Metabolism of Ronidazole (1-Methyl-5-nitroimidazol-2-ylmethyl Carbamate)

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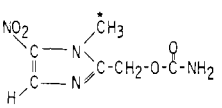
Ronidazole-^{14}C was administered to turkeys in diets containing 0.003–0.008% of drug. More than 80% of administered radioactivity was excreted and 1–2% was exhaled as $^{14}CO_2$. Metabolites were 2-hydroxymethyl-1-methyl-5-nitroimidazole in trace amounts. Also found were *N*-methylglycolamide, methylamine, and oxalic acid. Radioactivity in tissues was associated with a number of normal body metabolites, such as glutamic and aspartic acids and citric acid cycle intermediates. The presence of acetyl-^{14}C was demonstrated in urine and liver tissue. Radioactive protein, nucleic acid, and lipid fractions were found in whole liver, leading to the conclusion that extensive biodegradation of ronidazole-^{14}C allows the recombination of carbon-14 into body metabolites.

Ronidazole (I) is a nitroimidazole with antiparasitic activity useful in treatment of enterohepatitis in turkeys (Peterson, 1969). Radioactive indicator studies revealed that it undergoes rapid and complete biodegradation when administered to turkeys in feed. Similar degradation was observed when the labeled drug was incubated in liver homogenates and in urine. None of the tissues examined except muscle retained intact ronidazole or related compounds even during medication, despite considerable retention of radioactivity. Similar extensive biodegradation occurred in rats and dogs.

The extensive biodegradation of ronidazole is in marked contrast to the behavior of other nitroimidazoles. Typical is the behavior of dimetridazole (1,2-dimethyl-5-nitroimidazole), which yields seven structurally related metabolites, including the 2-hydroxymethyl derivatives in the turkey (Law *et al.*, 1963); metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] which is converted to five nitro-containing metabolites in man (Stambaugh *et al.*, 1967, 1968) and in mice (Stambaugh and Manthei, 1967), its major metabolite being the 2-hydroxymethyl derivative; and tinidazole (the ethane sulfonic ester of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), which is recoverable as unchanged drug in human plasma and urine (Taylor *et al.*, 1969).

EXPERIMENTAL METHODS

Tracers Employed. Two ^{14}C-labeled modifications were employed to study the metabolic disposition, excretion, $^{14}CO_2$ formation, and tissue retention of ronidazole. These radioactive forms were labeled in the 1-*N*-methyl group (I) and in the ring-2-C position (II).



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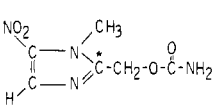

II

The specific activity of these preparations varied between ≃0.2 and ≃4.3 μCi/mg, depending on the purpose of the experiment. In most instances, sufficient tracer was blended by standard mixing procedures with several kilograms of base ration (a commercial turkey and game bird starter ration) to produce appropriate concentrations in feed. In single dose experiments the ronidazole-^{14}C was administered in aqueous solution, except in one massive dose experiment in which a solution in dilute lactic acid was employed.

Animal Studies. Day-old Beltsville (small white) turkey poults were obtained from a commercial hatchery and reared in electrically heated metal battery brooders with a wire floor, in a temperature controlled room. Water and feed were supplied *ad libitum*. The base ration was fed until the poults were 3 weeks of age. At this time they were weighed and distributed into groups equally balanced according to individual weights. Medicated diets containing 0.003, 0.006, and 0.008% ronidazole were fed for 4 days prior to withdrawal of medication and subsequent periodic sacrifice. Older turkeys, 5–30 weeks of age and with exteriorized colons, were utilized for certain metabolism and urinary excretion studies, whereas birds 2.5–4 weeks old were employed in the $^{14}CO_2$ exhalation experiments.

In the latter experiments poults were housed on wire in 12-in. desiccators through which a stream of cooled air was maintained by house vacuum. Exit air from each desiccator was passed through three successive absorbing towers equipped with fritted glass sparger plates, each tower containing 50 to 100 ml of 4 *N* NaOH. At the end of 4 hr each absorber train was replaced, and the experiment continued for another 20 hr.

Blood samples were obtained periodically from the brachial vein during the feeding of certain birds. In tissue retention experiments, birds were sacrificed at the time of withdrawal of medicated feeds and at specified times up to 5 days after withdrawal. Tissue samples were removed after sacrifice and stored frozen until assayed. Intact feces and urine were obtained from birds with artificial ani.

Radiometric Methods. PROCEDURE FOR URINE AND FECES. Radioactivity of urine and feces was measured by the method of Tocco *et al.* (1965).

PROCEDURE FOR TISSUES. Two methods were employed for the radiometric assay of tissue specimens. Kidney, liver, muscle, and plasma were measured by the Low Beta Geiger counter equipped with an ultra-thin window and characterized by a very low background. In most instances 4 g of wet tissue from individual birds was homogenized with 16 ml of water in a Virtis homogenizer and 1 g or 2 g of homogenate evaporated under an infrared lamp in 2.75 in. stainless steel planchets for counting. One-milliliter samples of plasma were similarly evaporated for assay. Standards were prepared and counted under identical conditions to obviate the necessity for making self-absorption corrections.

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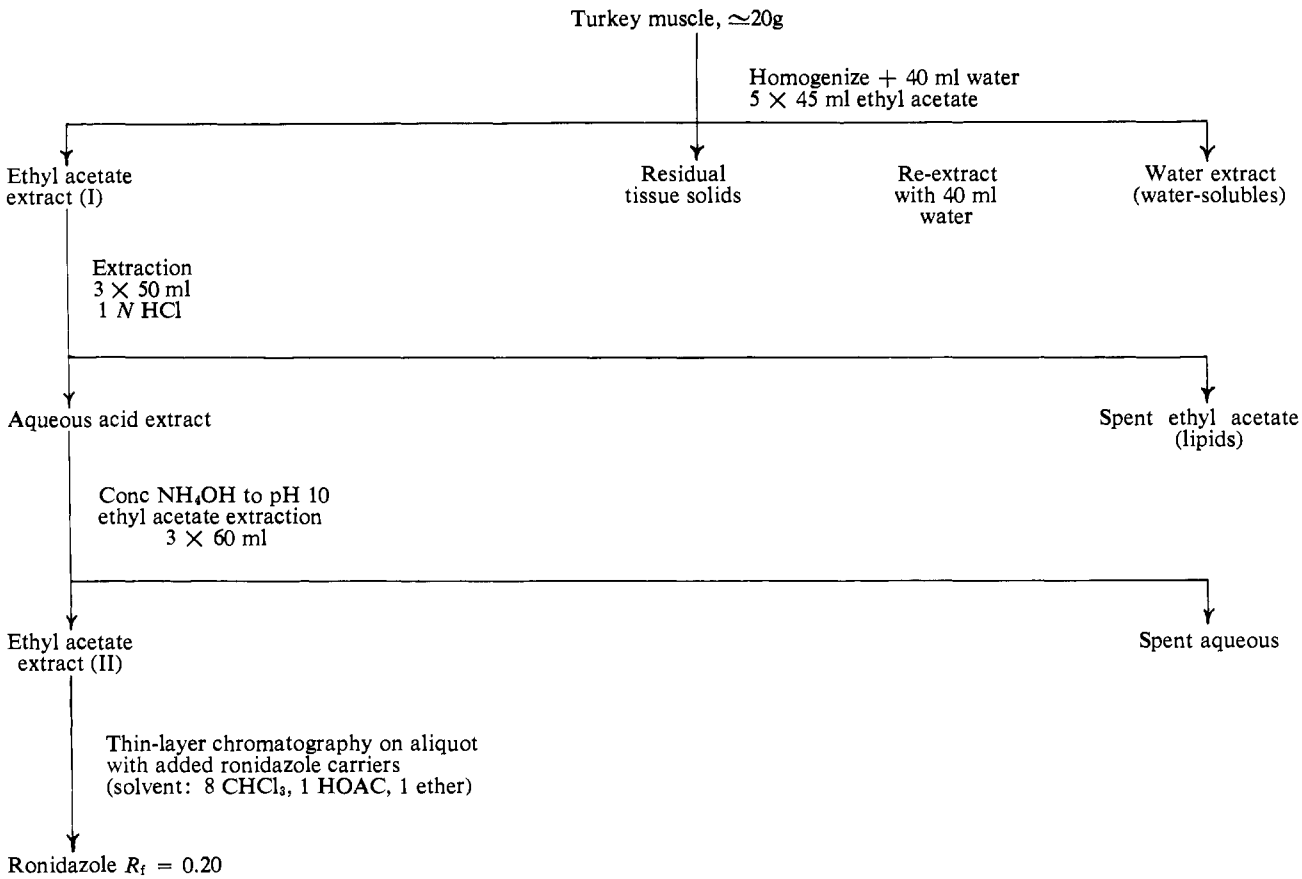

Figure 1. Scheme for recovery of Ronidazole and metabolites for assay

Skin samples and tissue homogenate samples (~0.2 g) were assayed by combustion in oxygen by the Schöniger method, and the $^{14}CO_2$ absorbed in 10 ml of 1 *M* methanolic hyamine for measurement by liquid scintillation counting. Half of this hyamine solution was added to 15 ml of scintillator solution in ethanol-free toluene.

