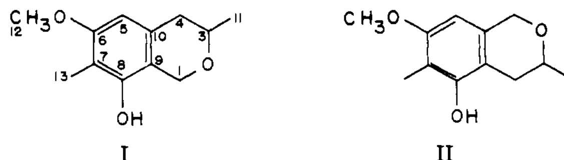
of I, along with the shift difference between phenol and phenyl acetate (Wehrli and Wirthlin, 1976), suggests that the ^{13}C peaks at 134.0 and 153.8 ppm are meta to the OH group in I, the peaks at 112.8 and 115.4 ppm are ortho to the OH group, and the unsubstituted carbon at 96.2 ppm is para to the OH group. Differences between the proton shifts of I and the acetate (III) (Table I) are consistent with these conclusions.

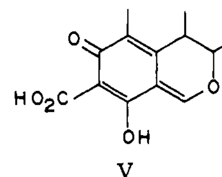
Thus, the data suggest the possibility of two structures for the toxin, I and II. Attempts to distinguish between



I and II using long-range, proton-carbon coupling constants obtained from the gated decoupled ^{13}C spectra of the toxin and its acetate were not successful. Only broad lines were observed for the substituted carbons of the aromatic ring. Furthermore, chemical shift differences for the aliphatic carbons between the toxin and its acetate (III) are not large enough to permit one to distinguish between I and II.

To further distinguish between I and II, the methoxy derivative (IV) of the toxin was prepared. Proton and ^{13}C NMR data for IV are given in Tables I and II, respectively. Differences between the ^{13}C chemical shifts of the toxin and IV (Table II) are consistent with the conclusions revealed previously on the basis of the ^{13}C chemical shift differences between the toxin and its acetate (III). When NMR spectra of IV (^{1}H and ^{13}C) were obtained upon successive additions of the lanthanide shift reagent Eu(fod)$_3$, the downfield induced shifts clearly showed that europium was complexing to any appreciable extent only with the aliphatic ether oxygen. Furthermore, the induced shift data are only consistent with structure I for the toxin and not with II. Thus, the structure of the toxin is established as 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (I) (Weast, 1968).

The structure of the toxin (I) is similar to that of another toxin, citrinin (V), isolated from *P. steckii* (Krogh, 1974).



A possible biosynthetic route to citrinin has been proposed (Curtis et al., 1968). However, without additional experiments, one can only speculate that I and V may be derived biosynthetically from a common pathway.

The toxin (I) had an LD_{50} of 800 mg/kg in day-old chickens. This demonstrates a case where a fungal metabolite is acutely toxic at relatively high levels and observed toxicity of the fungal extract was due to production of relatively large amounts of the metabolite. Also, a dramatic change in solubility as a result of purification may have contributed to reduced toxicity of the purified metabolite. No other toxins were detected during purification.

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Metabolism of [^{14}C]Mibolerone in the Chicken

George H. Dunn, Leo F. Krzeminski,* Ronald E. Gosline, and Charles J. Subacz

One-day-old chicks were primed on nonradioactive mibolerone for 21 days. [^{14}C]Mibolerone was then given orally for 28 days. Mibolerone was rapidly metabolized and eliminated. Highest concentrations, determined 1 h after the last ^{14}C dose, were found in bursa, followed by liver. Intact mibolerone was found in adipose tissues. Metabolites of mibolerone were detected in adipose tissues and liver, but were present in amounts too small for identification. There were no detectable residues in eggs collected 72 days after the last dose of [^{14}C]mibolerone. The method would have detected residues above 0.5 ppb.

Avian lymphoid leukosis (LL) is a bursa dependent lymphoid neoplasm (Kakuk et al., 1977) induced by the lymphoid leukosis viruses, a form of C-type RNA tumor virus (Romero et al., 1978a). The neoplasm occurs primarily in the bursa of fabricius. The disease progresses by metastasization of the malignant cells to visceral organs with eventual death (Burmester, 1969). Treatment has involved surgical removal of the bursa, treatment with cyclophosphamide or with androgens (Romero et al., 1978b). Associated drawbacks have included economics, survival, and loss of immunocompetance (Romero and Frank, 1977). The androgen analogue, Mibolerone (17β-hydroxy-7α,17-dimethylestr-4-en-3-one) administered

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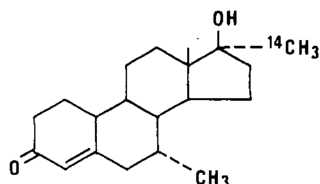

Figure 1. Mibolerone, labeled with ^{14}C methyl at the 17α position.

