Table IV. Human Urinary Metabolites from a Malathion Poisoning Case

Metabolite	Urine, human control, ppm	Urine, human exposed, ppm
DCA	<0.005	12
MCA	<0.005	223
DMTP	ND	96
DMDTP	ND	20
DMP	ND	50
MMP[a]	ND	8

[a] Monomethyl phosphate.

malathion. Carboxylic acids were found in most of the urines, the highest level being 0.01 ppm of the monoacid. The average of the ten, however, was less than 0.005 ppm of either acid.

Urine was available from a human poisoning case in which a man attempted suicide by drinking about 200 mL of 50% malathion preparation. Urine for the first 24 h was not available, but that from the second 24 h following exposure was analyzed. Table IV shows the results of the analysis for phosphorus-containing metabolites. Malathion monoacid is the most significant metabolite, followed by DMTP and DMP.

The method for the determination of DCA and MCA in urine has proven its value in monitoring animal and human exposure to malathion. In addition to the quantitative data, the analytical procedure provides confirmation of identity of the metabolites through the phosphorus-specific flame photometric detector and elution in the proper fraction from silica gel. Ethylation of MCA and DCA to form malathion provides additional confirmation.

This method provides a specific and sensitive procedure for the determination of malathion carboxylic acids in urine. The determination of such urinary metabolites helps provide an index of human exposure to organophosphorus pesticides which is essential to the human monitoring programs.

ACKNOWLEDGMENT

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Tissue Residue and Comparative Metabolism Studies on Tiazuril in the Chicken, Rat, Dog, and Monkey

Martin J. Lynch* and Sanford K. Figdor

The metabolism of the anticoccidial compound tiazuril (2-[3,5-dimethyl-4-(4-chlorophenylthio)-phenyl]-*as*-triazine-3,5(2*H*,4*H*)-dione) was studied in the chicken and laboratory animals. Following oral administration of tritium-labeled tiazuril (^{3}H-T) to the chicken at 1 mg/kg, ^{3}H-T equivalents of radioactivity was 0.06 ppm or less in edible tissues and plasma 5 days after dosing. Throughout the withdrawal period, radioactivity was higher in plasma, liver, and kidney than muscle, skin, and fat. Tiazuril (T) is metabolized by oxidation of the sulfur atom to T-SO and T-SO_2 and by hydroxylation of the chlorophenyl ring to HO-T, HO-T-SO, and HO-T-SO_2. T-SO_2 and HO-T-SO are the major metabolites in liver and excreta, respectively. Broilers maintained on T in feed at 0.0015% for 8 weeks showed less than 0.01 ppm of T and 0.03 ppm or less of T-SO_2 in plasma and edible tissues 4 days after drug withdrawal. In comparative metabolism studies, the dog is distinguished from the chicken, rat, and monkey by maintaining persistent concentrations of T in plasma and by its inability to hydroxylate the drug or its sulfoxidation products.

Tiazuril (2-[3,5-dimethyl-4-(4-chlorophenylthio)-phenyl]-*as*-triazine-3,5(2*H*,4*H*)-dione) belongs to a class of aryltriazines with structures similar to 6-azauracil that possess potent broad spectrum anticoccidial activity in the chicken (Miller, 1975; Miller et al., 1977; Mylari et al., 1977). It was proposed for treatment of broilers at a projected use level of 0.0010–0.0020% in feed. The anticoccidial activity, site of action, and metabolism of other potentially useful animal health products of this series have been described (Chappel et al., 1974; Ryley et al., 1974; Rash and Lynch, 1976).

The study reported here was part of a safety evaluation process incorporating suggestions of Perez and Weber (1975, 1976, 1977). The distribution and the rate of disappearance of metabolic residues of tiazuril remaining in edible tissues of the chicken under both laboratory and

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floor pen conditions was determined by radiotracer and chemical methods.

These studies were supplemented with a determination of the fate of tiazuril in the rat, dog, and monkey to test for similarity of metabolism in the use animal (chicken) and laboratory animals. Species showing the same metabolites as the chicken would be suitable animals for toxicological studies to assess risks to man, the eventual food consumer. The results of these comparative metabolism studies are also the subject of this report.

EXPERIMENTAL SECTION

Instruments. A MicroTek MT-220 gas chromatograph equipped with ^{63}Ni electron capture (ECD) and flame photometric (sulfur, FPD) detectors and set up for off-column injections was used to chromatograph tiazuril and metabolites. The carrier gas was argon–methane (9:1) at a flow rate of 100 mL/min. The instrumental settings were: inlet temperature, 300 °C; detector temperature, 300 °C; column temperature, 290 °C; pulse rate, 270 μs; pulse width, 3 μs; voltage, 55 V. The detector signal was recorded on a Westronics recorder and integrated by a Hewlett-Packard Model 3370A electronic integrator. The 1 m × 4 mm glass column contained 3% OV-17 on 60/80 mesh Gas-Chrom Q.

Mass spectra were obtained by gas chromatographic introduction of samples into a LKB Model 9000 mass spectrometer. Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation spectrometer. A Vanguard Model 880 Automatic Chromatogram Scanner was used to locate radioactvity on thin-layer plates.

Reagents and Reference Compounds. All reagents were reagent grade or glass-distilled. Diazomethane was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co., Inc.) as described by Grunwald et al. (1967). Reference compounds consisting of tiazuril or 2-[3,5-dimethyl-4-(4-chlorphenylthio)-phenyl]*as*-triazine-3,5(2*H*,4*H*)-dione (T), 2-[3,5-dimethyl-4-(4-chlorophenylsulfinyl)phenyl]-*as*-triazine-3,5(2*H*,4*H*)-dione (T-SO), and 2-[3,5-dimethyl-4-(4-chlorophenylsulfonyl)phenyl]-*as*-triazine-3,5(2*H*,4*H*)-dione ($T\text{-}SO_2$) were prepared by Dr. M. W. Miller (1975) and Dr. B. L. Mylari at Pfizer Central Research, Groton, Conn.

General Procedure for Methylating Reference Triazines. (1) To a solution of tiazuril (1.8 g, 0.005 mol) in methanol (50 mL) and 85% potassium hydroxide (0.36 g, 0.0055 mol) was added dimethyl sulfate (0.64 g, 0.005 mol). The reaction mixture was stirred at room temperature for 1.5 h. A white precipitate (1.3 g) was collected by filtration and recrystallized from methanol yielding 0.80 g of crystalline product: mp 129–130 °C; mass spectrum *m/e* 375 (40), 373 (100), 263 (11), 261 (31). A dried sample (at 0.1 mmHg, 80 °C, 2 h) was analyzed. Anal. Calcd for $C_{18}H_{16}O_2N_3SCl$: C, 57.83; H, 4.31; N, 11.24; S, 8.58; Cl, 9.48. Found: C, 57.91; H, 4.42; N, 11.23; S, 8.71; Cl, 9.47.

(2) Following the general methylation procedure using $T\text{-}SO_2$ (2.1 g, 0.005 mol) and dimethyl sulfate (0.64 g, 0.005 mol) in methanolic potassium hydroxide yielded 1.6 g of crude product. It was recrystallized from methanol to give 1.5 g: mp 182–183 °C; mass spectrum *m/e* 407 (13), 405 (34), 389 (41), 387 (100). Anal. Calcd for $C_{18}H_{16}O_4N_3SCl$: C, 53.27; H, 3.97; N, 10.35; S, 7.90; Cl, 8.74. Found: C, 53.13; H, 4.05; N, 10.26; S, 7.98; Cl, 8.70.

