Residues in Various Tissues of Steers Fed 4-Amino-3,5,6-trichloropicolinic Acid

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Nine young steers were fed the potassium salt of 4-amino-3,5,6-trichloropicolinic acid for at least 2 weeks at rates from 200 to 1600 p.p.m. in the total diet, equivalent to 2.6 to 23 mg. per kg. per day at time of sacrifice. Blood levels were determined frequently throughout the experiment. To obtain tissues for analysis, eight animals were slaughtered while on the chemical, while one was sacrificed after a 3-day withdrawal period. The concentration of the compound in blood reached a maximum within 3 days after feeding was begun and decreased rapidly after withdrawal. Residues in tissues from animals slaughtered without a withdrawal period were proportional to concentrations fed: <0.05 to 0.5 p.p.m. in muscle and fat, 0.12 to 2.0 p.p.m. in blood and liver, and 2 to 18 p.p.m. in kidney. These decreased to <0.1 p.p.m. in kidney and <0.05 p.p.m. in all other tissues within 3 days after withdrawal from the highest feeding level.

Tordon herbicides (Dow Chemical Co.), containing some form of the active ingredient 4-amino-3,5,6-trichloropicolinic acid, are potentially potent weapons for controlling woody plants and noxious broad-leaved weeds in pasture range lands (Gantz and Laning, 1963). Such use would inevitably result in limited deposition on adjacent areas of grass. Grass samples contained as much as 800 p.p.m. of the herbicide when taken from experimental plots immediately after application of 3 pounds per acre (Getzendaner and Herman, 1967).

In a commercial situation, cattle grazing on range land under treatment would have access to such newly treated grass. It was imperative, therefore, to determine to what extent the herbicide is deposited in the various body tissues when relatively large amounts are consumed. Metabolism studies (Redemann, 1965 a, b) have revealed no significant metabolites. Therefore, tissues were analyzed for only the parent compound.

EXPERIMENTAL PROCEDURES

Ten Hereford-Holstein steer calves, each weighing from 400 to 600 pounds, were confined in stanchions and conditioned for 3 weeks to a ration of equal parts, by weight, of grain mix and alfalfa hay. The grain ration was fed first from a 5-gallon pail. When all grain was consumed, the hay was made available in a feed box secured to the stanchion. The same rations were fed twice daily at about 9 A.M. and 3 P.M. The maximum quantity of the grain-hay ration that could be fed, so that all grain was consumed and no significant amount of hay remained at each successive feeding time, was determined for each animal. As the test progressed, rations were increased when necessary but the relative proportions of grain and hay remained constant at 1 to 1.

Blood samples were obtained at intervals throughout the experiment by venipuncture, using an 18-gage hypodermic needle to transfer 5 to 10 ml. directly into heparinized 1-ounce bottles. In a pilot experiment it was found that at a given ingestion level, concentrations of the herbicide found in blood varied widely when sampled at different hours of the day. Samples collected just prior to the morning feeding yielded the lowest and most consistent results. All sampling during this experiment was done at that time.

The formulation used in this study was a specially purified aqueous solution of the potassium salt of 4-amino-3,5,6-trichloropicolinic acid (22.8% acid equivalent). The material was incorporated into the grain ration by mixing the calculated quantity of solution with 10 pounds of grain, using a commercial food mixer, and blending the resulting concentrate with 90 pounds of grain in a vertical feed mixer. Grain rations were prepared to contain a concentration twice the desired feeding level to compensate for the equal quantity of hay fed which contained no added chemical.

At the conclusion of the conditioning period, two of the steers were maintained on control rations while the remaining eight were fed the herbicide at levels from 200 to 1600 p.p.m. (acid equivalents), following the schedule outlined in Table I. A pair of steers was sacrificed while on rations fortified with the herbicide at each level and tissues were collected for analysis.

One control animal was slaughtered about midway through the experiment to satisfy control tissue requirements. The other was continued on basal rations for 6 weeks, then fed 1600 p.p.m. of the compound for 4 weeks. Three days after withdrawing the herbicide the

Table I. Schedule for Steer Feeding Test

Steer No.	Feeding Rate,[a] Mg./Kg./Day	Lapsed Time at Feeding Rates (P.P.M. in Diet) Indicated: 2 weeks	2 weeks	2 weeks	2 weeks	2 weeks	3 days
1762	0	0	0[b]				
1753	3.2	200[b]					
1758	2.6	200[b]					
1755	6.9	200	400[b]				
1757	5.8	200	400[b]				
1754	13.4	200	400	800[b]			
1756	13.1	200	400	800[b]			
1759	22.5	200	400	800	1600[b]		
1760	22.8	200	400	800	1600[b]		
1761	23.0	0	0	0	1600	1600	0[b]

[a] Based on body weights at slaughter and feed consumption rates during week prior to slaughter.
[b] Sacrificed.