Samples of fat weighing 30–250 mg were dissolved directly in 5 ml of ethanol to which was added 15 ml of scintillator solution (100% toluene).

PROCEDURE FOR $^{14}CO_2$ IN EXPIRED AIR. Two milliliters were removed for assay from absorber traps containing 50–100 ml of 4 *N* NaOH. Excess sulfuric acid was added from a sidearm on an Erlenmeyer flask containing the sample to liberate $^{14}CO_2$, which was then allowed to diffuse to an adjoining Erlenmeyer flask containing 5 ml of hyamine solution (1 *M* in methanol). Absorption was hastened by rotary agitation and required ~3 hr for complete transfer. Three of the 5 ml of hyamine solution were added to 17 ml of ethanol-free scintillator solution in toluene for liquid scintillation counting. Quantitation was achieved by comparison with standard solutions in hyamine.

PROCEDURE FOR METABOLITES. Fractions isolated in connection with the studies of ronidazole metabolism were assayed radiometrically by direct liquid scintillation counting.

Demonstration of Metabolites. Ethyl Acetate Soluble Fraction. IN URINE. Metabolites formed from ronidazole were investigated by fractionation of urine free from fecal matter obtained from birds with artificial ani which had received ronidazole-^{14}C in feed or in single doses. (*In vitro* incubation of ronidazole-^{14}C with turkey liver homogenates were also studied. Results were in essential agreement with the *in vivo* studies.)

Urine was extracted exhaustively with ethyl acetate at pH 7–8. The aqueous phase was then processed as described in Figure 2. The following imidazoles related to ronidazole were subjected with the drug itself to the extraction procedure in Figure 1 to test the reliability of the assay method: (1) Ronidazole; (2) 2-hydroxymethyl-1-methyl-5-nitroimidazole; (3) 1-methyl-5-nitroimidazole-2-carboxylic acid; (4) 1-methyl-5-nitroimidazole; (5) hydroxycarbamic acid ester of 2-hydroxymethyl-1-methyl-5-nitroimidazole; and (6) ethereal sulfate of (2). All of the model compounds except (3) and (6) were extractable, hence assayable, by the radiometric method described above. Compound (3), *i.e.*, the free acid, was extractable at pH 3. It was not present, however, since no radioactivity was extracted from urine at this pH.

IN TISSUES. The tissue to be extracted was first ground in a meat chopper to insure homogeneity. A known weight of the ground tissue was homogenized with two volumes of water in a small Waring blender. A sample (3 ml) was taken for determination of total tissue radioactivity by the combustion method. The homogenate was then extracted and fractionated into an ethyl acetate extract, water-soluble components, a lipoidal fraction, and residual solids (Figure 1).

The recovery procedure detailed in Figure 1 was tested with control muscle to which had been added 4 μg of ronidazole-^{14}C with an activity of 12,000 cpm. A 90% recovery of ronidazole from muscle was realized.

ESTIMATION OF RONIDAZOLE AND METABOLITES. The identification of the recovered ronidazole and its metabolites was based upon a thin-layer chromatographic system using Analtech silica gel GF plates 5 × 20 cm. To an ethyl acetate extract of a tissue sample, carrier amounts of ronidazole, 2-

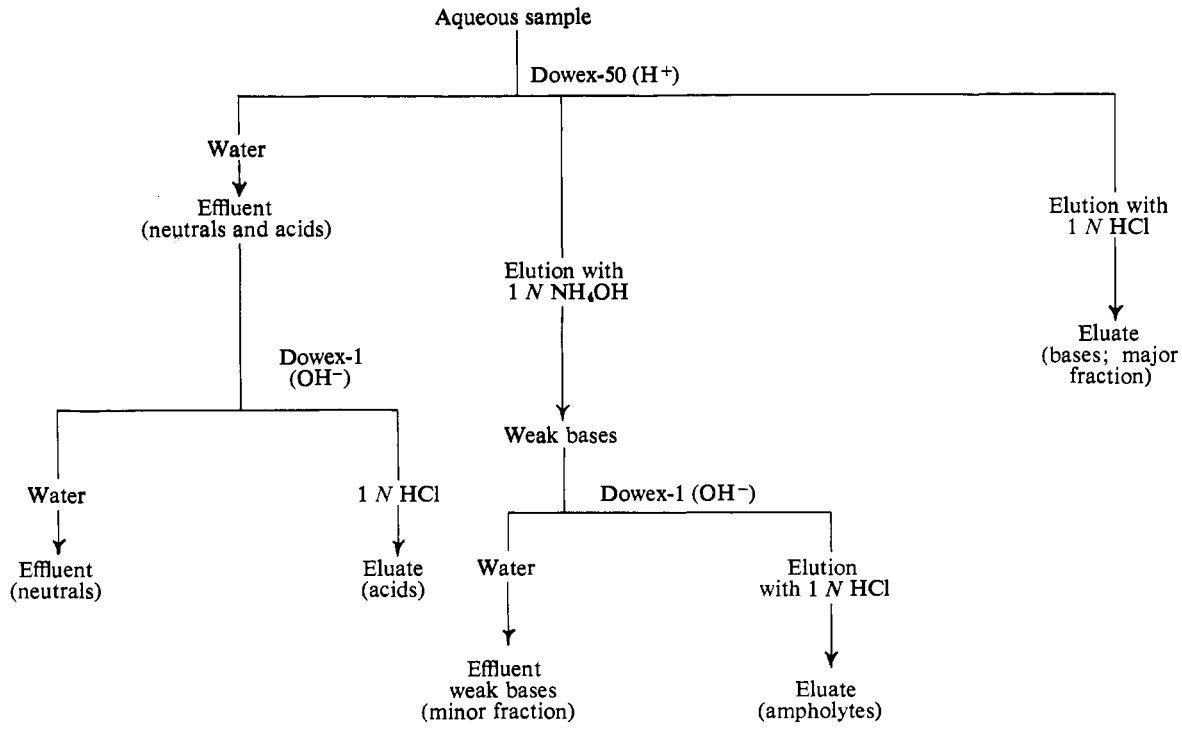

Figure 2. Fractionation procedure for water-soluble, ethyl acetate-insoluble, extracts of tissue and urine

hydroxymethyl-1-methyl-5-nitroimidazole and 1-methyl-5-nitroimidazole-2-carboxylic acid (about 20–30 μg each), were added. In the system chloroform–ether–acetic acid (80/10/10), the R_f values for these compounds were about 0.2, 0.4, and 0.6, respectively. After a plate was developed the carrier zones were clearly visible in uv light. Entire visualized zones were scraped off the plate and put in counting vials, while the remainder of the plate was scraped into vials in 1-cm increments. Recovery of ronidazole from tissue to thin-layer plate was $\simeq$90%. This thin-layer system was used as one radiometric assay procedure for ronidazole and metabolites in tissue.

As an alternative and confirmatory identification of drug residues, samples were subjected to paper electrophoresis using 0.5 *M* acetic acid as electrolyte. By adding the carrier compounds listed above, it was possible to locate the carrier samples on the dried paper strips in uv light. The absorbing areas were cut out and the papers placed in vials for scintillation counting. Electrophoresis yielded only two radioactive ultraviolet bands in any of the turkey tissues extracted, corresponding to the ronidazole band and the 2-hydroxymethyl-1-methyl-5-nitroimidazole band. A ronidazole-^{14}C standard showed only one radioactive band when added to control tissue, equivalent to a recovery of 92%.

Demonstration of Metabolites. Water Soluble Fraction. Urine retained a considerable fraction of the initial radioactivity after extraction with ethyl acetate. This water-soluble activity was examined by chromatography on ion-exchange resins [Dowex-50 (H^+) and Dowex-1 (OH^-)]. The scheme applied to urine and tissue extract is presented in Figure 2.

Demonstration of Metabolites. Residual Tissue Solids. Solids from liver and muscle of turkeys on diets containing 0.006% ronidazole-^{14}C of both labeled forms and at 2 and 5 days after withdrawal of diets were: (1) subjected to dialysis against water for 2 days; (2) extracted exhaustively with 50% ethanol; (3) treated with 10% TCA; and (4) fractionated into protein and nucleic acid. The first three treatments were designed to explore the existence of previously unreleased metabolic products or low molecular weight proteins. The last treatment showed how much activity was found in the two biological components, protein and nucleic acid.

