Table VI. Effect of Moisture and Time on the Disappearance of Adsorbed MBC from Three Soils[a]

Soil type	Moisture content	MBC residues, ppm				2-AB residues, ppm, after 9 months
		0[d] day	After 3 months	After 6 months	After 9 months	
Hamra	Dry[b]	2.1	1.9	1.8	1.7	0.2
	Field capacity (aerobic)	2.4	0.9	0.6	0.8	0.08
	Anaerobic[c]	2.4	1.5	0.4	0.6	0.06
Loess	Dry	2.8	2.0	2.5	1.8	0.2
	Field capacity (aerobic)	2.2	0.7	0.6	0.8	0.1
	Anaerobic	2.2	0.4	0.5	0.5	0.1
Hydromorphic grumusol	Dry	3.3	2.9	3.3	2.2	0.3
	Field capacity (aerobic)	3.2	1.3	0.9	0.5	0.1
	Anaerobic	3.2	2.1	0.7	0.6	0.1

[a] Recrystallized MBC was adsorbed to the soil. [b] Air-dried soil. [c] One centimeter of water above the soil. [d] Differences in the initial concentration due to different soil batches.

A possible degradation product of MBC, 2-aminobenzimidazole, was detected in the MBC-treated soil but in very small quantities. The results obtained in this laboratoy test are in close agreement with data reported by Baude et al. (1974) for benomyl in field experiments.

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Absorption, Excretion, and Metabolism of 1,3-Bis(*p*-chlorobenzylideneamino)guanidine Hydrochloride (Robenz Robenidine Hydrochloride) in the Chicken

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When chickens were given a single oral dose of robenidine hydrochloride labeled with ^{14}C in either the α-carbon atom of the *p*-chlorobenzylidene moiety or in the aminoguanidine carbon atom, they excreted 82% of the administered radioactivity within 24 hr. A major portion of this radioactivity corresponded to robenidine. Several major metabolites, retaining only the *p*-chlorobenzylidene-^{14}C label of robenidine, were found. These were isolated by solvent extraction and purified by column chromatography. Mass spectral analysis of the diazoethane derivatives of the metabolites indicated that they were mixed conjugates of ornithine and lysine containing *p*-chlorobenzoic acid with either benzoic acid or *p*-hydroxybenzoic acid. Robenidine and a metabolite identified as 3-amino-4-(*p*-chlorobenzylideneamino)-5-(*p*-chlorophenyl)-4*H*-1,2,4-triazole were found in fat, liver, and skin while robenidine and the ornithine and lysine conjugates of the acids were found in liver, kidney, and muscle.

The compound 1,3-bis(*p*-chlorobenzylideneamino)guanidine hydrochloride, the active ingredient in Robenz (registered trademark of American Cyanamid Co.) robenidine hydrochloride medicated premix coccidiostat manufactured by American Cyanamid Company, has been registered for use as an effective, safe feed additive product for the prevention of coccidiosis in broiler chickens. Robenidine hydrochloride is not related chemically to any of the previously or presently used anticoccidials and, therefore, represents an entirely new structure in the field of coccidiosis control. Robenidine hydrochloride is highly efficacious against the six major species of *Eimeria* that infect chickens (Kantor et al., 1970). It has a dual mode of action (Ryley and Wilson, 1971) in that it arrests the development

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of the first and second generation schizonts which are responsible for the major damage to the intestines of broilers and is effective against the sexual stage of coccidia development. Robenidine hydrochloride has been shown to be an inhibitor of oxidative phosphorylation (Wong et al., 1972) and is reported (Lee and Millard, 1972) to affect the protein metabolism or some other metabolic process of the parasite.

Robenidine hydrochloride was metabolized by the rat to *p*-chlorobenzoic acid and *p*-chlorohippuric acid (Zulalian and Gatterdam, 1973). Since the metabolism of this compound by chickens could be different from that observed in the rat, the metabolic fate of robenidine hydrochloride was investigated in chickens and the metabolites that were found in the excreta and tissues were isolated and identified. Robenidine hydrochloride labeled with ^{14}C in either the α-carbon atom of the *p*-chlorobenzylidene moiety or in the aminoguanidine carbon atom as shown in the structure was used for the metabolism studies.

ROBENZ robenidine hydrochloride

* Position of ^{14}C atom in the aminoguanidine tracer

* Position of ^{14}C atom in the benzylidene tracer

MATERIALS AND METHODS

Robenidine Hydrochloride-Aminoguanidine-^{14}C. Thiosemicarbazide-^{14}C (91 mg, 2.53 mCi/mmol), obtained from Mallinckrodt Nuclear, was dissolved in 15 ml of absolute ethanol containing 1.5 g of methyl chloride and placed into a 3-oz Aerosol vessel (Fischer-Porter, Model 110-007). The vessel was fitted with a screw cap pressure gauge, immersed in an oil bath, and heated to 110° for 2 hr. The mixture was allowed to cool and filtered, and the filtrate was concentrated in vacuo to yield methyl thiocarbazimidate-^{14}C (136 mg, 0.96 m*M*). This was then heated with 5.5 ml of 1% hydrazine hydrate in ethanol to yield 1,3-diaminoguanidine-^{14}C (83 mg, 0.66 m*M*). The radiolabeled aminoguanidine compound was dissolved in ethanol and treated with an excess of nonradioactive *p*-chlorobenzaldehyde to yield the guanidine tracer (78%) with a specific activity of 6.93 μCi/mg. The product had a radiochemical purity of 98.8% as determined by thin-layer chromatography.