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animal was sacrificed and tissues of interest were collected and analyzed.

Beginning one week prior to feeding the compound and continuing for the duration of the test, blood samples were collected from each animal on Monday, Wednesday, and Friday mornings prior to feeding. In addition, blood from the final steer was sampled at intervals during the 3-day withdrawal period.

Test animals were slaughtered in commercial fashion about 4 hours after eating their final grain ration. A blood sample was collected directly from the severed jugular vein in a heparinized bottle. A composite sample of lean muscle tissue was retained, consisting of several pounds each from the loin and front leg. Approximately a pound of perirenal fat was taken for analysis as well as half the liver and both kidneys. In addition, abdominal fat samples were collected from the steers sacrificed while on diets containing 200 p.p.m. and from both abdominal and subcutaneous fat from the steers receiving 1600 p.p.m. Blood samples were stored in a refrigerator until analyzed, usually within 24 hours. Those requiring longer storage were preserved in a freezer. All solid tissues were comminuted using a meat grinder, packaged in polyethylene bags, and stored in a freezer until analyzed. Tissues were analyzed within a few days of slaughter by one analyst and again after several weeks by a second analyst. No evidence of a decrease in apparent residue during storage was noted.

ANALYTICAL PROCEDURES

The apparatus, reagents, and gas chromatographic details are essentially as reported for the analysis of cereal grains (Bjerke *et al.,* 1967). Only newly developed extraction and cleanup procedures are presented here.

Liver. Step 1. Cut 10.0 grams of frozen tissue into small pieces and place in a 4-ounce wide-mouthed, screw-cap jar.

Step 2. Add 10 grams of diatomaceous earth, 5 grams of $NaHCO_3$, and 50 ml. of methanol. Attach the jar to a homogenizer and blend for 5 minutes at maximum speed, keeping the jar immersed in an ice bath. Filter the sample through a 60-ml. Büchner funnel having a coarse sintered glass disk covered with about 0.5 cm. of compressed diatomaceous earth. Wash the homogenizer spindle and jar three times with 10 ml. of methanol, putting the washings through the filter. Continue washing the filter cake with methanol until a total of 100.0 ml. of filtrate is collected. Avoid allowing the filter cake to go dry during filtration and washing.

Step 3. Pipet 15.0 ml. of the extract into a 35-ml. screw-cap centrifuge tube, add a boiling chip, and boil down on the steam bath until foaming occurs. Cool to room temperature.

Step 4. Add 10 ml. of water and acidify to pH <2 with 85% H_3PO_4. Add 4 grams of NaCl and 15.0 ml. of diethyl ether. Cap the tube, shake for 5 minutes, and separate the phases by centrifugation.

Step 5. Prepare a 1-gram column of Woelm basic alumina, activity grade I, in ether.

Step 6. Pipet 10.0 ml. of the ether phase from step 4 onto the column and allow it to run through. Follow, successively, with 20 ml. of ether and 20 ml. of acetone. Discard the effluent.

Step 7. Elute the column with 20 ml. of $0.25M$ $NaHCO_3$ solution, collecting the eluate in a 60-ml. separatory funnel.

Step 8. Acidify with 1.8 ml. of $5N$ H_2SO_4.

Step 9. Add 0.5 ml. of saturated $KMnO_4$ solution, mix, and allow to stand at room temperature for exactly 5 minutes. Add $5M$ $NaHSO_3$ solution dropwise with mixing until the solution is colorless.

Step 10. Dissolve 4 grams of NaCl in the solution.

Step 11. Extract the solution successively with 20 and 10 ml. of ether, combining the extracts in a 50-ml. beaker.

Step 12. Add about 0.1 gram of anhydrous Na_2SO_4, concentrate the ether solution to 1 to 2 ml. on a steam bath, and quantitatively transfer it to a 5-ml. volumetric flask, using ether for rinsing. Add a boiling chip and concentrate to about 1 ml. on the steam bath. Cool to room temperature.

Step 13. Add 0.5 ml. of diazomethane reagent (De Boer and Backer, 1963), 0.5 ml. of benzene, and a boiling chip and boil off the ether on a steam bath. Cool to room temperature and dilute to 5.0 ml. with benzene.