Table I. Amount of [^{14}C]Mibolerone per Capsule

week used for calc.	g of feed[a] consumed/day	μg of [^{14}C]-mibolerone/capsule	μg/g of feed equivalent	week dose administ.
4	36.0	67.6	1.9	5
5	44.5	67.3	1.5	6
6	51.2	68.0	1.3	7
			av 1.6	

[a] Average daily feed consumption per week.

via feed causes regression of the bursa of fabricius and thus prevents LL. The use of mibolerone does not adversely effect weight gain, egg production, or immunocompetance (Romero et al., 1977). Consequently, metabolism studies were initiated with carbon labeled mibolerone to determine whether eggs and tissues from layer hens contained residues of mibolerone or its metabolites.

EXPERIMENTAL SECTION

[^{14}C]Mibolerone. Mibolerone (17β-hydroxy-7α,17-dimethylester-4-en-3-one, Figure 1) labeled at the 17α methyl position with ^{14}C was obtained from New England Nuclear. This was purified on Alumina TLC plates (Analtech 250 μm thick) with chloroform as the mobile phase. Purity (specific activity) of the above was determined by high-pressure liquid chromatography (DuPont 820), with UV detector at 254 nm on a RP-8 (Anspec Co., Inc.) 25 cm × 4.6 mm i.d. column. The mobile phase was 47:53 acetonitrile/water (v/v). Specific activity was 394 dpm/ng or 53.6 mC/mM.

Reference Mibolerone. Unlabeled mibolerone (17β-hydroxy-7α,17-dimethylestr-4-en-3-one, 99% purity, The Upjohn Co.).

Reagents and Materials. Acetate buffer, prepared by dissolving 126 g of anhydrous sodium acetate and 13 mL of acetic acid in 220 mL of water; enzyme, Glusulase (Endo labs), 42 048 Fishman units (FU) of sulfatase/mL, 176 133 Fishman units (FU) of glucuronidase per milliliter; 0.20-μm filter unit (Nalge Co. No. 120); solvents, all solvents were "distilled in glass" grade (Burdick and Jackson) unless specified otherwise; TLC plates, 250-μm thickness, silica gel GF^{254} (Analtech); Florisil (Floridin Co.) XAD-2 resin (Rohm and Haas). The solvent was allowed to evaporate, and the top half of the capsule was slightly moistened with water and sealed.

Preparation of Labeled Dose. Labeled mibolerone was dissolved in ethanol and pipetted onto sucrose contained in the bottom half of a no. 3 gelatin capsule. The amount of labeled mibolerone added to each capsule was calculated from the average daily consumption of feed during the week prior to the administration (Table I). Calculations were based on feed containing 1.5 μg of mibolerone per gram of feed.

Administration of the Dose. The capsules were administered orally, one per bird, between 7:30–8:00 a.m. Control birds received capsules of sucrose (no mibolerone) on the same schedule as the treated birds.

Chicken Treatment. Approximately 100 White Rock strain chicks were divided into two groups (control and treated). The treated group was housed in a battery and fed for 1–28 days of age on a ration fortified with 1.5 μg of mibolerone/g of feed. The control group was housed in a similar manner and fed the same ration without mibolerone. Water was provided ad libitum.

At the end of three weeks, 20 chickens from the treated group were selected on the basis of body weights closest to the median weight of the group and transferred to metabolism cages (five birds/cage).

The [^{14}C]mibolerone was administered orally via the gelatin capsule to the treated group from 21–49 days of age. When the birds were 49 days of age (after 28 days [^{14}C]mibolerone treatment), the treated group was transferred to the same ration as the control group. Control birds received capsules prepared with only sucrose.

Sample Collection. Excreta was collected from the treated birds at the start of the radioactive dosing period and continued throughout the treatment period. Excreta from each cage of five birds were collected daily.

