

## DISCUSSION

The examination showed that, following dermal treatment, the active component is absorbed through the skin. However, from the results it can be derived that percutaneous absorption plays only a subordinate role in relation to the amount of Hoe 2910 which enters the body. More important is the oral intake of the active component, for example with the bath liquid, because of the good enteral absorption. But even with the selected high dose of 2.5 mg of Hoe 2910 per kg of body weight, which corresponds to an intake of almost 2.5 l. of the commercial 0.05% bath liquid by a cow, no adverse

effects were seen because orally administered Hoe 2910 was eliminated so rapidly and completely that already a short time after the treatment the residues found were very low. This especially applies to the organs intended for human consumption.

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## Metabolism of Bis(chloromethyl) Sulfone in Sheep and Cattle

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Bis(chloromethyl) sulfone is metabolized in sheep and cattle to form carbon dioxide in exhaled air. Chloromethanesulfinic and chloromethanesulfonic acids were identified in urine. Radioactive uric

acid and urea in urine and amino acids with carboxyl carbon labeling in liver and kidney tissues were identified as secondary metabolites.

**B**is(chloromethyl) sulfone is of interest for its effect on rumen fermentation. Recent work in this field is described by O'Connor *et al.* (1971) and Prins and Seekles (1968). A study of the metabolites present in the urine of sheep and cattle following oral dosing of the compound has been carried out. In addition, the nature of radioactive residues in the liver and kidney of sheep has also been investigated.

## METHODS

**Radiometric Methods.** Bis(chloromethyl) sulfone-<sup>14</sup>C was prepared by treating trithiane-<sup>14</sup>C with sulfur dichloride to form bis(chloromethyl) sulfide-<sup>14</sup>C (Mann and Pope, 1923). The latter was oxidized with hydrogen peroxide. For the animal studies a specific activity of 0.3 to 0.6  $\mu$ Ci/mg was employed. The radioactivity of urine samples and of isolated metabolites was determined by methods described by Tocco *et al.* (1965).

**Animal Studies.** For the isolation work described in this paper, a wether lamb weighing 31.5 kg was dosed orally with one capsule twice a day for 2 days. Each capsule contained 125 mg of bis(chloromethyl) sulfone-<sup>14</sup>C at 0.6  $\mu$ Ci/mg, a total of 500 mg and 300  $\mu$ Ci.

A Hereford steer weighing 264 kg was dosed with 1.2 g/day of bis(chloromethyl) sulfone-<sup>14</sup>C of specific activity 0.3  $\mu$ Ci/mg for 4 days by mixing the compound in the feed. The animal was preconditioned by feeding 0.03% of unlabeled compound in the diet for 5 days prior to dosing. Animals were housed in metabolism cages for separate and total collection of urine and feces.

Urine was collected and frozen until examined. Liver and

kidney from sheep were collected and frozen at sacrifice 4 days after dosing.

**Collection of Carbon Dioxide from Sheep and Steer.** Carbon dioxide was collected from a wether lamb for 4 days after receiving a single dose of 525 mg of bis(chloromethyl) sulfone-<sup>14</sup>C. The lamb was housed in an air-tight chamber which was ventilated at a rate of 60 l. of air per min. A small side stream of the expired air (2%) was drawn through alkali. A sample of this solution was acidified and the resulting CO<sub>2</sub> was trapped in Hyamine and counted by liquid scintillation procedures. A steer was similarly fitted with a mask and intermittent samples of expired air were obtained over a 4-day period after the animal received 15 g of the compound orally. Carbon dioxide was obtained and analyzed as described above. The total quantity of <sup>14</sup>C expired as <sup>14</sup>CO<sub>2</sub> was calculated by multiplying the average rate of expiration over the time increment between sampling periods.

**Separation Methods.** Urine from steer and sheep was fractionated by the scheme outlined in Figure 1. The pH 8 methylene chloride extract contained the unchanged drug ( $K_D$ , [solvent/water] = 14). The amount of intact sulfone present was determined by reverse isotope dilution analysis (Barker *et al.*, 1970). The aqueous effluent from Dowex 1 $\times$ 2 (Cl<sup>-</sup>) chromatography was evaporated to a small volume of water from which uric acid crystallized. Alternatively, when the effluent from Dowex 1 $\times$ 2 (Cl<sup>-</sup>) was passed through a column of Dowex 1 $\times$ 2 (OH<sup>-</sup>), the uric acid was retained and the aqueous effluent yielded crystalline urea upon evaporation.