Robenidine Hydrochloride-α-*p*-Chlorobenzylidene-^{14}C. *p*-Chlorobenzaldehyde-*carbonyl*-^{14}C, obtained from Mallinckrodt Nuclear, was allowed to react with nonradioactive 1,3-diaminoguanidine hydrochloride as described in the previous study (Zulalian and Gatterdam, 1973). The benzylidene tracer had a specific activity of 12.7 μCi/mg and was 99.2% pure, radiochemically.

Synthesis of Metabolites. The compounds prepared either as standards or identified as metabolites of robenidine hydrochloride are listed in Table I. All standards were authenticated by elemental analysis and characterized by infrared analysis and mass spectral analysis. *p*-Chlorobenzoic acid was obtained commercially.

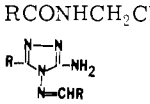
Compound 3. The N^2,N^5-bis(*p*-chlorobenzoyl)ornithine, prepared from *p*-chlorobenzoyl chloride and ornithine (Novello et al., 1926), had mp 212–214°.

Compounds 4 and 5. The N^5-benzyl ester of N^5-carboxyornithine, obtained from Nutritional Biochemicals Corp., was allowed to react with benzoyl chloride following the procedure used to prepare compound **3**. The product was treated with 32% hydrogen bromide in glacial acetic acid (Ben-Ishai and Berger, 1952) to remove the *N*-carbobenzoxy protecting group and afforded the N^2-benzoylornithine, isolated as its hydrobromide salt. The salt was treated with *p*-chlorobenzoyl chloride to obtain compound **4** (N^2-benzoyl-N^5-(*p*-chlorobenzoyl)ornithine), mp 199–201°. By using the N^2-benzyl ester of N^2-carboxyornithine and the same sequence of reaction steps, compound **5** (N^5-benzoyl-N^2-(*p*-chlorobenzoyl)ornithine), mp 196–197°, was prepared.

Compounds 6 and 7. The N^6-benzyl ester of N^6-carboxylysine, obtained from Mann Research Laboratories, was allowed to react with benzoyl chloride. The product was isolated and treated with 32% hydrogen bromide in glacial acetic acid to obtain the N^2-benzoyllysine, isolated as the hydrobromide salt. This was converted to compound **6** (N^2-benzoyl-N^6-(*p*-chlorobenzoyl)lysine), mp 158–159°, by reaction with *p*-chlorobenzoyl chloride. By using the N^2-benzyl ester of N^2-carboxylysine and the same sequence of reaction steps, compound **7** (N^6-benzoyl-N^2-(*p*-chlorobenzoyl)lysine), mp 184–184.5°, was obtained.

Compounds 8 and 9. The N^5-benzyl ester of N^5-carboxyornithine was allowed to react with *p*-chlorobenzoyl chloride followed by a removal of the *N*-carbobenzoxy protecting group to yield the N^2-(*p*-chlorobenzoyl)ornithine hydrobromide. The salt was treated with *p*-anisoyl chloride

Table I. Structures, Thin-Layer Chromatographic R_f Values,[a] and Amounts of Robenidine Hydrochloride and Its Metabolites Found in Chicken Excreta

Compd no.	Structure[b]	TLC coordinates: System I	TLC coordinates: System II	% contribution
1	$\lvert RCH{=}NNH\rvert_2C{=}NH \cdot HCl$ (robenidine hydrochloride)	75–70	73–69	60[c]
2	RCOOH	35–68	55–55	1[c]
3	$RCONHCH_2CH_2CH_2C(\text{–}NHCOR)HCOOH$	21–25	28–37	3[c]
4	$RCONHCH_2CH_2CH_2C(\text{–}NHCOR^1)HCOOH$	21–25	28–37	5[d]
5	$R^1CONHCH_2CH_2CH_2C(\text{–}NHCOR)HCOOH$	21–25	28–37	8[d]
6	$RCONHCH_2CH_2CH_2CH_2C(\text{–}NHCOR^1)HCOOH$	21–25	28–37	2[d]
7	$R^1CONHCH_2CH_2CH_2CH_2C(\text{–}NHCOR)HCOOH$	21–25	28–37	1[d]
8	$R^2CONHCH_2CH_2CH_2C(\text{–}NHCOR)HCOOH$	11–14	28–37	2[d]
9	$RCONHCH_2CH_2CH_2C(\text{–}NHCOR^2)HCOOH$	11–14	28–37	6[d]
10		50–51	50–51	[e]
				Total 88

[a] R_f values expressed as grid coordinates of the two-dimensional thin-layer plate on a grid scale 100 × 100 (15 × 15 cm solvent development in each direction). [b] R = C_6H_5Cl; R^1 = C_6H_5; and R^2 = C_6H_5HO. [c] From chickens in experiment I dosed with α-*p*-chlorobenzylidene-^{14}C labeled robenidine hydrochloride. [d] From chickens in experiment III. [e] Trace found in excreta, present in liver and fat.

