Metabolic Fate of the Herbicide Buthidazole in Lactating Cows and Laying Hens

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[^{14}C]Buthidazole [3-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-^{14}C-yl)-4-hydroxy-1-methyl-2-imidazolidinone] was administered in twice-daily oral doses for 14 consecutive days to cows at dosages equivalent to 0.5, 2.5, and 10 ppm in the diet and to hens at dosages equivalent to 0.3, 1.5, and 6 ppm in the diet. Within 12 h of the final [^{14}C]buthidazole dose, 80% of total administered ^{14}C was excreted in cow urine, 8% in feces, and 1% in milk; 82% was eliminated in hen excreta. Residues, as a function of dietary concentration, were about 1.4% for milk, 0.4% for eggs, 2% for cow muscle, and 0.2% for hen muscle. No residue was detected in milk, eggs, or tissues 7 days after treatment ceased. Only 0.1% of the radiocarbon excreted in cow urine was buthidazole. Hen excreta contained less than 1% unchanged buthidazole. Twelve metabolites were identified by thin-layer chromatography and mass spectrometry. The degradation pathway included hydroxylation, oxidation, demethylation, dehydration, ring opening, and hydrolysis. The major metabolites were *N*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]urea, 3-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-4-hydroxy-2-imidazolidinone, 3-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-4,5-dihydroxy-1-methyl-2-imidazolidinone, 3-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-4,5-dihydroxy-2-imidazolidinone, and *N'*-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-*N*-hydroxymethyl-*N*-methylurea.

The herbicide buthidazole [3-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)-4-hydroxy-1-methyl-2-imidazolidinone], code name VEL-5026, has shown promise for control of broadleaf and grassy weeds in corn, sugar cane, and certain tree crops and in noncropland. The current studies were undertaken to evaluate the metabolic fate and residue transfer to meat, milk, and eggs in dairy cows and laying hens which may be exposed to the herbicide in their feed and subsequently made available for human consumption.

MATERIALS AND METHODS

Chemicals. [^{14}C]Buthidazole was prepared by Pathfinder Laboratories, Inc., St. Louis, MO, and had a specific activity of 12.7 mCi/mmol and radiochemical purity of greater than 98% by thin-layer chromatography. Authentic reference standards of buthidazole and its metabolites (compounds I, IV, V, VII–XI, Table I) were synthesized by Velsicol Chemical Corp. Metabolite XII was isolated from dog urine during a [^{14}C]buthidazole metabolism study conducted in our laboratory (Yu and Atallah, 1976).

Treatment of Animals. *Cows.* Four lactating Holstein cows weighing between 402 and 479 kg were held in individual metabolism stalls and allowed to become acclimated for 1 week before treatment. The cows were given water ad libitum and maintained on a normal daily ration (14 kg) of hay and high protein dairy supplement during both the acclimation and treatment periods. Following acclimation, the cows were weighed and catheterized. The animals were given the appropriate dose of [^{14}C]buthidazole immediately after each morning and afternoon milking. Each dose was mixed with crushed grain, placed in a gelatin capsule, and administered with a balling gun. Dosage levels in the diet were 0.5 ppm (one cow), 2.5 ppm (two cows), and 10 ppm (one cow). [^{14}C]Buthidazole was diluted with analytical reference standard buthidazole to achieve a specific activity of 6.7 mCi/mmol for the 0.5 ppm dosing level and 2.4 mCi/mmol for the remaining dosing levels. Urine was collected via a catheter inserted in the bladder. Fecal material was collected in a trough below each metabolism stall. Milk was collected morning and evening with standard milking equipment. During the entire trial, 24-h composites of urine and feces from each animal were collected and weighed. A representative subsample was removed and frozen at −10 °C pending radioassay.

Daily dosing was continued for 14 days. Twelve hours after the final dosing, three cows (one at each dose level) were weighed and sacrificed. The remaining cow (2.5 ppm) was maintained on an untreated diet for 7 days to determine the rate of tissue clearance and was then weighed and sacrificed. Cows were sacrificed by exsanguination after being rendered unconscious with a stun gun. Samples of various tissues were collected and frozen (−10 °C) pending analysis. The stability of buthidazole residues during frozen storage was verified by periodic analysis of fortified samples.

Hens. Twenty-four white Leghorn hens weighing an average of 1.76 kg each were housed in individual metabolism cages to allow collection of total excreta. Following 10 days of acclimation, hens were divided into four groups of six hens each. One group was maintained as a control. The other three groups were respectively treated with [^{14}C]buthidazole (sp act., 12.7 mCi/mmol) at levels equivalent to 0.3, 1.5, and 6 ppm in the daily diet. The daily required amount of [^{14}C]buthidazole was administered to each bird as two equal doses at 12-h intervals. Each dose of buthidazole was mixed with laying mash, placed in a gelatin capsule, and administered orally. Daily dosing was continued for 14 days. At the end of the seventh day, two hens from each of the four groups were sacrificed 12 h after the last dose of [^{14}C]buthidazole had been given. Two more hens from each group were sacrificed after 14 days of treatment, 12 h after the final dose. The remaining eight hens were placed on an untreated diet and maintained for 7 days to determine tissue clearance before sacrifice.

During the experiment, eggs and excreta were collected from each hen at 24-h intervals and frozen at −10 °C pending analysis. The hens were sacrificed by cervical dislocation, and samples of various tissues were collected and frozen.