(3) T-SO (1.95 g) was reacted at room temperature with dimethyl sulfate (0.64 g, 0.005 mol) in methanolic potassium hydroxide to give 1.57 g of crude product. It was recrystallized from methanol to give 1.15 g: mp 174–175 °C; mass spectrum *m/e* 391 (11), 389 (30), 337 (20), 277 (100). Anal. Calcd for $C_{18}H_{16}O_3N_3SCl$: C, 55.46; H, 4.14; N, 10.78; S. 8.22; Cl, 9.09. Found: C, 55.59; H, 4.12; N, 10.71; S, 8.58; Cl, 8.94.

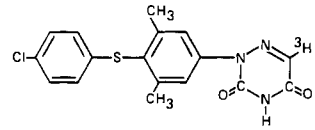

Figure 1. Structure of ^3H-T.

Preparation of Ring-Labeled ^3H-Tiazuril. Tiazuril labeled with tritium in the triazine ring was prepared by a previously described method (Rash and Lynch, 1976). One gram of the penultimate synthetic intermediate (Miller, 1975) 2-[3,5-dimethyl-4-(4-chlorophenylthio)-phenyl]-6-carboxy-*as*-triazine-3,5(2*H*,4*H*)-dione (provided through the courtesy of Dr. M. W. Miller at Pfizer Central Research) was stirred overnight with 50 mL of dry, redistilled ethyl acetate and 5 Ci of ^3H_2O. Excess water and tritium were removed by vacuum distillation and the tritium-labeled precursor decarboxylated by heating at 245 °C for 5 min in a constant-temperature oil bath. The product was passed over a column of 20 g of silica gel in benzene–acetic acid (95:5, v/v) and eluted with this solvent. Appropriate fractions (10 mL) were combined and concentrated to dryness. Darco treatment of a chloroform solution of the residue and filtration through Super-Cel followed. The filtrate was reduced in volume under nitrogen on a steam bath and crystallization started by the addition of hexane. Crystals were washed with hexane–chloroform and dried in vacuo over P_2O_5. The final purified labeled product (mp 134–135 °C) was synthesized in 38% yield as a homogeneous radiochemical compound as shown by autoradiography, and UV examination of TLC plates previously developed in a benzene–acetic acid (95:5) solvent system. The tritium-labeled tiazuril (^3H-T) had a final sp act. of 52.5 μCi/mg (Figure 1).

Incorporation of tritium into the 6 position of the triazine ring was confirmed by preparation of deuterated tiazuril (^2H-T) using deuterium oxide in place of ^3H_2O. Analysis of the mass spectrum of ^2H-T revealed 78% incorporation of deuterium into the triazine ring (Figure 2). No incorporation of deuterium was detected in the dimethylphenyl or chlorophenyl rings. To exclude interference by the N-H proton, the N-methylated analogue was prepared and examined by ^1H NMR (Figure 3). In this spectrum, the area under the signal (δ 7.6) corresponding to the proton in the 6 position of the triazine ring is quenched by 78%, indicating substitution of hydrogen by deuterium (Figure 3).

Animal Experiments. Several studies are reported with varying protocols. White Rock broilers, weighing 2.0 to 2.6 kg, were maintained on T in feed at either 0.0010% for 39 days, 0.0015% for 21 days, or 0.0020% for 25 days prior to oral administration of tritium-labeled compound (sp act., 52.5 μCi/mg). The labeled drug was administered as a suspension in 1% carboxymethylcellulose, 0.1% methocel, and 0.1% Tween-80 in water at dose levels equivalent to 1, 1.1, and 1.35 mg/kg b.w. for the three respective studies. After administration of labeled compound, birds were placed in metabolism cages with free access to nonmedicated food (Ralston-Purina Chick Startina) and water. Excreta were collected for analyses in 24-h intervals for 5 days following dosing with ^3H-T. The excreta were homogenized with water, lyophilized, and stored frozen. Blood samples were taken from broilers by endocardial puncture at timed intervals, treated with heparin, and separated plasma was frozen for later drug analysis. Edible tissues of chickens, including fat, skin, muscle (dark and light), liver, and kidney, were taken from

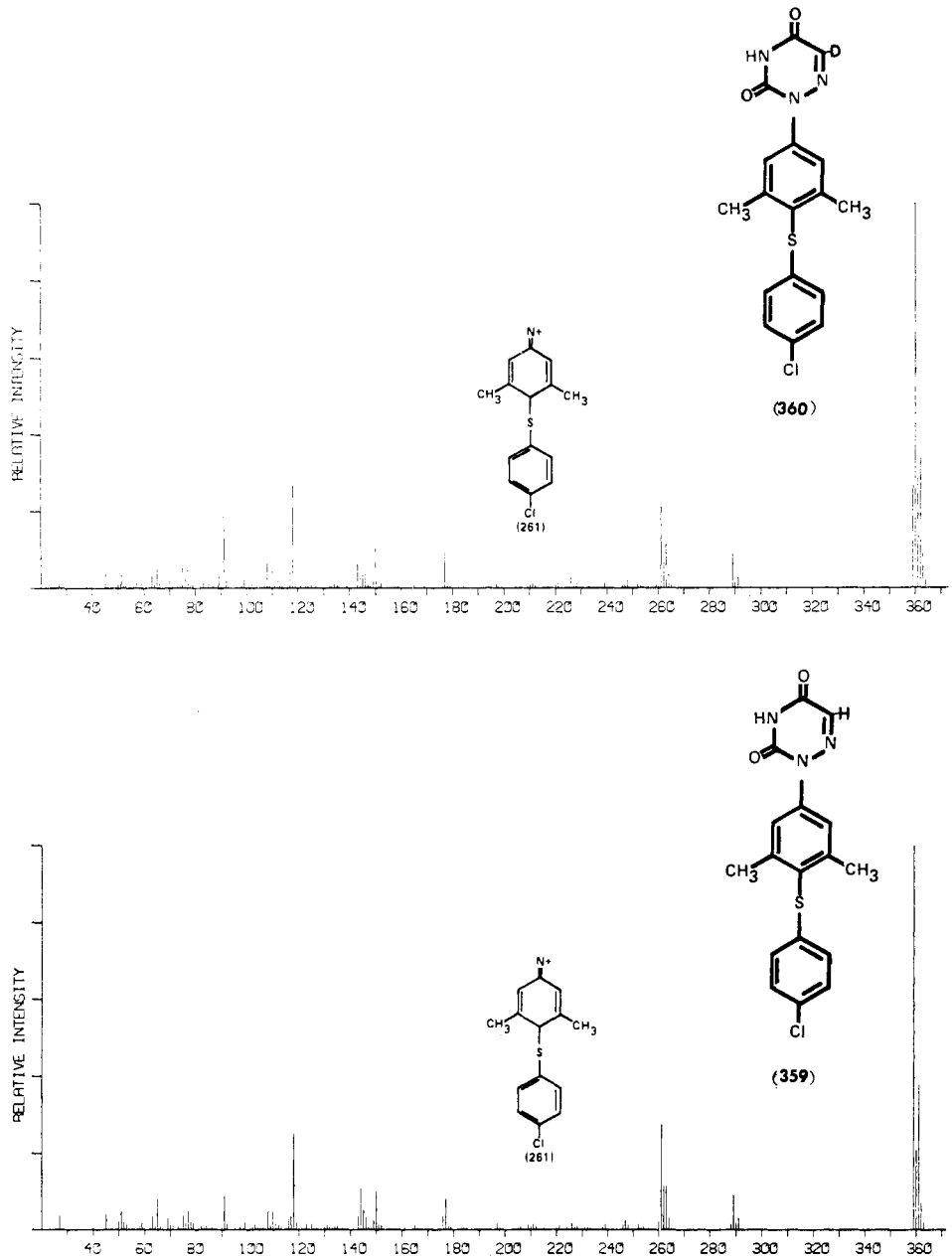

Figure 2. Mass spectra of deuterated T (upper) and T (lower).

