Feeding Studies with VCS-438 Herbicide in the Dairy Cow

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The herbicide VCS-438 [2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione] was fed to a lactating cow. Residues were not detected in milk, urine, or feces when 5 ppm were fed in the cattle ration. The compound was stable when incubated with the 10,000 × *g* supernatant fraction of beef liver. In rumen fluid, VCS-438 decomposed with production of a metabolite having an R_f value by thin-layer chromatography equal to that of 1-(3,4-dichlorophenyl)-3-methylurea. Mass spectrometric analysis also indicated the latter compound as the possible metabolite.

Velsicol Chemical Corporation's VCS-438 herbicide [2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione] (see Figure 1) controls various grasses and broadleaf weeds in alfalfa, corn, small grains, and other crops. Metabolic studies with this compound in biological systems have not been reported. This study was initiated to follow the possible elimination of residues of the herbicide in milk and excreta and to learn if it is metabolized in rumen fluid or by beef liver enzymes.

EXPERIMENTAL

Feeding Experiment. A Holstein cow weighing 534 kg and with a daily milk production of about 13.6 kg was catheterized and fed VCS-438 at a concentration of 5 ppm (based on a daily ration of 22.7 kg as feed) for 4 days. The 5-ppm feeding level was chosen as the maximum herbicide concentration that one would expect in herbage. The pure recrystallized 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 VCS-438 in the presence of fresh rumen fluid was studied. One milliliter of a solution of the herbicide in acetone (500 μg/ml) was thoroughly mixed with 100 ml of freshly filtered rumen fluid and held at 38°. At measured intervals 5 g were withdrawn and mixed with 5 ml of acetone. The mixture was filtered and the filter was rinsed with acetone to a total volume of 25 ml. One milliliter of the acetone filtrate was partitioned with 5 ml of benzene and 44 ml of 2% sodium sulfate solution. Up to 10 μl of the upper benzene layer were analyzed for the herbicide by electron affinity gas chromatography (EAGC).

The gas chromatograph was a Barber-Colman Model 10 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. per hr. The electrometer gain was 10,000. The column was U-shaped, made of borosilicate glass, 6 mm i.d., 61 cm long and containing a 1:1 by weight mixture of 10% OV-17 and 10% QF-1 on 80–100 mesh Gas Chrom Q. The operating temperatures for the column, flash heater, and detector were 175, 250, and 240°, respectively, and nitrogen (60 cm^3 per min) was the carrier gas. The retention time for VCS-438 was 12.6 min.

Liver. Possible metabolism of VCS-438 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 slaughtered 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* for 30 min. Incubation mixtures contained 10 μg of the herbicide (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 per minute 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 50-ml volumetric flask using 2 ml of acetone for rinsing. Benzene (5 ml) was added, the flask was made to volume with 2% sodium sulfate solution, and it was shaken vigorously for 1 min. Ten microliters of the upper benzene layer were analyzed for VCS-438 by EAGC.

EXTRACTION, ISOLATION, AND ANALYSIS OF VCS-438 FROM BODY FLUIDS

Milk. Twenty-five grams of milk were blended in an ice bath for 2 min with 65 ml of acetone and 2 g of Celite 545 filter aid. The mixture was filtered and the marc was rinsed with acetone until 100 ml of filtrate was collected. To the filtrate was added 500 ml of 2% sodium sulfate and the mixture was partitioned successively with one 50-ml and two 25-ml portions of hexane. The combined hexane extracts were dried by filtering through anhydrous sodium sulfate, evaporated with air, and made to a volume of 10 ml. The herbicide was isolated from this solution by column chromatography on a 40% diethyleneglycol–Celite column (Benziger, 1969). The column was 15 mm in diameter and contained 10 g of packing. Elution was performed using 135 ml of hexane in which only the last 75 ml was collected. This solution was evaporated to 5 ml and 10 μl were analyzed by EAGC.

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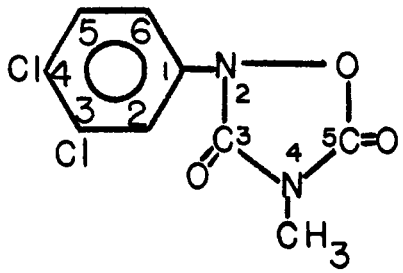

Figure 1. Molecular structure of VCS-438 herbicide

Table I. Recovery of VCS-438 from Samples

Sample	Added, ppm	Recovery, %	Estimated sensitivity, ppm
Milk	0.04	105, 100, 103	0.01
Urine	0.2	80, 95	0.1
Feces	0.2	80, 88	0.1
Rumen fluid	5	96	0.1
Liver 10,000 × *g* supernate	5	90	0.1

Urine. Urine (25 g) was similarly blended with acetone and filtered until 100 ml of filtrate were collected. Ten milliliters of the filtrate was partitioned with 2 ml of benzene and 88 ml of 2% sodium sulfate solution. VCS-438 was determined in 10 μl of the upper benzene layer by EAGC.