Radioassay. Liquid samples were radioassayed directly in Econofluor or Aquasol scintillation solution (New England Nuclear). Tissues and feces were combusted using a Packard Model 306 sample oxidizer. $^{14}CO_2$ was

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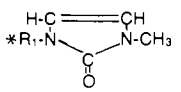
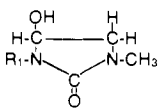
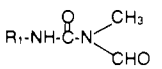
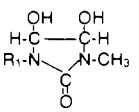
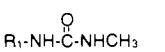
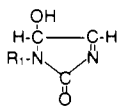
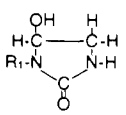
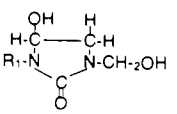
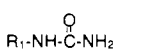
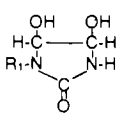
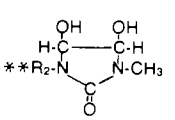
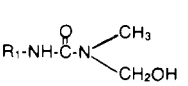
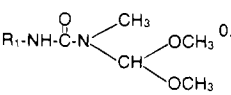
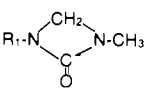
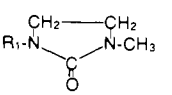
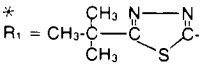
Table I. Structures and TLC R_f Values of Buthidazole and Its Metabolites in Cows and Hens

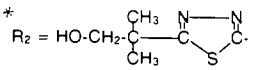
Designation and Trivial Name	Structure	R_f Values in Solvent System A	B	C	D	E	F
I. Dehydrate		0.62	0.40	0.34	0.40	0.37	0.62
II. Buthidazole		0.58	0.31	0.27	0.29	0.31	0.53
III. Methyl formyl		0.48	0.28	0.30	0.23	0.25	0.45
IV. Dihydroxy		0.41	0.50	0.20	0.59	—	0.62
V. Methyl urea		0.40	0.42	0.31	0.49	0.20	0.53
VI. Desmethyl dehydro		0.37	0.20	0.16	—	—	0.45
VII. Desmethyl		0.35	0.36	0.22	0.36	0.18	0.55
VIII. Methylol		0.32	0.31	0.16	0.35	0.17	0.55
IX. Amine	R_1-NH_2	0.20	0.30	0.29	0.49	0.16	0.47
X. Urea		0.18	0.28	0.22	0.47	0.17	0.47
XI. Dihydroxy desmethyl		0.20	0.26	0.10	0.50	0.15	0.56
XII. Dihydroxy hydroxy-*tert*-butyl		0.15	0.22	0.08	0.25	0.07	0.55
XIII. Methyl methylol		0.10	0.19	0.08	0.26	0.10	0.31

Designation and Trivial Name	Structure	R_f Values in Solvent System A	B	C	D	E	F
XIV. Unknown		0.80	0.85	—	—	—	—
XV. Unknown		0.65	0.55	0.29	0.63	0.37	0.65
XVI. Unknown		0.62	—	—	—	—	—
XVII. Unknown		0.55	0.54	0.14	0.70	0.23	0.65
XVIII. Unknown		0.55	0.45	0.11	0.43	0.16	0.60
XIX. Unknown		0.45	0.50	—	—	—	—
XX. Unknown		0.22	0.25	—	—	—	—
XXI. Unknown		0.18	0.15	—	—	—	—
XXII. Unknown		0.15	0.22	—	—	—	—
XXIII. Unknown		0.10	0.13	—	—	—	—
XXIV. Unknown		0.10	0.10	—	—	—	—
XXV. Unknown		0.05	0.15	—	—	—	—
XXVI. TLC origin		0	0	—	—	—	—
COMPOUND A		0.70	0.45	0.41	0.70	0.30	0.68
COMPOUND B		0.50	0.60	0.36	0.55	0.30	0.59
COMPOUND C (deoxy buthidazole)		0.60	0.25	0.25	—	—	0.50

Solvent System:

A — Methylene chloride/n-propanol (90:10)
B — Petroleum ether/diethyl ether/ethanol (50:35:15)
C — Benzene/dioxane/acetic acid (76:21:3)
D — Petroleum ether/diethyl ether/n-propanol (50:30:20)
E — Petroleum ether/chloroform/ethanol (70:20:10)
F — Acetonitrile/acetone/acetic acid (50:50:1)

* R_1 = 

** R_2 = 

trapped in Carbosorb and radioassayed in Permafluor V scintillation solution (Packard Instruments Co.). The efficiency of $^{14}CO_2$ recovery was determined by combusting a known amount of [^{14}C]hexadecane on filter paper. Recoveries were greater than 99%. All samples were counted in a Mark III liquid scintillation system, Model 6880 (Searle Analytic, Inc.) for 10 or 20 min. Counting efficiency was automatically determined by the external standard pulse method via an efficiency vs. external standard pulse curve stored in the microprocessor unit. The limit of detection ranged between 0.0013 and 0.0071 ppm depending on media analyzed and the specific activity of administered [^{14}C]buthidazole.

Thin-Layer Chromatography (TLC). Precoated silica gel G TLC chromatoplates (Sil G-UV 254, 0.25 mm, Macherey, Nagel & Co., distributed by Brinkmann Instruments, Inc.) were used for resolving buthidazole and its metabolites. The solvent systems and R_f values are presented in Table I. Both one- and two-dimensional TLC was used to identify the metabolites. For one-dimensional TLC, the plates were developed in a 9:1 mixture of methylene chloride and propanol (solvent system A). Two-dimensional TLC was accomplished using solvent system A and then a 50:35:15 mixture of petroleum ether, diethyl ether, and ethanol. The extracts were either spotted along or cochromatographed with seven authentic metabolites (compounds I, II, IV, V, VII, IX, X; Table I). For additional confirmation, ^{14}C metabolites were removed from the gel by extraction with acetone or methanol using a spot collector (Brinkmann Instruments, Inc.) and then cochromatographed with the suspected reference metabolites in several other solvent systems (Table I). The nonradioactive reference metabolites were detected under UV light and ^{14}C metabolites were detected by radioautography using Kodak blue brand X-ray film. To isolate sufficient quantities of metabolites for mass spectrometry analysis, extracts were applied as a band on 0.5-mm thick preparative TLC plates and developed in solvent system A. The ^{14}C bands were removed from the plate and eluted with acetone or methanol depending on polarity of the metabolite. Isolated metabolites were often further purified in other solvent systems.

Mass Spectrometry (MS). Mass spectra were obtained by direct inlet probe using a Hewlett Packard Model 5982 A quadrupole mass spectrometer in either the electron ionization (EI, 70 eV) or chemical ionization (CI, methane) mode. Data output from the MS was monitored with a Hewlett Packard Model 5934 A dual disc data system.