broilers at sacrifice and frozen until analyzed. For the determination of drug residues resulting from administration of the drug under floor pen conditions, 200 Penobscot strain boilers were fed ad libitum for 56 days with T in feed at 0.0015% and thereafter with a nonmedicated basal ration. Six birds each, three males and three females selected from the flock, were sacrificed on the last day of medication and on each of the following 5 days. Plasma and tissue samples were taken and stored for GLC analysis as in the radiotracer study.

Nonlabeled T was administered in feed to Charles River strain rats (0.25 kg) for 4 days at a dose level equivalent to 20 mg kg^{-1} day^{-1}. The animals were sacrificed on the fourth day and the heparinized plasma was pooled. ^{3}H-T (10 mg/kg, b.w.) was given to two rats by gavage (sp act. = 47.7 μCi/mg) in 1 mL of aqueous suspension formulated as described above. For collection of urine and feces, rats were placed in metabolism cages and fed Purina Lab Chow ad libitum. Water was provided throughout the study. Heparinized plasma was obtained from the tail of each animal at timed intervals through 168 h.

Adult beagle dogs (male and female) weighing 6.9 to 9.0 kg were dosed with nonlabeled T in capsule form. Two dogs were dosed daily for 5 consecutive days at 100 mg kg^{-1} day^{-1} and two more dogs for 3 days at 4 mg kg^{-1} day^{-1}. Heparinized plasma was obtained daily from each dog to 120 h postmedication. One female beagle (8.9 kg) with an external bile fistula was given by gavage ^{3}H-T 5 mg/kg (sp act. 47.7 μCi/mg) in 1 mL of aqueous suspension formulation described above. The animal was housed in a stainless steel metabolism cage for daily collection of bile, urine, and feces. Samples of heparinized plasma and excreta were collected to 168 h.

Two male rhesus monkeys (3 kg) were given single oral doses of nonlabeled T at 10 mg/kg in aqueous suspension. Heparinized plasma was obtained at periodic intervals out to 133 h.

Radiochemical Methods. Biological samples were counted in 15 mL of a liquid scintillation solution containing 0.3% 2,5-diphenyloxazole (PPO), and 0.01% bis[2-(5-phenyloxazolyl)]benzene (POPOP) in a mixture of 30% absolute ethanol and 70% toluene (30/70 scin-

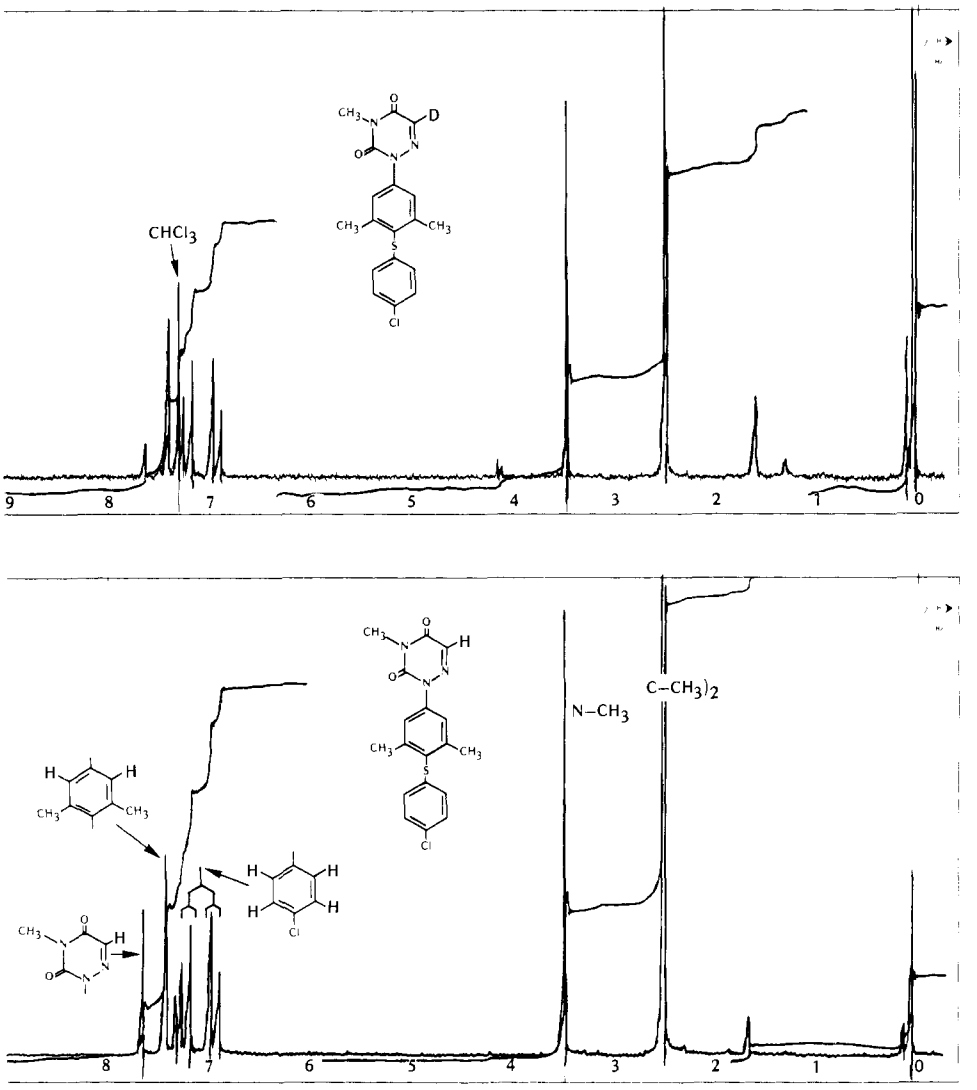

Figure 3. Proton magnetic resonance spectra of deuterated T (upper) and T (lower), each N-methylated.

tillator). Tritium radioactivity was measured and corrected for quenching using tritiated toluene as an internal standard.

Suitable aliquots (0.1–0.2 mL) of plasma or urine were dissolved in 0.5 mL of Nuclear Chicago solubilizing solution (NCS) with slight warming prior to counting in 30/70 scintillator. A 250-mg sample of tissue was added to 2.5 mL of "Soluene" (Packard Instrument International S.A.) in a liquid scintillation vial and placed in a Dubnoff shaker at 50 °C for 3 h to completely dissolve the tissue. The vial was cooled to room temperature, 15 mL of 30/70 scintillator added, and the sample counted as above. A lyophilized excreta sample (0.2–0.3 g) was compressed into a tablet and combusted in a Schoniger flask with $KClO_3$ over a solution of ethylene glycol monoethyl ether–ethanolamine (2:1). An aliquot of this trapping solvent was mixed with 30/70 scintillator and counted. Body water was assayed for tritium content by lyophilizing a portion of plasma or tissue and counting the trapped distillate.