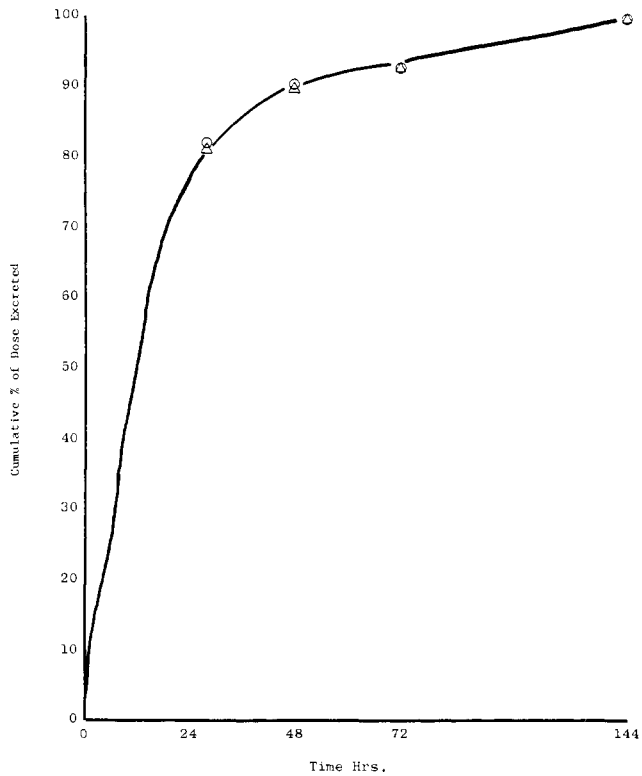


Figure 1. Excretion of carbon-14 by the chicken after a single oral dose of radiolabeled robenidine hydrochloride: (O) average of four chickens each receiving 2.5 mg (17.1 μCi of ^{14}C) of aminoguanidine-^{14}C labeled robenidine hydrochloride at 11.8 mg/kg; (Δ) average of four chickens each receiving 2.2 mg (28.4 μCi of ^{14}C) of α-*p*-chlorobenzylidene-^{14}C labeled robenidine hydrochloride at 10.7 mg/kg.

to afford N^5-(*p*-anisoyl)-N^2-(*p*-chlorobenzoyl)ornithine. This compound was treated with boron tribromide in methylene chloride (McOmie et al., 1968) at −78° in order to effect the demethylation of the *p*-methoxyl group. Compound 8 (N^2-(*p*-chlorobenzoyl)-N^5-(*p*-hydroxybenzoyl)ornithine) precipitated from the methylene chloride solution as a tan-colored solid, mp 228–230°. By using the N^2-benzyl ester of N^2-carboxyornithine and the same sequence of reaction steps, compound 9 (N^5-(*p*-chlorobenzoyl)-N^2-(*p*-hydroxybenzoyl)ornithine) was obtained, mp 227–229°.

Compound 10. Robenidine as the free base (33.4 g, 0.10 *M*) in 350 ml of dimethylformamide was heated to 80–95° on the steam bath as 190 ml (0.019 *M*) of 0.1 *N* iodine solution was added slowly with stirring. When all the iodine had been added, a total of 11 ml (0.13 *M*) of 30% hydrogen peroxide solution was slowly added dropwise with continued heating. When the addition was complete, the reaction mixture was heated with stirring an additional 40 min. There was then added 500 g of crushed ice, 500 ml of water, 5 ml of 10 *N* sodium hydroxide solution, and 10 ml of aqueous sodium thiosulfate solution. The resulting mixture was cooled to 10° by adding more crushed ice and then filtered to give a solid product. This was slurried with two 400-ml portions of water, collecting the product by filtration each time. It was then successively slurried and filtered with 200 ml of isopropyl alcohol, three 300-ml portions of ether, and a 200-ml portion of petroleum ether. After drying, there was obtained 28.5 g (86% yield) of compound 10 (3-amino-4-(*p*-chlorobenzylideneamino)-5-(*p*-chlorophenyl)-4*H*-1,2,4-triazole), mp 220–232°.

Treatment of Chickens. Three separate experiments were conducted. In experiment I, which was designed to measure the amount of ^{14}C eliminated in the excreta from chickens treated with either the guanidine or the benzylidene tracer, eight male Peterson-Cross chickens, each weighing 210–220 g, were divided into two groups of four. Chickens in one group were each given a single oral dose of the guanidine tracer. Chickens in the other group each received a single oral dose of the benzylidene tracer. Dosage rates are included in Figure 1. Each group of chickens was housed in separate galvanized metal wire-mesh cages (20 × 14 × 10 in.). Food and water were provided ad libitum. Excreta were collected on polyethylene sheets placed beneath each cage and collected at intervals as indicated in Figure 1 and were then homogenized with water and lyophilized.

Table II. Carbon-14 Residues in Tissues of Chickens after a Single Oral Dose of α-*p*-Chlorobenzylidene-^{14}C Labeled Robenidine Hydrochloride (Expressed as Parts per Million Equivalents of Robenidine Hydrochloride)

	Group, at withdrawal time of		
Tissue	A (dose, 20.0 mg/kg),[b] 4 hr	B (dose, 19.8 mg/kg),[c] 8 hr	C (dose, 13.2 mg/kg),[d] 24 hr
Liver	4.8	5.7	6.7
Kidney	1.2	3.6	2.9
Blood	0.3	0.5	0.3
Bile	21.1	37.1	52.9
Muscle	0.2	0.6	0.6
Skin[a]	0.1	0.5	0.8
Fat[a]	2.0	6.2	3.3

[a] Figures include the sum of extractable and unextractable residual radioactivity. [b] Each chicken received 6.2 mg of the drug equivalent to 78.6 μCi of carbon-14. [c] Each chicken received 6.1 mg of the drug equivalent to 77.4 μCi of carbon-14. [d] Each chicken received 4.2 mg of the drug equivalent to 53.1 μCi of carbon-14.