Two procedures were employed for fractionation. The method of Ogur and Rosen (1950) and Glick (1966) was applied to one sample, but was discontinued because the presence of perchloric acid in assay samples made combustion inadvisable. The method (Davis, 1962) applied to the remaining samples employed 10% sodium chloride instead of perchlorate as the protein precipitant. The nucleic acid and protein results may be low because of possible loss of radioactive polynucleotides and low molecular weight proteins in fractions such as dialysates, supernatants, and intermediate washes, which are usually discarded. In a test run with muscle, these fractions accounted for 8.6% of the solids' radioactivity which was in agreement with results of other assay methods (Table I).

RESULTS

Elimination of Radioactivity. Expiration of Radioactive Carbon Dioxide. The extent of radioactive carbon dioxide exhalation in 1–2 days by turkeys receiving single oral doses of labeled ronidazole equivalent to 6–9 mg/kg of body weight is reported in Table II. The birds converted both labeled forms to $^{14}CO_2$, exhaling 0.8–1% of the administered radioactivity in 24 hr. The ring-labeled tracer produced an additional 1–2% of dose in the second 24 hr collection period. In both instances, the rate of exhalation of $^{14}CO_2$ seemed to increase with time and was still proceeding after 2 days. Percentage figures listed in Table II are obviously minimal values. Carbon dioxide conversion must account for well over 3% of administered radioactivity. It is not surprising, in view of such extensive $^{14}CO_2$ production, that widespread distribution and prolonged retention of radioactivity occurred when ronidazole-^{14}C was fed to the test birds. This is typical of

Table I. Distribution of Radioactivity in Residual Solids from Liver and Muscle of Turkeys Consuming Medicated Diets,[a] % of Total Radioactivity

Labeled form	Tissue	Treatment	% Distribution			Extractable in 50% EtOH
			Protein	NA	Low mol wt protein[e]	
Ring-^{14}C	Liver[b]	1 day on	37	34	15	
Ring-^{14}C	Liver	3 days on	68	0.6		
Ring-^{14}C	Liver	3 days on				16
N-$^{14}CH_3$	Liver	3 days on				16
Ring-^{14}C	Liver	2 days off	84	1.1		
Ring-^{14}C	Liver	2 days off	54		3.5	5
N-$^{14}CH_3$	Liver	2 days off	70	9.3		10
Ring-^{14}C	Liver	5 days off	80	1.5	2.4	5
Ring-^{14}C	Muscle	3 days on	54	1.8		13
Ring-^{14}C	Muscle	2 days off	$\simeq$80[c]	4.9		14
Ring-^{14}C	Muscle	2 days off	88	1.4	8.6	
N-$^{14}CH_3$	Muscle	2 days off	74	6.0		9
Ring-^{14}C	Muscle	5 days off	$\geqslant$69[d]	2.7		12

[a] All diets contained 0.006% ronidazole. [b] Ogur–Rosen method of fractionation. [c] By difference. [d] Incomplete combustion. [e] Total activity in washings of nucleic acids.

Table II. $^{14}CO_2$ Exhalation by Turkey Poults Receiving Ronidazole-^{14}C

Ronidazole			% of dose as CO_2			
Labeled form	Dose, mg	Bird weight, g	0–4 hr	4–24 hr	24–48 hr	Total
2-^{14}C	3.92	484	0.05	0.93	0.98	1.96
	2.92	492	0.13	0.96	2.11	3.20
	Average	488	0.09	0.94	1.55	2.58
N-$^{14}CH_3$	2	216	0.05	0.66[a]		0.71
	2	232	0.04	0.80[a]		0.84
	Average	224	0.04	0.73[a]		0.77

[a] 4–22.5 hr collection.

Table III. Fecal and Urinary Excretion of Ronidazole-^{14}C by Turkeys

Bird		Ronidazole consumption				% of Dose							
No.	Age, weeks	Amount, mg	Label	Method of administration	Excreta	1 day on	2 days on	3 days on	1 day off	2 days off	3 days off	4 days off	Total
1	16	150	N-$^{14}CH_3$	Single oral dose in dilute lactic acid	Urine				30	3.2			33
					Feces				16	15			31
					Total				46	18			64
2	13	56.4	N-$^{14}CH_3$	0.006% Ronidazole	Urine	4.4	4.4	6.8	3.4				19
					Feces	3.3	10.2	10.9	10.4				35
					Mixed	3.5	3.1	7.9	3.2	6.4		3.7	28
					Total	11.2	17.7	25.6	17.0	6.4		3.7	82
2	18	28.8	N-$^{14}CH_3$	0.006% Ronidazole	Urine	2.3	11.4	11.7	1.2	0.3	0.2	0.1	27
					Feces	1.3	12.6	14.3	5.3	8.5	5.7	2.5	50
					Total[a]	3.6	24.0	26.0	6.5	8.8	5.9	2.6	77
2	25	27.1	Ring-^{14}C	0.006% Ronidazole	Urine	6.2	24.9		13.2	2.1	0.7	0.7	48
					Feces	3.2	8.0		8.4	3.4	4.3	4.4	32
					Total[b]	9.4	32.9		21.5	5.6	5.0	5.1	80

[a] Estimates of radioactivity excreted beyond 4 days suggest the additional elimination of $\simeq$1.0 mg, mostly in feces. [b] Estimates of radioactivity excreted beyond 4 days suggest the additional elimination of $\simeq$1.5 mg, mostly in feces.

the behavior of $NaH^{14}CO_3$ when administered to animals, and is presumably also true of the avian species (Skipper *et al.*, 1949a,b, 1952).

Excretion of Radioactivity in Urine and Feces. Table III summarizes the results of excreta measurements (in % of dose) obtained with Beltsville (small white) turkeys treated surgically to exteriorize their colons. Excretion by both routes was extensive, about 80% of dose being accounted for in 4 days; additional small amounts were detectable on subsequent days.

Chemical Nature of the Radioactivity in Urine. Birds with exteriorized colons provided urine uncontaminated by fecal matter. In an exploratory experiment, a single massive dose of 150 mg of ronidazole-N-$^{14}CH_3$, dissolved in a dilute lactic acid solution, was administered to a 16-week-old turkey. Assay of these urine samples for ronidazole and metabolites showed that 66–69% of the radioactivity excreted during the first 24 hr was due to ronidazole; and of the urinary radioactivity excreted during the second 24 hr, 43% was due to intact ronidazole and only 2% to 2-hydroxymethyl-1-methyl-5-nitroimidazole.

In a more extensive experiment, urine was obtained from

Table IV. Average Radioactivity Retention by Tissues at Sacrifice (0.006% Ronidazole in Diets)

Tissue	Ronidazole-^{14}C tracer	Equivalent μg/g or ml							
		0 days off[a]	2 days off	5 days off	10 days off	14 days off	21 days off	27 days off	35 days off
Kidney	Ring-2-^{14}C	5.1		0.38		0.08	0.0	0.0	0.0
	Ring-2-^{14}C	4.6		0.42		0.06	0.0	0.0	0.0
	Ring-2-^{14}C		0.87						
	N-$^{14}CH_3$	4.6			0.16		0.0		
	N-$^{14}CH_3$	4.6			0.11		0.0		
	N-$^{14}CH_3$		0.58						
		Avg 4.7							
Liver	Ring-2-^{14}C	4.9		0.18		0.0	0.0	0.0	0.0
	Ring-2-^{14}C	3.9		0.18		0.0	0.0	0.0	0.0
	Ring-2-^{14}C		0.48						
	N-$^{14}CH_3$	4.9			0.09		0.0		
	N-$^{14}CH_3$	4.1			0.0		0.0		
	N-$^{14}CH_3$		0.52						
		Avg 4.5							
Muscle	Ring-2-^{14}C	3.2		0.10		0.05	0.0	0.0	0.0
	Ring-2-^{14}C	2.5		0.07		0.0	0.0	0.0	0.0
	Ring-2-^{14}C		0.23						
	N-$^{14}CH_3$	3.4			0.34		0.08		
	N-$^{14}CH_3$	2.9			0.18		0.07		
	N-$^{14}CH_3$		0.32						
		Avg 3.0							
Plasma	Ring-2-^{14}C	3.1							
	Ring-2-^{14}C	2.3							
	Ring-2-^{14}C		0.09						
	N-$^{14}CH_3$	2.7			0.0		0.0		
	N-$^{14}CH_3$	2.3			0.0		0.0		
	N-$^{14}CH_3$		0.08						
		Avg 2.6							
Skin	Ring-2-^{14}C		0.52						
	N-$^{14}CH_3$		0.52						
Fat	Ring-2-^{14}C		0.41						
	N-$^{14}CH_3$		0.33						

[a] After 4 days on medicated feed.

birds being maintained for 2 days on a diet containing 0.006% ronidazole-N-$^{14}CH_3$. Fifty-one percent of the radioactivity in this urine was ronidazole and about 1% was 2-hydroxymethyl-1-methyl-5-nitroimidazole. It was further demonstrated that no glucuronide conjugates of ronidazole and related compounds were present in this urine (Tocco *et al.*, 1965).

