with a V-5500 control console, using the solid sample inlet system. Infrared spectra were obtained in micro-KBr pellets (10 mg KBr; 2 mm diameter pellets) using a Perkin-Elmer 337 infrared spectrometer equipped with a 4× beam condenser.

**Gel Filtration of Urinary Metabolites.** Sephadex LH-20 was equilibrated with water overnight and packed into two columns. The first column was a 110 × 2.5 cm column packed to 90 cm with LH-20. The second column was a 90 × 2.5 cm column fixed with low dead volume adapters and filled with LH-20. The bottoms of these columns were connected in series with 0.1 mm i.d. tubing so that the first column was eluted by descending elution into the bottom of the second column, which was eluted by ascending elution.

Samples (4-ml) of the sheep urine were applied to the column with nitrogen pressure, the sample was washed into the column with 2 ml of water, and the radioactivity eluted from the LH-20 with water pumped at 1.5 to 2 ml per min.

**Chromatographic Methods.** Paper chromatography (pc) and gas chromatography (glc) were performed as previously reported (Bakke *et al.*, 1971). Thin-layer chromatography (tlc) was done with precoated silica gel thin-layer plates (0.5-mm thickness, Brinkmann No. 5769/0020). Chromatographic plates were developed with chloroform–absolute ethanol (20:4, v/v), except in the one instance marked with an asterisk in Figure 2, where tetrahydrofuran was used.

**Isolation of Urinary Metabolites.** The flow diagram given in Figure 2 lists the combinations of methods applied to the isolation of each metabolite from the LH-20 column along with a more rapid method for the isolation of LH-20 fractions I–V by extraction of the urine with two volumes of a tetrahydrofuran–diethylether (9:1, v/v) mixture.

**Animal Treatment.** 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) cyclophosphamide-$^{14}$*C* (I). Rubber urinals on the wethers prevented contamination of urine with feces. The urine was collected at 4-hr intervals in an iced container. After assay for radioactivity in counting solution A (Bakke *et al.*, 1967), the urine was frozen in 4-ml aliquots and stored at −10 °C.

**Table I. Radioactivity Excreted in the Urine from Sheep Given Single Oral Doses of Cyclophosphamide-$^{14}C$**

| Collection interval, hr | % of $^{14}$C dose in the urine (cumulative) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ring-$^{14}$C | | | | Side chain-$^{14}$C | | | |
| | Sheep no. | | | | Sheep no. | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0–4 | *a* | *a* | *a* | 11.1 | 13.9 | *a* | 23.4 | 7.8 |
| 4–8 | 26.3 | 39.4 | 25.4 | 40.1 | 34.7 | 32.8 | 56.9 | 34.2 |
| 8–12 | 42.4 | 39.4[a] | 39.0 | 40.1 | 46.7 | 53.7 | 73.1 | 45.4 |
| 12–24 | 60.1 | 66.8 | 61.5 | 68.8 | 68.0 | 78.8 | 88.9 | 72.6 |
| 24–36 | *b* | 71.3 | 71.9 | 72.2 | *b* | 87.6 | 93.6 | 81.1 |
| 36–48 | | 72.7 | 75.5 | 79.8 | | 90.2 | 95.0 | 84.3 |
| 48–60 | | *b* | 77.7 | 81.4 | | *b* | 95.5 | 85.4 |
| 60–72 | | | 78.6 | 82.1 | | | 95.8 | 85.9 |
| 72–168 | | | *b* | 84.6 | | | *b* | 87.0 |

[a] No urine was excreted. [b] The experiments had been terminated.

RESULTS AND DISCUSSION

The excretions of radioactivity in the urine from sheep given single oral doses of either ring-$^{14}$C- or side chain-$^{14}$C-labeled cyclophosphamide (I) are given in Table I. In most cases the sheep did not urinate during the first 4 hr after dosing. The radioactivity from either label was, however, excreted mainly in the urine within the first 24 hr (60 to 90%). The urine samples used for the isolation of the metabolites were those collected during the first 12 hr after dosing.