Counting efficiencies for tritium in aliquots of plasma, liver, kidney, skin, muscle, fat, and excreta were 13.0 ± 1.6, 2.67 ± 0.69, 3.09 + 1.43, 10.4 ± 1.00, 11.1 ± 0.29, 11.2 ± 0.45, and 17.7 ± 1.6%, respectively.

Assay of T and T-SO_2 in Plasma and Tissues by Gas–Liquid Chromatography. Preparation of plasma samples and standards by the method of Rash and Lynch (1976) was followed. In this procedure a 0.5-mL aliquot of plasma was acidified with 1 mL of 0.1 M H_2SO_4 and incubated with 10.0 mL of benzene at 50 °C for 30 min. The mixture was stirred, cooled, and the benzene layer separated by centrifugation. A 5.0-mL aliquot of the benzene layer was methylated for GLC–ECD analysis with diazomethane according to Grunwald et al. (1967) and adjusted to a known volume. It was previously shown by Rash and Lynch (1976) that methylation of the triazine ring resulted in a derivative suitable for gas–liquid chromatography with detection by electron capture. The diazomethane method is recommended for assay by GLC–ECD after investigating several alkylation procedures (Gut et al., 1961) for 6-azauracil compounds.

Preparation of Tissue Samples for GLC–ECD Analysis. A 5.0-g sample of tissue was digested with 10 mL of 6 M HCl for 1 h at 100 °C, cooled, and extracted with two 15-mL portions of ethyl acetate. The extract was concentrated by rotary evaporation at 40 °C to an oily residue. This residue was reconstituted with 10 mL of benzene and then transferred to a glass column containing a glass wool plug, a benzene slurry of 1 g of silica gel as the lower layer, and 1 g of sodium sulfate as the upper layer. The column was washed with 10 mL of benzene, following addition of the sample. Tiazuril and metabolites were eluted from the column with 25–30 mL of benzene–methanol (9:1). The eluate was extracted with 10 mL of 0.1 M NaOH, clarified by centrifugation, and the organic layer removed by aspiration. The aqueous phase was neutralized (pH 6) with 4 mL of 1 M KH_2PO_4 and extracted with 10.0 mL of

benzene. A 5.0-mL aliquot of the benzene layer was methylated with diazomethane and adjusted to a known volume (by evaporation or dilution) prior to GLC–ECD analysis.

Gas–Liquid Chromatography of Plasma and Tissue Extracts. When aliquots (1–4 μL) of the methylated extracts of T and T-SO_2 or T-SO were injected into the GLC–ECD, they had retention times (t_R) of 1.2 and 2.1 min, respectively. The efficiency of the benzene extraction for T and T-SO_2 in plasma over the concentration range of 0.25 to 2.0 ppm was greater than 90%. Average recoveries (N = 55) of 62 ± 10 and 80 ± 5% were found for T and T-SO_2 supplemented into edible tissues over the concentration range 0.05 to 0.25 ppm. Therefore, drug concentrations above 0.01 μg/mL of plasma or 0.01 μg/g of tissue were determined by comparison with simultaneously run plasma and tissue homogenates containing known amounts of T and T-SO_2. The peak areas of standards per unit concentration were compared to that of the sample to determine its concentration. Results were reproducible within ±10%. No significant interference (<0.01 ppm) was found for control plasma, liver, kidney, muscle, fat, or skin samples.

TLC Reverse Isotope Dilution Analysis of Liver Samples. Since T-SO and T-SO_2 are unresolved by the above described GLC–ECD tissue residue assay procedure, a complementary method was employed to quantitate their levels in liver of broilers dosed and withdrawn from ^{3}H-T. In this procedure, 5 g of liver was fortified with 400 μg each of T, T-SO, and T-SO_2. These levels were required to detect T and metabolites on TLC plates by UV (254 nm) light. They also served to improve the recovery of radioactivity from tissue. Samples were carried through the digestion, extraction, column chromatography and methylation steps described for GLC–ECD assay. The final benzene extract was concentrated and applied in a band to a silica gel GF TLC plate (10 cm × 20 cm). Reference samples of T, T-SO, and T-SO_2, each N-methylated, were spotted on opposite sides of the plate, or developed as separate controls on a second TLC plate. The TLC plate was then developed by the "multiple pass" ascending technique of Thoma (1965) in a benzene–acetic acid (95:5) solvent system. Reference compounds were detected by their absorption under UV light (254 nm) and the corresponding sample zones excised from the silica gel adsorbent. The samples were eluted with methanol and radioactivity associated with eluates of each of the isolated thin-layer zones was quantitated. Aliquots of these eluates were examined by the specified GLC procedure to determine the recovery of each standard. Radioactivity in each eluate was corrected for recoveries of: 107, 87, and 89% for T, T-SO, and T-SO_2, respectively. In addition, zones from the remaining portions of the plate were excised, eluted, counted, and corrected for the recovery of added T to determine radioactivity associated with unidentified metabolites.

Metabolite Identification and Isolation. *Identification of Plasma Metabolites.* Plasma was acidified with 0.1 M H_2SO_4 and extracted with benzene according to the above described assay procedure. Benzene extracts of several plasma samples were combined, methylated with diazomethane, and concentrated by rotary evaporation to 0.1 mL. A 1–10-μL aliquot of the composite sample was examined by temperature-programmed GLC–MS analysis using standard samples of N-methylated T, T-SO, and T-SO_2 for comparison. Mass spectra were obtained for all major peaks, separated by GLC, and detected by the ion monitor.

Table I. Depletion of Plasma Radioactivity (Expressed in μg Equivalents of ^{3}H-T/mL) in the Chicken, Rat, and Dog following Oral Administration of Labeled Tiazuril (^{3}H-T)

Hours, postdose	Dose, mg/kg: Chicken 1.07[a]	Chicken 1.00[b]	Rat 10.0[c]	Dog 5.0[d]
7	2.28	2.18		15.6
24	1.17	1.36	6.87	13.1
48	0.56	0.41	1.92	7.6
72	0.13	0.06	1.21	7.6
96	0.05	0.05	0.93	5.6
120	0.02	<0.01		5.5
144				5.1
168			0.50	3.8

[a] Administered to six chickens maintained for 39 days with nonlabeled T in feed (0.001%) prior to administration of ^{3}H-T. Two birds were assayed at each time point. [b] Administered to chickens maintained for 21 days with nonlabeled T in feed (0.0015%) prior to administration of ^{3}H-T. Two birds were assayed at each time point. [c] Administered to two rats and results averaged. [d] Administered to a bile cannulated dog.

Identification of Tissue Metabolites. The GLC–ECD and TLC reverse isotope dilution procedures described above in the analysis section were utilized for this purpose.