One hour after the 21st and final radioactive dose, two treated and two control birds were slaughtered. Liver, fat, skin, kidney, and muscle were excised and frozen. The remainder of the chickens were continued on nonmedicated feed and two birds killed at 7, 14, 21, 28, 42, 56, and 120 days posttreatment. The six birds that remained were used for egg production.

The first six eggs from each of six mibolerone treated and five control birds were collected. The yolk was separated from the albumin and each weighed. Total radioactive residue was determined by combustion in a Packard TriCarb Biological Oxidizer. Approximately 100 mg of yolk and 1 g of albumin was used for each assay.

Florisil Column Chromatography. Florisil column chromatography was conducted as previously described (Krzeminski et al., 1976). In addition to the published elution sequence, methanol was used to recover additional radioactive compounds.

XAD-2 Column Chromatography. The XAD-2 resin (Rohm and Haas) was processed as follows: To a glass column (10 × 120 cm) fitted with a stopcock and a glass wool plug, 5 kg of resin was added as a slurry in water. A plug of glass wool was placed on top of the resin and the column was first washed with 12 L of acetone, then with 8 L of methanol, both at 60 mL/min. Finally, it was washed overnight at the same rate with deionized water. The resin was stored as a slurry in water and was used for the preparation of individual XAD-2 columns. Individual columns (1.9 × 35 cm) were packed as a slurry in water. A plug of glass wool was placed on top of the resin. The liver extracts were applied in 10 mL of water, the column was washed with 250 mL of water, and the radioactivity was then eluted with methanol. Additional radioactivity was sequentially eluted with 200-mL volumes of acidified methanol and acetone.

Enzyme Hydrolysis. Liver extracts believed to contain conjugated steroids were dissolved in 20 mL of deionized water. The pH was adjusted to 5.2 with acetate buffer and the solution sterilized by passage through a 0.20-μm filter. Four milliliters of sterile glusulase was directly added without filtration. A 40-h incubation was conducted at 37 °C.

Thin-Layer Chromatography. Silica gel plates (Analtech) were activated at 100 °C and stored over Drierite. Mobile phases used were: System I, 60:40 ethyl acetate/cyclohexane (v/v); system II, 90:10 methylene chloride/methanol (v/v). Each plate was automatically scraped (TLC Zonal Scraper, Analabs), and histograms were computer generated after liquid scintillation counting of the 0.5-cm bands.

