Metabolic Studies with Zytron Herbicide in a Lactating Cow

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Excretion and metabolism of the herbicide, Zytron [(*O*-(2,4-dichlorophenyl) *O*-methyl isopropylphosphoramidothioate], was studied in a lactating cow. At a herbicide level of 5 p.p.m. in the feed, a gas chromatographic peak with the identical retention time of 2,4-dichlorophenol was found in urine representing 102% of the total Zytron dose. Zytron disappeared when incubated with the 10,000 G max supernatant fraction of beef liver again with production of a peak with the retention time of 2,4-dichlorophenol. This conversion proceeded equally well in the presence of air or oxygen. The herbicide was stable when incubated with fresh rumen fluid. Zytron was not detected in milk or feces samples.

Zytron, [*O*-(2,4-dichlorophenyl) *O*-methyl isopropylphosphoramidothioate], is an effective herbicide for pre-emergence control of crabgrass and certain other weeds in turf. Interestingly it also provides control of ants, chinch bugs, and grubs in turf. Studies of the fate of this compound in the bovine have not been published. In the work reported, Zytron was fed to a lactating cow to study its excretion and metabolism.

EXPERIMENTAL PROCEDURES

Feeding Experiment. A Holstein cow weighing 1600 pounds and with a daily milk production of about 25 pounds was catheterized and fed Zytron at the 5-p.p.m. level (based on a daily ration of 50 pounds) for four days. This amounted to a daily dose of 113.5 mg. of Zytron. The pure, recrystallized compound in absolute ethanol was thoroughly mixed with the evening grain. Morning and evening subsamples of the total mixed milk were taken one day prior to 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.

In Vitro Studies. Stability of the herbicide was studied when incubated with rumen fluid. One milliliter of a solution of Zytron in acetone (500 μg. per ml.) was thoroughly mixed with 100 ml. of filtered rumen fluid (5 p.p.m.) and held at 38° C. At measured intervals, 5 ml. of the fluid were removed 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 sulfate solution was added, and the mixture was partitioned with 5 ml. of benzene. The benzene solution was analyzed for Zytron by gas chromatography using the operating parameters described.

Possible metabolism of Zytron was studied in the presence of the 10,000 G supernatant fraction of fresh beef liver which contains microsomes and soluble enzymes. A Holstein heifer 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 Zytron (100 μl. of a 50 μg. per ml. solution in acetone), 25 μmoles of magnesium chloride, 95 μmoles of tris 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 in a 25-ml. capped Erlenmeyer flask at 37° C. in an atmosphere of air or oxygen for 1 hour. The flasks contained a borosilicate marble 0.5 inch in diameter and 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 1 hour the reactions were terminated by the addition of 2 ml. of acetone and each incubation mixture was filtered into a 10-ml. volumetric flask and made to volume with acetone. The entire solution was partitioned with 5 ml. of benzene and 85 ml. of 2% sodium sulfate solution. The upper benzene solution was analyzed for Zytron and 2,4-dichlorophenol by gas chromatography as described.

Extraction and Isolation of Zytron. Analysis of milk, urine, and feces for Zytron was as follows: Twenty-five grams of the sample was extracted by blending with 80 ml. of acetone and 1 ml. of orthophosphoric acid. The mixture was filtered and rinsed with acetone to a total volume of 100 ml. Five milliliters (20 ml. was taken for milk analysis) of the acetone filtrate was transferred to a 100-ml. volumetric flask. Five milliliters of benzene was added and the flask was made to volume with 2% sodium sulfate solution and shaken for 1 minute. The upper benzene layer was analyzed for Zytron by gas chromatography as described.

Extraction and Isolation of 2,4-Dichlorophenol. For extraction of 2,4-dichlorophenol from milk, 20 ml. of the aqueous-acetone extracts of milk (those prepared for extraction of Zytron) was evaporated to about 10 ml. in a 100-ml. volumetric flask. Five milliliters of benzene was added, the flask was made to volume with 2% sodium sulfate, and the contents were shaken vigorously. The upper benzene layer was analyzed for 2,4-dichlorophenol by gas chromatography as described.