Identification of Excreta Metabolites. Chicken excreta was leached with 0.1 M NaOH–10% NaCl, filtered through diatomaceous earth (Super-Cel), and the filtrate acidified to pH 1 with concentrated HCl. This solution was digested under nitrogen for 3 h on a steam bath, cooled, filtered through glass wool, and applied to a XAD-2 column (250 mm × 20 mm i.d.). The column was washed in sequence with 5% NaCl and water, and eluted with methanol. The aqueous methanolic eluate was evaporated to remove methanol, adjusted to pH >12 with 0.1 M NaOH, washed with chloroform, neutralized to pH 6 with 1 M KH_2PO_4, and then extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness under vacuum, reconstituted with chloroform, and applied to a silica gel column (250 mm × 10 mm i.d.) packed to a height of 100 mm in chloroform. The column was washed with chloroform and eluted with chloroform–methanol (95:5) to recover radioactivity in excreta. The chloroform–methanol eluate was methylated with diazomethane and applied to a silica gel thin-layer plate. The plate was developed by the ascending multiple-pass technique for "three passes" in the solvent system benzene–acetic acid (95:5). Major radioactive zones were recorded with a chromatographic autoscanner. The silica gel layer was scraped from the developed plate in nine 10–15-mm zones, and each was eluted with ethyl acetate. Individual fractions were counted for radioactivity and aliquots injected, as described above, into a combined GLC–MS for further separation and identification. When metabolites were detected by this procedure, identification was verified by comparing R_f values (TLC) and retention times (GLC) to those found for authentic standards, where applicable, using electron capture and flame photometric (sulfur mode) detectors for GLC. Rat and dog urine were examined by the same procedures utilized for plasma.

RESULTS

Measurement, Identification, and Distribution of Radioactive Residues in Excreta, Plasma, and Tissues of the Chicken. Following a single oral dose of tritium-labeled tiazuril to the chicken at 1 mg/kg, plasma and tissue radioactivity (^{3}H) declined from 1–2 ppm during the first 24 h to 0.06 ppm or less 120 h after dosing (Tables I and II). The highest radioactivity was found in plasma,

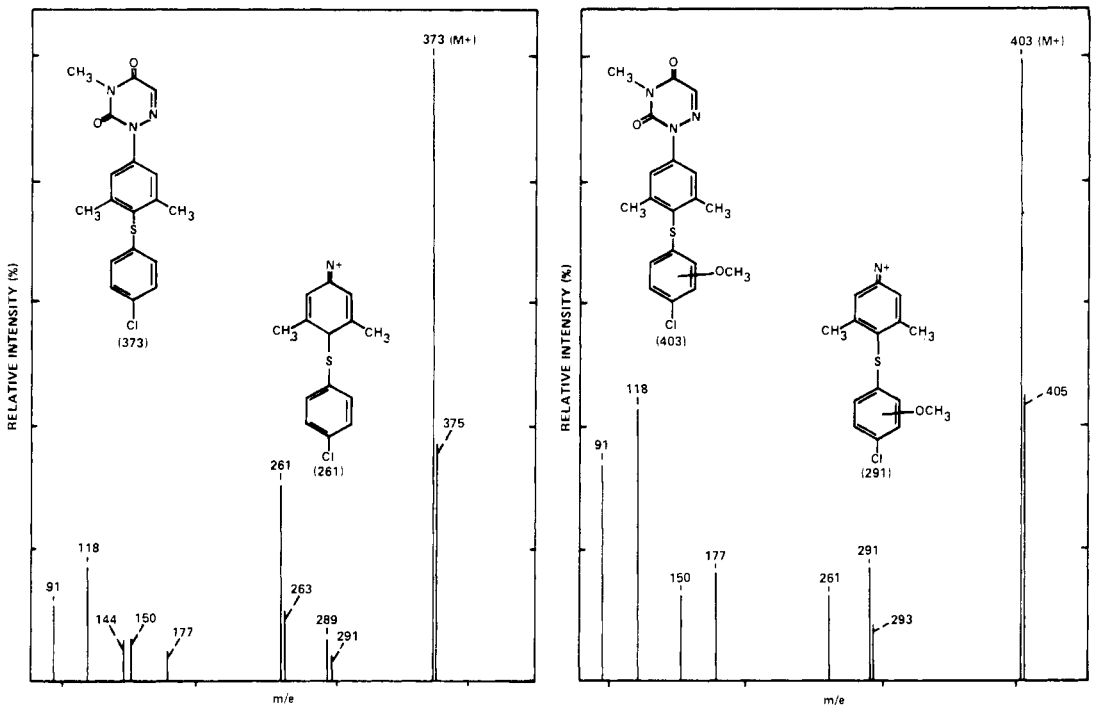

Figure 4. Mass spectra of N-methylated T and N- and O-methylated HO-T.

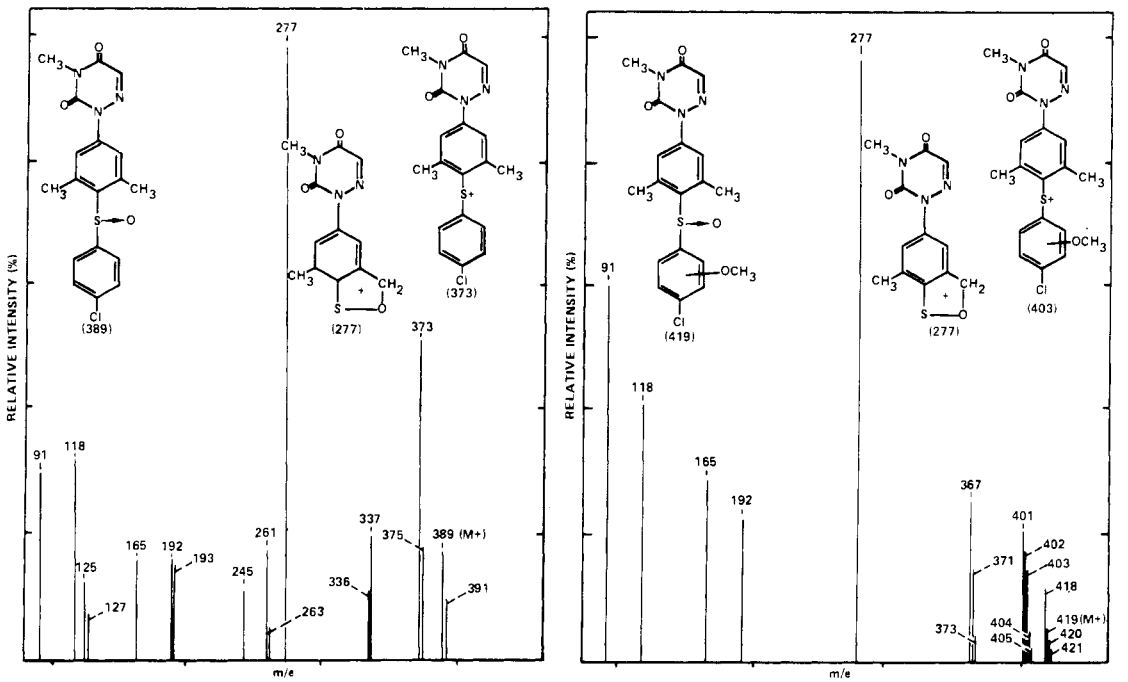

Figure 5. Mass spectra of N-methylated T-SO and N- and O-methylated HO-T-SO.

kidney, and liver (Table II). Radioactivity in tissues and plasma on day 5 of drug withdrawal was corrected for 50% tritium water content. Three to five days after dosing, 79 to 100% of the labeled dose was recovered in excreta (Table III).

