mono- and di-TMS derivatives of 2-methoxy-4,6-diamino-*s*-triazine (structure 5a, Figure 1; mass spectrum 5a, Table II).

During gas chromatography of the TMS derivative of metabolite 2, radioactivity bled from the column from 160° (the elution temperature for di-TMS-5a) until the peak appeared at 174°. The mass spectrum (2a, Table II) of this bleed prior to the peak at 174° was identical to that of di-TMS-5a (2-methoxy-4,6-diamino-*s*-triazine), indicating that the tri-TMS derivative of 2 is converted to di-TMS-5a during gas chromatography. It has not been excluded that this bleed is the di-TMS derivative of 2-hydroxy-4-amino-6-methylamino-*s*-triazine.

The tri-TMS derivative of metabolite 2 (2, Table II) had a molecular ion at *m/e* 373, with the most intense fragment ion at *m/e* 358 ($M\cdot^+ - 15$) and a fragment ion at ($M\cdot^+ - 31$). These ions had isotope clusters typical for silicon-containing ions, and their relative intensities indicated the presence of three silicon atoms. Subtracting three TMS groups would give the underivatized metabolite a mol wt of 157. The fragment ion at $M\cdot^+ - 31$ indicated the presence of a methoxyl moiety. The $M\cdot^+$ at an odd mass requires the presence of an odd number of nitrogens.

The evidence that the apparent mol wt of 2 (157) is 16 greater than that of 5a suggests that a metabolite is obtained from metabolite 2, whose structure is some hydroxylated form of 2-methoxy-4,6-diamino-*s*-triazine (structure 5a, Figure 1) or 2-hydroxy-4-amino-6-methylamino-*s*-triazine. A hydroxymethylamino group, a possibility from the latter structure, was not indicated, since most of the fragment ions in the mass spectrum of the TMS derivative of 2-chloro-4-hydroxymethylamino-6-isopropylamino-*s*-triazine (Bakke *et al.*, 1972) were attributed to fragmentations and/or rearrangements in the trimethylsilyloxymethylamino group, with the base peak in the spectrum being $[NHCH_2OSi(CH_3)_3]^+$. The corresponding fragment ions were not present in the mass spectrum of metabolite 2. A 2-TMS-methylenedioxy group was not indicated, since the free hemiacetal would not be stable under the ion-exchange conditions (approximately 2 hr at 65° and pH 3). This leaves the possibilities of either a hydroxyl on one of the ring nitrogens or a hydroxylamine structure.

The mass spectrum from the TMS derivative of metabolite 4 (4, Table I) had a molecular ion at *m/e* 313. Although this mass would suggest the di-TMS derivative of fraction 10, the fragmentation pattern and isotope peak intensity were not compatible with that structure. The M + 1 isotope peak intensity for the $M\cdot^+$ at 313 indicated the presence of one TMS moiety. This would give the underivatized metabolite a mol wt of 241, which is 16 amu greater than that for prometone, and indicates the metabolite is some oxygenated form of prometone. The location of the oxygen could not be determined from the fragment ions. It could not be present as a TMS ether of a primary alcohol on one of the isopropyl groups, for this structure gives rise to a large $M\cdot^+ - 103$ fragment ion. The base ion fragment at $M\cdot^+ - 73$ $[M\cdot^+ - Si(CH_3)_3]$ and the metastable ion at $M\cdot^+ - 89$ $[M\cdot^+ - OSi(CH_3)_3]$ lead one to speculate the existence of a $NOSi(CH_3)_3$ structure on either a ring or an alkyl nitrogen.

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Metabolic Studies with Chloropropylate Acaricide in the Dairy Cow

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Excretion and metabolism of the acaricide chloropropylate (isopropyl 4,4′-dichlorobenzilate) was studied in a dairy cow. At a level of 5 ppm in the feed for 4 days, 0.11 and 5.93% of the compound was excreted intact in the milk and feces, respectively. Major elimination occurred in the urine with excretion of 28.13 and 55.23% of the total chloropropylate dose, respectively, as 4,4′-dichlorobenzilic acid and conjugates of the latter compound. Excretion of 4,4′-dichlorobenzilic acid in feces represented 5.44% of the dose. Chloropropylate was stable in rumen fluid but it decomposed in the presence of the 10,000 × *g* supernatant fraction of beef liver.