In experiment II, which was designed to determine the radioactive residues in selected tissues of the chicken following treatment with the benzylidene tracer, nine male Peterson-Cross chickens, each weighing 300 g, were divided into three groups of three and given the doses of the ^{14}C compound as shown in Table II. At the time intervals indicated in the table, one group of three chickens was sacrificed and the liver, kidney, bile, blood, muscle, skin, and fat were removed. The same tissues within each group were pooled, homogenized with 5–10 ml of water/g of sample, and lyophilized. Fat and skin were extracted with benzene. The other tissues were extracted with ethanol. Excreta were extracted with ethanol and 5% hydrochloric acid in ethanol.

Experiment III was conducted to obtain sufficient quantities of the metabolites in the excreta for isolation and identification. Eight male Peterson-Cross chickens, each weighing 200 g, were dosed daily for 1 week by oral intubation at a rate of 25 mg/kg body weight of the benzylidene tracer which had been diluted with the nonradioactive drug so that the specific activity was 1.3 μCi/mg. The excreta were collected, homogenized with water, and lyophilized.

Determination of Radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer as previously described (Zulalian and Gatterdam, 1973). Radioactive spots on thin-layer chromatograms were located by means of autoradiography using Kodak Royal Pan professional grade photographic film. For purposes of quantitation, each radioactive zone was scraped from the plate and placed into a scintillation vial for direct counting. Radioactivity in the excreta and tissues was determined by a modification of the oxygen-flask combustion method (Kelly et al., 1961).

Thin-Layer Chromatography (TLC). Aliquots of the

Table III. Comparative Toxicities to Female Albino Mice of the Identified Metabolites and Robenidine Hydrochloride

Compd no.	Chemical name	LD_{50}, mg/kg
1	1,3-Bis(*p*-chlorobenzylideneamino)-guanidine hydrochloride [Robenz robenidine hydrochloride]	390
2	*p*-Chlorobenzoic acid	1170
3	N^2,N^5-Bis(*p*-chlorobenzoyl)-ornithine	>5000
4	N^2-Benzoyl-N^5-(*p*-chlorobenzoyl)-ornithine	>5000
5	N^5-Benzoyl-N^2-(*p*-chlorobenzoyl)-ornithine	>5000
6	N^2-Benzoyl-N^6-(*p*-chlorobenzoyl)-lysine	>5000
7	N^6-Benzoyl-N^2-(*p*-chlorobenzoyl)-lysine	>5000
8	N^2-(*p*-Chlorobenzoyl-N^5-(*p*-hydroxybenzoyl)ornithine	>5000
9	N^5-(*p*-Chlorobenzoyl)-N^2-(*p*-hydroxybenzoyl)ornithine	>5000
10	3-Amino-4-(*p*-chlorobenzylidene-amino)-5-(*p*-chlorophenyl)-4*H*-1,2,4-triazole	>4000

radioactive extracts were spotted at the corners of commercially available precoated 20 × 20 cm silica gel thin-layer plates scored for two-dimensional development. System I [chloroform–methanol–water (225:66:9, v/v/v)] vs. [chloroform–acetone–acetic acid (180:90:30, v/v/v)] and system II [ethyl acetate–1-propanol–water–formic acid (120:200:60:20, v/v/v/v)] vs. [ethyl acetate–1-propanol–water–ammonium hydroxide (120:200:60:20, v/v/v/v)] were the solvent systems.

Isolation of Metabolites. The lyophilized excreta from experiment III were extracted with ethanol and the extract taken to dryness. The radioactive residue was dissolved in 50 ml of a pH 3.3 buffer (39.8 ml of 0.1 *M* citric acid and 10.2 ml of 0.2 *M* dibasic sodium phosphate diluted to 100 ml) and transferred to a 5 × 15 cm column of Dowex-50 cation-exchange resin in the hydrogen ion form. The column was washed with 500 ml of fresh buffer and then with 500 ml of 10% ammonium hydroxide and the effluent was monitored in a Packard Tri-Carb liquid scintillation spectrometer. The radioactivity in the buffer wash was shown by TLC in system I to contain the principal metabolites while the ammonium hydroxide eluate was found to contain robenidine and one metabolite. The radioactivity in the buffer wash was taken to dryness. The radioactive residue was dissolved in a water–ethanol mixture (3:1) and transferred to a 2.54 × 78 cm column of Sephadex LH-20 lipophilic gel and the column washed with a mixture of water–ethanol (3:1). The flow rate was 15 ml/hr. Fractions were collected at 20-min intervals in an LKB 7000 Ultro-Rac (trademark of LKB-Produkter AB) fraction collector and aliquots counted to locate the radioactivity in the eluents. Four radioactive fractions were obtained; the second fraction to elute was shown to contain the principal metabolites. This fraction was concentrated to dryness and applied to a fresh Sephadex LH-20 column in water–ethanol and chromatographed as described earlier. Two radioactive fractions were obtained. Each appeared by TLC to contain a radiochemically pure metabolite; however, evaluation of the infrared spectra of the metabolites was inconclusive. Each was then allowed to react with diazoethane (Shafik and Enos, 1969) and the product was analyzed by mass spectrometry.