Elution of the Dowex 1 $\times$ 2 (Cl<sup>-</sup>) column was accomplished with either 1 M pyridine hydrochloride at pH 2.5 or with methanolic hydrochloric acid prepared by mixing concentrated hydrochloric acid and methanol (1:9). The eluate, consisting of strongly acidic metabolites, was extracted with ether, which removed a small amount of radioactivity (about 10%) which

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was not further characterized. A rough separation of chloromethanesulfinic acid from chloromethanesulfonic acid was achieved by the ethyl acetate extraction of the former from the Dowex 1×2 (Cl<sup>-</sup>) eluate.

**Countercurrent Distribution, Thin-Layer Chromatography.** Purification of the chloromethanesulfinic acid in the ethyl acetate extract (Figure 1) was achieved by countercurrent distribution between *n*-butanol and 0.1 *N* hydrochloric acid (12 stages) ( $K_D \sim 1$ ). Center-cut fractions from this distribution were evaporated and the residue was subjected to thin-layer chromatography on an Analtech silica gel G plate (2000  $\mu$ ). The  $R_f$  value is 0.3 with an acetone-water (96:4) developing system. Chloromethanesulfinic acid was eluted from silica gel with methanol. The methanol was evaporated and the residue in water was passed through a short column of Dowex 50×2 (H<sup>+</sup>) to remove extraneous cations. The resin effluent was converted to the benzhydramine salt. This salt was obtained for identification as a noncrystalline solid of about 75% purity.

**Urease Assay.** In order to confirm that the radioactivity found in isolated urea was present as <sup>14</sup>C in the molecule, an assay was carried out with urease. The crystalline urea isolated from urine was dissolved in 0.2 *M* pH 7 phosphate buffer and digested with jack bean meal (Calbiochem) for 1 hr at 37° in a modified Warburg flask. The mixture was acidified with 1 *N* hydrochloric acid and incubated overnight at 37°, allowing absorption of the carbon dioxide in 1 ml of Hyamine in the absorption cup. The Hyamine was then counted by scintillation counting under the usual control conditions.

**Identification and Determination of Chloromethanesulfonic Acid in Urine.** In a typical experiment 10 ml of sheep urine (containing 30,000 cpm by scintillation count) was treated with 173.5 mg of benzhydrammonium chloromethanesulfonate. The mixture was diluted with water to 20 ml and passed over a Dowex 50×2 (H<sup>+</sup>) column (100–200 mesh) followed by copious water wash to give 90 ml of aqueous effluent with quantitative recovery of radioactivity. This effluent was then adsorbed on a column of Dowex 1×2 (Cl<sup>-</sup>) resin (100–200 mesh) and washed until the radioactivity of the effluent dropped to essentially zero. The column was eluted with 100 ml of a mixture of methanol and concentrated hydrochloric acid (9:1), giving 18,000 cpm in the eluate.

The aqueous acid eluate was evaporated *in vacuo* to an acidic liquid residue which was subjected to thin-layer chromatography on an Analtech Silica gel G prep plate (20 × 20 cm) (2000  $\mu$ ) developed with a mixture of ethanol (2B absolute), water, and ammonium hydroxide (7 *N*) (100:20:4.5). The radioactive zone at  $R_f$  0.9 was scraped from the plate and eluted with methanol. The solvent was replaced by water and the acidic solution was titrated with benzhydramine to pH 6.4. The aqueous solution was treated with 52 mg of charcoal and the filtrate was evaporated *in vacuo*. The crystalline residue of benzhydrammonium chloromethanesulfonate was recrystallized from acetonitrile to constant count-to-weight ratio.

**Ninhydrin Reaction.** The radioactivity incorporated into the 1-carboxyl group of amino acids was determined by the ninhydrin reaction. The reaction was carried out by the method of Schach von Wittenau (1967). Liver and kidney tissues were used for these experiments.

**Synthesis of Metabolites.** CHLOROMETHANESULFONIC ACID was prepared by the method of Schoellkopf *et al.* (1963) from methylene chloride and sodium sulfite. The sodium salt was converted to the free acid which was, in turn, converted

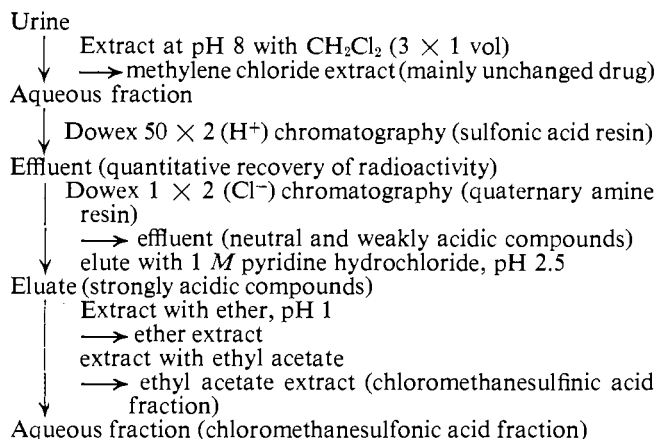


Figure 1. Urine fractionation scheme

to the benzhydramine salt and crystallized from acetonitrile, mp 173–175°.