The gel filtration column (LH-20) separated the urinary radioactivity from either ring-$^{14}$C- or side chain-$^{14}$C-labeled I into 11 radioactive fractions. The elution patterns and quantitation of the recovery of radioactivity in each fraction are given in Figure 3. All fractions from this column could be freeze-dried without loss of radioactivity.

The elution volumes of fractions I through VI from both $^{14}$C labels were identical. The quantities of fractions I through VI from both $^{14}$C labels excreted in the urine, based on the radioactivity applied to the column, were similar. Their migrations on paper or thin-layer chromatography were identical. As will be shown later, these fractions contained the same metabolites. The early fractions (VII–XI) from the column had different elution volumes, depending upon the position of the $^{14}$C label. Of this group, only fraction VII, containing ring-$^{14}$C-labeled metabolites (VII-R), was characterized.

The chromatographic data for each metabolite and/or its derivatives are given in the isolation flow diagram (Figure 2). The radioactive fraction listed as VII-R in Figure 2 was found only in urine from sheep given ring-labeled I. The remaining metabolites (I–VI) and/or derivatives of metabolites listed in Figure 2 were isolated from the urine of sheep given either $^{14}$C label. The roman numerals in the center of Figure 2 correlate the LH-20 fractions in Figure 3 with the mass spectra (Table II) obtained from the isolated metabolites, and with the identified or proposed structures for the metabolites and/or derivatives given in Figure 1. Where derivatives of metabolites were isolated, the roman numerals are followed by a letter.

Fraction I from the LH-20 column was identified by mass spectrometry to be cyclophosphamide.

The identification of the metabolites in sheep urine fractions II and VI have been previously reported (Bakke *et al.*, 1971). These have also been identified by Hill *et al.* (1970) and Struck (1971) in the urine from dogs given I intravenously.

A sample of synthetic VI (supplied by R. F. Struck), when methylated, gave a mass spectrum (VIa, Table II) identical with methylated VI isolated from sheep urine.

Authentic compound II and the corresponding sheep metabolite (Fraction II, Figure 3), when treated with diazomethane, would gas chromatograph. The mass and infrared spectra (Figure 4) of these methyl derivatives (compound IIa) trapped from the gas chromatograph were identical. It is assumed that the methyl group replaced the hydrogen on the nitrogen in the ring, since the infrared band at 1695 cm$^{-1}$ was present after methylation, and the strong bands at 1630 and 1615 cm$^{-1}$ in the spectra of the unmethylated compound disappeared upon methylation. The 1630 and 1615 cm$^{-1}$ bands were suggested by Hill *et al.* (1970) to arise from ketoenol tautomerism. Methylation at the ring nitrogen would effectively stop tautomerization, thereby stabilizing the carbonyl group, which is assumed to absorb at 1695 cm$^{-1}$.

Fraction III from LH-20 was shown to be cyclophosphamide, with one of the 2-chloroethyl groups replaced by a hydrogen (compound III, Figure 1). The synthesized metabolite had mass and infrared spectra (Figure 4) identical with those isolated from sheep urine.

Fractions IV and V were not completely separated on the LH-20 column and appeared as an unresolved doublet. The freeze-dried residue from these combined fractions (Fraction IV + V) gave only one radioactive component by paper chromatography ($R_f$ 0.5). Equal mixtures, based on radioactivity, of ring- and side chain-labeled fraction IV + V cochromatographed on paper at $R_f$ 0.5. This fraction (IV + V), when eluted from paper, was too contaminated to obtain any useful mass spectral data. Methylation of the radioactive fraction eluted from the paper with diazomethane apparently removed this contamination upon evaporation of the reaction mixture and/or released the radioactivity from some complex because subsequent mass spectrometry of the diazomethane-treated residue gave a molecular ion and a fragmentation pattern that could be interpreted (Spectrum V, Table II). The fragmentation pattern is compatible with structure V in Figure 1, due to the presence of a molecular ion