Identification of Metabolites. As an aid to the identification of metabolites, authentic standards prepared as potential metabolites were cochromatographed with the radiometabolite mixture in systems I and II. Coincident spots of the radiometabolite detected by autoradiography and the standard visualized on the plate under ultraviolet light (254 nm) provided tentative identification. Confirmation was achieved through the isotope dilution technique via cocrystallization of the synthetically prepared standard with the radioactive metabolite to achieve a constant specific activity.

Hydrolysis of Metabolites. The metabolites were hydrolyzed with 1 *N* sodium hydroxide at 90° for 6 hr, acidified to pH 1.5, and extracted with ethyl acetate. The radioactivity that was recovered in the extract was examined by thin-layer chromatography with system I.

Instrumental Analysis. Infrared spectra were taken with a Perkin-Elmer Infracord spectrophotometer using the multiple internal reflectance attachment from Wilks Scientific Co. and KRS-5 plates. Mass spectra were taken on the CEC 2110B high-resolution spectrometer.

Toxicity of Robenidine Hydrochloride and Its Metabolites. Oral LD_{50} values were determined in female albino mice. Robenidine hydrochloride and metabolites listed in Table I were fed via stomach tube as 1-ml aqueous suspensions to each mouse. In the LD_{50} region, five replicates were used. The mortality counts were taken 24 hr after treatment and the LD_{50} values are given in Table III.

RESULTS AND DISCUSSION

Excretion and Tissue Retention. From the published data for the dietary intake for chickens, it was estimated that a 200-g chicken consumed about 25 g of food daily (experiment I) and a 300-g chicken about 37.5 g (experiment II). Based on these daily consumptions, it was calculated that the single oral dosage rates used in experiments I and II corresponded to a concentration in the diet of 90 and 160 ppm of robenidine hydrochloride. This dose is about three to five times higher than the recommended dose of 30 ppm of robenidine hydrochloride in the medicated feed ration. However, for the metabolism study a high dose of the radiolabeled compound was desirable in order to provide sufficient amounts of radioactivity in the extracts of excreta and tissues so as to facilitate two-dimensional thin-layer chromatographic analysis with minimum cleanup of the extracts.

In experiment I, about 99.8% of the radioactivity was recovered in the excreta within 144 hr after treatment with a single oral dose of either the benzylidene or the guanidine tracer (Fig. 1). About 82% of the radioactivity was excreted during the 24-hr period after dosing.

The residues of radioactivity in the tissues, calculated as parts per million equivalents of robenidine hydrochloride, are given in Table II. The highest levels of radioactive residues occurred in kidney, fat, and muscle at 8 hr and in liver and skin at 24 hr.

Extraction and Thin-Layer Chromatography of Radioactivity in Excreta. The 0–24-hr samples, representing about 82% of the radioactivity from each tracer, were extracted with ethanol and with 5% hydrochloric acid–ethanol. About 79% of the radioactivity in the excreta from the benzylidene tracer experiment and about 73% of that from the guanidine tracer experiment could be extracted with ethanol; about 14 and 20%, respectively, were subsequently extracted with 5% hydrochloric acid–ethanol, and about 7% remained unextracted.

By TLC in system I, it was found that a major portion of the ethanol-soluble radioactivity was due to unchanged robenidine. This amounted to about 60% of the radioactivity from the benzylidene tracer extract and about 76% from

the guanidine tracer extract. Therefore, robenidine hydrochloride had been incompletely absorbed within the body of the chicken and a major portion of the dose passed through the gastrointestinal tract unchanged.

When excreta from an unmedicated chicken was fortified with either of the tracers and extracted, about 92% of the guanidine tracer and about 98% of the benzylidene tracer were recovered with ethanol and the remainder of the radioactivity was recovered with 5% hydrochloric acid–ethanol. TLC of the ethanol extract (system I) showed that robenidine hydrochloride was stable and did not undergo chemical decomposition.

Qualitatively, it was found that several metabolites retained only the *p*-chlorobenzylidene-^{14}C label of robenidine hydrochloride, since they were absent from the chromatogram of the radiometabolites derived from the guanidine tracer. These metabolites, when combined, accounted for about 20% of the ethanol-soluble radioactivity and were considered to be the major metabolites of robenidine hydrochloride. The combination of these metabolites and robenidine hydrochloride accounted for about 80% of the total ethanol-soluble radioactivity.

There were also several minor metabolites that appeared on the chromatograms of the extracts from both tracers, indicating that they retained at least one of the benzylidene ^{14}C atoms as well as the center aminoguanidine ^{14}C atom. About 10% of the radioactivity in the extract from the benzylidene tracer and 14% of the extract from the guanidine tracer could be accounted for by these metabolites. In addition, about 10% of the radioactivity was too polar to migrate well on the thin-layer chromatograms under the conditions of system I and remained unresolved at or near the origin. By TLC of the ethanol-soluble radioactivity in system II, it was possible to resolve the ethanol-soluble radiometabolite mixture into a minimum of 12 discrete radiometabolites in addition to parent robenidine. Negligible amounts of radioactivity remained at the origin. There did not appear to be any significant metabolites which retained only the guanidine ^{14}C atom. The acid–ethanol extracts of both tracers were also found to contain metabolites that were chromatographically similar to those in the ethanol extract. Since the ethanol extract represented the major radioactive fraction, it was used in the subsequent work. No attempt was made to determine the fate of the aminoguanidine moiety of robenidine hydrochloride or to obtain any tissue residue data for this ^{14}C label in chickens.