Feces. The method for feces was similar to that for milk, except that 10 g of sample were extracted and the final hexane eluate was evaporated to 10 ml prior to analysis.

EXTRACTION, ISOLATION, AND ANALYSIS OF METABOLITES OF VCS-438

Rumen Fluid. Possible metabolites of the herbicide such as 3-(3,4-dichlorophenyl)-1-methylurea (I), 3-(3,4-dichlorophenyl)urea (II), and 3,4-dichloroaniline (III) were extracted and isolated by an adaptation of the thin-layer chromatographic procedure of Katz (1967), as used for separating metabolites of linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea]. Two milliliters of a solution of VCS-438 in acetone (500 μg per ml) was incubated with 100 ml of rumen fluid. (This concentration of acetone in the rumen fluid has shown no inhibitory effects in many studies in this laboratory of possible reductive and hydrolytic degradation of toxicants. Dickson and Webb (1964) also state that low concentrations of solvents such as acetone do not inactivate enzymes. Acetone would be converted to isopropanol in rumen fluid.) At measured intervals, 5 g were withdrawn and mixed with 5 ml of acetone. The solution was filtered and the acetone was evaporated (as judged by odor) with air. The aqueous solution was adjusted to pH 10 with ammonium hydroxide and equilibrated with 5 ml of ethyl acetate and 90 ml of saturated sodium chloride. One milliliter of the ethyl acetate layer was evaporated to about 0.1 ml and separated by thin-layer chromatography on silica gel (Katz, 1967). This fraction contained the possible metabolites I and II. For metabolite III, another 5-ml portion of rumen fluid was similarly extracted with acetone and, after evaporation of acetone, the aqueous solution was adjusted to pH 7 with hydrochloric acid and extracted with benzene in place of ethyl acetate. The latter solution was similarly analyzed by thin-layer chromatography. The R_f values for VCS-438 and metabolites I, II, and III were, respectively, 0.97, 0.43, 0.14, and 0.77.

Urine. Five grams of urine were extracted with acetone and analyzed by the procedure used for rumen fluid.

Feces. Feces was analyzed for the possible presence of metabolite III by the following procedure. Ten grams of feces were refluxed for 1 hr with 40 ml of 9 *M* sulfuric acid. After dilution with 100 ml of water and neutralization with sodium hydroxide, the solution was distilled and 50 ml of distillate were collected in 25 ml of 0.5 *N* hydrochloric acid. The distillate was adjusted to pH 7 and extracted with 5 ml of benzene. One milliliter of the benzene extract was evaporated and spotted for thin-layer chromatography.

RESULTS AND DISCUSSION

No residues of VCS-438 or its metabolites were detected in milk, urine, or feces. Although the acid hydrolysis method used for herbicide metabolites in feces would only allow detection of 3,4-dichloroaniline (III), the absence of it would necessitate the absence of VCS-438, I, and II, which would all probably be converted to III by the hydrolysis. The herbicide was stable when incubated with the liver preparation. Table I lists the recoveries of VCS-438 from control samples and the estimated sensitivity of the method. Qualitative recoveries were obtained when metabolites I, II, and III were added to urine, feces, and rumen fluid and were carried through the thin-layer chromatographic procedure. The herbicide completely disappeared within 20 min when incubated with rumen fluid, as determined by thin-layer chromatography. A new spot appeared, however, having the R_f value of metabolite I [3-(3,4-dichlorophenyl)-1-methylurea]. This metabolite was analyzed by mass spectrometry. A portion of the spot was therefore scraped off and the silica gel was transferred to a borosilicate capillary tube. This tube was placed in the heated direct solids probe of a Model MS-902 (Associated Electrical Industries, Ltd.) mass spectrometer equipped with a conventional electron impact source. This technique was adopted since the metabolite was lost by vaporization when attempting to extract it from the silica gel and concentrate the extract. The mass spectrum showed an isotopic cluster of peaks at masses 161 ($C_6H_5N^{35}Cl_2$), 163 ($C_6H_5N^{35}Cl^{37}Cl$), and 165 ($C_6H_5N^{37}Cl_2$), corresponding to the molecular weight of dichloroaniline (III). No cluster of peaks was observed at masses 218, 220, and 222 which would have corresponded to the molecular ion of 3-(3,4-dichlorophenyl)-1-methylurea (I). However, when a known sample of 3-(3,4-dichlorophenyl)-1-methylurea (I) was deposited on silica gel and similarly vaporized into the mass spectrometer, it exhibited an identical spectrum. Again no molecular ion was observed. Introduction of the known urea standard (I) directly into the mass spectrometer (not vaporized from silica gel) showed not only the 161, 163, and 165 peaks but also the molecular ion cluster at masses 218, 220, and 222. The latter evidence proves that the known urea is decomposing (probably owing to the presence of the silica gel) to the dichloroaniline before being ionized in the mass spectrometer. In light of this, the detection of dichloroaniline in the mass spectrometer (vaporized from silica gel) may be used as evidence for the presence of the corresponding urea (I). A comparison of R_f values during thin-layer chromatography proves the metabolite was not 3,4-dichloroaniline. The above mass spectral analyses were replicated and the same results reproduced three times.

