Excretory Pathway of Amiben in a Lactating Cow

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The excretion and metabolism of amiben (3-amino-2,5-dichlorobenzoic acid) herbicide in a lactating cow was studied. At a herbicide level of 5 ppm in the feed, a gas chromatographic peak with the retention time of amiben methyl ester was present in the chromatograms of hydrolyzed urine, unhydrolyzed urine, and unhydrolyzed feces. It represented, respectively, 17.8% (as the conjugated herbicide acid) and 70.7% and 4.6% (each as the free acid) or a total of 93.1% of the entire herbicide dose. Amiben residues were absent in hydrolyzed and unhydrolyzed milk samples. The herbicide was stable when incubated with rumen fluid for 24 hr.

The herbicide, amiben (3-amino-2,5-dichlorobenzoic acid) controls weeds in vegetables such as tomatoes, soybeans, and certain other crops. Although many studies have been conducted on the fate of amiben in plants (Ashton, 1966; Bache *et al.*, 1964; Colby, 1965; Colby *et al.*, 1964; Frear *et al.*, 1967), soils (Donaldson and Foy, 1965; Wildung *et al.*, 1968) and as affected by light (Crosby and Leitis, 1969; Plimmer and Hummer, 1969), the metabolism of the compound in animals has received only very limited attention (Amchem Products, Inc., 1967). The possibility of its entry into dairy cattle rations as a result of deliberate herbicide application or from drift contamination prompted this study of its fate in a dairy cow.

EXPERIMENTAL

Feeding Experiment. A Holstein cow weighing 1520 pounds and with a daily average milk production of 69 lb was catheterized and fed amiben at the 5 ppm level (based on a daily ration of 50 lb) for 4 days. This amounted to a 4-day total herbicide dose of 454 mg. 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 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 **Rumen Study.** Stability of the herbicide was studied when incubated with rumen fluid. One milliliter of a solution of amiben in acetone (500 μg per ml) was thoroughly mixed with 100 ml of freshly filtered rumen fluid and held at 38° C. At measured intervals, 5 ml of the fluid were removed for analysis.

Extraction and Isolation of Amiben Residues. Analysis of milk and feces for free amiben was performed by the following procedure. Twenty-five grams of well-mixed whole milk or feces were blended with 70 ml of acetone containing 1 ml of *ortho* phosphoric acid. The mixture was filtered and rinsed with acetone to a total filtrate volume of 100 ml. Up to 20 ml of the filtrate were transferred to a 25-ml volumetric flask and the acetone was evaporated (as judged by odor) with air. Four milliliters of diethyl ether were added, the flask was made to volume with saturated sodium chloride solution, and the contents were shaken vigorously. Two milliliters of the upper diethyl ether layer were transferred to a 10-ml volumetric flask and the solution was evaporated. For methylation of amiben, 2 ml of a solution of boron trifluoride in methanol (125 g per l.) were added and the flask was held in a boiling water bath for 2 min with frequent swirling. The flask was cooled under tap water. The addition of 2 ml of boron trifluoride solution with heating was repeated once more. After cooling, 1 ml of hexane was added, the flask was made to volume with 2% sodium sulfate solution, and shaken vigorously. Up to 10 μl of the hexane layer were injected for gas chromatographic analysis.

The determination of free amiben in urine was as follows. Four grams of urine were placed in a 10-ml volumetric flask containing 1 ml of 1N hydrochloric acid and 4 ml of diethyl ether. The flask was made to volume with saturated sodium chloride and thoroughly shaken. A 2-ml aliquot of the ether layer was pipetted into a 10-ml volumetric flask containing 1 ml of benzene, and the mixture was evaporated. The remainder of the procedure including methylation of the evaporated residue and gas chromatographic analysis was the same as that described above.