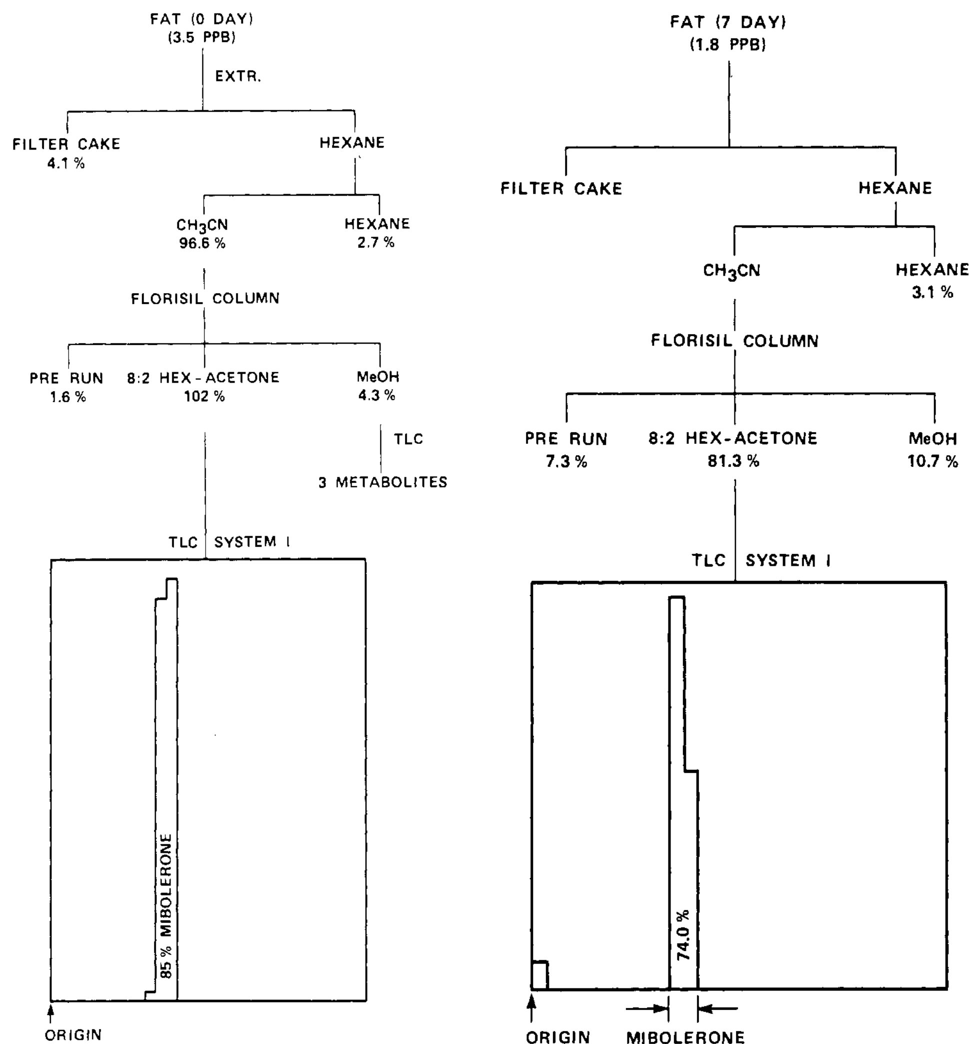

Figure 2. Fractionation of fat and 0 and 7 days posttreatment.

Radioactive Determination. Total radioactivity was determined by combustion of tissue aliquots in a Packard TriCarb Oxidizer (Model 306), corrected for quenching and efficiency by internal standard techniques. Total counts were converted to [^{14}C]mibolerone equivalents.

Liver Preparation for Histograms. A single liver (no. 5358) was homogenized in a blender with acetonitrile (3 × 100 mL) and the combined extracts partitioned against hexane (3 × 200 mL). The acetonitrile layer was rotary evaporated and the residue chromatographed on a XAD-2 resin column. The residue from the methanol eluate was partitioned between ethyl ether and water. The radioactivity contained in the other eluates were pooled and dried by rotary evaporation. This residue was then partitioned between ethyl ether and water. The residues contained in the water phase were enzymatically hydrolyzed with glusulase. The radioactive fractions were recovered by XAD-2 column chromatography. The methanol eluate was then roto-dried and the residue reconstituted to 1 mL for TLC.

Liver Preparation for Mibolerone Determination. A liver (no. 5438) from a second chicken was homogenized in a blender with acetonitrile (3 × 200 mL). The combined extracts were partitioned against hexane (3 × 200 mL). The acetonitrile layer was rotodried and chromatographed on Florisil. Residues from both the 8:2 hexane/acetone (v/v) and the methanol eluates were partitioned between hexane and 7:3 methanol/water (v/v). The methanol/water was then partitioned against methylene chloride. The methylene chloride layer was transferred to 0.3-mL reaction vials for subsequent thin-layer chromatography.

Fat Preparation. Fat was admixed with twice its weight of sand and ground with a mortar and pestle. The fat–sand mixture was extracted with hot hexane (4 × 14 mL). The hexane solution was partitioned (2×) against acetonitrile. The residue contained in acetonitrile was chromatographed on a Florisil column. Radioactive eluates were chromatographed on thin-layer plates.

Fat Extraction from the Carcass. The carcass was cut into manageable pieces, placed in a 4-L stainless steel beaker and autoclaved for 90 min at 120 °C and 15 psi. Then 100 mL of deionized water was added and the carcass stored for 18 h at 4 °C. The solidified fat was skimmed off the top. The gelatinous material that remained was heated on a steam bath until it liquidified. It was then extracted thrice with hot hexane. The hexane was filtered through sodium sulfate and combined with the skimmed fat. The hexane solution was partitioned against acetonitrile. The acetonitrile residue was chromatographed on Florisil and the radioactive 8:2 hexane/acetone eluates examined on TLC plates.