If the first metabolite of VCS-438 is I, the ultimate fate of the latter is speculative. Acid hydrolysis of rumen fluid and urine samples was conducted to liberate possible complexes of III which could have been produced by further metabolic hydrolysis and conjugative reactions of I, but III was not de-

tected. No abnormal symptoms or effects on milk production were observed while conducting the feeding experiments.

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Mechanism of Dieldrin-Induced Fat Accumulation in Rat Liver

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Dieldrin was administered orally to male albino rats at a dose level of 30 mg/kg and the effects on hepatic lipid metabolism were determined. Liver total lipid content was increased ($p < 0.05$) and this change was confined only to the triglyceride fraction; phospholipid and cholesterol levels remained unaltered. This was paralleled by an increase in incorporation of glucose-^{14}C into glyceride-glycerol. The incorporation of the isotope into fatty acids and the activity of hepatic fatty acid synthetase were significantly reduced in insecticide-administered rats, indicating an inhibition of lipogenesis by dieldrin. The secretion of triglycerides into plasma is unaffected. Hence, the accumulation of fat in the liver during dieldrin toxicity is ascribed to enhanced hepatic synthesis of triglycerides, due to increased availability of free fatty acids and α-glycerophosphate.

Studies on the effect of chlorinated hydrocarbon insecticides on mammalian biochemical functions have, of late, generated considerable interest. In recent reports from this laboratory we demonstrated manifold disturbances in intermediary metabolism of rats receiving a single oral dose of dieldrin (Bhatia *et al.*, 1971, 1972a,b; Bhatia, 1972). The findings included hyperglycemia, lowered glucose tolerance, enhanced hepatic gluconeogenesis, deposition of glycogen in the liver, and an increase in plasma nonesterified fatty acid (NEFA) level. Stimulation of lipolysis resulting in elevated plasma NEFA has also been observed in DDT-intoxicated rats (Schwabe, 1964). The enhanced mobilization of fat, among other factors, is generally associated with conditions which lead to accumulation of fat in the liver (Isselbacher and Greenberger, 1964; Lombardi, 1966; Farber, 1967). In this context it is of great interest to examine the effect of dieldrin on hepatic lipid metabolism.

Our preliminary studies indicated a slight but significant increase in the liver total lipids of insecticide-treated rats. In this paper we have attempted to elucidate the mechanism by which dieldrin induces lipid accumulation in the liver.

EXPERIMENTAL

Wistar strain male albino rats weighing 100–120 g were used in the present investigation. The experimental rats were orally administered dieldrin dissolved in groundnut oil at a dose level of 30 mg/kg body weight. The corresponding group of control aimals received 0.2–0.3 ml of groundnut oil only. The rats were sacrificed 24 hr after treatment. During the posttreatment period the animals were kept fasted but had free access to water.

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For studies involving the incorporation of glucose-*U*-^{14}*C* into liver lipids, 20–22 hr-fasted animals were orally administered glucose-*U*-^{14}*C* (specific activity 30 μCi/200 mg) at a dose level of 30 μCi/100 g body weight and sacrificed 3 hr thereafter.

Rats were stunned by a blow on the head and blood was collected in heparinized tubes by cutting the jugular vein. Liver was removed and freed of adhering materials by dipping in chilled normal saline. Samples of the tissue (1–2 g) were dropped in 20 volumes of chloroform–methanol (2:1, v/v) mixture and finely ground with acid-washed sand. In the case of plasma, 1–2 ml of freshly separated plasma was pipetted out dropwise into a flask containing 20 ml of the chloroform–methanol mixture. Lipids were extracted and isolated according to the procedure of Folch *et al.* (1957). Final lipid solutions in chloroform were stored in sealed stoppered tubes at −20° until required for further estimations.

Total lipid content was determined gravimetrically. Phospholipid phosphorus was estimated according to the method of Bartlett (1959) as modified by Marinetti (1962). The phospholipid content was calculated by multiplying the phosphorus value by the factor of 25. Total cholesterol was determined by the procedure of Hanel and Dam (1955). Neutral glycerides, comprised predominantly of triglycerides, were calculated by substracting the sum of cholesterol and phospholipids from total lipid. The results are expressed as mg of glycerides/g of fresh liver.

For determination of radioactivity of glucose-*U*-^{14}*C* incorporated into various lipid fractions, an aliquot of the lipid solution in chloroform was plated in stainless steel planchets and radioactivity was measured in a windowless gas flow counter. Another aliquot containing 5–7 mg of lipid was evaporated to dryness under nitrogen and lipids were saponified (Fain *et al.*, 1963). Nonsaponifiable materials and fatty acids were extracted into petroleum ether (boiling range 40–60°) from the alkaline alcoholic digest