Metabolic Studies with Chloroneb Fungicide in a Lactating Cow

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The cotton fungicide, Chloroneb (1,4-dichloro-2,5-dimethoxybenzene), was fed to a dairy cow. No residues of Chloroneb were detected in milk or feces when 5 p.p.m. were fed in the cattle ration. A metabolite was produced when the fungicide was incubated with the 10,000-G supernatant fraction of beef liver. Its gas chromatographic retention time was identical to that of the 2,5-dichloro-4-methoxyphenol, a known metabolite of Chloroneb in animals. A peak having the identical retention time of this phenol was also observed in hydrolyzed urine samples which, assuming it was the phenol, represented 44.3% of the total Chloroneb dose. The fungicide was stable in the presence of rumen fluid.

Chloroneb (1,4-dichloro-2,5-dimethoxybenzene) is the active ingredient of du Pont's Demosan 65 W fungicide formulation for control of cotton seedling diseases. The possibility of residues of the compound in cottonseed meal used in dairy rations prompted this investigation of its metabolism in a lactating cow. The presence of a metabolite of Chloroneb, 2,5-dichloro-4-methoxyphenol, in animals has been reported in a paper describing an analytical method based on microcoulometric gas chromatography for determining both compounds (Pease, 1967). In the work reported, electron affinity gas chromatography has been applied to analysis of these compounds in milk, urine, feces, rumen fluid, and liver preparations.

EXPERIMENTAL PROCEDURE

A Holstein cow was catheterized and fed the fungicide at the 5 p.p.m. level (based on a daily ration of 22.7 kg. for four days). The pure, recrystallized compound in absolute ethyl alcohol was thoroughly mixed with the grain. Morning and evening subsamples of the total mixed milk were taken one day before feeding (control sample), daily throughout the feeding period, and for six 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.

Analysis of milk, urine, and feces for Chloroneb was as follows: Twenty-five grams of the sample was extracted by blending with 60 ml. of acetone and 1 ml. of orthophosphoric acid. (For extraction of feces 10 grams of Supercel and 20 ml. of water were added prior to blending.) The mixture was filtered and rinsed with acetone to a total volume of 100 ml. The filtrate was partitioned successively with 50, 20, and 10 ml. of hexane, and the combined hexane extracts were evaporated to about 20 ml. using a gentle air stream. One teaspoon of anhydrous sodium sulfate was added to the solution, and the solution was mixed and filtered. The filtrate was evaporated as above and made to a final volume of exactly 10 ml. with hexane. The solution was analyzed by electron affinity gas chromatography. The column was 1.83 meters long, containing 10% OV-17 on 80- to 100-mesh Gas Chrom Q and operated at 180° C. The instrument and other operating parameters were identical to those described earlier (Gutenmann *et al.*, 1963). The retention time of Chloroneb was 10.6 minutes.

Analysis of the phenolic metabolite of Chloroneb, 2,5-dichloro-4-methoxyphenol, possibly present as a conjugate in urine, was as follows: One gram of urine was mixed with 4 ml. of concentrated hydrochloric acid and the solution was allowed to stand for 3 hours. The solution was carefully neutralized with 50% sodium hydroxide solution with chilling (ice bath) to pH 9.6. The solution was transferred to a 50-ml. volumetric flask using 2 to 3 ml. of water for rinsing. Five milliliters of a 20% solution of ethyl acetate in diethyl ether was added, and the flask was made to volume with saturated sodium chloride. The mixture was shaken vigorously, and the upper organic layer was analyzed by gas chromatography according to the procedure described for Chloroneb. The retention time of the phenol was 7.5 minutes.

The following procedure was used to analyze feces for phenolic conjugates: The acetone was evaporated from a 4-ml. portion of the acetone extract of each feces sample using a gentle air stream until the solvent could no longer be detected by odor. Remaining water in the sample was not evaporated. Four milliliters of concentrated hydrochloric acid was added, and the solution was allowed to stand for 3 hours. The remainder of the procedure was the same as that used above for analysis of phenolic conjugates in urine.

