The Excretion of Hexachlorophene in the Dairy Cow

Leigh E. St. John, Jr. and Donald J. Lisk*

A feeding experiment was conducted to study the excretion and possible metabolism of hexachlorophene in a lactating cow. At a level of 5 ppm in the daily ration, no residues of hexachlorophene were found in milk. Excretion of the intact compound in the urine and feces accounted, respectively, for 0.24 and 63.8% of the total dose. Hexachlorophene did not decompose when incubated with rumen fluid and beef liver 10,000 × *g* supernate.

Hexachlorophene [2,2′-methylene-bis-(3,4,6-trichlorophenol)] is a compound commonly added to soap as a bactericide and used in skin preparations as a disinfectant. It is also effective for controlling fungus and bacterial diseases of plants such as *Helminthosporium* blight of corn and bacterial spot of tomatoes. Gump (1969) has reviewed the toxicological properties of the compound. Recent concern has been expressed about absorption of the compound through skin and possible resultant neurological damage (*Chem. Eng. News*, 1971).

Excretion of the compound has been studied in feeding studies with rats, rabbits, and cattle (Wit and Van Genderen, 1962) and in humans and cats (Chung *et al.*, 1963). From these studies it appears that hexachlorophene is mainly excreted unchanged in the feces with minor urinary excretion of the intact compound. No excretion of the compound in cow's milk was noted, but the sensitivity of the method used was only 0.8 ppm. In the work reported, hexachlorophene was fed to a dairy cow to study its possible elimination in milk and excreta and stability in the presence of rumen fluid and liver enzymes.

EXPERIMENTAL PROCEDURE

A Holstein cow weighing 572 kg and with an average daily milk production of about 16 kg (4.1% butterfat) was catheterized and fed hexachlorophene at the 5 ppm level (based on a daily ration of 22.7 kg) for 4 days. This fungicide concentration was considered a maximum level which could occur on foraged crops as a result of direct spray applications or drift contamination. 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 hexachlorophene in the presence of fresh rumen fluid was studied. One milliliter of a solution of hexachlorophene in acetone, 500 μg per ml, was thoroughly mixed with 100 ml of fresh filtered rumen fluid and held at 38°C. At measured intervals up to 7 hr, 5 ml of fluid were removed and transferred to a 15-ml centrifuge tube containing 5 ml of acetone. The resulting mixture was filtered into a 25-ml volumetric flask and the flask was made to volume with acetone. A 4-ml aliquot of the aqueous–acetone filtrate was transferred to a 10-ml volumetric flask and the acetone was evaporated with air (as judged by odor). Two milliliters of benzene were added to the remaining solution, the flask was made to volume with 2% sodium sulfate solution, and the contents were shaken vigorously. The mixture was centrifuged and 1 ml of the benzene layer was transferred to a glass-stoppered 8-ml test tube and evaporated to near dryness. The residue was dissolved in 0.22 ml of methanol, 2 ml of diethyl ether were added to the tube, and the sample was methylated by bubbling diazomethane gas into the solution for 10 min by the procedure of Schlenk and Gellerman (1960). The solution was then evaporated with air, the residue redissolved in 2 ml of ethyl acetate, and up to 5 μl was analyzed by gas chromatography. The methylation and gas chromatographic operating parameters described here have been previously used for analysis of hexachlorophene in various agricultural commodities (Gutenmann and Lisk, 1970).

Liver. Possible metabolism of hexachlorophene was studied in the presence of the 10,000 × *g* supernatant fraction of fresh beef liver which contains microsomes and soluble enzymes. One-tenth microgram of hexachlorophene was incubated for 1 hr using the procedure described by Gutenmann and Lisk (1969). Following incubation the reaction mixture was quenched with 3 ml of acetone and then filtered into a 25-ml volumetric flask. The filter was rinsed with a small amount of acetone and the acetone evaporated with air. The remainder of the procedure, consisting of partitioning hexachlorophene into benzene followed by methylation and gas chromatography, was conducted as described for rumen fluid, beginning with the evaporation of the acetone filtrate solution.

EXTRACTION, ISOLATION AND ANALYSIS OF HEXACHLOROPHENE IN OTHER BODY FLUIDS

Milk and Urine. Twenty-five grams of milk or urine was blended with 65 ml of acetone in an ice bath for 3 min. The mixture was filtered and the filter was rinsed with acetone until the total filtrate volume was 100 ml. The filtrate was transferred to a 250-ml separatory funnel and partitioned successively with 50, 25, and 10 ml of chloroform. After each partitioning the lower chloroform–acetone layer was filtered through anhydrous sodium sulfate and the filtrates were combined in a 300-ml round-bottomed flask and evapo-

Pesticide Residue Laboratory, Department of Entomology, Cornell University, Ithaca, New York 14850.