Following acid hydrolysis, 78% of the total radioactivity in 0–24 h excreta was isolated for separation by TLC and only 11% when the sample was not hydrolyzed, indicating conjugation of residues. Radioactivity isolated after acid hydrolysis, purification and methylation yielded several components by TLC and GLC–ECD (Table IV). Some of these products were subsequently identified by GLC–MS (Figures 4–6) as originating from the oxidation of the sulfur atom and hydroxylation of the chlorophenyl ring of T. They were designated T-SO, T-SO_2, HO-T, HO-T-SO, and HO-T-SO_2. The proposed biotransformation pathway for T is presented in Figure 7. The major

Table II. Depletion of Radioactivity (Expressed in μg Equivalent of ^{3}H-T/g) in Chicken Tissues[a] after a Single Oral 1 mg/kg Dose of ^{3}H-T to Chickens Pretreated with Nonlabeled T in Feed[b]

Time, h	Liver	Skin	Muscle	Fat	Kidney
24	1.16	0.43	0.16	0.62	1.39
72	0.22	0.07	0.06	0.05	0.21
120	0.03	0.02	0.02	<0.01	0.06

[a] Average result of tissues taken from two chickens. [b] Chickens were maintained on tiazuril in feed at 0.0010–0.0015% for 21 to 39 days prior to administration of ^{3}H-T.

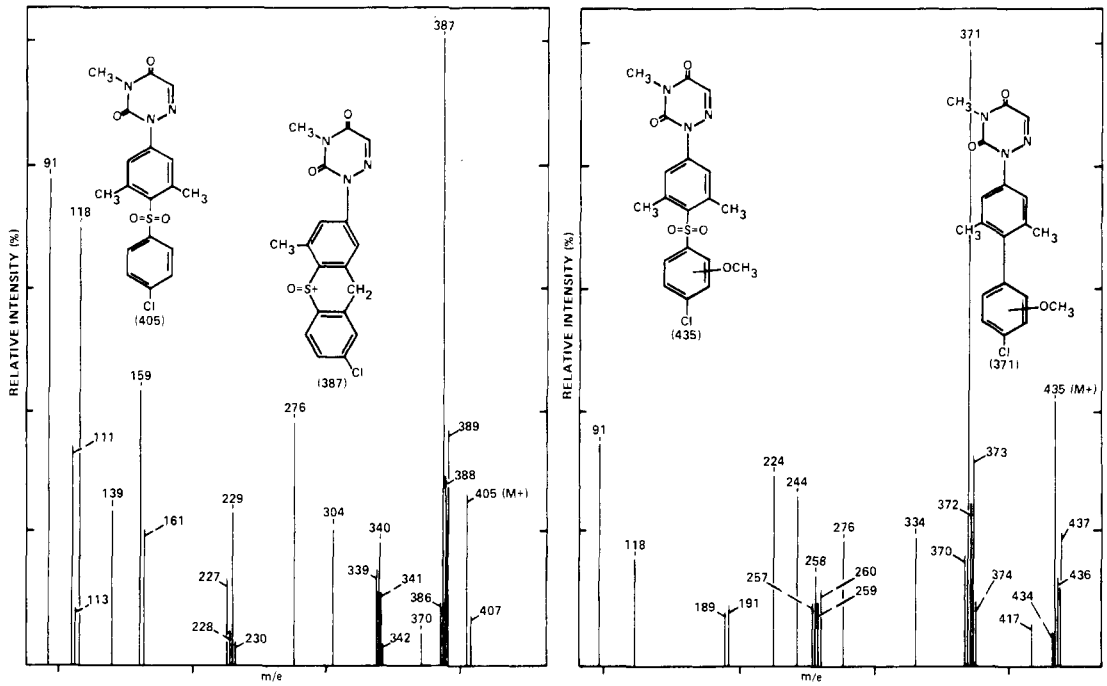

Figure 6. Mass spectra of N-methylated T-SO_2 and N- and O-methylated HO-T-SO_2.

Table III. Excretion of ^3H in the Chicken, Rat, and Dog after Oral Administration of ^3H-T

Animal tested	^3H-T dose, mg/kg	Hours postdose	^3H recovered (% of dose) Urine	Feces	Bile	Total of excreta
Chicken	1.07	0-24				36.2
		24-48				23.0
		48-72				14.9
		72-96				3.6
		96-120				1.3
						79.0
Chicken	1.0	0-24				50.5
		24-48				44.1
		48-72				6.0
						100.6
Rat	10.0	0-24	10.4	42.8		53.2
		24-48	3.1	20.7		23.8
		48-72	0.9	2.2		3.1
			14.4	65.7		80.1
Dog (bile cannulated)	5.0	0-24	0.4	0.1	4.7	5.2
		24-48	0.9	8.3	4.3	13.5
		48-72	0.5	3.4	3.0	6.9
		72-96	0.5	1.4[a]	2.3	
		96-120	1.4		2.7[b]	9.3[c]
		120-144	1.0			
		144-168	0.6		2.1	2.7
			5.3	13.2	19.1	37.6

[a] 72-168 h collection. [b] 96-144 h collection. [c] 96-144 h collection.

metabolite in acid hydrolyzed 0–24 h excreta was identified as HO-T-SO or 2-[3,5-dimethyl-4-(2- or 3-hydroxy-4-chlorophenylsulfinyl)phenyl]-*as*-triazine-3,5(2*H*,4*H*)-dione. As seen in Table IV, approximately 33% of the radioactivity recovered by TLC was not identified, but may represent more polar metabolites or incomplete alkylation of the hydroxylated compounds by diazomethane to O-methylated ethers (Kosak et al., 1954).

Radioactivity amounting to 70% of the total was isolated from a composite of plasma extracts of 1–24-h samples. It was separated and identified by programmed GLC–MS

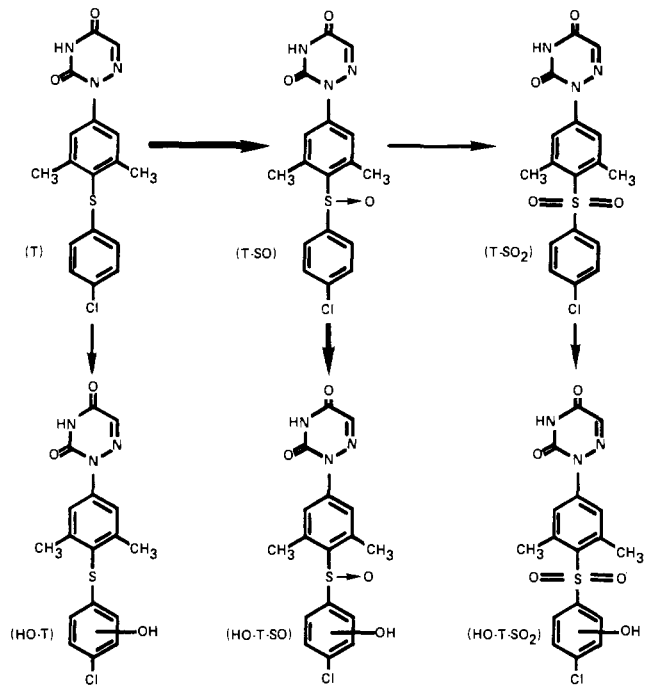

Figure 7. Proposed biotransformation pathway for T in the chicken.

as containing T, T-SO, and T-SO_2. The major component of this mixture was T-SO_2. The distribution of T and T-SO_2 was determined by GLC–ECD assay and corresponds to total radioactivity by the third and fifth days of drug withdrawal (Table V). Apparently earlier withdrawal samples contain nonlabeled residues from the stressing period as well as labeled compounds.