Chloropropylate (isopropyl 4,4′-dichlorobenzilate) is an effective acaricide for control of various mites on apples and pears. The use of pesticides on fruit always raises the possibility of cattle forage contamination through drift or residues in fruit pomace, which is sometimes used in dairy rations. Investigations in this laboratory have shown that virtually all of the chloropropylate in apples remains in the pomace after juice expression (Gutenmann and Lisk, 1972). No published work has appeared on the fate of chloropropylate in the bovine. Bourke *et al.* (1970) studied the fate of chloropropylate in rats. Using the carbon-14 (acetate)-labeled compound administered *via* stomach tube and measuring total radioactivity, elimination was shown to occur in the feces (64.5%) and urine (5.1%), with tissue storage in the liver (8.9%) and gastrointestinal tract (8.4%) and minor amounts in other organs and expired carbon dioxide. In the work reported, a study of the passage of chloropropylate from apples into pomace and the fate of the pure compound in a lactating cow has been made.

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EXPERIMENTAL SECTION

Feeding Experiment. A Holstein cow weighing 500 kg and with a daily average milk production of 15.5 kg was catheterized and fed chloropropylate at the 5-ppm level (based on a daily ration of 22.7 kg) for 4 days. The pure compound in acetone was thoroughly mixed with the evening grain. Morning and evening subsamples of the total mixed milk were taken 1 day prior to feeding (control sample) daily throughout the feeding period and for 6 days thereafter. The total daily urine and manure samples were similarly collected, weighed, mixed, and subsampled during the same test period. The manure samples were collected in specially constructed trays. All samples were immediately frozen prior to analysis.

In Vitro STUDIES

Rumen Fluid. The stability of chloropropylate in the presence of fresh rumen fluid was studied. One milliliter of a solution of chloropropylate in acetone (500 μg/ml) was thoroughly mixed with 100 ml of freshly filtered rumen fluid and held at 38°. At measured intervals, 5 ml of fluid was removed and immediately mixed with 5 ml of acetone and filtered. The filtrate was diluted to 25 ml with acetone. Five milliliters of the diluted filtrate was partitioned with 5 ml of *n*-hexane and 90 ml of 2% sodium sulfate solution. The upper hexane solution was analyzed by electron affinity gas chromatography.

Liver. Possible metabolism of chloropropylate was studied in the presence of the 10,000 × *g* supernatant fraction of fresh beef liver which contains microsomes and soluble enzymes. An Angus steer was sacrificed and the liver was immediately removed. A portion was immersed in 0.25 *M* sucrose solution at 0° and all further processing for enzyme preparation was conducted in the cold (0–4°). A 20% liver homogenate in the sucrose solution was prepared using a Dounce homogenizer. The homogenate was centrifuged at 10,000 × *g* max for 30 min. Incubation mixtures contained 10 μg of the acaricide (20 μl of a 500 μg/ml solution in ethyl acetate), 25 μmol of magnesium chloride, 95 μmol of Tris buffer, pH 7.4, 20 μmol of glucose 6-phosphate, 1.5 μmol of TPN (NADP), and 1 ml of the enzyme (10,000 × *g* supernate) preparation in a total volume of 5 ml. Incubations were carried out in a 25-ml Erlenmeyer flask at 37° in an atmosphere of air for 30 min. The flasks were mechanically shaken 100 times/min on a reciprocating shaker during incubation. (These samples as well as the controls, which included either no enzyme or no substrate, were carried through the procedure in triplicate.) After 30 min the reactions were terminated by the addition of 3 ml of acetone and each incubation mixture was transferred to a 100-ml volumetric flask using 2 ml of acetone for rinsing. Hexane (5 ml) was added, the flask was made to volume with 2% sodium sulfate solution, and it was shaken vigorously for 1 min. The layers were separated by centrifugation. The upper hexane layer was analyzed for chloropropylate by electron affinity gas chromatography. Analysis was also performed for 4,4′-dichlorobenzilic acid as a possible hydrolytic metabolite of chloropropylate. This was accomplished by acidification of the final acetone solution (that used to stop the enzyme reaction) with 5 ml of concentrated HCl and analysis as in the procedure described further on for this metabolite in urine.