Table I. Daily Excretion Pattern of Hexachlorophene in Urine and Feces

Day	% of total dose (454 mg) excreted	
	Urine	Feces
1[a]	Nd	Nd
2	Nd	0.6
3	Nd	8.6
4[b]	0.03	8.7
5	0.05	17.1
6	0.06	17.9
7	0.05	7.2
8	0.03	2.3
9	Nd	0.9
10	0.02	0.5
	Total 0.24	63.8

[a] First day of feeding hexachlorophene. [b] Last day of feeding hexachlorophene. Nd, not detectable.

Table II. Recovery of Hexachlorophene from Samples

Sample	Added, ppm	Recovery, %	Sensitivity, ppm
Milk	0.02	60	0.02
	0.04	80	
	0.08	120	
Urine (unhydrolyzed)	0.04	68	0.02
Urine (hydrolyzed)	0.2	71, 83	0.05
Feces	0.08	125	0.1
	0.4	80	
	1.2	100	
	3.2	100	
Rumen fluid	5	116, 96	
Liver 10,000 × *g* supernate	5	110	

rated to dryness using rotary evaporation in a 40°C water bath. The residue was dissolved in exactly 10 ml of ethyl acetate. A portion of the ethyl acetate solution (5 ml) was evaporated in a 10-ml volumetric flask and the residue methylated as described for rumen fluid, beginning with the addition of 0.22 ml of methanol.

Feces. Twenty-five grams of feces was taken for analysis. The extraction procedure was basically that used for milk and urine, with certain exceptions. Celite 545 (10 g) was added to the blender and the mixture was filtered by suction. Water (20 ml) was added to the 100 ml of acetone filtrate resulting from the initial feces extraction. The residue remaining after evaporation of the chloroform solution was dissolved in 25 ml of carbon tetrachloride, 18 g of a mixture of sulfuric acid on Celite 545 (6 ml per 7 g) was added, and the mixture was allowed to stand with intermittent swirling for 20 min. The mixture was filtered by suction through a 0.25-in. pad of Celite 545 and the filter was rinsed with 50 ml of carbon tetrachloride. The filtrate was evaporated by rotary evaporation and the residue redissolved in 10 ml of ethyl acetate. Five milliliters of the ethyl acetate solution was evaporated in a 10-ml volumetric flask and the residue was methylated and chromatographed.

HYDROLYSIS OF URINE FOR POSSIBLE CONJUGATES OF HEXACHLOROPHENE

Five grams of urine was mixed with 5 ml of concentrated hydrochloric acid in a 50-ml volumetric flask and allowed to stand at room temperature overnight. Acetone (1 ml) and 10 ml of 20% ethyl acetate in diethyl ether were added, the flask was made to volume with water, and the contents were shaken vigorously. After centrifuging, the upper organic layer was transferred to a 10-ml volumetric flask, evaporated, and the residue methylated and chromatographed.

DETERMINATION

Final analysis for hexachlorophene was done by electron affinity gas chromatography. The gas chromatograph was a Barber-Colman Model 10 equipped with a battery operated No. A-4071 electron affinity detector 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. per hr. The electrometer gain was 10,000. Nitrogen (60 cm^3 per min) was the carrier gas. The column was U-shaped, made of borosilicate glass, 6 mm i.d., 2 ft long, and containing 10% DC-200 on 80 to 100 mesh Gas Chrom Q. The operating temperatures of the column, detector, and flash heater were, respectively, 205, 235, and 248°C. The retention time for hexachlorophene was 10.3 min.

RESULTS AND DISCUSSION

No residues of hexachlorophene were detected in milk. About 0.24 and 63.8% of the total dose of hexachlorophene fed (454 mg) was excreted intact in the urine and feces, respectively. The daily urinary and fecal excretion patterns are presented in Table I. No attempt was made to correct these data for percent recovery. No increase in the amount of hexachlorophene excreted in urine was observed following acid hydrolysis, therefore indicating the absence of conjugates. The sulfuric acid–Celite procedure was necessary to sufficiently isolate hexachlorophene from feces. Hexachlorophene did not decompose in the presence of rumen fluid for 7 hr or when incubated with the beef liver 10,000 × *g* supernatant fraction for 1 hr. Table II lists the percent recoveries and estimated sensitivities (based on a 10% full scale recorder deflection) of the method for various samples.

The results of this study thus confirm those reported previously for hexachlorophene in cattle and other animals (Wit and Van Genderen, 1962; Chung *et al.*, 1963). Major elimination of the compound occurs in feces with only minor urinary excretion. It is interesting that Wit and Van Genderen (1962) reported elimination of 63% of the total single oral dose of hexachlorophene in the feces of cows, whereas we found 63.8%.