The above observations have been confirmed with pooled urine from birds receiving diets supplemented with both labeled forms of ronidazole-^{14}C. Birds receiving a diet containing 0.006% ronidazole-2-^{14}C for 1 day excreted a urine from which 45% of the radioactivity was extractable by ethyl acetate. The water soluble radioactivity left after the ethyl acetate extractions was subfractionated by the scheme outlined in Figure 2. The base fraction, which was significant in amount only when N-$^{14}CH_3$-labeled drug was used, was shown to be >85% methylamine by converting the volatile count (at pH 10–100° C) to a benzoic acid salt whence on heating the dry residue in a sealed tube at 150° C for a few hours water was eliminated and the corresponding N-$^{14}CH_3$ benzamide was produced. Comparison with a known sample by tlc indicated identity.

The neutral fraction from turkey urine was significant only when ring-labeled drug was used. Here 87% of the radioactivity was shown by a reverse isotope dilution method to be in the form of an acetyl group hydrolyzable to acetic acid. Attempts to identify the complete structure of this acetylated substance were not successful and many of the likely possibilities such as acetamide were proved to be absent. In particular no N-methyl glycolamide was found, even though it was demonstrated that at pH 10–10.5 at 100° C 0.45 mol % of N-methyl glycolamide is formed in a few hours from ronidazole. Obviously therefore it might be an expected urinary excretion product of the drug metabolism. A subsequent ronidazole metabolism study in the pig yielded a large neutral fraction which proved to be mainly labeled acetamide.

Retention of Radioactivity in Tissues. LEVELS OF RADIOACTIVITY. Residual radioactivity in kidney, liver, muscle, plasma, skin, and fat of poults maintained on 0.006% medicated feed for 4 days prior to withdrawal is reported in Table IV in terms of ronidazole (μg/g or ml) equivalent to the group average carbon-14 content of these tissues. For other feed concentrations, overall equivalent "on drug" levels were approximately proportional to drug content. For example, drug residues in plasma after 4 days of feeding were 1.5 μg/ml, 2.6 μg/ml, and 3.6 μg/ml, respectively, for the 0.003, 0.006, and 0.008% feeds. Elimination of ronidazole-^{14}C from the diet was accompanied by a prompt drop in tissue radioactivity to a level indistinguishable from that of control tissue by ≃21 days after removal of drug. Both labeled forms of ronidazole suggested the same tissue retention pattern.

ETHYL ACETATE SOLUBLE RADIOACTIVITY FROM TISSUE. Paper electrophoresis and thin-layer chromatography of the ethyl acetate tissue extract were shown to isolate all intact imidazole conceivably present. Only ronidazole and 2-hydroxymethyl-1-methyl-5-nitroimidazole, however, were actually found by this assay method to be present in tissue or urine of birds treated with ronidazole-^{14}C. Results of such radiometric assays for ronidazole and metabolites in three tissues from turkeys being maintained on ronidazole, and for several withdrawal periods thereafter, are compiled in Table V for

birds receiving both labeled forms of ronidazole. Results are reported in terms of μg of ronidazole equivalent to radioactivity residing in a gram of tissue.

The possibility that ronidazole might exist in tissue as a conjugate, despite its demonstrated absence in urine, was eliminated by hydrolyzing residual muscle which had already been analyzed for ronidazole by the scheme in Figure 1. Hydrolysis failed to liberate significant radioactivity extractable by the ethyl acetate procedure, thereby indicating the absence in tissue of conjugates of ronidazole or its metabolites.

It is clear from the results in Table V that, of the several turkey tissues examined, only muscle retained appreciable quantities of ronidazole-^{14}C, and even that only while the birds were still consuming a radioactive diet. The nature of the residues in these tissues has been confirmed by a polarographic assay method based on the reduction of the nitro group (Wittick, 1969).

WATER SOLUBLE RADIOACTIVITY FROM TISSUE. The water-soluble, ethyl acetate-insoluble extracts of tissues from turkeys reported in Table VI were, like urine, fractionated further by the scheme described in Figure 2 to obtain the four classes of radioactive water-soluble compounds described in Table VII, the chemical nature of which is described below.

Table V. Equivalent Concentration of Ronidazole and Metabolite in Turkey Tissue (Diets Contain 0.006% Ronidazole-^{14}C)

Treatment	Tissue	Total radioactivity, μg/g	Tissue residue, μg/g[a]	
			Ronidazole	2-$HOCH_2$ deriv
1 day on drug ring-2-^{14}C	Liver	0.893	[<0.02]	
3 days on drug ring-2-^{14}C	Liver	2.65	[<0.04]	
3 days on drug ring-2-^{14}C	Kidney	4.43	0.09	0.0
	Liver	3.77	0.01	0.0
	Muscle	2.05	1.6	0.03
2 days off drug ring-2-^{14}C	Kidney	0.9	0.0	0.0
	Liver	0.38	0.0	0.0
	Muscle	0.15	0.007	0.0001
5 days off drug ring-2-^{14}C	Kidney	0.44	0.0	0.0
	Liver	0.134	0.0	0.0
	Muscle	0.067	0.0	0.0
3 days on drug N-$^{14}CH_3$	Kidney	4.00	<0.03	0.0
	Liver	4.15	<0.02	0.0
	Muscle	2.58	1.5	0.1

[a] Average of tlc and electrophoretic results.

Table VI. Fractionation of Tissue Radioactivity

Treatment,[a] tracer	Tissue	Total radioactivity, μg/g	Tissue ronidazole + metabolite, μg/g	Water soluble[b]		Residual solids		Lipid fraction	
				μg/g	% of total	μg/g	% of total	μg/g	% of total
Day 1 on drug (Ronidazole-2-^{14}C)	Liver	0.89	<0.02	0.77	86	0.10	11		
Day 3 on drug (Ronidazole-2-^{14}C)	Liver	2.65	<0.04	2.30	87	0.30	11		
Day 3 on drug (Ronidazole-2-^{14}C)	Kidney	4.43	0.09	3.15	71	0.8	18	0.33	7
	Liver	3.77	0.01	3.08	82	0.5	13	0.11	3
	Muscle	2.05	1.63	0.45	22	0.1	5	0.02	1
Day 2 off drug (Ronidazole-2-^{14}C)	Kidney	0.9	0.0	0.46	51	0.2	22	0.22	25
	Liver	0.38	0.0	0.20	53	0.14	37	0.05	10
	Muscle	0.15	0.007	0.091	61	0.04	27	0.01	7
Day 5 off drug (Ronidazole-2-^{14}C)	Kidney	0.44	0.0	0.23	53	0.075	17	0.11	26
	Liver	0.134	0.0	0.051	38	0.064	48	0.01	10
	Muscle	0.067	0.0	0.032	48	0.028	42	0.005	8
Day 3 on drug (Ronidazole-N-$^{14}CH_3$)	Kidney	4.00	<0.03	2.67	67	1.3	33		
	Liver	4.15	<0.02	3.09	75	1.03	24	0.02	0.5
	Muscle	2.58	1.5	0.11	4	0.7	27	0.02	1

[a] All diets contained 0.006% ronidazole. [b] Ethyl acetate insoluble.

Table VII. Chromatographic Fractionation of Water Solubles from Turkey Tissue[a]

Treatment,[b] tracer	Tissue	Radioactivity in water solubles		% of total radioactivity in fraction			
		Equiv, μg/g	% of total	Neutral	Acid	Base	Ampholyte
Day 1 on drug (Ronidazole-2-^{14}C)	Liver	0.77	86	9	34	2	42
Day 3 on drug (Ronidazole-2-^{14}C)	Liver	2.30	87	3	39	4	41
Day 3 on drug (Ronidazole-2-^{14}C)	Kidney	3.15	71	6	17	10	34
	Liver	3.08	82	9	14[c]	2	44
	Muscle	0.45	22	5	7	1	6
Day 2 off drug (Ronidazole-2-^{14}C)	Kidney	0.46	51	8	3	4	24
	Liver	0.20	53	18	16	3	15
	Muscle	0.09	61	15	12	0	32
Days 5 off drug (Ronidazole-2-^{14}C)	Kidney	0.23	53	5	7	5	35
	Liver	0.051	38	5	10	4	18
	Muscle	0.032	48	8	15	2	23
Day 3 on drug (Ronidazole-N-$^{14}CH_3$)	Liver	3.09	75	20	2	35	18

[a] % values are % of total radioactivity in the tissue and are directly convertible into equivalent μg/g. [b] All diets contained 0.006% ronidazole. [c] Value probably in error due to loss of ≃9% nonelutable activity.