*Anal.* Calcd for C<sub>14</sub>H<sub>16</sub>NSClO<sub>3</sub>: C, 53.58; H, 5.14; S, 10.22; N, 4.46. Found: C, 53.65; H, 5.04; S, 10.41; N, 4.43.

CHLOROMETHANESULFINIC ACID was prepared by the method of McGowan (1884) from chloromethanesulfonyl chloride and sulfur dioxide in ethanol at -5°. Isolation of the chloromethanesulfinic acid as the benzhydramine salt gave a crystalline product contaminated by chloromethanesulfonic acid. Nmr spectrometry indicated it to be an approximately 1:1 mixture. Separation of the two acids was achieved on a silica gel plate using acetone-water (96:4) as the developing solvent. Elution of the chloromethanesulfinic acid ( $R_f$  0.3) and conversion to the benzhydramine salt again gave a mixture of similar character. It is apparent that oxidation takes place during the manipulations. Mass spectrometric evidence was unequivocal for the identity of the chloromethanesulfinic acid.

**Gas Chromatography and Mass Spectrometry.** Gas chromatography was carried out with a Barber-Colman Model 5000 instrument (hydrogen flame ionization detector). A 6 ft × 4 mm i.d. glass column containing 5% SE-30 packing was temperature programmed from 70 to 200° at 10°/min. The LKB Model 9000 instrument was employed for direct probe mass spectrometry and combined gas chromatography-mass spectrometry. The column conditions were essentially the same as those described above. The spectrometer conditions included: electron energy, 70 eV; source temperature, 270°; accelerating voltage, 3.5 kV; trap current, 60  $\mu$ A. Trimethylsilylation was achieved by treating the sample with bis(trimethylsilyl)acetamide (BSA) or BSA-*d*<sub>18</sub> for 15 hr at room temperature.

## RESULTS AND DISCUSSION

**Identification of Metabolites.** CHLOROMETHANESULFONIC ACID. Chloromethanesulfonic acid was found on tlc to possess the same  $R_f$  as one of the strongly acidic metabolites in the Dowex 1×2 (Cl<sup>-</sup>) eluate. Reverse isotope dilution analyses were carried out on this eluate and on urine samples directly. Chloromethanesulfonic acid was found to account for 25% of the radioactivity in the 24-hr sheep urine and 12% of the radioactivity in the 24-hr steer urine.

CHLOROMETHANESULFINIC ACID. The crude benzhydramine salt of the isolated chloromethanesulfinic acid was dissolved in deuteriomethanol for examination by nmr spectroscopy. The spectrum showed resonance at  $\tau$  6.35 with reference to tetramethylsilane. For comparison, resonance

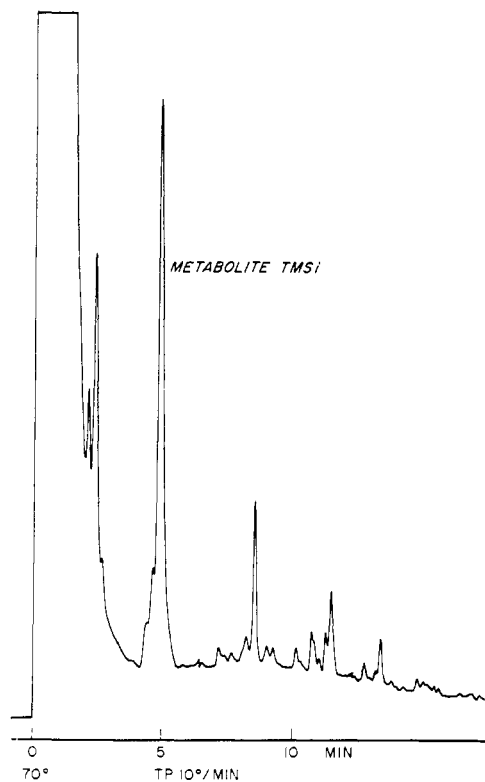


Figure 2. Gas chromatogram resulting from analysis of trimethylsilylated (BSA) acidic metabolite (eventually shown to be chloromethanesulfonic acid). Column conditions given in experimental section