Residues in tissues were too low to be identified by their mass spectra. However, through a combination of GLC-ECD (Table V) and TLC reverse isotope dilution techniques, the magnitude and distribution of radioactivity in liver, a "target tissue", was assigned among T, T-SO, T-SO_2 and unidentified polar metabolites (Table VI). T-SO_2 was detected as the major metabolite in 1- and 3-day

Table IV. Thin-Layer Distribution of Radioactivity and Gas-Liquid Chromatographic-Mass Spectrometric Identification of Methylated Tiazuril Metabolites in Chicken Excreta 0–24 h after Oral Administration of Tritium-Labeled Drug[a]

TLC distribution			GC-MS		
Zones	Percent of TLC radio-activity	R_S	t_R	M^+ (m/e)	Metabolite identity in TLC zones N- and O-methylated
I	5	1.0	1.6	403	HO-T
II	12	0.8	2.1, 3.2	405, 435	T-SO_2 (minor), HO-T-SO_2 (major)
III	51	0.6	2.1, 3.2	389, 419	T-SO (minor), HO-T-SO (major)
IV	10	0.3	nd		Unidentified
V	23	0.2	nd		Unidentified
N-Methylated Stds					
T		1.0	1.2	373	
T-SO_2		0.8	2.1	405	
T-SO		0.6	2.1	389	

[a] R_S = thin-layer chromatographic mobility relative to tiazuril (R_S = 1.0) in the system: benzene–acetic acid (95:5)/silica gel GF; t_R = gas-liquid chromatographic retention in min on a 4 mm × 1 m U-shaped glass column packed with Gas-Chrom Q, 60–80 mesh, coated with OV-17 (3%) at a temperature of 290 °C and argon–methane (9:1) flow of 100 mL/min; nd = not detected; HO-T = hydroxylated tiazuril; T-SO_2 = tiazuril sulfone; T-SO = tiazuril sulfoxide; HO-T-SO_2 = hydroxylated tiazuril sulfone; HO-T-SO = hydroxylated tiazuril sulfoxide.

Table V. GLC-ECD Assays in ppm for T and T-SO_2 in Plasma and Liver of Chickens Orally Dosed with Labeled Drug[a]

Time, h	T	T-SO_2	Total	Radio-activity (^3H)[b]
		Plasma		
7	1.91	1.60	3.51	2.28
24	0.45	0.86	1.31	1.17
48	0.28	0.64	0.92	0.56
72	0.01	0.12	0.13	0.13
96	nd	0.08	0.08	0.05
120	nd	0.02	0.02	0.02
		Liver		
24	0.37	1.10	1.47	1.16
72	nd	0.23	0.23	0.22
120	nd	0.03	0.03	0.03

[a] Six birds were maintained on nonlabeled T in feed (0.001%) for 39 days prior to oral administration of tritium-labeled drug at a dose level equivalent to 1 mg/kg; nd = none detected. [b] Expressed in μg equivalents of ^3H-T/mL of plasma or per gram of tissue (ppm). Radioactivity in plasma was corrected for tritium water content ranging from <0.01 ppm at 7 h to 0.02 ppm at 120 h. The day-5 liver sample was corrected for tritium water content of 0.03 ppm.

liver withdrawal samples; it accounted for 63% of total radioactivity by the third day of drug withdrawal (Table VI). No hydroxylated metabolites were detected by GLC–ECD in plasma, liver, or other tissues.

Tiazuril Depletion Studies in Chickens Fed in a Floor Pen Trial. Tissue residues were determined by GLC–ECD analysis for unchanged drug and metabolites in broilers fed with feed containing T at 0.0015% for 56 days. T depleted to less than 0.01 ppm in plasma and tissues by the second day of drug withdrawal and to 0.03 ppm or less of T-SO_2 by the fourth or fifth day of withdrawal (Table VII).

Comparative Metabolism Studies in the Chicken, Rat, Monkey, and Dog. Table VIII shows the plasma levels of T and T-SO_2, as determined by GLC–ECD assay, following single and multiple oral administration of nonlabeled T to the chicken, rat, monkey, and dog. Essentially no conversion of T to T-SO or T-SO_2 is detected in plasma of the dog unless the drug is administered daily at high levels. The half-life for T in the dog (about 80 h) is much greater than seen in the chicken, rat, or monkey (6–13 h).

Table VI. Reverse Isotope Dilution Analysis and Thin-Layer Chromatographic Distribution of Tritium Radioactivity in Liver of Chickens 1 and 3 Days after Oral Administration of ^3H-T to Prestressed Birds[a]

	Day 1		Day 3	
	ppm	%	ppm	%
T	0.36	23	0.01	5
T-SO_2	0.60	38	0.12	63
T-SO	0.26	16	0.01	5
Unidentified polar metabolite(s)	0.21	13	0.05	26
Total	1.43	90	0.19	99
Radiochemical assay of sample	1.59		0.19	

[a] Chickens were maintained on nonlabeled T in feed (0.002%) for 25 days prior to oral administration of tritium-labeled drug at a dose level equivalent to 1.35 mg/kg; ppm = μg equivalents of ^3H-T/g of tissue.

The persistence of T in the dog is also seen in radiotracer comparative metabolism studies (Tables I and III). Radioactivity is slowly excreted by a bile cannulated dog with concomitant persistence of the drug in plasma. The rat is more nearly like the chicken in the depletion of radioactivity in plasma and the recovery of the dose in excreta (Tables I and III).

No metabolites were identified in urine of either the rat or the dog (Table IX). Major metabolites found in 0–24 h rat feces consist primarily of HO-T-SO and HO-T-SO_2 (Table IX). Dog feces only contained unchanged T and some unidentified polar radioactivity; bile, however, showed equal amounts of T and T-SO plus T-SO_2 with possible traces of hydroxylated metabolites (Table IX).

DISCUSSION

Under commercial conditions, or chronic administration of the coccidiostat at low levels (0.0015%) in feed, the concentrations of T and T-SO_2 in edible tissues are 0.1–0.6 and 0.2–1.4 ppm, respectively, on the day of withdrawal. These levels decline to less than 0.01 ppm for T and 0.03 ppm for T-SO_2 by the second and fourth days, respectively. Therefore, in observing a 5-day withdrawal period, the meat consumer would not be exposed to residues of T above 0.1 ppm. Since kidney and liver contain the highest residues, GLC–ECD assays for T-SO_2 would detect its presence above 0.1 ppm and therefore, provide assurance that the coccidiostat was withdrawn for 3 or more days.