Table VIII. Demonstration of the Presence of *N*-Methylglycolamide-^{14}C in the Neutral Fraction from Liver Aqueous Extracts

Sample	Source	R_f values				
		Solvent I	Solvent II	Solvent III	Solvent IV	Solvent V
N-Methylformamide	Model compound	0.53	0.56	0.51		
N-Methyloxamide	Model compound	0.35			0.12	
N-Methylglycolamide	Model compound	0.49	0.42	0.45	0.48	
Neutral fraction 2-^{14}C	0.006% in diet	0.49 (≃50%)	0.42 (≃50%)		0.50	
		0.66				
Neutral fraction 2-^{14}C	0.006% in diet		0.34	0.25		
			0.42 (≃50%)	0.45		
			0.74			
N-Methylglycolamide	Model compound				0.62[a]	0.71
Neutral fraction, *N*-$^{14}CH_3$	0.006% in diet				0.42[a]	0.77
					0.62[a] (≃20%)	

[a] Chromatography in Eastman Silica Gel plate. Solvent I: *N*-propanol/0.5% HCl (4:1). Solvent II: *sec*-butanol/3% NH_4OH (25:11). Solvent III: acetonitrile/water (9:1). Solvent IV: 1-butanol/acetic acid/water (4:1:1). Solvent V: ethanol/MeEt ketone/formic acid/water (50:30:10:20) at 50° C.

Neutral Aqueous Fraction from Tissue. IDENTIFICATION OF *N*-METHYLGLYCOLAMIDE. The observation that both labeled forms produced a radioactive neutral fraction suggests, after consideration of the ronidazole structure, that *N*-methylglycolamide ($HOCH_2CONHCH_3$) could be responsible, in part, for this radioactivity. The presence of this compound was suggested by thin-layer chromatography of the neutral fraction in turkey liver which was first purified on a large scale using silica gel GF plates developed with the solvent system 4 1-butanol/1 acetic acid/1 H_2O.

The *N*-methylglycolamide was located on the plate by comparison with a reference sample of the compound. The desired zone was scraped from the glass plate and eluted with ethanol. In this way a fairly pure sample was obtained containing 20 to 25% of the radioactivity of the neutral fraction from ring-labeled ronidazole. This and additional samples of partially purified neutral fractions, obtained from experiments with both ring-labeled ronidazole-^{14}C and *N*-methyl-labeled ronidazole-^{14}C, were compared with reference samples of *N*-methylglycolamide-^{14}C and of other possible amides such as *N*-methylformamide-^{14}C and *N*-methyloxamide-^{14}C for identification purposes, using a series of five developing solvents. The percentages of applied radioactivity which were recovered from the *N*-methylglycolamide spots are reported in parentheses in Table VIII. These experiments with the water-soluble extract of turkey liver suggest that one component of the aqueous neutral fraction from liver is *N*-methylglycolamide, which accounts for ≃10% of the total neutral radioactive products from ring-labeled ronidazole and for ≃6% from the *N*-methyl-labeled drug.

IDENTIFICATION OF ACETYL. The metabolic degradation of ronidazole to acetyl-containing derivatives detectable in turkey urine strongly suggests that similar water-soluble radioactive derivatives exist also in tissue. This was confirmed by examination of the water-soluble extracts of turkey liver by the same reverse isotope dilution method described above for urine. Two sets of birds maintained for 3 days on 0.006% ronidazole ring-2-^{14}C provided tissues for extraction, hydrolysis, distillation, and benzhydrylamide formation. The water-soluble extract from one set was washed with ethyl acetate before hydrolysis, which provided residual acetic acid to serve as carrier. The amount of volatile acid present in the aqueous extracts accounted for ≃21% of the radioactivity therein (average of 16% and 26%). For identification the volatile acid was converted to the crystalline benzhydrylammonium acetate, 3.88 g ≃10,400 cpm. The salt was then pyrolyzed to the amide in a sealed tube at 154° C. The radioactive amide was purified further by column chromatography on neutral alumina, using 2% methanol–diisopropyl ether as the developing solvent. This system was found to separate the *N*-benzhydrylacetamide from higher aliphatic analogs, even when the *N*-benzhydrylacetamide was present only in trace amounts. The single radioactive component revealed by this technique coincided with the elution behavior of synthetic *N*-benzhydrylacetamide and was thus indistinguishable therefrom. Three cuts were assayed radiometrically by the combustion method, and specific activities compared with that of the starting material. Values are recorded in Table IX. Although radioactivity is low because of the large amount of carrier present, the specific activities of starting and of purified materials are obviously constant and equal to 2.56 cpm/mg. Since the amide equivalent of the 3.88 g of benzhydrylammonium acetate initially present is 3.60 g, the radioactivity residing in the acetate initially present is 3600 × 2.56 = 9200 cpm or at least ≃88% of the total volatile acid. Turkey tissue is thus shown, by the reverse isotope dilution method, to resemble turkey urine in that it contains a considerable amount (≃19%) of acetyl derivatives in the water-soluble extracts from liver.

Qualitative experiments with the aqueous extracts from livers of turkeys fed ronidazole-*N*-$^{14}CH_3$ revealed that this labeled modification also produced a considerable quantity of radioactive volatile acid (acetic acid) which could be liberated by alkaline hydrolysis. Although not specifically identified, it may reasonably be presumed to indicate the presence of acetyl derivatives.

Acid Aqueous Fraction From Tissue. IDENTIFICATION OF

Table IX. Specific Activities of *N*-Benzhydrylacetamides

Sample	No.	Net cpm[a]	Specific activity/ cpm/mg
Before chromatography	A	25.2 ± 0.89	2.62
	B	33.1 ± 0.93	2.49
	Avg		2.56
After chromatography	Cut 1	19.0 ± 0.85	2.47
	Cut 2	20.7 ± 0.87	2.70
	Cut 3	18.9 ± 0.85	2.50
	Avg		2.56

[a] Net cpm ± standard deviation of the net; background 26.8 cpm ± 0.52.

Table X. Analysis for Oxalic Acid-^{14}C from Liver of Turkeys Fed Ring-Labeled Ronidazole

Method	Sample No.	Sample Amount	Net cpm ± σ_{cpm}	cpm per sample	Total oxalic acid, cpm	% oxalic acid
Oxalate decarboxylase	524A[b]	0.5 ml (11.7 mg)	2.6 ± 0.5	7.1[a]	830	2.4
	524J[b]	10.8 mg	2.1 ± 0.5	5.9[a]	750	2.1
	524J[b]	13.6 mg	2.6 ± 0.5	7.2[a]	720	2.0
Permanganate oxidation	524J[d]	31.7 mg	13.5 ± 0.8	16.2[c]	700	1.9
	524J[d]	23.2 mg	11.7 ± 0.8	14.0	820	2.3

[a] Corrected for 5 out of 6 ml hyamine, and for enzymatic CO_2 recovery of 43%. [b] Initial total activity in the aqueous acidic fraction was 35,410 cpm on date of measurement. [c] Corrected for 5 out of 6 ml hyamine. [d] Initial total activity in the aqueous acidic fraction was 36,130 cpm on the date these measurements were performed.

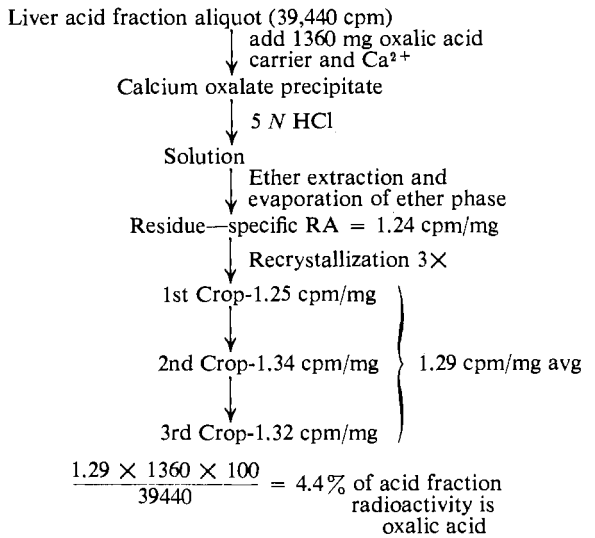

Figure 3. Procedure for the determination of oxalic acid-^{14}C in turkey liver tissue acidic fraction

OXALIC ACID. Reverse Isotope Dilution Assay. Radioactive oxalic acid was found to be present in the aqueous extract from liver of turkeys fed ronidazole-ring-2-^{14}C. The identification was made by reverse isotope dilution and confirmed by enzymatic decarboxylation with oxalate decarboxylase and permanganate–sulfuric acid oxidation. The liver tissue employed was derived from turkeys maintained on 0.006% feed for 1 day. It was extracted and fractionated to yield the acidic fraction. The procedure used is outlined in Figure 3.