Step 14. Chromatograph 2.0 μl. of the benzene solution and determine its concentration by referring the height of the peak obtained to a calibration curve prepared as previously described (Bjerke *et al.,* 1967).

Kidney. For concentrations in the range from 0 to 1 p.p.m., follow the procedure for liver analysis, omitting step 9. For concentrations ranging from 1 to 10 p.p.m., follow the procedure for liver analysis through step 11, omitting step 9 and combining the ether extracts in step 11 in a 50-ml. volumetric flask instead of a beaker. Dilute to volume with ether. Pipet a 2-ml. aliquot into a 5-ml. volumetric flask. Complete the analysis as for liver analysis, beginning with step 13. For concentrations ranging from 10 to 20 p.p.m., follow the procedure for 1 to 10 p.p.m., but use 7.5 ml. of methanol extract instead of 15 ml. (step 3 of the procedure for liver analysis).

Muscle. Follow the procedure for the analysis of liver with the following modifications: Take a 20-gram sample of muscle tissue in step 1, use 9 grams of diatomaceous earth and 8 grams of $NaHCO_3$ in step 2, and take a 7.5-ml. aliquot of methanol extract in step 3.

Fat. Weigh 10.0 grams of fat into a 4-ounce jar, add 25.0 ml. of $0.25N$ $NaHCO_3$, and place in a 60–65° water bath for 5 minutes with occasional swirling. Immediately add 4 grams of diatomaceous earth, attach the jar to a homogenizer, and blend at maximum speed for 1 minute. Cool to 0° in an ice bath. Filter the sample through a 60-ml. Büchner funnel having a coarse sintered glass disk covered with about 0.5 cm. of compressed diatomaceous earth, collecting about 11 ml. of filtrate. Pipet 5.0 ml. of the filtrate into a 4-dram screw-cap vial. Cautiously add 0.2 ml. of H_3PO_4 and swirl to expel CO_2. Add 4 grams of NaCl and 4.0 ml. of ether. Cap the vial and shake vigorously for 2 minutes. Separate the phases by centrifugation. Pipet

2.0 ml. of the ether phase into a 5-ml. volumetric flask. Complete the analysis as in the procedure for liver, beginning with step 13.

Blood. Pipet 2.0 grams into a 4-dram vial. Add 4 ml. of H_2O, mix, and let stand 2 minutes to hemolyze cells. Add 0.1 ml. of H_3PO_4, 4.0 ml. of ether, and 3 grams of NaCl, in the order given. Cap the vial immediately and shake vigorously for 2 minutes. Separate the phases by centrifugation. For concentrations of 4-amino-3,5,6-trichloropicolinic acid less than 1 p.p.m., pipet 2.0 ml. of the ether phase into a 5-ml. volumetric flask. For concentrations greater than 1 p.p.m. take a 1-ml. aliquot. Complete the analysis as in the procedure for liver, beginning with step 13.

Representative chromatograms resulting from analyses of various tissues from the control steer and those fed 4-amino-3,5,6-trichloropicolinic acid, potassium salt, are shown in Figure 1.

RESULTS AND DISCUSSION

Samples of the tissues of interest were collected from the control steer and analyzed to determine blank values. Although, for most tissues, slight inflections occurred on the chromatograms at the retention time for the methyl ester of 4-amino-3,5,6-trichloropicolinic acid, in no case was the estimated blank equivalent to more than 0.01 p.p.m.

The efficiency of each method was determined by fortifying control tissues with known amounts of the herbicide and applying the appropriate analytical procedure. Table II shows the results of these analyses, indicating average recoveries ranging from 87% for blood and liver to 97% for muscle and kidney tissues.

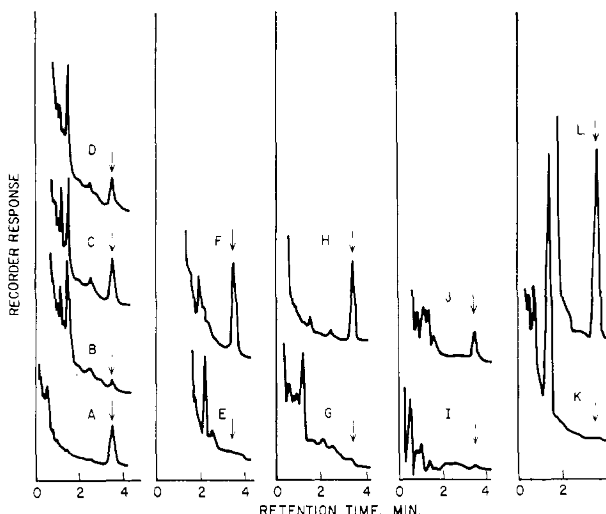

Figure 1. Representative chromatograms from analyses for residues of 4-amino-3,5,6-trichloropicolinic acid in bovine tissues