Isolation and Identification of Metabolites in Excreta. The conjugation of aromatic acids in birds by ornithine (Jaffe, 1877; Baldwin et al., 1960), by lysine (Efimochkina, 1951), and by glutamine (Hiratani, 1957) and the metabolism of robenidine hydrochloride by rats to *p*-chlorobenzoic acid and *p*-chlorohippuric acid (Zulalian and Gatterdam, 1973) suggested that chickens would metabolically oxidize robenidine hydrochloride to *p*-chlorobenzoic acid for conversion to the glutamine, lysine, and ornithine conjugates. By TLC (systems I and II), it was found that the two-dimensional chromatographic migration coordinates of one of the minor metabolites (Table I) matched with that of *p*-chlorobenzoic acid (compound **2**). In addition, the major chicken metabolite, amounting to about 15% of the radioactivity, chromatographically matched with the synthetic N^2,N^5-bis(*p*-chlorobenzoyl)ornithine (compound **3**). Further confirmation of this identification was sought.

The synthetic compound (compound **3**) and the metabolite, obtained from the silica gel scraping of a TLC isolation, were mixed and repeatedly recrystallized until a constant specific activity was achieved, thus demonstrating the presence of N^2,N^5-bis(*p*-chlorobenzoyl)ornithine in the isolate. However, only 13% of the metabolite radioactivity (about 3% of the total radioactivity extracted from the excreta with ethanol) had cocrystallized with the carrier compound, showing that the isolate contained a large amount of some other compound. The identification of robenidine hydrochloride, *p*-chlorobenzoic acid, and N^2,N^5-bis(*p*-chlorobenzoyl)ornithuric acid accounted for only 64% of the excreta radioactivity in experiment I (Table I). When the *p*-chlorobenzoic conjugates of glutamine and lysine were synthesized and analyzed by TLC, it was found that they did not correspond to any of the metabolites.

The radioactivity was extracted from the excreta of experiment III and the metabolites separated from robenidine by chromatography on Dowex-50 resin (H^+ ion form). They were further purified by chromatography on Sephadex LH-20 lipophilic gel columns prior to treatment with diazoethane.

The mass spectrum of metabolite–isolate (fraction A) after ethylation with diazoethane showed ions at *m/e* 402, 412, 416, and 456. These all appeared to be molecular ions and, based on the high-resolution data, the probable elemental compositions for these ions were $C_{21}H_{23}N_2O_4Cl$ (*m/e* 402), $C_{23}H_{28}N_2O_5$ (*m/e* 412), $C_{22}H_{25}N_2O_4Cl$ (*m/e* 416), and $C_{25}H_{32}N_2O_6$ (*m/e* 456). An ornithine conjugate involving benzoic acid and *p*-chlorobenzoic acid was suggested for the ion at *m/e* 402 and a lysine conjugate involving benzoic acid and *p*-chlorobenzoic acid was suggested for the ion at *m/e* 416. For the ions at *m/e* 412 and 456, conjugates of ornithine involving benzoic acid and *p*-hydroxybenzoic acid were suggested. The mass spectrum of the second metabolite–isolate (fraction B) after ethylation with diazoethane showed a parent ion at *m/e* 446. This metabolite was suggested to be a conjugate of ornithine involving *p*-chlorobenzoic acid and *p*-hydroxybenzoic acid. The benzoic acid and *p*-hydroxybenzoic acid portions of these metabolites were not derived from the robenidine hydrochloride (α-*p*-chlorobenzylidene-^{14}C label) since hydrolysis of the conjugates followed by thin-layer chromatography indicated that of the aromatic acids released only the *p*-chlorobenzoic acid was radioactive. Therefore, the benzoic acid and the *p*-hydroxybenzoic acid must be arising from sources that are endogenous to the chicken. The metabolites corresponding to *m/e* 412 and 456 were regarded as nonradiolabeled contaminants in fraction A. It was not possible to differentiate by mass spectrometry the position of substitution of the aromatic acids on the amino groups of these amino acids. Positional substitution isomers were considered likely. The chemical synthesis of these mixed conjugates was undertaken to confirm the identifications of the assigned structures and for subsequent evaluation of their toxicological properties.

As shown in Table I, compounds **3**–**7** all had identical thin-layer chromatographic properties with the principal excreta metabolite in system I. By isotope dilution cocrystallization studies, it was shown that the two ornithine conjugates (**4** and **5**) accounted for about 13% of the radioactivity in the excreta, and the lysine conjugates (**6** and **7**) accounted for about 3% of the radioactivity. Compound **5** (N^5-benzoyl-N^2-(*p*-chlorobenzoyl)ornithine) was the favored isomer by about a 2:1 ratio for the ornithine conjugates involving both *p*-chlorobenzoic acid and benzoic acid. Compound **3** (N^2,N^5-bis(*p*-chlorobenzoyl)ornithine) accounted for the remainder of the radioactivity in the Sephadex LH-20 fraction, based on the earlier isotope dilution cocrystallization study.

Compounds **8** and **9** were shown to be identical with the metabolite in the other Sephadex LH-20 fraction. By isotope dilution cocrystallization studies, it was shown that these two compounds accounted for all of the radioactivity in this fraction and for about 8% of the radioactivity in the excreta. Compound **9** (N^5-(*p*-chlorobenzoyl)-N^2-(*p*-hydroxybenzoyl)ornithine) occurred as the favored isomer by about a 3:1 ratio.