ENZYMATIC ASSAY OF OXALIC ACID. The enzymatic decarboxylation of oxalic acid as explained by Shimazono and Hayaishi (1957) yields formic acid and carbon dioxide.

The incubation procedure used (Mayer *et al.*, 1963) was developed and tested using authentic oxalic acid-^{14}C and Worthington oxalate decarboxylase. The liberated CO_2 (pH 3.4) was generated in one-half of a double Erlenmeyer flask and collected in hyamine in the second half for scintillation counting. The observed performance of this system was found to be 92% of theory. Duplicate acidic fraction aliquots were submitted to the identical procedure with the details and results presented in Table X.

PERMANGANATE OXIDATION ASSAY. Oxalic acid can be oxidized by permanganate in sulfuric acid to yield CO_2 quantitatively (Kolthoff and Sandell, 1936). The method was tested with authentic oxalic acid-^{14}C and modified until quantitative recovery was achieved. The procedure utilized the same double Erlenmeyer flask, as did the enzyme analysis for transferring the evolved CO_2 into the hyamine for scintillation counting. Results are presented in Table X. The amount of oxalate thus lies between 2 and 4%, depending on the method employed.

Identification of Other Carboxylic Acids. Evidence has been accumulated for the presence of at least six radioactive carboxylic acids besides oxalic acid in liver of turkeys maintained for 3 days on feed containing 0.006% ronidazole-2-^{14}C. These are fumaric, succinic, glycolic, malic, α-ketoglutaric, and citric and/or oxaloacetic. The identification and estimation of these acid constituents was successfully performed by two-dimensional chromatography on 8 in. × 8 in. Gelman itlc type fiberglass thin-layer chromatography plates impregnated with silica gel (Bleiweis *et al.*, 1967). Solvents used were: (I) 28 petroleum ether/12 anhydrous ether/1 formic acid; followed by (II) 80 chloroform/1 methanol/1 formic acid. After development, acids were visualized by spraying with 0.04% bromophenol blue in ethanol, which forms a yellow spot at the locations of the carboxylic acids. The radioactivity residing in the carboxylic acids was determined by placing the yellow spots cut into small pieces into 70/30 phosphor for liquid scintillation counting. The behavior of the radioactive acid fraction was compared with a mixture of the following typical (nonradioactive) carboxylic acids: fumaric, succinic, glycolic, α-ketoglutaric, malic, oxalic, citric, and oxalacetic. The unknown fraction was charged with 3 μg of each of these eight acids. In addition, a standard chromatogram was prepared from a mixture of 3 μg of each of these acids. Background activity was measured at random areas removed from the fiberglass sheet at a distance from the active spots and from the actual carboxylic acid spots on the standard chromatogram. The developed standard acid spots contributed no extraneous activity.

The chromatogram of the mixture of the eight standard carboxylic acids was essentially identical to Figure 4, which is a typical chromatogram of the acid fraction from turkey livers. In all cases, only five of the acids are readily distinguished. The mixture of citric, oxalic, and oxaloacetic acids is either not separated or only poorly separated in these chromatograms. The radioactivity residing in the yellow spots obtained from the chromatograms produced by the acid fraction was measured by liquid scintillation counting. Observations of duplicate chromatograms (A and B) are reported as net cpm in Table XI. A $2\sigma_{net}$ of 2.0 cpm was estimated. An unidentified acid was observed with the sample from turkey liver. Since all the other acids associated with radioactivity appear to be normal carboxylic acids, it is very probable that the unidentified component is also a component of normal tissue. Of a total of $\simeq$100 cpm charged to the fiberglass sheets, some 70% was accounted for in the areas analyzed. Since oxalic acid was shown to account for 2–4% of the acid fraction, we may conclude that citric acid plus oxaloacetic acid account for $\simeq$38% of the acidic con-

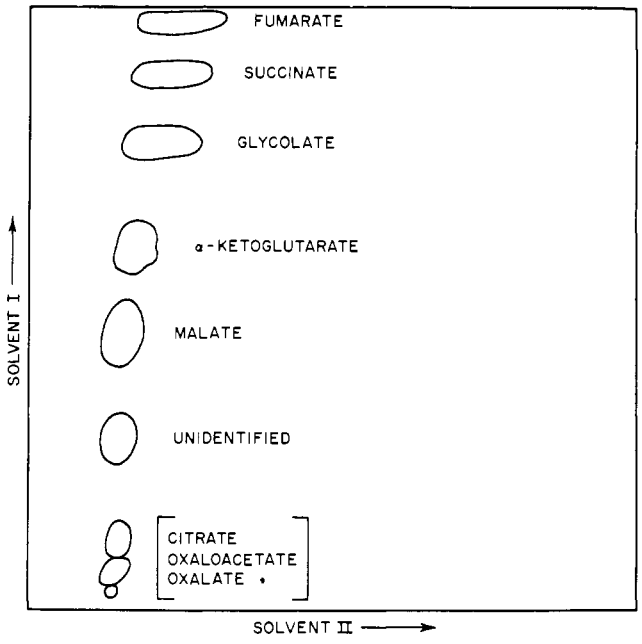

Figure 4. Two-dimensional radio chromatogram of acid fraction from liver (on fiberglass)

Table XI. Radioactivity of Carboxylic Acids in Turkey Tissue

Acid	Net cpm A	Net cpm B	Net cpm Average	% of Total[a]
Fumaric	10.6	(1.3)	6.0	6
Succinic	5.8	2.4	4.1	4
Glycolic	3.1	5.3	4.2	4
α-Ketoglutaric	4.4	13.3	8.9	9
Malic	6.7	4.0	5.4	5
Unidentified	9.0	0.0	4.5	5
Citric Oxaloacetic Oxalic	45.3	31.2	38.3	38
Total	85	58	71	71

[a] Total activity charged was ≃100 cpm.

stituents of turkey liver. All of the radioactive acids identified are participants in the tricarboxylic acid cycle and are constituents of normal tissue.

Basic Aqueous Fraction From Tissue. IDENTIFICATION OF METHYLAMINE. Radioactivity residing in the basic fractions from *in vitro* liver homogenates, and in urine and liver from medicated turkeys, represented a much greater proportion of total activity when ronidazole was labeled in the N-CH_3 position than in the ring, *i.e.*, ≃12/1 for turkey liver (Table VII). The obvious reason is the extensive cleavage of the imidazole ring to an amine such as methylamine, which would be radioactive when *N*-methyl-labeled ronidazole was employed but which would be nonradioactive with the other labeled form.

The presence of a volatilizable radioactive component was examined in the water-soluble fraction from pooled livers of turkeys maintained for 3 days on 0.006% *N*-methyl-labeled ronidazole. To 3 ml of aqueous extract was added 20–30 mg of methylamine hydrochloride as carrier. The solution was made alkaline and the free bases were removed by a stream of nitrogen and collected in an acid trap. Results obtained with the aqueous extracts from the two labeled forms are reported in Table XII. The expected behavior was realized. A radioactive volatile alkaline component was demonstrable only with tissue from birds dosed with *N*-$^{14}CH_3$-labeled ronidazole. This fraction accounted for 32% of total radioactivity in the aqueous extract.