Sample Analysis. One hundred milliliters of cow urine was extracted three times with 100 mL of ethyl acetate. The ^{14}C in the combined organic layer was designated as "free metabolites". The aqueous layer was adjusted to 0.1 N HCl, refluxed for 30 min, and then extracted three times with 100 mL of ethyl acetate. The ethyl acetate layer was designated as "acid-released metabolites". The remaining aqueous layer was adjusted to 0.1 N NaOH, refluxed for 30 min, and then extracted three times with ethyl acetate. The combined ethyl acetate extracts were designated "base-released metabolites". ^{14}C remaining in the aqueous layer was designated "water-soluble metabolites". Duplicate 1-mL samples of the organic and aqueous layers from the various steps were radioassayed. The organic extracts were dried over anhydrous sodium sulfate and filtered, and the solvent was removed in a rotary evaporator. A small amount of acetone was added to the evaporation flask and the extract was subjected to TLC analysis.

Samples of 100 g each of cow feces, hen excreta, or tissue except fat, were blended in a Waring blender for about 4 min with 300 mL of acetonitrile/water (1:1), and the mixture was centrifuged. The supernatant was decanted and saved. The solid residue was extracted two more times. The acetonitrile in the combined extracts was evaporated. The remaining aqueous solution was extracted three times with 200 mL of ethyl acetate. The aqueous layer was adjusted to 0.2 N with HCl and then added to the solid residue. This mixture was refluxed for 30 min and the mixture centrifuged. The supernatant was extracted three times with 200 mL of ethyl acetate. The organic extracts were dried with anhydrous sodium sulfate, then concentrated to a volume suitable for TLC analysis.

Radiocarbon in the milk was fractionated into oil-soluble, organosoluble, and water-soluble metabolites according to techniques used by Atallah et al. (1976). However, in addition, the aqueous layer was further adjusted to 0.1 N with HCl, refluxed for 30 min, and then extracted two times with 150 mL of diethyl ether/pentane (2:1). This organic layer was designated "acid-released metabolites". The aqueous layer formed a gel-like material which was broken by adding acetone. The organosoluble and acid-released metabolites were analyzed by TLC.

Fifty grams of eggs were blended three times with 200 mL of acetonitrile/water (1:1) and centrifuged. Acetonitrile in the combined supernatant was completely removed using a rotary evaporator. The remaining aqueous solution was extracted three times with 100 mL of ethyl acetate. The ethyl acetate extracts from the various steps were combined and dried over anhydrous sodium sulfate and filtered, and the solvent was completely removed with a rotary evaporator. One hundred milliliters of hexane was added to the remaining oily residue and the resultant solution was extracted three times with 50 mL of acetonitrile. The acetonitrile layer was subjected to TLC analysis. The aqueous layer was combined with the solid residue, adjusted to 0.2 N with HCl, refluxed for 30 min, and centrifuged. The supernatant was extracted three times with ethyl acetate. The combined ethyl acetate extracts were radioassayed and subjected to TLC analysis. The solid residue and aqueous layer were separately radioassayed.

Ten grams of each fat sample was blended twice with 50 mL of hexane. The supernatant was decanted and saved. The solid residue was again homogenized twice with 50 mL of acetonitrile. The combined hexane extracts were extracted two times with acetonitrile. The acetonitrile extracts were combined and radioassayed as were the hexane layer and unextracted solid residue.

RESULTS AND DISSCUSSION

Absorption and Excretion. Oral doses of [^{14}C]buthidazole were rapidly absorbed and efficiently voided from the body of lactating cows (Table II). A near equilibrium between intake and excretion was reached within 5 days. At the end of the 14-day treatment period, about 90% of the dose had been accounted for in excreta. The urine provided the major route of elimination, accounting for about 80% of the dose; 6–13% was voided in the feces. Only about 0.9–1.8% of the dose was eliminated via the milk. The cow that was returned to herbicide-free diet had no detectable residue in the milk 48 h after the last dose. In this cow, elimination via the urine and feces was completed within 2 days after the last dose.

[^{14}C]Buthidazole orally administered to hens was also rapidly eliminated in the excreta (Table II); 80–92% of the dose was eliminated after only 1 day. In hens that were dosed for 14 days and then returned to an untreated diet

Table II. Percent Radiocarbon in the Excreta and Milk from Cows and Hens Administered [*thiadiazol*-2-^{14}C]Buthidazole in the Diet for 14 Days

	feeding level, ppm	days after initiation of treatment/cumulative percent of dose						
		1	5	10	14	15	16	21
cows								
urine	0.5	70.4	75.0	76.3	79.6			
	2.5	78.5	80.5	80.4	75.0			
	2.5	93.2	81.3	82.4	85.7	86.0	86.0	86.1
	10.0	71.8	78.3	77.7	76.2			
feces	0.5	4.1	8.0	8.1	8.7			
	2.5	4.5	4.4	9.7	11.0			
	2.5	6.0	6.0	6.1	6.0	6.1	6.2	6.2
	10.0	4.3	6.0	6.2	7.8			
milk	0.5	1.46	1.64	1.70	1.78			
	2.5	0.90	1.06	1.04	1.01			
	2.5	0.92	0.96	0.98	1.01	1.01	1.01	1.02
	10.0	0.80	0.88	0.89	0.91			
hens								
excreta	0.3	79.6	75.6	79.6	80.6	81.1	81.3	81.7
	1.5	88.4	86.5	86.8	92.1	92.6	92.7	93.0
	6.0	91.5	77.6	74.0	74.5	74.8	75.0	75.3