Based on the results of experiments I and III, 88% of the ethanol-soluble radioactivity was identified (Table I). The remainder of the radioactivity was divided among numer-

ous separable radioactive compounds which have not been identified.

Tissues. In order to establish the relevance of the metabolites that were identified in excreta to those occurring in the tissues, the radioactivity in selected tissues (experiment II) was isolated by extraction and chromatographed in system I. Robenidine hydrochloride, *p*-chlorobenzoic acid, and the mixtures of conjugates of ornithine were identified as metabolites occurring in the liver, kidney, and muscle. There was an unidentified metabolite in liver which was similar to the metabolite found in the 10% ammonium hydroxide eluate of the Dowex-50 resin. Robenidine hydrochloride and this unidentified metabolite were also found in the extracts of fat and skin. Since liver, fat, and skin represent edible portions of the chicken, the presence of the metabolite in these tissues required that it be identified.

When an ethanol solution of radiochemically pure robenidine hydrochloride was allowed to age and was analyzed by TLC for possible chemical breakdown, it was shown to contain a compound that was chromatographically similar to the metabolite occurring in these tissues. The compound was isolated from the solution by TLC and analyzed by mass spectrometry. The mass spectrum suggested that the compound was *p*-chlorobenzonitrile; however, this compound was subsequently shown not to be the metabolite. Coincident with this result was the result of a study (Wayne and Gund, 1970) of the oxidative ring closure reaction of robenidine in the presence of iodine and hydrogen peroxide. The compound produced from this reaction was also found to be identical by TLC with the metabolite in liver. The mass spectral analysis of the compound obtained from the iodine–hydrogen peroxide reaction was consistent with the structure 3-amino-4-(*p*-chlorobenzylideneamino)-5-(*p*-chlorophenyl)-4*H*-1,2,4-triazole (**10**). The final confirmation of this compound as a metabolite in liver and fat was made by isotope dilution via cocrystallization of the synthetic carrier compound and the radioactivity extracted from liver. The mass spectrum of **10** also gave a peak for a compound identified as *p*-chlorobenzonitrile. The formation of *p*-chlorobenzonitrile was interpreted as being generated from the thermal breakdown of **10**.

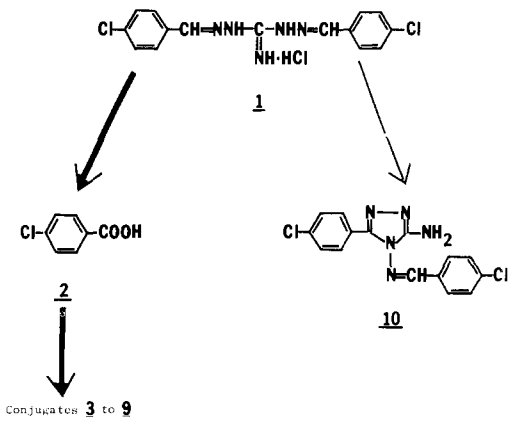


Figure 2. Metabolic route for Robenz robenidine hydrochloride in the chicken.

CONCLUSION

Benzoic acid has been shown to be metabolized by the chicken to ornithuric acid (Baldwin et al., 1960). In addition, enzyme systems have been found in chicken kidney which catalyze the formation of ornithuric acid (McGilvery and Cohen, 1950; Schacter et al., 1955), the synthesis of mono- and dibenzoylornithines (Marshall and Koeppe, 1964), and the benzoylation of ornithine (Marshall, 1966). Since robenidine was metabolized by the rat to *p*-chlorobenzoic acid and *p*-chlorohippuric acid (Zulalian and Gatterdam, 1973), it was not surprising, therefore, to find *p*-chlorobenzoic acid and N^2,N^5-bis(*p*-chlorobenzoyl)ornithine (compounds **2** and **3**) as the metabolites of robenidine in the chicken. However, the formation of the triazole metabolite (**10**) and the conjugates of ornithine and lysine containing *p*-chlorobenzoic acid with either benzoic acid or *p*-hydroxybenzoic acid (**4–9**) in the chicken were considered to be novel.

For the comparative metabolism of robenidine hydrochloride in rat vs. chicken, there was a species difference in the amino acids used to form the conjugates of *p*-chlorobenzoic acid. The triazole metabolite of robenidine was sought but could not be found in liver, kidney, and muscle tissue of rats. However, there was an indication of its presence in very low concentration in fat based on a study with α-*p*-chlorobenzylidene-^{14}C labeled robenidine hydrochloride. No data are available on the comparative metabolism of rat vs. chicken vs. man. However, it is expected that robenidine hydrochloride would be metabolized in man to yield *p*-chlorobenzoic acid and *p*-chlorohippuric acid similar to that observed for the in vivo study with rats. The in vitro organ maintenance technique (Sullivan et al., 1972) offers promise of a method to perform in depth studies of the comparative metabolism of man vs. animal without direct dosing of human subjects.

In addition to the identification of robenidine hydrochloride, *p*-chlorobenzoic acid, and the conjugates (**3–9**) as metabolites in liver, kidney, and muscle, 3-amino-4-(*p*-chlorobenzylideneamino)-5-(*p*-chlorophenyl)-4*H*-1,2,4-triazole (**10**) was also identified as a metabolite in liver, amounting to 12% of the radioactivity. Robenidine hydrochloride and the triazole metabolite were the principal radioactive residues found in fat and skin. Based on these findings, a scheme for the metabolism of robenidine hydrochloride by the chicken is presented in Figure 2.