Arrows indicate retention time of methyl-4-amino-3,5,6-trichloropicolinate

A. Ester equivalent to 0.02 ng. of 4-amino-3,5,6-trichloropicolinic acid
B. Control muscle
C. Muscle fortified with 0.05 p.p.m. of 4-amino-3,5,6-trichloropicolinic acid
D. Muscle from steer fed 200 p.p.m.
E. Control liver
F. Liver from steer fed 200 p.p.m.
G. Control kidney
H. Kidney from steer fed 200 p.p.m.
I. Control fat
J. Fat from steer fed 200 p.p.m.
K. Control blood
L. Blood from steer fed 200 p.p.m.

Table II. Recovery of 4-Amino-3,5,6-trichloropicolinic Acid from Fortified Bovine Tissues

	Blood		**Muscle**		**Liver**		**Kidney**		**Fat**	
P.P.M. Added	**P.p.m. found**	**% recov.**	**P.p.m. found**	**% recov.**	**P.p.m. found**	**% recov.**	**P.p.m. found**	**% recov.**	**P.p.m. found**	**% recov.**
0.05	0.045	90	0.055	110	0.045	90	0.047	94	0.045	90
	0.040	80	0.05	100	0.045	90	0.042	84	0.050	100
	—	—	—	—	0.050	100	—	—	—	—
0.1	0.095	95	0.10	100	0.085	85	0.095	95	0.090	90
	0.095	95	0.10	100	0.085	85	0.100	100	0.095	95
0.2	0.175	88	0.18	90	0.16	80	0.190	95	0.180	90
	0.175	88	0.19	95	0.175	88	0.195	98	0.170	85
	—	—	—	—	—	—	0.190	95	—	—
0.3	—	—	0.25	83	—	—	—	—	—	—
	—	—	0.26	87	—	—	—	—	—	—
	—	—	0.32	107	—	—	—	—	—	—
	—	—	0.27	90	—	—	—	—	—	—
0.5	0.435	87	0.49	98	0.40	80	0.475	95	0.48	96
	0.435	87	0.52	104	0.39	78	—	—	0.50	100
	—	—	—	—	—	—	—	—	0.55	110
1.0	0.80	80	—	—	0.89	89	1.00	100	0.94	94
	0.86	86	—	—	—	—	—	—	1.04	104
	—	—	—	—	—	—	—	—	0.93	93
1.5	—	—	—	—	1.41	94	—	—	—	—
2.0	1.75	88	—	—	1.59	80	—	—	—	—
	1.72	86	—	—	—	—	—	—	—	—
5.0	—	—	—	—	—	—	4.98	100	—	—
10.0	—	—	—	—	—	—	10.0	100	—	—
20.0	—	—	—	—	—	—	20.7	104	—	—
Av. % recovery		87		97		87		97		96

Table III. Concentrations of 4-Amino-3,5,6-trichloropicolinic Acid in Bovine Blood for Several Ingestion Levels

Ingestion Level, P.P.M.	No. of Days at Given Level	No. of Steers Averaged	Average P.P.M. in Blood: On given sampling day	Average P.P.M. in Blood: At given ingestion level
0		10	0	0
		10	0	
		10	0	
200	3	8	0.18	0.17
	5	7[a]	0.18	
	7	7[a]	0.18	
	10	8	0.18	
	12	8	0.17	
	14	8	0.15	
400	3	6	0.30	0.32
	5	6	0.35	
	7	6	0.34	
	12	6	0.31	
	14	6	0.30	
800	3	4	0.51	0.61
	5	4	0.56	
	7	4	0.68	
	10	4	0.72	
	12	4	0.62	
	14	4	0.55	
1600	3	2	1.18	1.14
	5	2	1.07	
	7	2	1.22	
	10	2	1.18	
	12	2	1.03	
	14	2	1.16	

[a] One animal, temporarily off feed due to bloat, not included in average.