Analysis of the milk and urine samples for possible conjugates of amiben was performed using an acid hydrolysis procedure. Twenty-five grams of the sample were transferred to a 250-ml Erlenmeyer flask with a 24/40 standard-taper joint. Five milliliters of 5N hydrochloric acid were added, a 3-ball Snyder column was attached, and the contents were refluxed for 30 min. The mixture was filtered into a 50-ml, volumetric flask containing 5 ml of 5N sodium hydroxide, and the filter was rinsed with 0.1N hydrochloric acid until 50 ml of filtrate were collected. Five milliliters of the filtrate were transferred to a 10-ml volumetric flask. The pH of the solution was adjusted to between 1 and 2 using hydrochloric acid. Four milliliters of diethyl ether were added, the flask was made to

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volume with saturated sodium chloride, and the contents were shaken vigorously. Two milliliters of the ether layer were transferred to a 10-ml volumetric flask containing 1 ml of benzene and the mixture was evaporated to dryness. The residue was methylated with the boron trifluoride–methanol solution and analyzed by gas chromatography by the procedure described for analysis of unconjugated amiben in milk and feces.

Urine samples were also subjected to alkaline hydrolysis to study possible conjugate formation. Twenty-five grams of urine were refluxed as in the acid hydrolysis procedure with 4 ml of 5N sodium hydroxide for 30 min. The mixture was filtered into a 50-ml volumetric flask containing 4 ml of 5N hydrochloric acid. The filter was rinsed with 0.1N sodium hydroxide until 50 ml of filtrate were collected. The remainder of the procedure beginning with partitioning of 5 ml of the filtrate (adjusted to pH 1 to 2) with ether was identical to that used for acid hydrolysis of samples.

The determination of amiben in rumen fluid was carried out by the following procedure. The 5 ml of rumen fluid which were withdrawn for analysis were immediately mixed with 5 ml of acetone to stop the reaction. The mixture was filtered, and the filter was rinsed with acetone to yield a total filtrate volume of 25 ml. A 4-ml aliquot of the filtrate was pipetted into a 10-ml volumetric flask, and the acetone was evaporated (as judged by odor) with air. One milliliter of 0.1N hydrochloric acid and 4 ml of ether were added, the flask was made to volume with saturated sodium chloride, and the contents shaken thoroughly. Two milliliters of the ether layer were transferred to a 10-ml volumetric flask containing 1 ml of benzene, and the solution was evaporated. Methylation of the residue and gas chromatographic analysis were completed by the procedure used for analysis of free amiben in milk and feces.

Determination. Final analysis for amiben 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 μc of radium^{226}. The recorder was a Wheelco, 0–50 mv equipped with 10-in. chart paper, running 10 in. per hr. The electrometer gain setting was 10,000. The column was U-shaped, made of borosilicate glass, 6 mm, i.d., 6 ft long, and containing 10% DC-200 on 80- to 100-mesh Gas Chrom Q. The operating temperatures for the column, flash heater, and detector were 192°, 230°, and 200° C, respectively, and nitrogen (60 cc per min) was the carrier gas. The retention time for amiben methyl ester was 4.6 min.

RESULTS AND DISCUSSION

A gas chromatographic peak with the identical retention time of amiben methyl ester (4.6 min) was observed in unhydrolyzed urine and in urine which was hydrolyzed with base. A peak with the same retention time was also observed in unhydrolyzed feces samples. Table I lists the daily urinary and fecal excretion pattern in units of percent of the total herbicide dose (454 mg). The sum of these three excretory routes, therefore, accounted for 93.1% of the total amiben which the cow ingested. Acid hydrolysis of urine and the analysis showed excretion of the herbicide in almost identical daily amounts as when unhydrolyzed urine was analyzed. This indicated that digestion with acid did not hydrolyze amiben conjugates which were, however, cleaved with alkali. The low level of conjugated herbicide found in the urine on the fourth day could not be explained. Duplicate analyses verified this level of conjugated amiben. Figure 1 shows chromatograms of the peak in unhydrolyzed urine 1 day after feeding of amiben began and control urine.