Similar behavior was exhibited by the major basic component obtained after fractionation of the water-soluble extract on Dowex-50 (H^+) (*cf.* scheme in Figure 2). This basic component contained 41% of the total water-soluble radioactivity. An aliquot was made alkaline to pH 14 in the same closed system, with nitrogen passing through at 60° C. No carrier methylamine was added in this experiment. About 91% of the initial activity in the base fraction was trapped, which was equivalent to ≃37% of total initial radioactivity. It was identified as methylamine-^{14}C by benzoylating an aliquot of this fraction with benzoyl chloride under Schotten–Baumann conditions. The benzamides produced were dissolved in ether, which was evaporated to yield a crystalline product. Extraction with a small volume of benzene removed most of the radioactivity (80%). The mass of crystals consisted of benzamide derived from ammonium chloride present in the acid eluate. The identity of the *N*-methyl-^{14}C-benzamide (≃0.25 μg) was arrived at by comparison with a synthetic sample, first by thin-layer chromatography on Silica G plates after addition of 2.5 μg of nonradioactive *N*-methylbenzamide as carrier. Replicate plates developed in 95/5 benzene/methanol all showed a single radioactive spot with R_f comparable to that of a known sample. The radioactive sections of the plates were separated and combined for elution with methanol to yield 2.3 mg of total eluate containing 0.2–0.3 μg of ^{14}C-labeled form of *N*-methylbenzamide. By radioactive gas–liquid chromatography, it was found that the retention time of the sample did not differ from that of *N*-methylbenzamide.

Ampholyte Aqueous Fraction from Tissue. IDENTIFICATION OF AMINO ACIDS. Preliminary examination suggested that the ampholyte fraction contained known amino acids.

Table XII. Distillation of Water-Soluble Volatile Component in Basic Fraction of Turkey Liver (3 Days on 0.006% Drug)

Label	Sample	pH during distillation	Initial reaction, dpm	Radioactivity in distillate, dpm	% volatile
N-$^{14}CH_3$	Aqueous extract	1–2	2510	0	0
		10–11	2510	801	32
Ring-^{14}C	Aqueous extract	1–2	211[a]	0	0
		10–11	211	0	0
N-$^{14}CH_3$	Major basic fraction	14			37[b]
Ring-^{14}C	Major basic fraction	14		0	0

[a] Detection sensitivity ≃2%. [b] Based on total radioactivity in the water-soluble fraction.

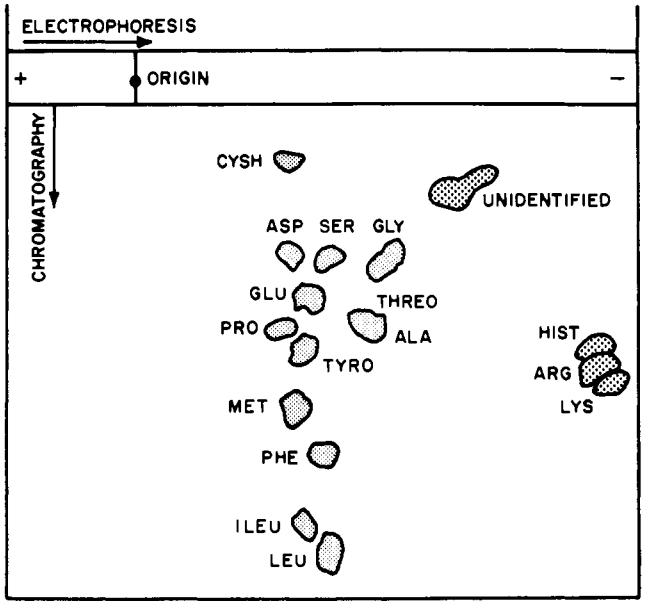

Figure 5. Fingerprint of standard mixture of amino acids

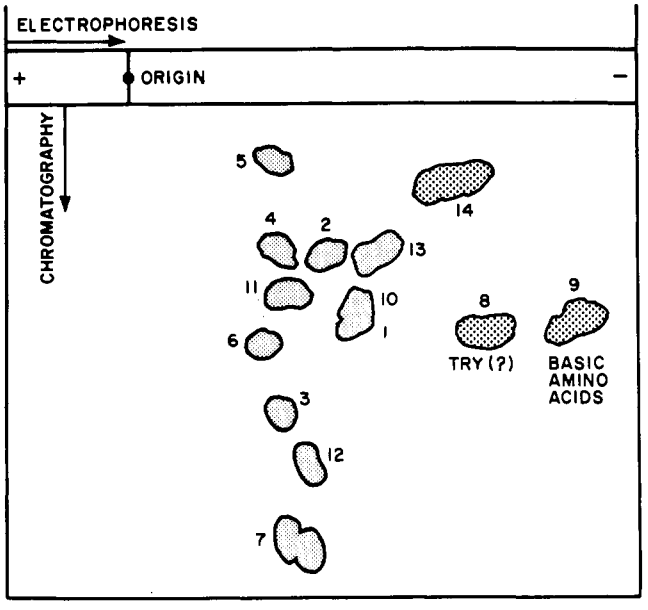

Figure 6. Fingerprint of ampholyte fraction from liver

Accordingly, Ingram's (1958) "finger printing" method was applied to the total ampholyte fraction obtained from livers of birds maintained for 3 days on 0.006% ronidazole-ring-2-^{14}C. The ampholyte fraction was concentrated, and 5λ placed on 15 in. × 18 in. Whatman 3MM paper for electrophoresis at 2000 V in 4% (v/v) aqueous formic acid (pH 2.2) and 0.3% pyridine. After 1.05 hr the paper was removed and dried, and the sheet turned at right angles for descending chromatography, using the solvent system 19:1:5:25 1-butanol/butyl acetate/acetic acid/water. Chromatography was terminated after 20 hr, and the paper dried and sprayed with ninhydrin reagent. Three to five runs were performed to provide sufficient activity for reliable measurement. Ninhydrin positive spots were associated with known amino acids where possible by reference to standard patterns (Richmond and Hartley, 1959) of mixtures of amino acids. Colored spots were removed, eluted, and lyophilized, and residues transferred to 3 in. stainless steel planchets for radiometric assay in the Low Beta counter. Equivalent amino acids or peptides from three papers were combined for this purpose.

A Beckman Amino Acid Calibration Mixture (Type 1) of 17 amino acids was employed to obtain a fingerprint pattern with model compounds for comparison (Figure 5). The fingerprint of the ampholyte fraction is shown in Figure 6. The "unidentified" spot in the standard pattern must be a basic substance (an amine?) produced in the standard mixture, which also contains ammonium ion. The ampholyte pattern was practically identical with that of the standard mixture, except for the appearance of a new amino acid, identified tentatively as tryptophane, and the absence of tyrosine, which may be associated with the phenylalanine. Also, the combined arginine, lysine, histidine spot was poorly resolved, and is referred to as "Basic Amino Acids." All of these amino acids were radioactive, glutamic acid being most active. The actual activity (in cpm) of each component is recorded in Table XIII. It is to be noted that the total radioactivity of these substances amounted to over 80% of the radioactivity charged to the three papers employed. It is clear that practically all of the radioactivity in the ampholyte fraction was associated with known amino acids, and that relatively little radioactivity was incorporated in other types of ampholytes.

Table XIII. Amino Acids in Ampholyte Fractions from Pooled Turkey Livers

Component no.	Amino acid	cpm[a]	% of total radioactivity
1	Alanine	1.3	3
2	Serine	1.7	4
3	Methionine	1.9	4
4	Aspartic acid	3.0	7
5	Cysteine	3.7	9
6	Proline	1.5	4
7	Leucine and isoleucine	1.5	4
8	Tryptophane (?)	0.6	1
9	Basic amino acids[b]	0.9	2
10	Threonine	2.2	5
11	Glutamic acid	13.3	31
12	Tyrosine (?) and phenylalanine	1.6	4
13	Glycine	1.6	3
14	Unidentified	0.8	2

[a] Detection limit (2σ net) $\simeq$ 0.3 cpm; total cpm applied was 43 cpm to three sheets in all cases except for glycine assay where radioactivity was 37 cpm applied to two sheets. [b] Although not resolved, this component is either arginine and/or lysine and/or histidine.

Residual Solids from Tissue. The radioactivity distribution resulting from the fractionation of residual solids from liver and muscle of turkeys, while receiving medication and after withdrawal of medicated diets, is reported in Table I.

It is clear that the radioactivity present in the residual tissue solids is firmly incorporated. The major component is found in the protein fraction, which accounts for 80% of the total by 2 days after withdrawal. An overall average of $\simeq$4% of radioactivity is found in the nucleic acid portion once medication is discontinued. The small amount of extractable material is probably low molecular protein solubilized by aqueous ethanol, since prior treatment of tissue would have removed any significant amount of other low molecular weight metabolites. The above observations suggest that adventitious carbon-14 has been firmly incorporated into normal tissue components.