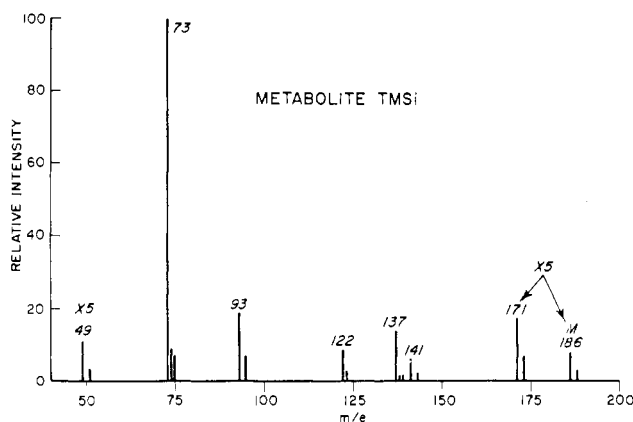


Figure 3. Mass spectrum of trimethylsilylated (BSA) acidic metabolite obtained *via* combined gas chromatography-mass spectrometry. Instrument conditions given in experimental section

was observed at  $\tau$  5.7 for the benzhydrylamine salt of a synthetic sample of chloromethanesulfonic acid.

Further confirmation of structure was obtained by treating the deuteriomethanol solution of chloromethanesulfonic acid with excess 30% hydrogen peroxide. Disappearance of the  $\tau$  6.35 resonance and simultaneous development of the  $\tau$  5.7 resonance was observed over a 48-hr period at room temperature.

The direct probe mass spectrum of the unknown acidic metabolite displayed pairs of signals exhibiting the monochloro isotope cluster at  $m/e$  114/116 and 49/51. An intense ion of  $m/e$  65 was also observed. When the metabolite fraction was subjected to trimethylsilylation and then analyzed by gas chromatography, a major peak was observed at a

retention time of 5 min (Figure 2). This sample was then analyzed by combined gas chromatography-mass spectrometry; the mass spectrum of the major component is presented in Figure 3. The molecular ion with monochloro isotope cluster was found at  $m/e$  186/188, an increase of 72 mass units (hence monotrimethylsilylation). Other fragment ions exhibiting the monochloro isotope cluster include those at  $m/e$  49/51 and 171/173, the latter corresponding to loss of a methyl group from the molecular ion. A chlorine-free fragment was observed at  $m/e$  137. These data are consistent with ions expected from  $\text{ClCH}_2\text{SO}_2\text{H}$ , chloromethanesulfonic acid (and its trimethylsilyl ester). The mass spectrum of the acid (molecular weight 114) would be expected to show a molecular ion of  $m/e$  114/116 and fragment ions of  $m/e$  49/51  $[\text{ClCH}_2]^+$  and 65  $[\text{SO}_2\text{H}]^+$ , resulting from scission of the carbon-sulfur bond. The trimethylsilyl ester ( $m/e$  186/188) should yield analogous fragment ions of  $m/e$  49/51 and 137  $[\text{SO}_2\text{Si}(\text{CH}_3)_3]^+$ . The ion of  $m/e$  122 is  $m/e$  137 minus a methyl group. A sample of synthetic chloromethanesulfonic acid was converted to the trimethylsilyl ester and analyzed by combined gas chromatography-mass spectrometry. Both its retention time and spectrum were identical to those of the trimethylsilylated metabolite. In order to further verify the structure of the chloromethanesulfonic acid and to identify additional fragment ions of the trimethylsilyl ester, the acid was derivatized with bis(trimethylsilyl)acetamide- $d_{18}$  (McCloskey *et al.*, 1968) and the resulting trimethylsilyl- $d_9$  ester was subjected to combined gas chromatography-mass spectrometry. The observed shifts (trimethylsilyl  $\rightarrow$  trimethylsilyl- $d_9$ ) in  $m/e$  values (186/188  $\rightarrow$  195/197, 171/173  $\rightarrow$  177/179, 137  $\rightarrow$  146, 122  $\rightarrow$  128, 73  $\rightarrow$  82,  $[\text{Si}(\text{CH}_3)_3]^+ \rightarrow [\text{Si}(\text{CD}_3)_3]^+$ ) 49/51  $\rightarrow$  49/51) confirm the proposed ion structures. The  $m/e$  values of the two unidentified fragment ions from the trimethylsilyl ester,  $m/e$  141/143 and 93/95, were each observed to shift by 6 mass units, to 147/149 and 99/101, respectively. These are apparently rearrangement ions,  $[\text{ClSi}(\text{CH}_3)_2]^+$  and  $[\text{ClSi}(\text{CH}_3)_2\text{OS}]^+$ .