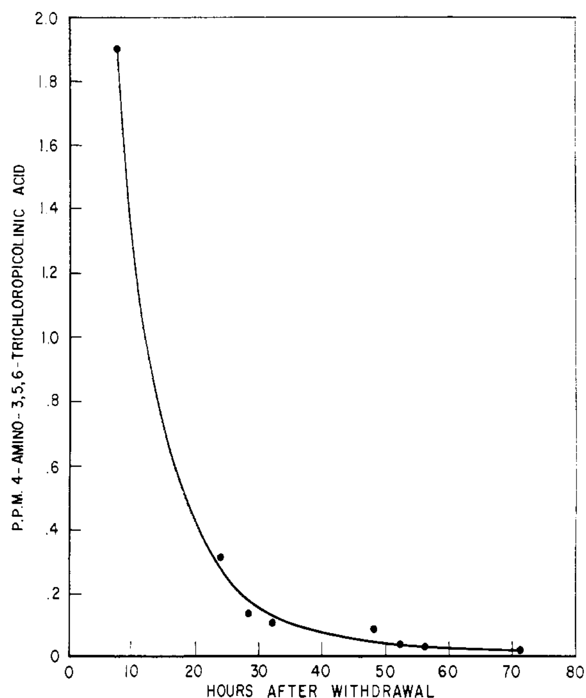

Figure 2. Disappearance of 4-amino-3,5,6-trichloropicolinic acid from blood of steer 1762 (1600 p.p.m. fed) after withdrawal from diet

Table IV. Residues of 4-Amino-3,5,6-trichloropicolinic Acid in Body Tissues of Steers

Steer No.	P.P.M. in Diet	Residue, P.P.M.: Blood	Muscle	Liver	Kidney	Perirenal fat	Subcutaneous fat	Abdominal fat
1762	0	<0.05	<0.05	<0.05	<0.05	<0.05	—	—
1761	1600[a]	<0.05	<0.05	<0.05	0.06	<0.05	<0.05	<0.05
1753	200	0.23	<0.05	0.19	2.6	0.26[b]	—	0.06
1758	200	0.18	<0.05	0.12	2.0	0.07	—	0.06
1755	400	0.37	0.07	0.30	3.9	0.12	—	—
1757	400	0.44	0.05	0.35	4.0	0.06	—	—
1754	800	0.85	0.20	0.65	8.5	0.12	—	—
1756	800	0.73	0.32	0.74	8.8	0.13	—	—
1759	1600	2.0	0.30	1.61	18.0	0.29	0.35	0.28
1760	1600	1.4	0.29	1.14	15.1	0.45	0.29	0.23

[a] Compound withdrawn from diet 3 days prior to slaughter.
[b] Sample appeared to be contaminated with kidney tissue.

Figure 1 shows chromatograms from each type of tissue fortified with the compound.

Data concerning levels of 4-amino-3,5,6-trichloropicolinic acid in blood from all animals for each sampling date were averaged (Table III). Maxima occurred at all levels of ingestion within 3 days after feeding began. Average blood levels for all animals resulting from each feeding level indicate that, over the range studied, concentrations of 4-amino-3,5,6-trichloropicolinic acid in the blood are nearly directly proportional to concentrations in the feed. Figure 2 shows the rate of disappearance of the compound from blood after the herbicide has been withdrawn from the diet. In the animal studied, ingesting 1600 p.p.m. of the compound at the time of withdrawal, the blood level declined rapidly, reaching a value well below 0.05 p.p.m. within

72 hours. Metabolism studies have shown that the compound is excreted extremely rapidly and efficiently in the urine of mammals (Redemann, 1965 a, b).

The results of analyses of blood, muscle, liver, kidney, and fat from the control animal and those fed the herbicide are tabulated in Table IV. All values are corrected for any significant blank and for recovery.

As would be expected, kidney tissues from animals sacrificed while on diets containing the compound contained the greatest residues, averaging 1% of the level in the diet. However, when the herbicide was withdrawn from the diet this value dropped to less than 0.1 p.p.m. within 3 days.

Residues found in liver tissues correspond closely with levels found in blood at slaughter, averaging approximately 0.1% of the concentration fed. Lean muscle samples contained the least residue of any tissue analyzed, ranging from <0.05 p.p.m. at the 200-p.p.m. level of ingestion to about 0.3 p.p.m. in animals fed at the highest level. Residues in fat were of the same order of magnitude as those in muscle. Residues in all tissues except kidney were well below the measurable level of 0.05 p.p.m. in animals slaughtered 3 days after withdrawing the compound from the diet. Kidney tissue contained 0.06 p.p.m. at that time.

ACKNOWLEDGMENT

The authors thank personnel of the Animal Science Section, Agricultural Products Department, for assistance in conducting this experiment, and Marguerite Leng for reviewing the manuscript.

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Received for review April 18, 1968. Accepted January 6, 1969.