Feces samples were processed as follows: A 20-ml. portion of the aqueous-acetone extracts of feces (those prepared for extraction of Zytron) was transferred to a 50-ml. volumetric flask. The acetone was evaporated (as judged by odor) using air. One milliliter of 5*N* hydrochloric acid and 5 ml. of a 20% solution of ethyl acetate in diethyl ether were added to the remaining aqueous extract. The flask was made to volume with saturated sodium chloride and shaken vigorously. The upper organic layer was analyzed for 2,4-dichlorophenol by gas chromatography as described.

For extraction of urine, 5 ml. of urine was transferred to a 50-ml. volumetric flask. The remainder of the procedure beginning with the addition of 1 ml. of 5*N* hydrochloric acid

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Table I. Gas Chromatographic Operating Parameters

Sample	Column Substrate	Temperature, ° C. Column	Flash Heater	Detector	Retention Time, Minutes
Zytron					
Milk, urine, feces, rumen fluid, liver 10,000 G supernate	10% DC-200 on 80- to 100-mesh Gas-Chrom Q	190	245	200	13.0
2,4-Dichlorophenol					
Milk, feces, liver 10,000 G supernate	10% OV-17 on 80- to 100-mesh Gas-Chrom Q	140	245	200	6.0
Urine	10% DC-200 on 80- to 100-mesh Gas-Chrom Q	110	200	180	7.1

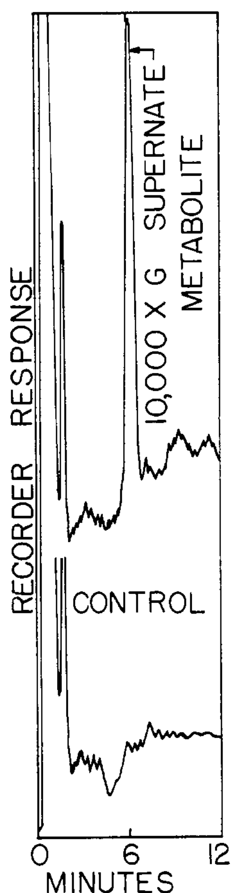

Figure 1. Chromatograms showing metabolite peak after one hour of incubation of Zytron with the 10,000 G supernate fraction of beef liver and control

was identical to that described above for extraction and analysis of 2,4-dichlorophenol in feces.

DETERMINATION

Final analysis for Zytron and 2,4-dichlorophenol was made by electron affinity gas chromatography. The gas chromatograph was a Barber-Colman Model 10 with a battery-operated No. A-4071, 6 cc. electron affinity detector containing 56 μCi of radium-226. The recorder was a Wheelco, 0 to 50 mV., equipped with 10-inch chart paper, running 10 inches per hour. The electrometer gain setting was 10,000. Nitrogen (60 cc. per minute) was the carrier gas. The columns were U-shaped, made of borosilicate glass, 6 mm. i.d. and 6 feet long. Table I lists the other operating parameters.

Table II. Daily Pattern of Metabolite Excretion in Cow Urine

Day	% of Total Zytron Excreted as the Metabolite
1[a]	Not detectable
2	4.0
3	25.0
4[b]	25.3
5	22.1
6	21.0
7	2.8
8	1.8
9	Not detectable
10	Not detectable

[a] First day of feeding Zytron.
[b] Last day of feeding Zytron.

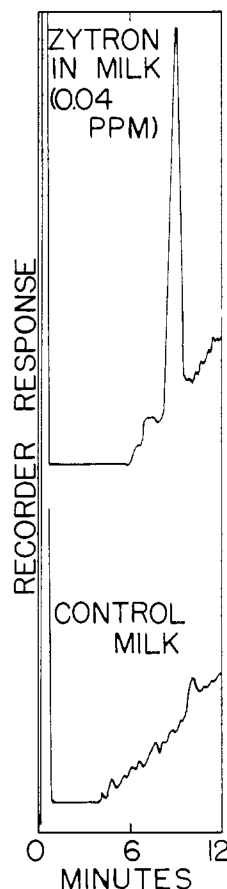

Figure 2. Chromatograms of recovery of Zytron from milk and control

Table III. Results of Incubation of Zytron with 10,000 G Supernate Fraction of Beef Liver in the Presence of Air or Oxygen