Table III. Residue Levels (ppm Equivalent of Buthidazole) in the Tissues, Milk, and Eggs after Oral Administration of [*thiadiazole*-^{14}C]Buthidazole to Cows and Hens for 14 Days

sampling day and feeding level (ppm)		muscle	liver	kidney	fat	heart	gizzard	milk	eggs[a]
cows									
day 7	0.5							0.011	
	2.5							0.031	
	10.0							0.18	
day 14	0.5	<0.0037	0.036	0.028	<0.0026			0.013	
	2.5	<0.0071	0.066	0.059	<0.0071			0.022	
	10.0	0.0211	0.31	0.21	<0.0071			0.15	
day 21	2.5	<0.0071	0.0082	<0.0071	<0.0071			<0.0014	
hens									
day 7	0.3	<0.0013	0.0038	0.0026	<0.0013	<0.0013	<0.0013		<0.0013
	1.5	0.0017	0.0134	0.0085	<0.0013	0.0021	0.0011		0.0078
	6.0	0.0124	0.0723	0.0541	0.0025	0.0172	0.0105		0.025
day 14	0.3	<0.0013	0.0031	0.0026	<0.0013	<0.0013	<0.0013		0.0014
	1.5	0.0021	0.0158	0.0121	<0.0013	0.0031	0.0019		0.0069
	6.0	0.0106	0.0605	0.0457	0.0026	0.0131	0.0092		0.0252
day 21	0.3	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013		<0.0013
	1.5	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013		<0.0013
	6.0	<0.0013	0.0036	0.0019	<0.0013	<0.0013	<0.0013		0.0016

[a] Radiocarbon residue in the day 16 egg samples (14 days of treatment followed by 2 days on normal diet) were <0.0013, 0.0035, and 0.0109 ppm for feeding levels at 0.3, 1.5, 6.0 ppm.

for 7 days, 75–93% of the administered ^{14}C was accounted for in excreta. Elimination via the excreta was completed within 2 days after the last dose.

^{14}C Residues in Tissues, Milk, and Eggs. Maximum total residue present in the body of any of the cows at the end of the 14-day treatment period was 2% of the dose; the residue was only 0.01% for the cow fed on a herbicide-free diet for 7 days. The tissue distribution of administered radiocarbon is shown in Table III. In cows, the liver and kidney were the only tissues that contained detectable levels of residues at all dosage levels. No detectable residues were found in visceral fat regardless of dosage level. Muscle had detectable residues (0.02 ppm) only at the 10-ppm dosage level; the 0.5- and 2.5-ppm dosages produced no detectable residue. The average concentrations of [^{14}C]buthidazole equivalents in whole milk of cows fed the herbicide at 0.5, 2.5, and 10 ppm for 14 days were 0.01, 0.02, and 0.15 ppm, respectively (Table III). There was no sign of any increase in concentrations as daily exposure proceeded. Centrifugal separation of the whole milk into butter fat and skim milk showed that the residues were almost totally presented in the aqueous solution. There was rapid dissipation of residue from cow tissues and milk upon removal of the herbicide from the diet. In the cow that was maintained on a herbicide-free diet for 1 week following the last dosage, no residue was detected in any of the analyzed samples except the liver, which contained extremely low levels (0.008 ppm).

Residues in the tissues of laying hens were even lower than those in cow tissues (Table III). Residues in the birds' liver and kidney were 1.0 and 0.8%, respectively, of the [^{14}C]buthidazole concentration in the diet. Residues in the tissues did not increase as the treatment period increased from 7 to 14 days. Residues were depleted from hen tissues when birds were returned to an untreated diet for 7 days after being exposed to buthidazole for 14 days. ^{14}C residues reached a plateau in eggs about 7 days after initiation of the dosing (Table III). The residue level in the eggs at plateau was about 0.4% of the daily dietary level and reached nondetectable levels within a few days after treatment ceased.

The relationship between the feeding levels and residues in tissues, milk, and eggs is presented in Figure 1. These residues increased almost proportionally to the increase in dose.

Extraction Characteristics of ^{14}C Residues. Table IV shows the extraction characteristics of radiocarbon residues in the excreta, tissues, milk, and eggs. No consistent variations among the sampling intervals or dosage levels were observed. Therefore, the data in Table IV were

Table IV. Extraction Characteristics of Radiocarbon in Excreta, Tissues, Milk, and Eggs from Cows and Hens Orally Administered [*thiadiazole*-2-^{14}C]Buthidazole Daily for 14 Days

sample and feeding level (ppm)		percent of total ^{14}C in the sample						
			free					
		free	in hexane	in acetonitrile	acid-released	base-released	water-soluble	unextracted
cows								
urine[a]	0.5	54.5			30.5	2.0	11.2	
	2.5	55.6			17.3	2.7	22.7	
	10.0	64.9			12.7	1.8	16.6	
feces[a]	0.5	34.4			19.3	14.7	20.4	16.9
	2.5	35.9			17.3	14.9	24.5	13.2
	10.0	48.6			21.3	16.2	15.7	15.5
liver[b]	0.5	20.6			4.9	11.6	34.0	27.0
	2.5	11.2			9.2	4.3	23.9	51.9
	10.0	15.8			19.5	8.6	26.4	26.6
kidney[b]	0.5	24.6			18.0	9.8	40.0	8.5
	2.5	17.8			15.9	5.3	30.6	8.1
	10.0	51.3			23.9	6.7	20.9	8.4
muscle[b]	10.0	60.6			4.9		15.7	15.3
milk[a]	0.5		0.9	16.7	28.9		55.0	
	2.5		0.4	17.5	28.3		56.4	
	10.0		1.1	14.2	36.3		43.0	9.4
hens								
excreta[a]	0.3	31.3			22.3	13.4	23.3	8.1
	1.5	25.3			18.9	11.1	26.1	8.1
	6.0	26.1			21.0	12.7	30.8	21.2
liver[c]	0.3	22.6			18.1	15.9	30.2	14.4
	1.5	20.9			17.9	14.2	32.0	14.4
	6.0	14.4			7.3	10.2	20.0	27.5
kidney[c]	0.3	34.6			15.3	13.1	18.0	19.4
	1.5	31.7			15.4	8.8	20.5	19.7
	6.0	33.2			15.1	7.9	15.5	32.1
heart[c]	6.0	52.2			15.4	14.0	11.5	6.6
breast[c]	6.0	55.0			9.4	9.0	15.2	15.1
thigh[c]	6.0	57.3			11.2	9.7	14.2	14.6
gizzard[c]	6.0	50.4			20.0	7.5	17.1	6.7
fat[c]	6.0		19.9	41.6				11.5
eggs[d]	1.5		0.4	49.3	17.4		20.2	13.0
	6.0		0.2	31.5	25.7		31.1	7.9

[a] Average of day 1, day 4, day 8, and day 14 samples. [b] Day 14 sample. [c] Average of day 7 and day 14 samples. [d] Average of day 4, day 9, day 14, and day 16 samples.

averages of samples taken at different time intervals for each dosage level.