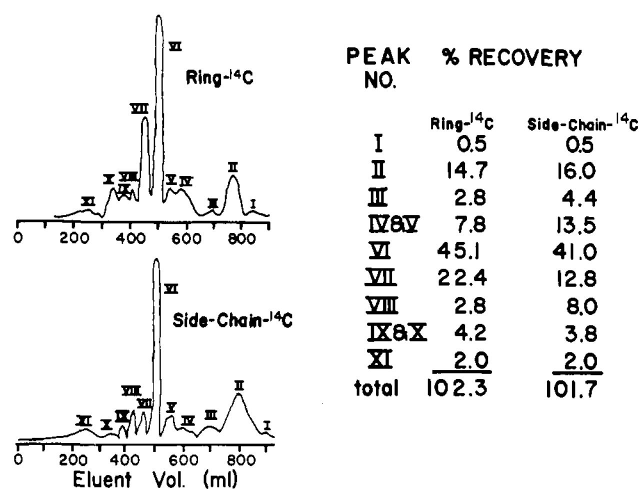


Figure 3. Gel filtration of the sheep urinary radioactivity after a single dose of ring-$^{14}$C-labeled and side chain-$^{14}$C-labeled cyclophosphamide. The fractions were numbered in an inverse consecutive order such that cyclophosphamide (the last fraction of the column) would be number I. Fractions VII through XI from ring-$^{14}$C and side chain-$^{14}$C did not have the same elution volumes; fractions I through VI had identical elution volumes. The percent recoveries are percent of total activity excreted in the urine

Table II. Mass Spectra of Cyclophosphamide Metabolites and Derivatives of the Metabolites[a]

| m/e | Relative abundance | Fragment description[b] |
|---|---|---|
| **I** | | |
| 260 (2 Cl) | 0.8 | $M^{\cdot +}$ |
| 225 (Cl) | 3 | $M^{\cdot +} - Cl$ |
| 224 (Cl) | 9 | $M^{\cdot +} - HCl$ |
| 211 (Cl) | 100 | $M^{\cdot +} - CH_2Cl$ |
| 175 | 98 | $M^{\cdot +} - (HCl + CH_2Cl)$ |
| 120 | 43 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 92 (Cl) | 26 | $[ClCH_2CH_2N(H)CH_2]^+$ |
| **II** | | |
| 274 (2 Cl) | 0.3 | $M^{\cdot +}$ |
| 239 (Cl) | 0.9 | $M^{\cdot +} - Cl$ |
| 238 (Cl) | 0.8 | $M^{\cdot +} - HCl$ |
| 225 (Cl) | 100 | $M^{\cdot +} - CH_2Cl$ |
| 189 | 23 | $M^{\cdot +} - (HCl + CH_2Cl)$ |
| 134 | 5 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 92 (Cl) | 39 | $[ClCH_2CH_2N(H)CH_2]^+$ |
| **IIa** | | |
| 288 (2 Cl) | 0.3 | $M^{\cdot +}$ |
| 253 (Cl) | 1.2 | $M^{\cdot +} - Cl$ |
| 239 (Cl) | 100 | $M^{\cdot +} - CH_2Cl$ |
| 203 | 1 | $M^{\cdot +} - (HCl + CH_2Cl)$ |
| 148 | 35 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 92 (Cl) | 19 | $[ClCH_2CH_2N(H)CH_2]^+$ |
| **III** | | |
| 198 (Cl) | 3.6 | $M^{\cdot +}$ |
| 163 | 2.4 | $M^{\cdot +} - Cl$ |
| 162 | 1.1 | $M^{\cdot +} - HCl$ |
| 149 | 100 | $M^{\cdot +} - CH_2Cl$ |
| 120 | 25 | $M^{\cdot +} - HNCH_2CH_2Cl$ |
| **IV** | | |
| 278 | | $M^{\cdot +}$ |
| 247 (2 Cl) | 2.2 | $M^{\cdot +} - CH_2OH$ |
| 242 (Cl) | 1.2 | $M^{\cdot +} - HCl$ |
| 229 (Cl) | 17.5 | $M^{\cdot +} - CH_2Cl$ |
| 221 (2 Cl) | 5 | |
| 212 (Cl) | 3.7 | 247 − Cl |
| 193 | 2.3 | $M^{\cdot +} - (CH_2Cl + HCl)$ |
| 185 (Cl) | 2.5 | |
| 171 (Cl) | 20.5 | |
| 138 | 100 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 135 | 4 | |
| 92 (Cl) | 34 | $[ClCH_2CH_2N(H)CH_2]^+$ |