Amiben residues were not detected in milk (unhydrolyzed or hydrolyzed). The herbicide was stable when incubated with rumen fluid for 24 hr. Table II lists the recoveries of

Table I. Daily Excretion of Amiben Residues in Urine and Feces by a Cow Receiving[a] Dietary Intake of 5 ppm Amiben

Days	Excreted as Unconjugated herbicide (% of total dose)	Excreted as Conjugated herbicide (% of total dose)	Excreted as Unconjugated herbicide (% of total dose)
	Urinary excretion		Fecal excretion
1[a]	nd[b]	nd	nd
2	9.7	5.2	0.5
3	18.3	6.2	1.2
4[c]	19.6	1.1	0.9
5	18.8	3.8	0.5
6	3.7	0.9	0.5
7	0.3	0.5	0.3
8	0.2	0.1	0.3
9	0.1	nd	0.1
10	nd	nd	0.3
Totals	70.7	17.8	4.6

[a] First day of feeding amiben. [b] Not detectable. [c] Last day of feeding amiben.

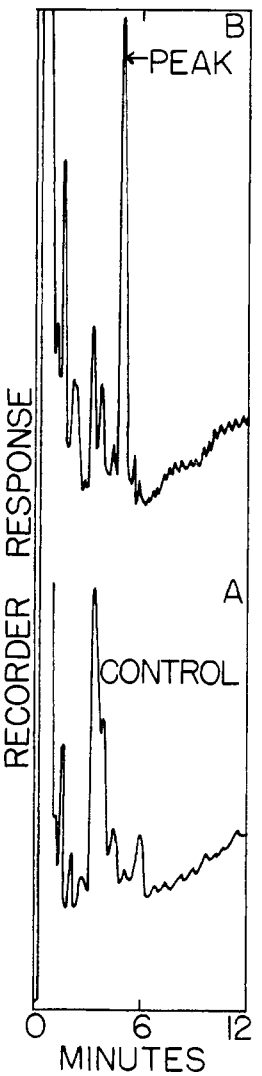

Figure 1. Chromatograms of unhydrolyzed urine from a cow (A) before and (B) 1 day after beginning a dietary intake of 5 ppm amiben

Table II. Recovery of Amiben From Control Samples

Sample	Amiben added (based on sample fresh weight) (ppm)	Recovery (%)
Milk (unhydrolyzed)	0.04	108, 94, 108
	0.08	90, 70, 64
Milk (acid hydrolyzed)	0.2	90
	0.4	105
	0.8	79, 92
Urine (unhydrolyzed)	0.05	100
Urine (acid hydrolyzed)	0.08	62
	0.8	89, 97
Urine (base hydrolyzed)	0.8	108
Feces (unhydrolyzed)	0.08	75
	0.16	65
Rumen fluid (unhydrolyzed)	5.0	84

the herbicide added to samples prior to extraction or digestion. The sensitivity of the method for amiben in milk, urine, or feces was about 0.02 ppm.

Hydrolysis of milk with base or digestion of feces with acid or base released interfering substances which prevented analysis for other herbicide conjugates which may have been present in these samples. Attempts to further isolate amiben from these sample digests prior to gas chromatographic analysis were unsuccessful.

Owing to experimental errors, especially those of sample dilution prior to sensitive electron affinity detection, the 93.1% of the total herbicide dose accounted for may, in truth, have represented total excretion of amiben in urine and feces. More likely, a small additional quantity was eliminated as a conjugate in the feces. The only other study of the metabolism of amiben in animals employed dogs (Amchem Products, Inc., 1967). The compound was also largely excreted in urine and feces. No residues of amiben were found in samples of tissues taken from the dogs. The level of amiben fed (5 ppm) probably represented the maximum level that would ever occur in cattle forage as a result of drift contamination.

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Received for review January 12, 1970. Accepted March 5, 1970.