The relevance of utilizing the excretion products of an animal as a source of metabolites for isolation and identification depends upon assessing the metabolite composition residing in edible tissues. In this study, the finding of the triazole metabolite in liver, fat, and skin but not in excreta emphasizes the importance of evaluating residual tissue metabolites. However, since all of the identified metabolites have been shown to be less toxic than robenidine hydrochloride (Table III) and robenidine hydrochloride was the significant residue in all the tissues, it would appear that residue methods responding only to robenidine hydrochloride may provide the most realistic means of evaluating the levels of toxicologically significant residues which may result from the field use of this anticoccidial compound.

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Absorption of Herbicides by Wheat as Influenced by the Phenoxy Compound

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Herbicide mixtures of the sodium salt of trichloroacetic acid (Cl_3CCOOH) and the amine salt of a phenoxy herbicide as post-emergence treatment in the greenhouse study improved wheat tolerance to Cl_3CCOOH. The physiological basis for tolerance, as measured by absorption, accumulation, and distribution of Cl_3CCOOH-^{14}C in intact wheat seedlings and excised root segments, was investigated. In a series of experiments wheat seedlings absorbed reduced amounts of ^{14}C-labeled Cl_3CCOOH and other herbicides from solutions containing one of five phenoxy compounds (10^{-4} to 10^{-6} M). Results also suggested that Cl_3CCOOH absorption by wheat roots behaved like an active process requiring a metabolic energy supply and that 2,4-dichlorophenoxyacetic acid (2,4-D) inhibited the stimulatory effect of adenosine 5′-triphosphate (ATP) on Cl_3CCOOH absorption and acted as a noncompetitive inhibitor for root entrance and movement of Cl_3CCOOH to the shoots of seedlings. Based on this investigation, improvement of wheat tolerance to Cl_3CCOOH with Cl_3CCOOH–phenoxy mixtures was mainly due to physiological restraint and noncompetitive inhibition of absorption and subsequent translocation of Cl_3CCOOH in the wheat plant by the phenoxy herbicide.

Herbicide mixtures are becoming more popular today allowing a reduction in the rate of a single herbicide, resulting in less environmental problems (Caseley, 1971), broadening the spectrum of weed control, and reducing the cost of some newly developed herbicides by partial substitute of a lower cost herbicide. The early developed herbicides, particularly 2,4-dichlorophenoxyacetic acid (2,4-D) and [(4-chloro-*o*-tolyl)oxy]acetic acid (MCPA), have such characteristics as good broadleaf weed control and low cost. Thus, these two phenoxy herbicides in single application or in combination with other herbicides have been widely used in small grain production and ranked the highest in usage. In 1972 11 million pounds were applied on about 28 million acres in the Canadian Prairie provinces.

Through field and greenhouse experiments we observed (Chow and Dryden, 1973) that under some conditions Cl_3CCOOH in the phenoxy herbicide mixtures exhibited better weed control and higher crop tolerance as well. The studies described herein report that wheat treated with a phenoxy compound mixture accumulated less herbicide as compared with a herbicide applied alone at equal rates. The results offer a possible explanation why wheat tolerance was improved by applying a phenoxy herbicide mixture.

MATERIALS AND METHODS

Chemicals and Radioassay Instrument. In the greenhouse pot study, commercial herbicide products (73% sodium salt of Cl_3CCOOH, 50% amine salt of 2,4-D, and 50% amine salt of MCPA) were investigated. For other experiments, nonlabeled and ^{14}C-labeled herbicides and chemicals used are listed in Table I, along with common and chemical names, specific activities, purities, and sources.

Labeled compounds were dissolved in 50% ethanol as stock solutions. A certain amount of these stock solutions, alone or in combination with a nonlabeled compound, was diluted with distilled water to a desired concentration to form the treatment solution. The pH of the treatment solutions was adjusted prior to application.

Radioactivity in extracts was measured at ambient temperature by using a Liquimat 220 (Picker Nuclear) liquid scintillation spectrometer equipped with a ^{137}Cs external standard and photomultiplier tubes of a bialkali photocathode type. For toluene-^{14}C the instrument had a counting efficiency of 90% accompanied by about 20 cpm of background counting rate.

Greenhouse Pot Study; Wheat Tolerance to Herbicide Mixture. In the greenhouse pot tests with wheat (*Triticum aestivum* (L.) var. "Manitou"), 12 seeds were sown in pots containing 1500 g of clay loam soil (26.7% clay, 33.0% silt, 40.3% sand, 5.6% organic matter, and pH 7.5). Fluorescent light at about 16,500 lx for a 16-hr photoperiod was provided. After germination, seedlings were thinned to six plants per pot for treatment. The treatment solutions were sprayed on the foliage of seedlings as well as the soil surface at the rate of 262 l./ha of water at a pressure of 2.1 kg/cm^2 (30 psi) at the three-leaf stage of wheat. All rates of commercial products of herbicides were applied in terms of active ingredients in kilograms per hectare. Water was added to the surface of the soil in pots as required by the growth of seedlings. All treatments were arranged in a randomized block design with four replicates and pots on the bench were rearranged once a week in order to receive uniform light exposure and air circulation. The test was terminated at the end of 4 weeks with a visual rating of wheat tolerance to Cl_3CCOOH and the fresh weight yield of shoots.

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