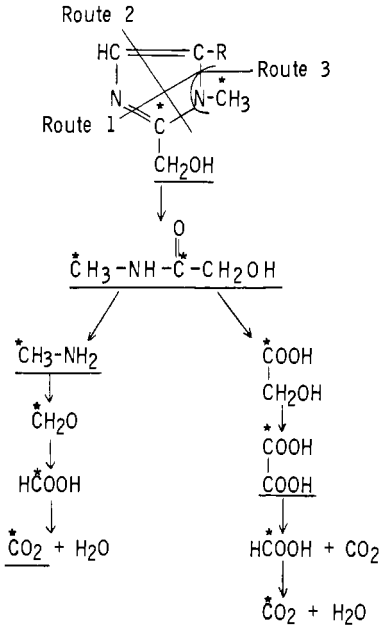

Figure 7. Ring scission of Ronidazole. Compounds underlined have been identified in tissue or exhaled air

The higher radioactivity incorporation into nucleic acids observed with ronidazole-*N*-$^{14}CH_3$ is in accord with this conclusion, as is the gradual buildup of percent radioactivity in residual solids of liver and muscle when medication is discontinued.

DISCUSSION

Biogradation Mechanisms. Ronidazole appears to be rapidly and extensively degraded to "nonmetabolite" products related to the parent compound (Rosenblum, 1965) and in this way to differ from other nitroimidazoles which have been reported to resist metabolic ring scission. Only muscle was found to contain ronidazole and 2-hydroxymethyl-1-methyl-5-nitroimidazole and this only while turkeys were consuming medicated diets. The study of on-drug liver indicated that all identifiable radioactive components were compounds considered innocuous substances or common components of tissue. Plausible major metabolic pathways accounting for these radioactive components, as intermediates or as end products of ronidazole metabolism, may be deduced from the results of the tracer experiments described above. These biotransformations, which may occur successively or simultaneously, are simply enumerated below, without reference to the sequential relation between them.

The carbamoyl group is removed by hydrolysis to produce 2-hydroxymethyl-1-methyl-5-nitroimidazole. This derivative has been identified and is produced by both labeled forms of ronidazole, as one would expect. It has moreover been shown to be metabolized more rapidly and more completely than ronidazole, which accounts for its small stationary state concentration (Trenner, 1967).

Ring scission may occur by several routes, as seen in Figure 7. Routes 1 and 2 both account for the formation of *N*-methylglycolamide. Route 1 is perhaps more likely, since it must be operative in the case of *N*-$^{14}CH_3$-labeled ronidazole, and route 2 would require prior ring reduction. Figure 7 is a schematic representation of ring scission to form *N*-methylglycolamide, followed by two series of sequential reactions, each terminating in formation of $^{14}CO_2$. The presence of radioactive *N*-methylglycolamide in the tissues of birds receiving both forms of labeled ronidazole is in agreement with this degradative scheme. Methylamine-^{14}C and oxalic acid-^{14}C have both been isolated from liver tissue and identified; and considerable $^{14}CO_2$ was found in the exhalations by turkey poults for several days after oral administration of labeled drugs. The two sequences are accepted pathways for the metabolism of methylamine and glycolic acid. The scheme in Figure 7 accounts further for the observations that: (1) both labeled forms of drug produced $^{14}CO_2$; (2) the percent radioactivity in the basic fraction from tissue of turkeys maintained on ronidazole-*N*-$^{14}CH_3$ was far greater than when ronidazole-2-^{14}C was used, and that methylamine-^{14}C was found only with the former tracer; and (3) the acid fraction from the 2-^{14}C-tracer contained a much greater percent of total radioactivity (water-soluble) than did the acid fraction from the *N*-$^{14}CH_3$-labeled drug. Scission of the ring with direct elimination of CH_3NH_2 (route 3) is of course not excluded. The greater radioactivity of the nucleic acid fraction from ronidazole-*N*-$^{14}CH_3$ is in accord with this expectation.

The NO_2 group in the imidazole is written as *R* since it disappears rapidly in tissue and liver homogenates. This group is probably hydrolyzed or, if reduced to $-NH_2$, converts in turn to –OH or simply –H.

Labeled nucleic acids are produced from precursor labeled purines biosynthesized *in situ* from labeled carbon dioxide and formate by the accepted pathway. This accounts for the radioactive nucleic acids in tissue originating from both labeled forms of ronidazole.

Radioactive amino acids and proteins are formed by the fixation of $^{14}CO_2$ through the citric acid cycle which encompasses the formation of aspartic acid-^{14}C and glutamic acid-^{14}C, from which other amino acids are derived, and which are known to be present in proteins. Worthy of note is the fact that the radioactivity residing in the ampholyte fraction is about the same for both labeled forms of the drug. This is in accord with the citric acid cycle mechanism recalling that both labeled forms produced comparable quantities of $^{14}CO_2$.

It is noteworthy that all of the amino acids identified or thought to be present are normal α-amino acids, commonly present in body tissues and fluids exhibiting no abnormal structural features. The absence of abnormal *N*-methyl acids is in accord with the similar degradative paths observed with both ronidazole-^{14}C tracers, as suggested by the comparable ampholyte contents of tissue and of tissue homogenates.

Radioactive acetyl is easily explained in terms of acetyl-^{14}C-CoA formation from citric acid-^{14}C. The acetyl-^{14}C can be transferred by a transacetylation mechanism from the acetyl coenzymes to *N*-acetylglucosamine or other unspecified neutral substances.

Radioactive lipid formation is also mediated by acetyl-CoA which is involved in the synthesis of fatty acids. Radioactivity from acetyl-^{14}C-CoA will, accordingly, find its way into lipids by this path.

Accountability. The biogradation of ronidazole and the incorporation of its degradation products into biochemical components of tissue as lipid, protein, and nucleic acid is so extensive that only the water-soluble fraction can reasonably be suspected of containing unusual decomposition product. The maximum concentration of these extraneous substances can be estimated from the composition of the water-soluble extract from liver. It was reported above that: (1) radioactivity in the neutral fraction from birds fed ronidazole-2-^{14}C was completely (94–98%) accounted for as acetyl derivative and *N*-methylglycolamide; (2) methylamine-^{14}C accounted for 78–90% of the radioactivity in the basic fraction

from birds maintained on *N*-^{14}CH-ronidazole; (3) radioactivity in the ampholyte fraction (from both tracers) was associated mostly (≃83%) with amino acids, with the remainder undoubtedly including other ampholytic substances; and (4) the acid fraction was shown to contain six or eight known carboxylic acids, which accounts for ≃70% of total radioactivity. This amounts virtually to complete accountability for the ronidazole molecule in terms of known substances.

Virtually all of the retained radioactivity is due to simple compounds commonly found in normal tissue or which are common dietary components.

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Effect of Testosterone on Metabolism of ^{14}C-Photodieldrin in Normal, Castrated, and Oophorectomized Rats

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Subacute levels (5 μg per day) of ^{14}C-labeled photodieldrin were administered orally to normal adult rats of both sexes and to rats that had been castrated or oöphorectomized within 3 days of birth. Urinary and fecal excretion patterns of ^{14}C activity were determined before and after administration of testosterone to all of the rats. Testosterone greatly enhanced excretion of ^{14}C-labeled metabolites in control females and sex hormone-deficient groups. The major metabolite found in the urine of these animals after testosterone treatment was ^{14}C-ketodieldrin, which was present in only trace amounts before the steroid was given. Testosterone did not affect the levels of ^{14}C-ketodieldrin excreted by control males. Tissue levels of ^{14}C activity in females and sex hormone-deficient males and females after 6 weeks of testosterone administration were comparable to those found in control males. The highest concentrations of ^{14}C activity were found in the kidneys of all four groups. High levels of labeled compounds found in biopsy samples of adipose tissue from females and sex hormone-deficient groups before testosterone administration were not evident at autopsy.

Dailey *et al.* (1970) recently reported a striking sex difference in the excretion, distribution, and storage of ^{14}C-photodieldrin (Figure 1) in rats. From three to ten times as much ^{14}C activity was retained by females as compared to males in all tissues monitored except in kidney, which appeared to be a major storage site in male rats. Adipose tissue was the major storage depot of ^{14}C activity in females. A definite sex difference was also apparent in the metabolites of ^{14}C-photodieldrin isolated from the urine of these rats, as was reported by Klein *et al.* (1970). The principal metabolite found in urine of males was ketodieldrin (Figure 1) but female rats excreted at least four very polar, nonvolatile metabolites, none of which were identified as ketodieldrin by the methods employed. Because of these findings, it became of interest to determine if the excretion, distribution, or storage of ^{14}C-photodieldrin, given in subacute doses, could be altered by administration of testosterone to normal rats and rats deficient in sex hormones.

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METHODS AND MATERIALS

The ^{14}C-photodieldrin, unlabeled photodieldrin, and ketodieldrin used in this study were part of the stock chemicals described by Dailey *et al.* (1970) and Klein *et al.* (1970). The instruments used to isolate, identify, and measure the metabolites and compounds of interest are listed in the same references and, in addition, a gas microcoulometer chromato-