Over 50% of the cow urinary radiocarbon was present as "free" metabolites, 20% was released upon acid hydrolysis, and only a small amount (2%) was released by base hydrolysis. The water-soluble metabolites accounted for about 17% of urinary radiocarbon. An average of about 40% of the cow fecal radiocarbon could be readily extracted by ethyl acetate. Acid and base hydrolysis released 20 and 15% of the fecal ^{14}C, respectively. Approximately 17% of the radiocarbon in milk was extracted with the organic solvents. When the extracted residues were partitioned between acetonitrile and hexane, most of the radiocarbon (16%) was located in the acetonitrile and less than 1% in the hexane fraction (fat-soluble). One-third of the radiocarbon residues remaining in the aqueous phase were rendered organosoluble upon acidification. These acid-released metabolites comprised an average of 31% of total milk radiocarbon. The remaining unextracted unhydrolyzed residues (average 51%) were in the aqueous layer. Of the total ^{14}C residues, the free and acid-released metabolites constituted 27, 51, and 66% in the cow liver, kidney, and muscle, respectively. The water-soluble metabolites in liver, kidney, and muscle were about 28, 30, and 15% of total residues, respectively. About one-third of the radiocarbon residues in the liver were initially unextracted from solids. Subsequent exhaustive extractions of these solids further removed a major part of the residues. This suggested that a portion of the liver residues was physically trapped rather than "bound". Remaining in kidney and muscle solids (bound residues) were 8 and 15%, respectively, of total radiocarbon present in the sample.

The extraction characteristics of ^{14}C in hen excreta were similar to that of cow feces. About 30% of the radiocarbon in the excreta was free metabolites, 20% was acid-released, and 10% was base-released. Water-soluble and unextracted ^{14}C accounted for 30 and 10% of the total radiocarbon, respectively. About 40% of the radiocarbon in eggs was partitioned into acetonitrile and less than 1% into the hexane fraction. This was similar to the behavior of ^{14}C in milk. Acid-released metabolites accounted for 20% of the ^{14}C. Water-soluble and unextracted metabolites constituted 25 and 10% of the radiocarbon present in the sample, respectively. The extraction characteristics of ^{14}C in hen tissues were also very similar to the respective cow tissues. The free metabolites accounted for 20% of the ^{14}C in the hen liver and 30–60% in hen kidney and muscle.

Metabolite Identification. From cows treated with 10 ppm and hens treated with 6 ppm, metabolites that were "free" or were rendered organosoluble by acid and base treatment were further analyzed by TLC (Table V). With only a few exceptions, which will be discussed, no qualitative differences in the distribution of metabolites among the free, acid-, and base-released metabolites were observed. Therefore, the data in Table V represent the total extracted ^{14}C in the free, acid-, and base-released fractions. Also, there were no significant quantitative

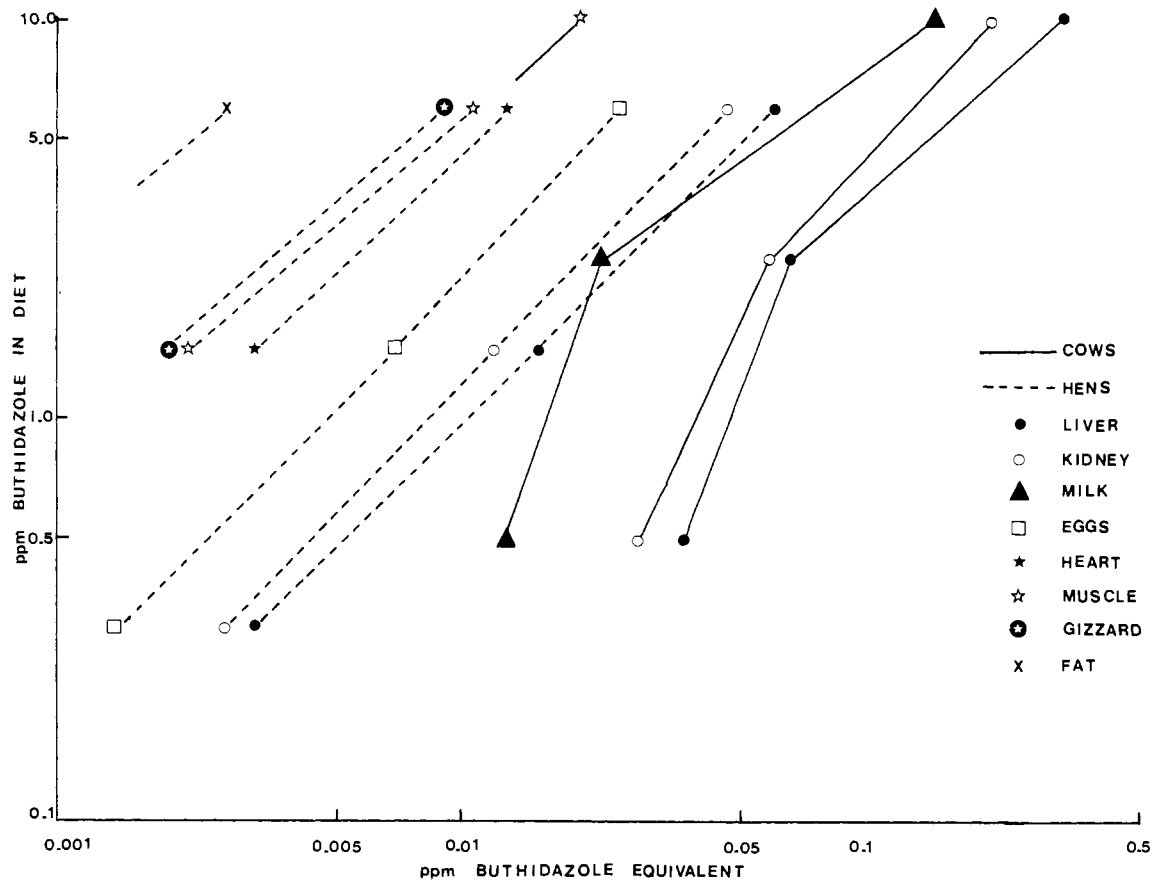


Figure 1. Residue of [^{14}C]buthidazole in tissues, milk, and eggs following continuous feeding of the herbicide to cows and hens for 14 days.