| m/e | Relative abundance | Fragment description[b] |
|---|---|---|
| **IVa** | | |
| 320 | | $M^{\cdot +}$ |
| 284 (Cl) | 1 | $M^{\cdot +} - HCl$ |
| 271 (Cl) | 6.5 | $M^{\cdot +} - CH_2Cl$ |
| 247 (2 Cl) | 15.5 | $M^{\cdot +} - CH_2OCOCH_3$ |
| 235 | 2.5 | $M^{\cdot +} - (HCl + CH_2Cl)$ |
| 229 (Cl) | 10 | $M^{\cdot +} - (CH_2Cl + COCH_3)$ |
| 221 (2 Cl) | 5.5 | $[NH_2(OH)_2P\text{–}N(CH_2CH_2Cl)_2]^+$ |
| 193 | 4 | 229 − HCl |
| 185 (Cl) | 4 | 221 − HCl |
| 180 | 11 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 171 (Cl) | 21 | |
| 138 | 100 | $180 - CH_2{=}C{=}O$ |
| 135 | 14 | 171 − HCl |
| 101 | 100 | $[CH_3OCOCH_2CH_2CH_2O]^-$ |
| 92 (Cl) | 44 | $[ClCH_2CH_2N(H)CH_2]^+$ |
| **V** | | |
| 238 | <0.1 | $M^{\cdot +}$ |
| 221 | 1 | $M^{\cdot +} - OH$ |
| 207 | 2 | $M^{\cdot +} - CH_2OH$ |
| 193 | 7.5 | $M^{\cdot +} - CH_2CH_2OH$ |
| 134 | 20 | $M^{\cdot +} - N(CH_2CH_2OH)_2$ |
| 105 | 100 | $[HN(CH_2CH_2OH)_2]^+$ |
| **VIa** | | |
| 306 | | $M^{\cdot +}$ |
| 275 (2 Cl) | 2 | $M^{\cdot +} - OCH_3$ |
| 270 (Cl) | 1.5 | $M^{\cdot +} - HCl$ |
| 257 (Cl) | 6 | $M^{\cdot +} - CH_2Cl$ |
| 239 (Cl) | 1 | $M^{\cdot +} - (HCl + OCH_3)$ |
| 223 (Cl) | 3 | |
| 221 | 6.5 | $M^{\cdot +} - (HCl + CH_2Cl)$ |
| 185 (Cl) | 2 | |
| 171 (Cl) | 2.5 | |
| 166 | 100 | $M^{\cdot +} - N(CH_2CH_2Cl)_2$ |
| 135 | 9 | |
| 92 (Cl) | 10 | $[ClCH_2CH_2N(H)CH_2]^+$ |
| **VIIa** | | |
| 212 | | $M^{\cdot +}$ |
| 181 | 12 | $M^{\cdot +} - OCH_3$ |
| 153 | 35 | $M^{\cdot +} - COOCH_3$ |
| 127 | 100 | $[(CH_3O)_2P(OH)_2]^+$ |
| 109 | 42 | $[(CH_3O)_2PO]^+$ |
| **VIIb** | | |
| 256 | 10 | $M^{\cdot +}$ |
| 241 | 100 | $M^{\cdot +} - CH_3$ |
| 225 | 5 | $M^{\cdot +} - OCH_3$ |
| 153 | 5 | $M^{\cdot +} - CH_2OSi(CH_3)_3$ |