Residues of the compound were found to be absent in milk in our study using a method with 40 times the sensitivity of that reported previously (Wit and Van Genderen, 1962). These workers administered one oral dose to each of five cows at a level of about 20 times that used by us. They analyzed only the milk collected 24 hr following dosing and hexachlorophene was not detected.

The fate of the remainder of the hexachlorophene dose is in question. In a study with ^{14}C-labeled hexachlorophene, Wit and Van Genderen (1962) reported 24.5 to 41.8% of the oral dose present in urine and feces of rabbits, and 26.4 to 35.3% of the oral dose in the feces of rats as unidentified metabolites of hexachlorophene based on measurements of total radioactivity. It is therefore possible that other metabolites of hexachlorophene were present which were not detected by gas chromatography.

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Metabolism of Xenobiotics in Ruminants. IV.

Storage and Excretion of HEOD in Holstein Cows

Kim A. Wilson* and Robert M. Cook

The recovery of HEOD in milk, urine, blood, feces, and body fat was determined when the pesticide was orally administered in gelatin capsules to Holstein cows at the level of 0.1 mg/kg of body weight for 3 and 6 weeks. Forty to 50% of the pesticide could be accounted for as HEOD at the end of 6 weeks. Most of this was in the feces. HEOD was not detected in urine. By analyzing wet instead of dry feces, HEOD levels were four to seven times higher than when dried feces were analyzed. The main route of HEOD elimination from the body was through the feces and not through the milk. Fifty to 60% of the HEOD unaccounted for was believed to be in the form of hydroxylated metabolites. Thus, 90% of the pesticide can be accounted for in the feces and as metabolites. Phenobarbital lowered HEOD levels in milk and body fat.

As discussed in a previous publication in this series (Cook, 1970), the contamination of livestock with chlorinated pesticides is a serious problem in animal agriculture. In addition to the presence of the parent pesticide compound, metabolites of some pesticides are present in meat and milk. It has been clearly established that DDT is changed in the environment to two major metabolites, DDD and DDE, and these metabolites are present in the blood and tissues of animals and man. Metabolites of DDT are easy to measure. However, metabolites of other pesticides that are hydroxylated are not readily detected. Several years ago HEOD was shown to be metabolized to several different compounds in rats and rabbits (Korte and Arent, 1965; Klein *et al.*, 1968). Recently it was shown that HEOD is readily metabolized to several metabolites in sheep (Hedde *et al.*, 1970). The fact that HEOD is metabolized in the sheep suggests that metabolites of this insecticide may be present in animal food products.

We conducted a HEOD balance trial using lactating Holstein cows with two objectives: to determine the recovery of an oral dose of HEOD in milk, blood, urine, feces, and body fat and the effect of phenobarbital on these measurements; and to determine the recovery of HEOD metabolites in milk, blood, urine, feces, and body fat and the effect of phenobarbital on these measurements. The first phase of the study has been completed and the results are reported in this communication.

Dairy Science Department, Michigan State University, East Lansing, Michigan 48823.

EXPERIMENTAL PROCEDURE

Two groups of four lactating Holstein cows each were contaminated orally for 3 weeks with HEOD at the level of 0.1 mg per kg of body weight per day. During the second 3-week period, two cows in each group continued to receive the pesticide, while the remaining two cows were not given HEOD (Table I). In addition, phenobarbital was superimposed on one group of four cows for the entire 6-week period at the level of 10.0 mg per kg of body weight per day. Daily samples of milk, feces, and urine were collected during the experimental period. During weeks 2 and 6 total urine was collected for 5 and 6 days, respectively. Body fat samples were obtained at 0, 2, 4, and 6 weeks of the experiment; blood samples were obtained at 0, 2, 4, and 5 weeks. All samples were stored at −20°C until prepared for analysis. The animals were fed a ration of hay, corn silage, and grain that was balanced to meet the requirements of maintenance and lactation. Phenobarbital and HEOD were administered orally in a gelatin capsule which also contained 15 g of chromic oxide for determining total fecal dry matter.

Milk and blood were prepared for analysis according to the method described by Crosby and Archer (1966), except that hexane was used instead of pentane. The milk fat percentage was determined by the Babcock method. Shoulder fat was prepared by homogenizing 1 part fat to 10 parts hexane in a glass tissue grinder. The homogenate was dried, using anhydrous sodium sulfate. Urine was extracted with three successive portions of hexane (2 parts urine:1 part hexane). The extracts were concentrated to 1 ml on a rotary evaporator under vacuum at 40°C.

Daily samples of feces were thoroughly mixed and aliquots