Sample	Replicate No.	Incubation Time, Minutes	% Zytron Recovered		% Zytron Converted to Metabolite	
			Air	Oxygen	Air	Oxygen
Complete incubation mixture	1	0	94	88	Nil	Nil
	2	0	105	86	Nil	Nil
	3	0	97	86	Nil	Nil
Complete incubation mixture	1	60	6	8	98	94
	2	60	8	Nil	91	87
	3	60	8	6	91	87
Complete incubation mixture minus enzyme	1	60	96	80	Nil	Nil
	2	60	86	83	Nil	Nil
	3	60	86	83	Nil	Nil
Complete incubation mixture minus Zytron (control)	1	60	Nil	Nil	Nil	Nil

Table IV. Recovery of Zytron and 2,4-dichlorophenol from Samples

Sample	Zytron		2,4-dichlorophenol	
	Added, p.p.m.	Recovery, %	Added, p.p.m.	Recovery, %
Milk	0.04	80, 80, 98, 86	0.1	50
	0.1	96, 78, 91	0.5	100
Urine	1	87	0.5	94, 88
			2.5	101
Feces	4	85	2	72

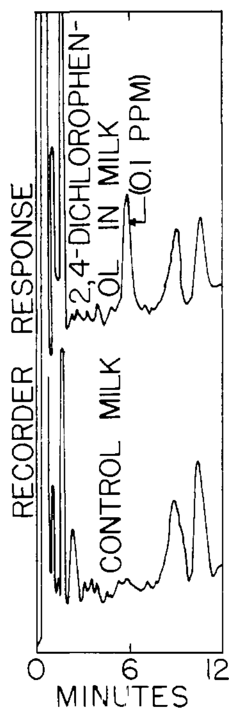

Figure 3. Chromatograms of recovery of 2,4-dichlorophenol from milk and control

RESULTS AND DISCUSSION

A gas chromatographic peak with the identical retention time of 2,4-dichlorophenol, a possible hydrolysis product of Zytron, was observed in the urine samples. Assuming it was 2,4-dichlorophenol, it represented 102% of the total Zytron dose (454 milligrams) on an equivalent basis. Table II lists the daily percentages of total equivalent Zytron excreted.

Zytron disappeared almost completely in 1 hour of incubation with the 10,000 G max supernatant fraction of beef liver again with production of a peak with the retention time of 2,4-dichlorophenol. Figure 1 shows chromatograms with this peak reproduced and the control without Zytron. The rate of disappearance of Zytron appeared to be about the same in air or oxygen. Table II lists the results of the incubation study. Nearly all of the herbicide was converted to the metabolite during the incubation. Similar hydrolysis of aryl substituted organophosphorous compounds in cattle has been reported (Pankaskie *et al.*, 1952).

No chromatographic peaks indicating Zytron or 2,4-dichlorophenol were observed in milk or feces samples. Figures 2 and 3 show chromatograms of the recovery of Zytron and 2,4-dichlorophenol from milk and the corresponding controls. The methods were sensitive to about 0.01 and 0.05 p.p.m. of Zytron and 2,4-dichlorophenol, respectively, in milk. Zytron was stable when incubated with rumen fluid. Table IV lists the recoveries of Zytron and 2,4-dichlorophenol from various samples.

The form of the metabolite of Zytron in the urine can only be speculated upon. Possibly the metabolite may have been 2,4-dichlorophenol present in urine as a salt. The existence of the metabolite as a conjugate of 2,4-dichlorophenol was also possible. If a conjugate were present, it was presumably either rapidly hydrolyzed initially by the addition of hydrochloric acid during extraction or may have been thermally degraded to the phenol during gas chromatography.

The excretion of 2,4-dichlorophenol as a glucuronide by rabbits has been reported (Deichmann and Thomas, 1943). Excretion of 2,4-dichlorophenol as an ethereal sulfate in rabbits has also been shown (Anderton *et al.*, 1948). These workers concluded that chlorinated phenols with a pK_a less than 7, (the pK_a of 2,4-dichlorophenol is 7.7) do not form ethereal sulfates in the rabbit. The nature of the possible conjugate(s) of the metabolite in cow urine is not known.

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