[a] Structures given in Figure 1. [b] Fragment descriptions are tentative assignments.

at *m/e* 238 and its associated losses of OH, $CH_2OH$, and $CH_2CH_2OH$. The presence of a fragment at *m/e* 134 indicated either the presence of an oxidized ring as was found for metabolite II or that the ring nitrogen had been methylated. Methylation of the ring nitrogen is not expected, however, because diazomethane would not methylate cyclophosphamide or metabolite III.

Assuming that the oxidized ring is present in metabolite V, the methylation study also indicated that the oxidation did not take place at the 4 position, for if the hydrogen on the ring nitrogen of metabolite II could be replaced with a methyl group from diazomethane, it would be expected that this metabolite would methylate and give a fragment for the ring at *m/e* 148 rather than at *m/e* 134. It is possible that the ring is oxidized at another position, *i.e.*, carbons 5 or 6, either of which would not produce a mixed diamide having a proton reactive to diazomethane. Attempts to purify the metabolite for infrared analysis to determine the presence of absorption in the carbonyl region have been unsuccessful.

The structure of the side chain moiety of metabolite V can be inferred from the presence of the intense fragment ion at *m/e* 105. This ion, which would correspond to the molecular ion for diethanolamine, could arise from either a fragmentation with rearrangement of a proton or from thermal decomposition of the proposed metabolite. The thermal decomposition is not indicated since the fragmentation pattern from known diethanolamine was not present in the mass spectrum.

Attempts to acylate the proposed metabolite (metabolite V) with acylation reagents (trifluoroacetic anhydride, acetic anhydride, and acetyl chloride) were unsuccessful. Treatment of the eluate from paper chromatography with acetyl chloride