RESULTS AND DISCUSSION

This study was conducted in "replacement chickens" (*Federal Register*, 1973). These are chickens raised to replace chickens involved in egg production. Chickens for this application would be most likely to contract lymphoid leukosis. The White Rock strain was selected as it was

Table II. Accountability of the Dose in the Excreta

treatment[a] day	radioactivity in excreta (dpm × 10^9)					% of administ. dose
	pen 1[b]	pen 2	pen 3	pen 4	av	
1	1.43	1.33	1.89	1.76	1.60	120.5
2	1.81	1.38	1.25	1.75	1.55	116.4
3	1.89	1.38	2.42	1.99	1.92	144.4
4	1.62	1.21	1.66	1.45	1.49	111.7
5	1.90	1.42	2.20	1.74	1.82	136.5
6	1.28	0.99	1.45	1.42	1.29	96.6
7	1.78	1.26	1.78	1.77	1.65	123.9
8	1.72	1.47	2.06	1.65	1.73	130.7
9	1.39	0.96	1.26	1.27	1.22	92.4
10	1.53	1.07	1.57	1.45	1.41	106.4
11	1.74	1.18	1.66	1.32	1.48	111.7
12	1.47	1.22	1.61	1.39	1.42	107.8
13	1.48	1.29	1.69	0.88	1.34	101.1
14	1.43	1.19	1.50	1.30	1.36	102.7
15	1.66	1.20	1.80	0.90	1.39	103.7
16	1.41	1.24	1.67	1.46	1.45	107.8
17	1.52	1.47	1.50	0.97	1.37	101.9
18	1.42	1.16	1.76	0.85	1.30	96.8
19	1.13	1.44	1.46	1.28	1.33	99.1
20	1.66	1.42	1.20	1.31	1.40	104.3
21	1.22	1.06	1.67	1.68	1.41	105.0
total	32.47	26.34	35.06	29.59		
% dose	116.3	94.3	125.6	106.0		
accountability	110.6 %					

[a] Days 1-7, dose/five birds = 1.33 × 10^9 dpm/day; days 8-14, dose/birds = 1.32 × 10^9 dpm/day; days 15-21 dose/birds = 1.34 × 10^9 dpm/day. [b] Five birds per pen.