EXTRACTION AND ISOLATION OF CHLOROPROPYLATE

Milk. The determination of residues of chloropropylate in milk was performed by an adaptation of the method of Ciba-Geigy Corp. (Analytical Bulletin No 9, 1970). It consisted of hexane extraction, partitioning chloropropylate into acetonitrile, isolation using column chromatography on alumina, and analysis by electron affinity–gas chromatography.

Urine and Feces. Twenty-five grams of sample were extracted by blending with acetone. The mixture was filtered and the filter was rinsed with acetone until 100 ml of filtrate was collected. Ten milliliters of the extract was partitioned with 5 ml of hexane and 85 ml of 2% sodium sulfate. The upper hexane solution was analyzed by electron affinity–gas chromatography.

EXTRACTION AND ISOLATION OF 4,4′-DICHLOROBENZILIC ACID METABOLITE

A possible metabolite resulting from hydrolysis of chloropropylate is 4,4′-dichlorobenzilic acid. Urine and feces were analyzed for it.

Urine. Five grams of urine was acidified with 1 ml of 4 *N* hydrochloric acid and then partitioned with 5 ml of diethyl ether and 90 ml of saturated sodium chloride. After centrifugation the upper ether layer was removed and concentrated to 2 ml and 0.22 ml of methanol was added. The solution was then methylated by the procedure of Schlenk and Gellerman (1960) and analyzed by electron affinity–gas chromatography.

Feces. Ten grams of feces was acidified with 20 ml of 6 *N* hydrochloric acid. After being thoroughly mixed and allowed to stand for 20 min, the mixture was shaken with 10 ml of ether and 50 ml of saturated sodium chloride. The mixture was centrifuged and 2 ml of the ether was removed and mixed with 0.22 ml of methanol. This solution was then methylated as above and analyzed by gas chromatography.

EXTRACTION AND ISOLATION OF CONJUGATES OF 4,4′-DICHLOROBENZILIC ACID FROM URINE

Five grams of urine was heated at 100° for 20 min with 5 ml of concentrated hydrochloric acid. After cooling, the solution was partitioned with 5 ml of ether and 85 ml of saturated sodium chloride. The ether solution was separated by centrifugation and methylated as above prior to gas chromatographic analysis. The extent of conjugation was determined by the difference between the amount of 4,4′-dichlorobenzilic acid extracted before hydrolysis and the quantity extracted after.

Table I. Daily Elimination of Chloropropylate and Metabolites (Expressed as Equivalent Chloropropylate) in Body Secretions as Percent of Total (454 mg) Dose

Days	Chloropropylate in milk	4,4′-Dichlorobenzilic acid in urine[a]	4,4′-Dichlorobenzilic acid conjugates in urine	Chloropropylate in feces	4,4′-Dichlorobenzilic acid in feces
1[b]	nd[c]	nd	nd	nd	nd
2	nd	1.03	1.36	nd	nd
3	nd	4.48	4.97	0.74	0.09
4[d]	nd	4.80	10.78	1.29	1.59
5	0.03	7.42	22.77	2.02	0.60
6	0.07	7.19	13.57	1.31	1.83
7	0.01	2.24	1.17	0.32	0.48
8	nd	0.53	0.15	0.15	0.15
9	nd	0.22	0.25	0.10	0.10
10	nd	0.22	0.21	nd	nd
Total	0.11	28.13	55.23	5.93	5.44
				Grand total	94.84

[a] Chloropropylate itself was not detectable in urine. [b] First day of feeding chloropropylate. [c] Not detectable. [d] Last day of feeding chloropropylate.