**CARBON DIOXIDE.** About 16% of dosed  $^{14}\text{C}$  was expired as  $\text{CO}_2$  in the sheep in 4 days, whereas in the steer the analogous value was only 4.5%. A considerable amount of radioactivity, 0.05% of the dose/day, was still in the expired air of both animals after the 4 days. Previous results with rats had indicated 10–20% of the dose was expired as  $^{14}\text{CO}_2$ . These data indicate that the compound was extensively broken down by the animals.

**AMINO ACIDS.** A further indication of the extensive breakdown is pointed out by the nature of radioactivity remaining in tissues of sheep dosed with the compound. Ninhydrin oxidation of liver and kidney tissue yielded 2 and 4%, respectively, of the radioactivity as  $\text{CO}_2$ , presumably present in the 1-carboxyl group of amino acids.

**Urea and Uric Acid.** Evaporation of the Dowex 1  $\times$  2 ( $\text{OH}^-$ ) aqueous effluent from sheep urine left a crystalline residue of urea which was recrystallized from ethyl acetate to melt at 130–133°. Scintillation count was 36 cpm/mg. Determination of radioactive carbon dioxide by urease digestion gave a value of 34 cpm/mg. Determination of radioactivity released as carbon dioxide by urease in urine gave a value of about 1% of total urinary radioactivity.

Uric acid was obtained as a crystalline residue upon evaporation of the effluent from Dowex 1  $\times$  2 ( $\text{Cl}^-$ ). A sample recrystallized from water was identified by comparison of its uv spectrum with that of authentic uric acid ( $\lambda_{\text{max}} = 295 \text{ nm}$ , purity 85%). Combustion analysis gave a specific activity of 315 cpm/mg.

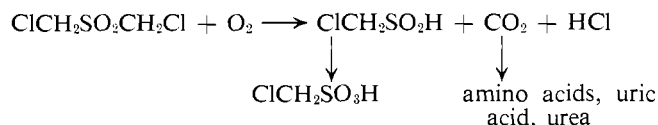
Table I. Separation of Metabolites in Urine

	Sheep urine, %	Steer urine, %
Unchanged bis(chloromethyl) sulfone	2	15
Weak acid and neutral fraction (uric acid and urea, etc.)	11	18
Strongly acid fraction (Dowex 1×2 [Cl <sup>-</sup> ] adsorbate)	70	66
A. Ether-soluble strong acid fraction (unidentified)	3	10
B. Ethyl acetate-soluble strong acid fraction (mainly chloromethane- sulfinic acid)	30	24
C. Water-soluble strong acid fraction (mainly chloromethanesulfonic acid)	34	25
Excretion of radioactivity by animals		
Urine	17	43
Feces	3	8

Further proof of the identity of the urinary uric acid was furnished by a tlc comparison with an authentic sample using methods described by Gordon *et al.* (1962). Brinkmann silica gel F-254 plates (5 × 20 cm) were spotted with 50–60 μg each of: (1) urinary uric acid sample; (2) reference uric acid sample; and (3) a mixture of the two samples. The plates were then developed in the three solvent systems. The developed plates were observed in uv light, and the uv absorbing spot from sample 1 was scraped into a counting vial for scintillation counting.

In the tlc comparison, identical  $R_f$  values were observed for all three samples with each solvent system. The uv absorbing spots from sample 1 contained essentially all the radioactivity placed on the plates.

**Metabolic Pathway.** The metabolic products identified in the urine of ruminants are consistent with the following metabolic pathway.



The identification of chloromethanesulfinic acid establishes it as an essential intermediate which is easily oxidized to chloro-

methanesulfonic acid. Whether this latter oxidation is metabolic or artifactual (taking place after the urine is voided) is unknown. Because of the unstable nature of the chloromethanesulfinic acid, the amounts of this metabolite in urine and the oxidation product, chloromethanesulfonic acid, are difficult to determine. A rough separation of these two was made by extraction of an acidic aqueous solution with ethyl acetate, with chloromethanesulfinic acid being the more solvent soluble. Table I contains data on the amounts of the various fractions which were separated by the scheme of Figure 1. Extensive degradation of bis(chloromethyl) sulfone resulted in the formation of CO<sub>2</sub>, some of which was incorporated into amino acid, uric acid, and urea. Thus the "nonmetabolite" products (Rosenblum, 1965) become an important part of the overall metabolism of this compound.

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