T and its metabolites need to be considered in a safety evaluation of the drug since they constitute more than 70% of the tissue residues in the chicken throughout the first

Table VII. GLC-ECD Assays in ppm for T and T-SO_2 in Plasma and Edible Tissues of Chickens Fed (ad libitum) T in Medicated Feed (0.0015%) for 56 Days

Withdrawal time, h	Plasma	Liver	Skin	Muscle	Fat	Kidney
	ppm T[a]					
0	0.58 ± 0.19	0.34 ± 0.15	0.19 ± 0.12	0.09 ± 0.05	0.18 ± 0.14	0.27 ± 0.11
24	0.07 ± 0.08	0.02 ± 0.03	<0.01	<0.01	<0.01	<0.01
48	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	T-SO_2[a]					
0	1.36 ± 0.13	1.15 ± 0.28	0.23 ± 0.05	0.18 ± 0.04	0.20 ± 0.08	0.83 ± 0.19
24	0.51 ± 0.40	0.32 ± 0.23	0.06 ± 0.05	0.04 ± 0.05	0.07 ± 0.04	0.22 ± 0.13
48	0.12 ± 0.13	0.08 ± 0.09	0.02 ± 0.03	<0.01	0.02 ± 0.03	0.09 ± 0.09
72	0.06 ± 0.04	0.03 ± 0.02	na	na	na	0.04 ± 0.03
96	0.01 ± 0.02	<0.01	na	na	na	<0.01
120	0.03 ± 0.04	<0.01	na	na	na	<0.01

[a] Average result of single assays on plasma and tissues taken from six chickens at each withdrawal period with standard deviation of results; na = not assayed.

Table VIII. Plasma Concentrations (μg/mL) of T and T-SO_2[a] in the Chicken, Rat, Monkey, and Dog after Oral Administration of Nonlabeled Drug

	Animal						
	Chicken		Rat		Monkey	Dog	
	Dose, mg/kg						
	4	1	10	20	10	4	100
	Formulation						
	Suspension	Feed	Suspension	Feed	Suspension	Capsule	Capsule
	Days dosed						
	3	56	1	4	1	3	5
Hours, postdose	T/T-SO_2 concentrations, μg/mL						
0-8	4.8/3.4	0.58/1.36	19.1/7.4	50/114	18.4/1.8	25.2/Tr	
24	1.5/2.6	0.07/0.56	2.4/6.9		6.5/6.8	21.3/Tr	229/42
48	0.19/1.0	<0.01/0.12			1.7/4.6	14.4/Tr	198/64
72	0.03/0.4	nd/0.06				12.4/Tr	217/64
96	nd/0.1	nd/0.01				12.9/Tr	201/74
120	nd/0.05					9.5/Tr	173/71
133					nd/0.7		
Tiazuril plasma half-life, h	9		~6		13	80	

[a] Plasma samples contain T-SO_2 unresolved from T-SO; nd = none detected; Tr = trace; suspension = tiazuril suspended in 1% carboxycellulose, 0.1% methocel, and 0.1% Tween-80.

Table IX. Approximate Chromatographic Distribution of Radioactivity in Chicken, Rat, and Dog Excreta after Oral Administration of ^{3}H-T

Animal tested	Hours, postdose	Specimen	Methods of analysis	Percent distribution					
				T	T-SO plus T-SO_2	HO-T	HO-T-SO	HO-T-SO_2	Unidentified polar compd
Chicken	0-24	Excreta	*a, b, c*	<1	~2	~4	~50	~10	~33
Rat	0-24	Urine	*a, b*	nd[e]	nd	nd	nd	nd	*d*
	0-24	Feces	*a, b, c*	~3	~6	~1	~35	~41	~14
Dog (bile cannulated)	24-48	Urine	*a, b*	nd	nd	nd	nd	nd	*d*
	24-48	Feces	*b*	~62	~1	nd	nd	nd	~37
	96-144	Bile	*b*	~44	~45	(possible traces)			~11

[a] Separation of radioactive components after methylation by TLC using a benzene-acetic acid (95:5)/silica gel GF system and estimation by scintillation counting. [b] Examination by electron capture and flame photometric GLC to estimate T, T-SO plus T-SO_2 and to identify hydroxylated metabolites. [c] Identification of components by mass spectrometry. [d] Radioactivity remained at the "origin" of the plate. [e] nd = none detected.

3 days of drug withdrawal. Consequently, toxicology studies should be performed in species in which the drug is readily metabolized to metabolites of T. This will ensure self-exposure of laboratory animals to metabolic residues man might ingest when consuming meat taken from chickens maintained but not properly withdrawn from T.

Among the three species of laboratory animals proposed for use in subacute or chronic toxicology studies, the rat and possibly the monkey, metabolize T like the chicken. The dog is distinguished from the chicken and the rat by maintaining persistent high unchanged drug concentrations in plasma, a significantly slower rate of excretion of radioactivity, and no more than possible traces of hydroxylated metabolites in excreta. The presence of T-SO and T-SO_2 in bile of the dog after a single oral dose and concomitant appearance of these metabolites in dog plasma following daily administration of nonlabeled T suggests enterohepatic circulation of these compounds.

However, it would also appear that hydroxylation of the chlorophenyl ring as well as oxidation of the sulfur atom of T, as seen in the chicken and the rat, is required for rapid elimination of the drug from animals.

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Comparison of Cholinesterase Activity, Residue Levels, and Urinary Metabolite Excretion of Rats Exposed to Organophosphorus Pesticides

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Blood cholinesterase activity, urinary levels of phenolic and organophosphorus metabolites, and residues of intact compounds in blood and fat were determined following exposure of rats to organophosphorus pesticides. The eight pesticides studied included representative halogenated compounds (carbophenothion, dichlofenthion, ronnel, and leptophos) and nonhalogenated compounds (parathion, EPN, dimethoate, and dichlorvos). Cholinesterase activity was determined by gas chromatography. Metabolites were extracted, derivatized, subjected to silica gel chromatography, and quantitated by FP–GC and EC–GC. Residues were extracted, cleaned up with silica gel chromatography, and quantitated with EC–GC. Data comparing these various exposure indicators are presented.

A number of methods are available for monitoring exposure to degradable pesticides. These include methodology for determining blood cholinesterase levels, urinary alkyl phosphate metabolites, urinary phenol metabolites, and residues of the intact compounds in blood and in adipose and other tissues. These methods have been used singly to monitor exposure to organophosphorus pesticides. However, it would be valuable to have a comparison of the effectiveness of the four indices of exposure for application to future studies. The work reported in this paper gives a comparison of the four methods, using rats as models, in a feeding study involving eight organophosphorus pesticides.

The pesticides selected for the study are shown in Table I. They include aromatic and aliphatic organophosphate compounds as well as two organophosphonate pesticides. The eight compounds may also be classified by the presence or absence of halogenated moieties. The compounds were selected to provide an opportunity to compare cholinesterase inhibition, tissue residue levels, and urinary excretion of metabolites in rats exposed to different classes of organophosphorus pesticides. Hopefully, it would also give an insight into possible delayed excretion of halogenated organophosphorus pesticides.

Table I. Pesticides Selected

Compound		Halogenated	
Class	Name	Yes	No
Aliphatic organophosphate	Dichlorvos	X	
	Dimethoate		X
Aromatic organophosphate	Carbophenothion	X	
	Dichlofenthion	X	
	Ronnel	X	
	Parathion		X
Organophosphonate	Leptophos	X	
	EPN		X

EXPERIMENTAL SECTION

Male Sprague-Dawley rats, weighing 160–185 g each, were used in the study. So that the rats could be treated as individuals for cholinesterase determinations, they were distinguished by ear notching. They were housed in pairs in stainless steel metabolism cages which were cleaned daily to minimize fecal contamination. Standard laboratory chow and water were furnished ad libitum.

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