Stability of Chloroneb was studied when incubated with rumen fluid. The compound in 1 ml. of ethanol was thoroughly mixed with 100 ml. of freshly filtered rumen fluid (5 p.p.m.) and held at 38° C. At measured intervals, 5 ml. of the fluid were withdrawn and immediately mixed with 5 ml. of acetone. The mixture was filtered and the filter rinsed with acetone to a total volume of 25 ml. Five milliliters of this solution was removed, 40 ml. of 2% sodium

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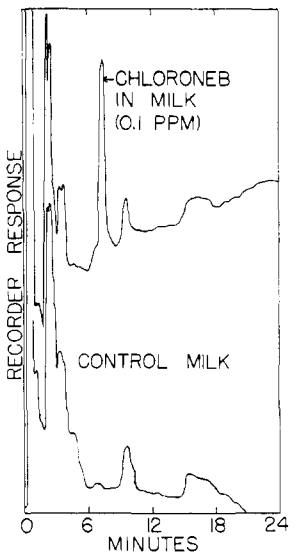

Figure 1. Chromatograms of recovery of 0.1 p.p.m. of Chloroneb from milk and control milk

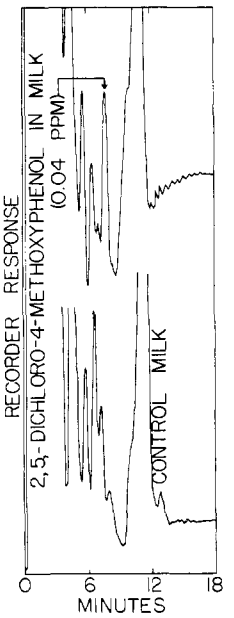

Figure 2. Chromatograms of recovery of 0.04 p.p.m. of 2,5-dichloro - 4 - methoxyphenol from milk and control milk

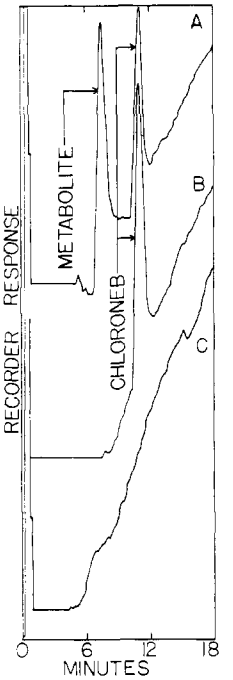

Figure 3. Chromatograms showing (A) remaining Chloroneb and production of metabolite after incubation for 1 hour with 90,000 G supernate of beef liver, (B) corresponding control without enzyme, and (C) corresponding control without substrate (Chloroneb)

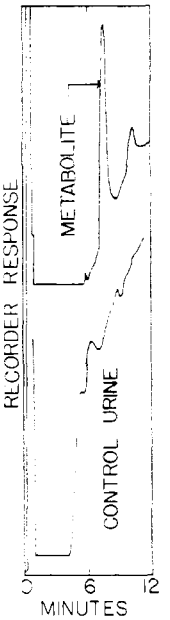

Figure 4. Chromatograms showing Chloroneb metabolite in hydrolyzed urine one day after feeding began and control urine

sulfate solution was added, and the mixture was partitioned with 5 ml. of hexane. The hexane solution was analyzed by gas chromatography as described.

The stability of Chloroneb in the presence of the 10,000-G supernate fraction of fresh beef liver was studied. An Angus steer was sacrificed and the liver was immediately removed. A portion was immersed in 0.25*M* sucrose solution at 0° C. and all further processing for enzyme preparation was conducted in the cold (0–4° C.). 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 minutes. Incubation mixtures contained 5 μg. of Chloroneb (100 μl. of a 50 μg. per ml. solution in acetone), 5 μmoles of nicotinamide, 25 μmoles of magnesium chloride, 140 μmoles of phosphate buffer, pH 7.4, 20 μmoles of glucose-6-phosphate, 1.5 μmoles of TPN, and 1 ml. of the enzyme (10,000-G supernate) preparation in a total volume of 5.0 ml. Incubations were carried out at 37° C. in an atmosphere of air for 1 hour. (These samples as well as the controls which included either no enzyme or no substrate were carried through the procedure in triplicate.) After 1 hour the reactions were terminated by the addition of 3 ml. of acetone and each incubation mixture was filtered into a 100-ml. volumetric flask and rinsed with 5 ml. of acetone. Five milliliters of ethyl acetate was added, and the flask was made to volume with saturated sodium chloride solution. The upper organic layer was appropriately diluted and analyzed by gas chromatography as described.