Table II. Recovery of Chloropropylate and 4,4′-Dichlorobenzilic Acid from Control Samples

Sample	Chloropropylate		Estimated sensitivity, ppb	4,4′-Dichlorobenzilic acid		Estimated sensitivity, ppb
	Added, ppm	Recovery, %		Added, ppm	Recovery, %	
Milk	0.05	60, 77	2			
	0.1	85, 80				
Urine	1.0	81, 93, 108	20	0.2	106	
				2.0	94	20
Urine (hydrolyzed)				0.4	150	20
				1.0	100	
				2.0	65	
Feces	0.2	80	20	1.0	100, 84	50
	1.0	92				
Rumen fluid	5	85, 100	50			
Liver 10,000 × g supernate	5	84, 80, 100	100			

GAS CHROMATOGRAPHIC ANALYSIS

Final analysis was made using a Barber-Colman Model 10 gas chromatograph equipped with an electron affinity detector. The detector was a battery-operated No. A-4071, of 6 cm^3 volume and containing 56 μCi of radium-226. The recorder was a Wheelco, 0 to 50 mV, equipped with 10-in. chart paper running 10 in./hr. The electrometer gain was 10,000. The columns were U-shaped, made of borosilicate glass, and 6 mm i.d. The column for chloropropylate was 0.92 m long and contained 10% DC-200 on 80 to 100 mesh Gas Chrom Q. The column for 4,4′-dichlorobenzilic acid (methyl ester) was 1.83 m long and contained 5% Carbowax 20M on 80 to 100 mesh Chromosorb W. The latter column caused slower elution of the 4,4′-dichlorobenzilic acid methyl ester and efficient separation from earlier eluting matrix compounds. The operating temperatures for the columns, flash heater, and detector were 200, 250, and 245°, respectively, and nitrogen (60 cm^3/min) was the carrier gas. The retention times for chloropropylate and 4,4′-dichlorobenzilic acid methyl ester were 4.8 and 20.3 min, respectively.

RESULTS AND DISCUSSION

Table I summarizes the occurrence of chloropropylate and metabolites as found in body secretions. Residues of intact chloropropylate in small amounts were indicated in milk. The concentrations in milk on days 5, 6, and 7 of the experiment were 9, 18, and 4 ppb, respectively. Since residues of chlorinated hydrocarbons in milk up to 0.3 ppm (in butterfat) are not necessarily actionable (Kilpatrick, 1972) and since considerable dilution of residues in milk normally occurs during plant processing, these concentrations of chloropropylate in milk would probably be reduced far below actionable levels. Although orchard drift contamination of adjacent forage by chloropropylate could conceivably reach levels of 5 ppm (the dose level used in this study), this would undoubtedly occur only in isolated instances. If apple pomace containing 5 ppm of chloropropylate was fed to cows, it would only constitute a portion (usually up to about 50%) of the cattle ration. At the present time there is little chance of milk contamination from this latter source since feeding pomace to cows is still illegal in New York owing to the continuing presence of other fat-soluble chlorinated hydrocarbons in it.

The major portion of chloropropylate was excreted in urine as 4,4′-dichlorobenzilic acid (28.13%) and conjugates of the latter compounds (55.23%), with smaller quantities of chloropropylate (5.93%) and its acid metabolite (5.44%) eliminated in feces. These results would tend to confirm the speculation of Horn *et al.* (1955) that chlorobenzilate (ethyl 4,4′-dichlorobenzilate), a very similar compound, might be excreted by dogs or rats as the hydrolysis product 4,4′-dichlorobenzilic acid.

Chloropropylate was found to be stable in rumen fluid when incubated up to 7 hr. The compound (partially) decomposed or was rendered unextractable in liver with only 42 to 46% of intact chloropropylate determinable after 30 min. No peaks were noted to indicate production of 4,4′-dichlorobenzilic acid or other metabolites. These findings indicate that chloropropylate is stable in the rumen but undergoes either decomposition or other reactions in liver, which may or may not be related to the other observed routes of excretion. Table II lists the recoveries of chloropropylate and 4,4′-dichlorobenzilic acid added before extraction to control samples.

As shown in Table I, 94.84% of the total amount of chloropropylate fed was accounted for. Small amounts of the compound may have been deposited in body tissues. The cow used was not sacrificed and therefore tissue analysis was not performed. It is also probable that small additional quantities of 4,4′-dichlorobenzilic acid were excreted freely and conjugated in the urine after urine collection ended on the tenth day of the experiment (see Table I).

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