Table V. Identity of Radiocarbon in the Excreta, Tissues, Milk, and Eggs from Cows and Hens Orally Administered [*thiadiazole*-2-^{14}C]Buthidazole Daily for 14 Days

	percent of ^{14}C in the sample analyzed								
	cows administered 10 ppm in the diet					hens administered 6 ppm in the diet			
compound	urine[a]	feces[b]	milk[b]	liver[c]	kidney[c]	excreta[d]	liver[e]	kidney[f]	eggs[c]
I, dehydrate	0.1		0.4	0.3	0.0				
II, buthidazole	0.1	6.5	1.9	0.4	2.1	1.0	0.0	2.9	1.4
III, methyl formyl						9.7			
IV, dihydroxy	1.7	9.2	4.6	1.8	1.7	1.9			4.2
V, methylurea	0.2					1.2			
VI, desmethyl dehydro			23.1						
VII, desmethyl	19.1	11.3	4.7	0.0	9.3	1.3	0.7		4.8
VIII, methylol			2.9	1.9	4.4	0.4			
IX, amine	0.4	17.6		1.6	1.4	2.8	0.8		
X, urea	17.4	13.4	8.3	7.1	22.3	14.9	14.8	8.5	23.0
XI, dihydroxy desmethyl	12.2	7.1	0.4	2.3	22.1	10.4		10.5	5.8
XII, dihydroxy hydroxy-*tert*-butyl						4.3			2.5
XIII, methyl methylol	8.6	11.2	2.9	2.0	5.2	2.5	2.9	3.0	1.5
XIV		4.4					5.6	1.4	0.7
XV	0.0		0.1	1.4	0.0	1.0	1.0		1.1
XVI	0.1		0.7	0.3	0.0	0.2	0.3		
XVII						1.1		1.1	
XVIII						0.5			1.5
IX	0.1					0.8		3.4	1.5
XX						3.1			
XXI	0.7			13.0	0.0	1.5			1.5
XXII	1.2			1.8	7.1				
XXIII	0.5								
XXIV	1.2								
XXV	1.6			1.7	1.0	1.6			
XXVI, origin	12.8	8.0	0.7	8.6	5.3	1.1	4.3	8.8	5.3

[a] Average of day 1, day 8, and day 14 samples. [b] Average of day 1, day 4, day 8, and day 14 samples. [c] Day 14 sample. [d] Average of day 4, day 14, and day 16 samples. [e] Average of day 7 and day 14 samples. [f] Day 7 sample.

differences among the samples taken at different time intervals, hence an average of the samples taken was used.

The desmethyl metabolite [3-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)-4-hydroxy-2-imidazolidinone] con-

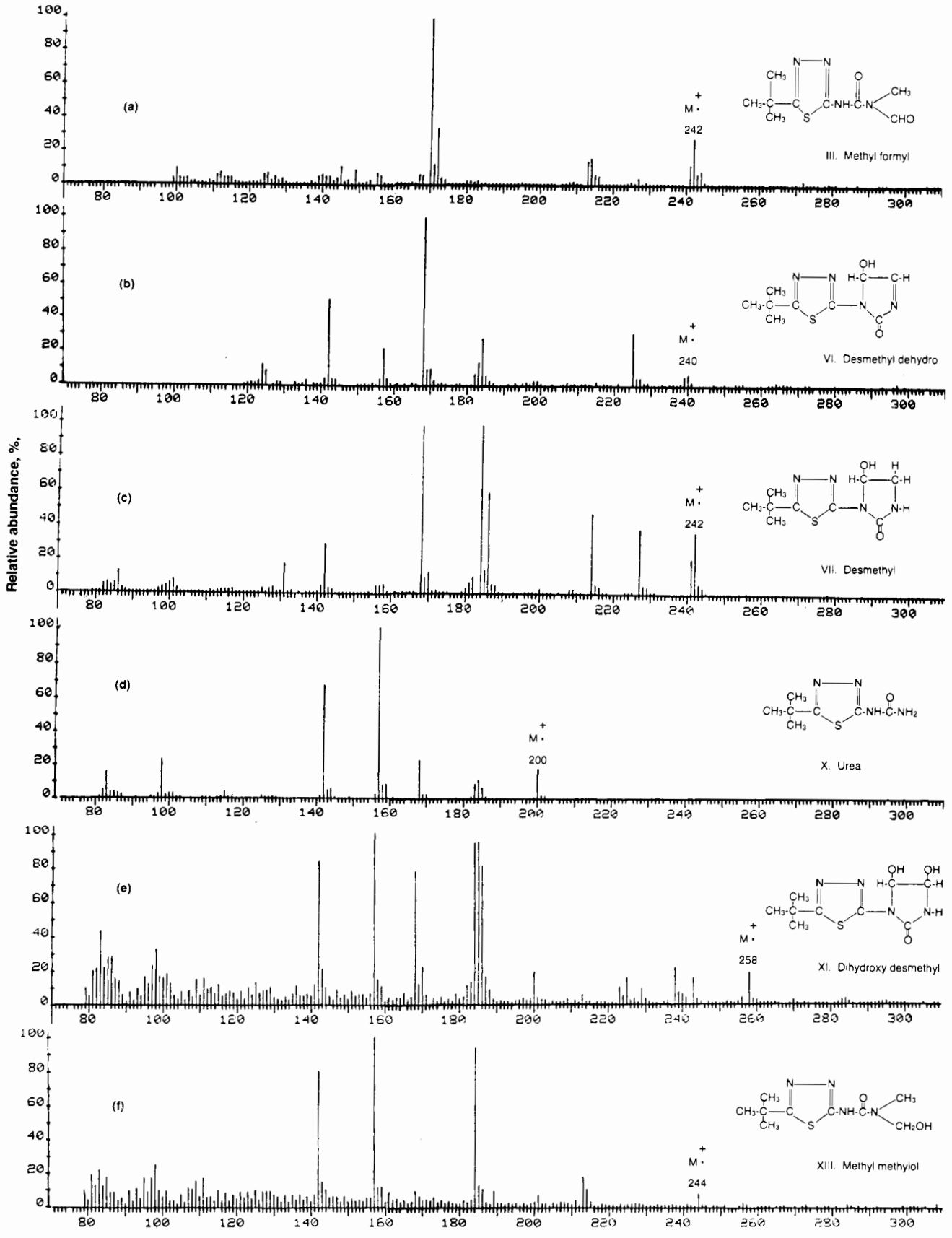


Figure 2. Mass spectra (EI) of major buthidazole metabolites in cows and hens.