RESULTS

No peaks representing residues of intact Chloroneb or the free phenol were observed in unhydrolyzed milk, urine, or feces samples. Chloroneb was stable when incubated with rumen fluid up to 6 hours. Figures 1 and 2 show chromatograms of the recoveries, respectively, of Chloroneb and 2,5-dichloro-4-methoxyphenol from milk and of the corresponding control samples. The recoveries of Chloroneb and its phenol from samples are listed in Table I. The sensitivity of the method for Chloroneb or the phenol was about 0.02 p.p.m.

Chloroneb disappeared in the 10,000-G supernate fraction of beef liver with the production of a new peak having a retention time identical to 2,5-dichloro-4-methoxyphenol. Chloroneb decreased from a total of 5 μg. per 5 ml. incubate to a range of 2.0 to 2.5 μg. during 1 hour of incubation. Assuming the new peak was 2,5-dichloro-4-methoxyphenol, it reached a concentration of 3.1 to 3.5 μg. per 5 ml. of incubate in 1 hour. Figure 3 shows chromatograms of Chloroneb and the metabolite in the total incubation mixture and of the controls containing either no enzyme or no substrate.

The Chloroneb metabolite was not detected in the hydrolyzed fecal samples. In hydrolyzed urine, however, a peak again with the same retention time as 2,5-dichloro-4-methoxyphenol appeared in those samples collected on the first,

Table I. Recovery of Chloroneb and Its Phenol in Bovine Tissues, Fluids, and Excreta

	Chloroneb		Phenol	
Sample	Added, p.p.m.	Recovery, %	Added, p.p.m.	Recovery, %
Milk	0.1	80	0.04	88, 75
	0.2	85, 65	1	70
Urine	0.2	60, 65, 55	5	104, 108, 104, 102
Feces	0.6	73	5	95
Rumen fluid	5.0	84		
Liver 10,000-G supernate	5.0	96, 96, 96		

second, third, and fourth days after feeding of Chloroneb began. Assuming the peak represented the phenol, its excretion as a conjugate in the urine represented 44.3% of the total Chloroneb dose (454 mg.). In Figure 4, chromatograms of the metabolite are illustrated in hydrolyzed urine 1 day after the feeding of Chloroneb began and control urine.

The fate of the remaining 55.7% of the Chloroneb dose is unknown. A metabolite such as 1,4-dichloro-2,5-dihydroxybenzene resulting from conversion of both methoxy groups to phenols should have been able to be detected since it would probably have separated on the OV-17 column and would have possessed sufficient electron affinity for detection. Metabolism involving hydroxylation or reduction with elimination of one or both chlorine atoms would have produced a degradation product with insufficient detector response to be observed.

In the hydrolysis procedure developed for isolation of the metabolite from urine, room temperature digestion of urine with hydrochloric acid for 3 hours was satisfactory. Digestion up to 24 hours did not increase the quantity of the metabolite found. Heating the acidified urine resulted in complete loss of the metabolite presumably by volatilization. A pH of 9.6 was optimum for extraction of the metabolite into the ethyl acetate–ether solution.

Federal tolerances of 2 p.p.m. of Chloroneb in or on cotton forage and vines (forage) of beans and soybeans, 0.1 p.p.m. in or on beans, cottonseed, soybeans, and sugarbeets (roots and tops) and 0.05 p.p.m. in milk were recently established (Kirk, 1969). The feeding level (5 p.p.m.) used in this study would therefore represent an exaggerated dose.

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