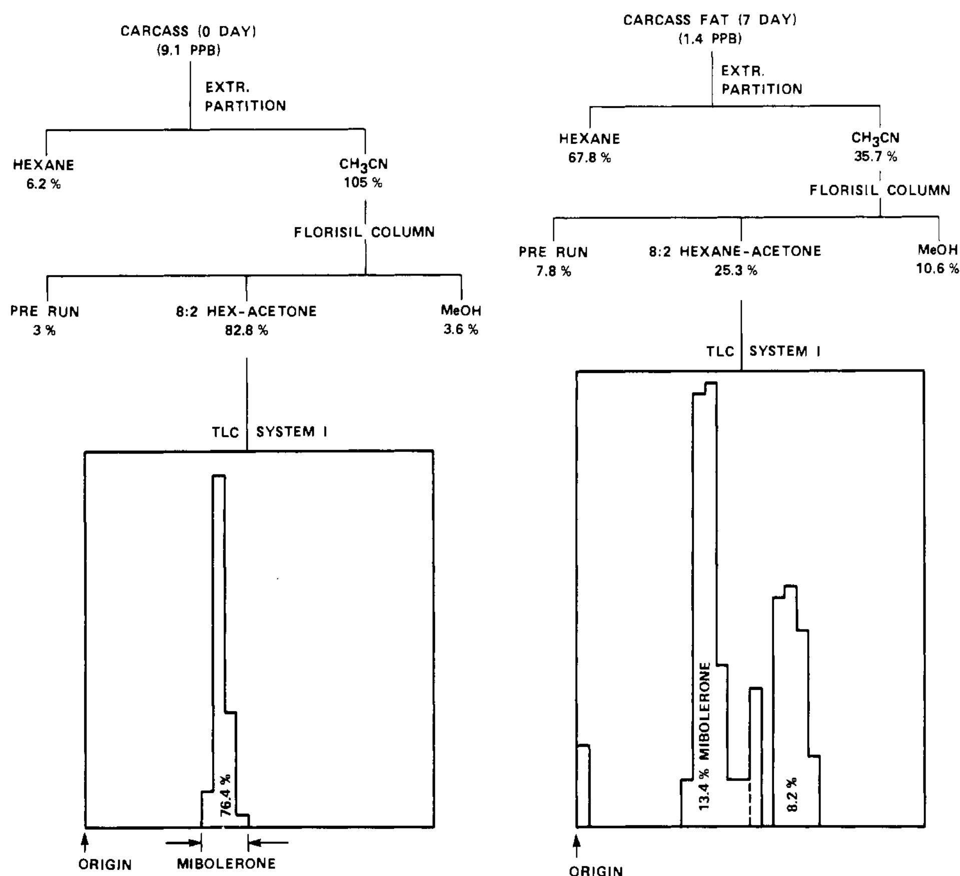

Figure 3. Fractionation of carcass extract at 0 and 7 days posttreatment.

noted for its high fat content. This was thought to be advantageous in the conduction of metabolism studies that involved fat.

Excretion. Excretion of the radioactivity via the excreta was rapid. Accountability was 111% for the 28-day period during which [^{14}C]mibolerone was administered (Table II). The high bias was observed in earlier studies (Ogilvie, 1977; Gosline et al., 1977). The relatively large positive bias is believed due to small daily biases which are accumulative over the several weeks that the radioactivity of the excreta was determined. Possibly there was a problem in the estimation of combustion efficiency. Nevertheless the results indicate rapid obtainment of equilibrium for ^{14}C radioactivity excretion.

Tissue Levels. Measurement of radioactivity 1 h (0 day) after the last dose showed liver contained the

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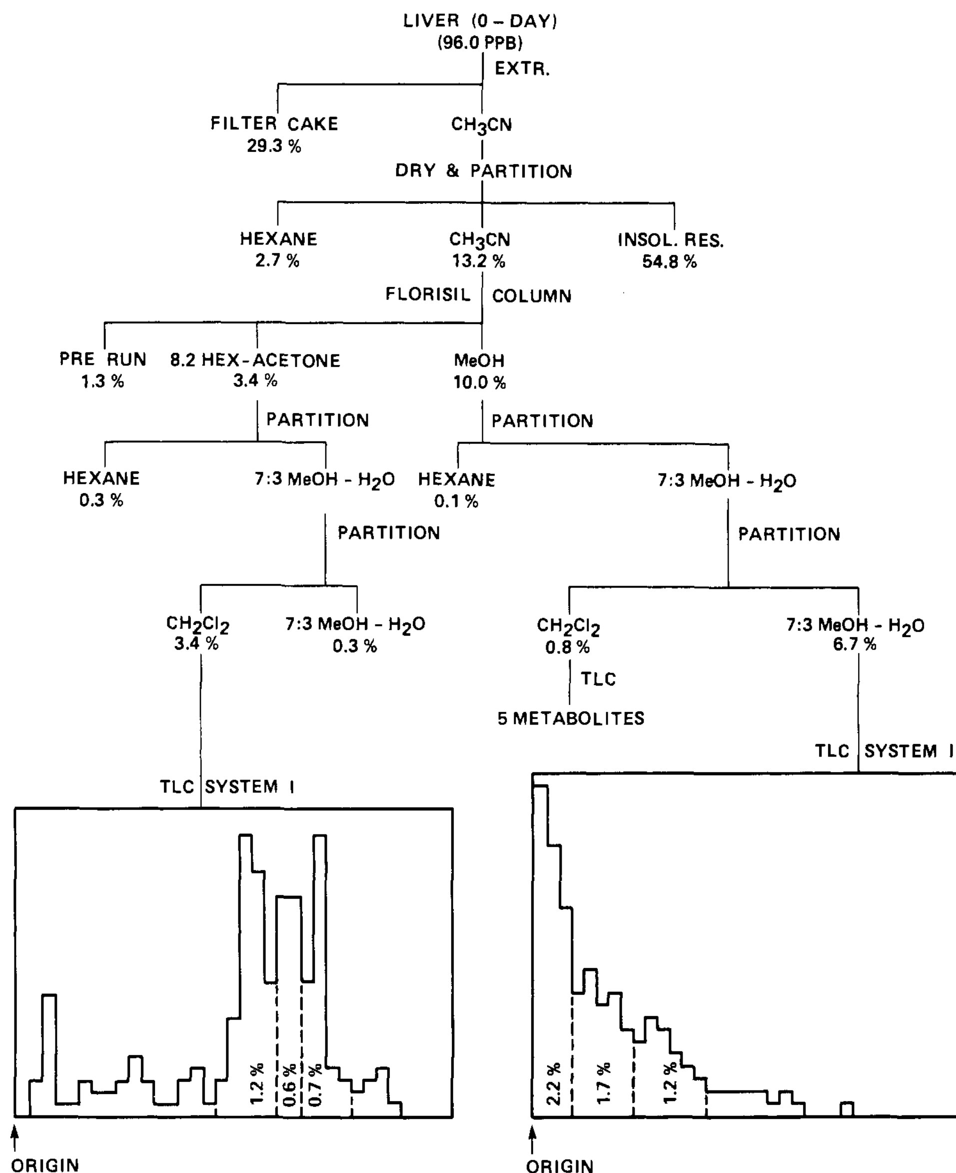