stituted 19% of cow urinary radiocarbon and was the major urinary metabolite. Also detected were the urea [*N*-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)urea, 17%], dihydroxy desmethyl [3-(5-(1,1-dimethylethyl)-1,3,6-thiadiazol-2-yl)-4,5-dihydroxy-2-imidazolidinone, 12%], methyl methylol [*N'*-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)-*N*-hydroxymethyl-*N*-methylurea, 9%], dihydroxy [3-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)-4,5-di-

hydroxy-1-methyl-2-imidazolidinone, 2%], and small amounts of unchanged buthidazole (II), dehydrate (I, see Table I for designation), methylurea (V), and amine (IX). Mass spectra of the desmethyl, urea, and dihydroxy desmethyl isolated from cow urine (Figure 2) were identical with the corresponding authentic reference standards. The structure of methyl methylol shown in Table I was confirmed by mass spectrometry (Figure 2). When stored in methanol solution for a few days, this metabolite formed two major products as detected by TLC (Table I). One of the products (designated compound A) had a molecular ion m/e 288. The other product (designated compound B) had a molecular ion m/e 226. The structures of A and B were proposed in Table I. The amine (18%) was the major fecal metabolite in cows. The urea (13%), desmethyl (11%), methyl methylol (11%), dihydroxy (9%), dihydroxy desmethyl (7%), and unchanged buthidazole (6%) were also detected. Metabolite VI constituted 23% of the radiocarbon in the milk. A mass spectrum of VI showed a molecular ion of m/e 240 (Figure 2). The TLC R_f value and mass spectrum of VI was different from that of authentic compound C which also had a molecular ion m/e 240 (Table I). The structure of VI was therefore proposed as desmethyl dehydro and is shown in Table I. This metabolite was probably a conjugate in milk because it could be extracted only after acid hydrolysis. The other metabolites in milk were the urea (8% of total ^{14}C), desmethyl (5%), dihydroxy (5%), methylol (3%), methyl methylol (3%), buthidazole (2%), and dehydrate (0.4%). Metabolites detected in cow liver were the urea (7% of total ^{14}C), dihydroxy desmethyl (2%), methylol (2%), methyl methylol (2%), dihydroxy (2%), amine (2%), buthidazole (0.4%), and dehydrate (0.3%). The urea (22% of total ^{14}C) and dihydroxy desmethyl (22%) were the major metabolites in cow kidney. Desmethyl (9%), methyl methylol (5%), methylol (4%), buthidazole (2%), dihydroxy (2%), and amine (1%) were also detected.

It appeared that buthidazole was also rapidly metabolized in the laying hens. Only 1% of the radiocarbon in the excreta was unchanged buthidazole. There were 19 metabolites detected by TLC. The major metabolite was urea (15%). The dihydroxy desmethyl metabolite accounted for 10% of the excreted ^{14}C. A new metabolite (III), which had not been detected in previous animal and plant metabolism studies (unpublished data from this laboratory), also constituted 10% of the ^{14}C. This metabolite was isolated and analyzed by MS. The MS spectrum (Figure 2) showed that III had a molecular ion m/e 242. The structure of III was identified and is shown in Table I as methyl formyl. Metabolite III was probably a conjugate and could be extracted only after acid or base hydrolysis. Other identified metabolites each constituting 0.5–4% of the total excreta ^{14}C were dihydroxy, methylurea, desmethyl, methylol, amine, dihydroxy-*tert*-butyl, and methyl methylol. There were also nine other metabolites, the identities of which were undetermined principally because they were present only in very minor amounts. Urea (23%) was the major metabolite in the eggs. Dihydroxy desmethyl (6%), desmethyl (5%), dihydroxy (4%), dihydroxy hydroxy-*tert*-butyl (2.5%), methyl methylol (1.5%), buthidazole (1%), and six minor unidentified metabolites were detected. Urea (15%) was also the major metabolite in hen liver. Methyl methylol (2.9%), desmethyl (1.4%), and four unidentified metabolites were also detected. No unchanged buthidazole was found. Urea (9%) and dihydroxy desmethyl (10.5%) were the major metabolites found in the hen kidney. Methyl methylol (3%), buthidazole (3%), and four unidentified