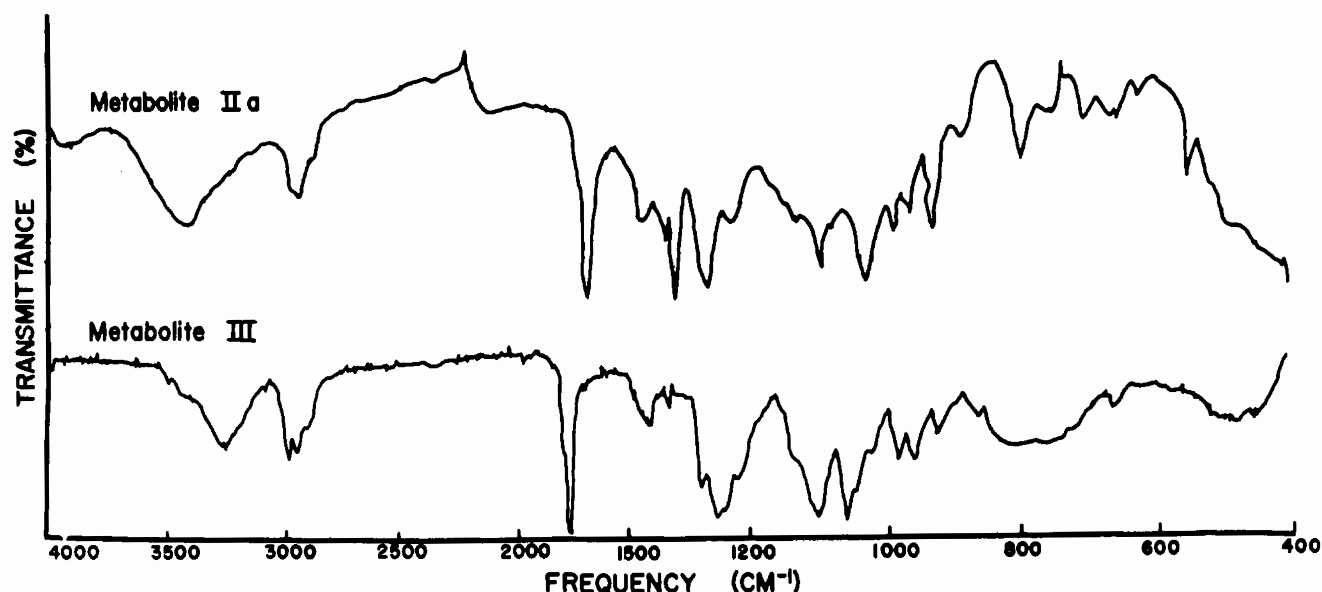


**Figure 4. Infrared spectra of Metabolites IIa and III**

and evaporating the reaction mixture to dryness changed the paper $R_f$ from 0.5 to 0.9 but had no effect on the mass spectrum. The second metabolite in LH-20 fraction IV + V was isolated by treatment of the fraction with acetyl chloride. Thin-layer chromatography of the treated sample, after removal of the excess reagent, separated two components. One component ($R_f$ 0.6) again gave mass spectrum V; the other component gave mass spectrum IVa. Mass spectrum IVa contained fragment ions with chlorine isotope peaks, and the fragmentation pattern was compatible with the mass spectrum expected for an acetyl derivative of one of the ring-opened forms of cyclophosphamide.

The underivatized form of metabolite IVa, along with I and metabolites II, III, and Va, was isolated by tlc from the tetrahydrofuran–diethylether extracts of sheep urine (Figure 2). Metabolite V again had to be treated with diazomethane to obtain mass spectrum V. Metabolite IV migrated with an $R_f$ of 0.37 on tlc and gave mass spectrum IV. The molecular ion and fragmentation pattern are consistent with a ring-opened form of cyclophosphamide (I) and also consistent with that expected from the unacetylated analog of compound IVa isolated from the LH-20 column eluate, as described above. The ring of cyclophosphamide could hydrolyze to yield three isomers, of which two would be acids and one a phosphoramide. The latter isomer, structure IV (Figure 1), was assigned to the metabolite because neither the mass spectrum nor the migrations on paper or thin-layer chromatograms changed upon treatment of this metabolite with diazomethane, which should have methylated the acid functions of the other two isomers.

Two metabolites (VIIa and VIIb) were isolated as derivatives from LH-20 fraction VII-R from the urine of sheep given the ring-$^{14}C$-labeled I. These two derivatives were separated as the methyl esters by tlc of the diazomethane-treated LH-20 fraction. The compound with the $R_f$ of 0.8 gave a mass spectrum and had a glc elution temperature identical with synthetic VIIa (Bakke *et al.*, 1971). The methylated metabolite at the tlc origin after silanizing (Regisil) and gas chromatography gave a mass spectrum (VIIb) compatible with the silyl ether of 3-dimethylphosphoryl propanol (VIIb, Figure 1). The major component in this LH-20 fraction was VIIa.

Cyclophosphamide, its metabolites, and metabolite derivatives, which accounted for 96% (60–90% of the dose) of the radioactivity, excreted in the urine from sheep given single oral doses of ring-$^{14}C$-labeled cyclophosphamide, were either identified (compounds I, II, III, VI, and VIIa, Figure 2) or characterized (compounds IV, V, and VIIb, Figure 1) by mass spectrometry. Of these only those represented by LH-20 fraction VII did not contain radioactivity from side chain-labeled cyclophosphamide.

The uncharacterized fractions (fractions VII–XI) in the urine from sheep dosed with side chain-labeled I had small, but apparently real, differences in elution volumes from those observed for the ring-labeled fractions VII through XI. These fractions possibly represent either hydrolysis products of the other metabolites or incorporation of $^{14}C$ into natural products. None of these are *nor*-nitrogen mustard, since it elutes from the LH-20 column in the area of metabolites I and II.

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