Figure 4. Nonhydrolysis fractionation of a liver (no. 5318).

equivalent of 94.4 ppb mibolerone based on the initial specific activity of [^{14}C]mibolerone. Thereafter, the radioactivity declined to 2.2, 0.6, and 0.5 ppb at 7, 14, and 21 days posttreatment (Table III).

Levels of radioactivity in adipose tissue (fat) declined from 3.5 ppb (1-h posttreatment) to 1.8, 1.0, and 0.5 ppb at 7, 14, and 21 days posttreatment (Table III). Carcass lipids (total extractable body fat) were somewhat higher in radioactivity at day zero but declined at a rate that paralleled the adipose tissue. Although liver was initially higher than fat, after 2 weeks the radioactive concentration in the fat exceeded the concentration in liver. This "cross-over" in the concentration curves was observed in earlier preliminary experiments as well (Dunn, 1977).

There were no residues above the limit of detection (0.5 ppb) in blood sera, kidney, muscle, or skin at 7 days posttreatment. The birds were 56 days of age at this time. The first three eggs produced by each of six chickens were assayed for radioactive content. There were no detectable residues in any egg. At the time of egg production the birds were approximately 120 days of age, 70 days since the last mibolerone dose.

Metabolism in Fat. ^{14}C radioactivity in fat was separated into two fractions based on Florisil column elution (Figure 2). The ^{14}C radioactivity from the 8:2 hexane/acetone elution migrated as a single bond on a silica TLC

Table III. ppb Mibolerone Equivalents of [^{14}C]Radioactivity in Selected Organs and Tissues[a]

tissue		days posttreatment 0	7	14	21
blood, sera	control	<0.5	<0.5	<0.5	<0.5
	treated	4.9	<0.5	<0.5	<0.5
bursa	control	<0.5	<0.5	<0.5	<0.5
	treated	308.3	<0.5	<0.5	<0.5
adipose tissue	control	0.8	<0.5	<0.5	<0.5
	treated	3.5	1.8	1.0	<0.5
kidney	control	<0.5	<0.5	<0.5	<0.5
	treated	4.8	<0.5	<0.5	<0.5
liver	control	<0.5	<0.5	<0.5	<0.5
	treated	94.4	2.2	0.6	<0.5
muscle, red[b]	control	<0.5	<0.5	<0.5	<0.5
	treated	3.3	<0.5	<0.5	<0.5
muscle, white[c]	control	<0.5	<0.5	<0.5	<0.5
	treated	<0.5	<0.5	<0.5	<0.5
skin	control	<0.5	<0.5	<0.5	<0.5
	treated	<0.5	<0.5	<0.5	<0.5
carcass fat	treated	9.1	1.4		

[a] Based on the average of two birds. Radioactivity expressed as ppb mibolerone equivalents. [b] Leg and thigh muscle. [c] Breast muscle.