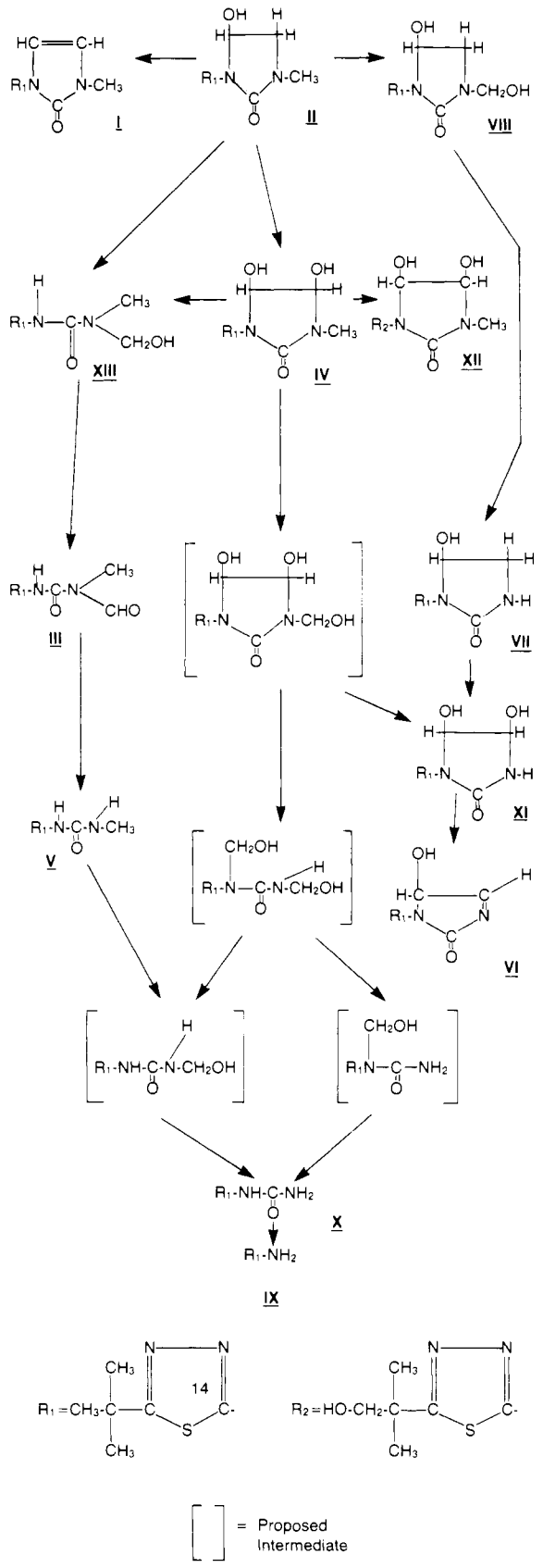


Figure 3. Proposed metabolic pathway of buthidazole in cows and hens.

metabolites were also detected.

Only a very small amount of unchanged buthidazole was detected in any of the analyzed samples. These results show that cows and hens metabolized buthidazole very rapidly and extensively. While there were quantitative

differences in the metabolites isolated from different samples in the same animal species, no qualitative differences were observed except that desmethyl dehydro was found only in milk and methyl formyl only in hen excreta.

The metabolic pathway of buthidazole in cows and hens is shown in Figure 3. This pathway involved hydroxylation, oxidation, demethylation, dehydration, ring opening, and hydrolysis.

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Metabolism of [^{14}C]Zearalenone in Laying Hens

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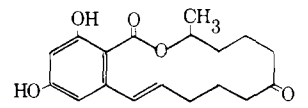
A single dose of 10 mg of [^{14}C]zearalenone/kg was administered by gavage to White Leghorn laying hens, and its absorption, distribution, and excretion at 2, 4, 24, 48, and 72 h after dosage were studied. ^{14}C-Labeled residues in excreta, bile, egg yolk, clutch, and liver were partially characterized. About 94% of the administered ^{14}C activity was eliminated via the excreta within 72 h of dosing. About one-third of the dose was excreted as unchanged [^{14}C]zearalenone, while another one-third appeared as a polar metabolite. No major retention sites of ^{14}C activity were found in edible muscle tissues but persistent levels of lipophilic metabolite(s) were detected in egg yolk at a concentration of 195 μg equiv/100 g of wet weight (about 2 ppm) 72 h after dosing.

Zearalenone, a mycotoxin produced by some strains of



Fusarium roseum, *F. oxysporum*, *F. tricinctum*, and *F. moniliforme* (Steele et al., 1976), has been found in corn infested with these fungi. When the 1973 corn crop was surveyed, zearalenone was detected in 6% of the marketable corn from the Corn Belt area, which includes nine midwestern states (Stoloff et al., 1976). Zearalenone and its uterotropically active derivatives are classified as estrogens since they produce estrus, i.e., cornification of the vagina in adult mice. The biological effect of zearalenone on the metabolism of various animal species has been reviewed (Mirocha et al., 1977). Swine, poultry, and cattle appear to be affected by the presence of zearalenone in the diet. Swine are possibly most sensitive to the estrogenic activity of this compound, which can contribute to infertility in sows by its effect on the ovaries. Since chickens are relatively resistant to the physiological effects of zearalenone (Mirocha et al., 1977), the likelihood that contaminated grain might be used as a feedstock for them is increased. Therefore zearalenone might enter the human food chain via chicken meat and eggs.

This study was undertaken to determine the distribution of zearalenone and its metabolites in eggs and edible tissues of laying hens dosed with [^{14}C]zearalenone and to partially characterize some of the metabolites.

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MATERIALS AND METHODS

Radiolabeled Zearalenone. The uniformly labeled [^{14}C]zearalenone was prepared by Anver Bioscience Design, Inc., Sierra Madre, CA. Purity, reported to be 99%, was verified by the high-pressure liquid chromatographic procedure of Ware and Thorpe (1978). A total of 400 mg (61.6 μCi), having a sp act. of 0.154 μCi/mg, was available for this study.

Experimental Animals. White Leghorn laying hens (Truslow Farms, Chestertown, MD) were not less than 26 or more than 39 weeks of age and had an egg-laying efficiency of 70% or more. Hens were housed individually in metal cages in a room in which the temperature was maintained at 70–72 °F, relative humidity at 30–50%, and a light/dark cycle of 16/8 h. The hens were fed laying mash (Truslow Farms, Chestertown, MD) and water ad libitum. Each hen was weighed weekly, and daily egg production was recorded.

At 8:00 a.m. on the day that a hen was selected for use in the metabolic study it was fasted for 1 h before being dosed (by gavage into the crop) with 10 mg/kg (1.54 μCi/kg) of [^{14}C]zearalenone dissolved in propylene glycol at a concentration of 10 mg/mL. The hen was then placed immediately in a Delmar-Roth metabolic chamber (Delmar Scientific Glass Products of Coleman Instruments, Maywood, IL) from which excreta and expired CO_2 could be collected quantitatively. Feed and water were available at all times. Each hen was kept in the metabolic chamber until the selected time interval after dosing had elapsed. Four hens were used for each of five different time in-

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