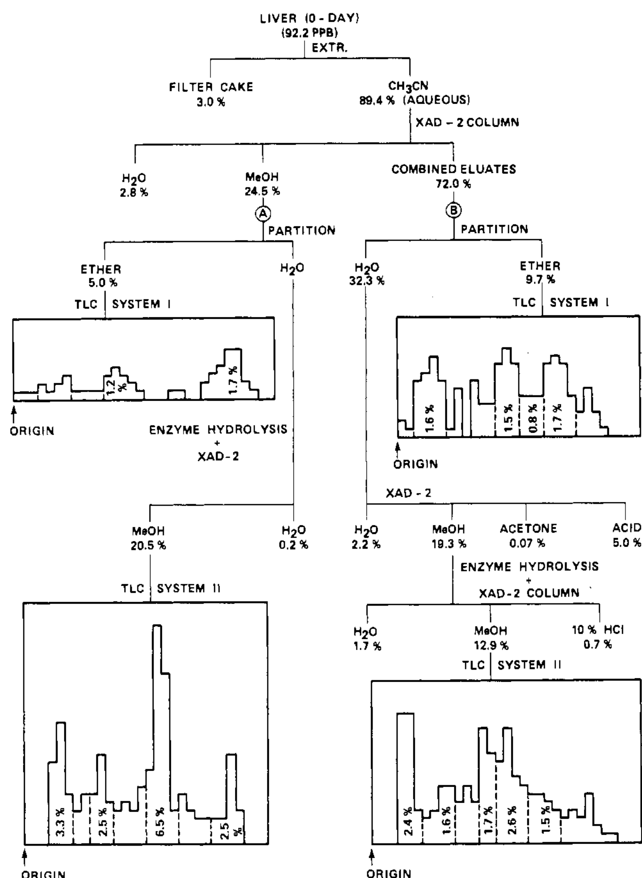

Figure 5. Hydrolytic fractionation of a liver (no. 5358).

plate. This was true both for the zero-day and 7-day posttreatment sample. The R_f was similar to that of a mibolerone standard. Minor metabolites were evident in the methanol eluate of florisil. Three were evident at the zero-day time period. There was insufficient material to make a similar determination at the 7-day posttreatment period. The presence of mibolerone as the only significant metabolite in fat had been previously confirmed by GC/MS (Jaglan, 1977). The carcass was rendered in an attempt to obtain additional lipid material for metabolite isolation characterization. An ad hoc recovery study was conducted. A carcass of a chicken that did not receive any mibolerone was fortified with [^{14}C]mibolerone. Approximately 98% was recovered. No attempt was made to prevent or determine if the autoclave technique caused degradation of metabolites. There was a close similarity to fat harvested at the zero necropsy in terms of fractionation (Figure 3) and subsequent thin-layer chromatographic profile. This would indicate the preparative technique was suitable. At the 7-day necropsy it was observed that a nonpolar metabolite was present. It was not observed in the other carcass of fat extracts. Its concentration was quite low and that may account for it not being observed elsewhere.

Metabolism in the Liver. Livers were harvested from two chickens 1 h after each had received their final [^{14}C]mibolerone dose. One liver (no. 5318) was processed without hydrolysis (Figure 4). The unconjugated metabolites contained 13.2% of the total. At least five distinct metabolites were evident after florisil column chromatography, solvent partition, and chromatography on thin-layer plates. Mibolerone was estimated to be present at slightly over 1% (1 ppm) of the total.

A second liver (no. 5358) was prepared for enzymatic hydrolysis (Figure 5). Several metabolites were demonstrated. The fractionation into many individual metabolites of low concentration precluded further characterization. It was for this reason no attempt was conducted to fractionate liver from the 7-day necropsy period.

CONCLUSION

[^{14}C]Mibolerone, given orally to chickens, was rapidly metabolized and eliminated. Highest concentrations of radioactive material were found in bursa, followed by liver. Intact mibolerone was found in adipose tissues. Metabolites of mibolerone were detected in adipose tissue and liver, but were present in amounts too small for identification. Residues in detectable amounts were not found in eggs collected 72 days after the last dose of [^{14}C]mibolerone. The methods would have detected residues above 0.5 ppb.

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