sulfamethazine can be seen. Lanes c and d are respectively the partitioned $CHCl_3$ extracts of [^{14}C]sulfamethazine and of [^{14}C]sulfamethazine with glucose spiked into 80% methanol control tissue homogenates. Several new radioactive compounds can be seen, among them the N^4-glucopyranosylsulfamethazine. Similar results were obtained from the MEK fractions. The glucose adduct was also formed when the $CHCl_3$ fraction from control liver was spiked and evaporated to dryness and the residue chromatographed on a TLC plate.

These studies do not preclude the possibility that the sugar adduct and other observed compounds may actually exist in vivo at lower concentrations and were artificially enhanced by the fractionation procedure. In any case, investigators should be cognizant of these phenomena as other tissue residue studies are undertaken with this class of compounds. Sulfonamides are freely reactive with endogenous tissue extract components, complicating what might be a relatively simple residue profile with an array of artifacts.

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Metabolism of [^{14}C]Sulfamethazine in Swine

D. D. Giera,* R. F. Sieck, L. K. Graper, J. L. Mertz, and R. J. Herberg

Following oral administration of [^{14}C]sulfamethazine to a barrow for 2 days, the animal was slaughtered 1 day after treatment. Sulfamethazine and N^4-acetylsulfamethazine accounted for 58, 58, 65, and 40% of the total ^{14}C-labeled tissue residues in liver, kidney, muscle, and fat, respectively. Approximately 14% of the total ^{14}C-labeled residue in liver and muscle was found to be N^4-glucopyranosylsulfamethazine, the genesis of which was not definitively determined. A second animal was dosed for 4 days with essentially all of the dose being excreted in 15 days. Approximately 84% of the dose was eliminated in urine and 16% in feces. Sulfamethazine, N^4-acetylsulfamethazine, and a sulfamethazine sulfate conjugate accounted for 35, 45, and 5% of the total urinary ^{14}C-labeled residue. Sulfamethazine, N^4-acetylsulfamethazine, polar metabolities, and nonextractable residue accounted for 7, 10, 34, and 37%, respectively, of the total fecal ^{14}C-labeled residue. Fifteen days posttreatment, muscle and fat tissues had no detectable ^{14}C-labeled residue, and liver and kidney tissue levels were well below the 100-ppb established tolerance for sulfamethazine. Approximately 29% of the total hepatic ^{14}C-labeled residue was sulfamethazine.

Between 1974 and 1977, 12.4% of the swine tissue samples examined by the U.S. Department of Agriculture were found to contain sulfonamide residues above the established tolerance of 100 ppb (Trabosh, 1978). Improper drug withdrawal procedures, inadvertent contamination of unmedicated feed in feed mills, and contamination of feeding pens have been cited as factors that may contribute to the violation rate. Many of the violative residues were in the 100-ppb range, a level of sensitivity at which the reliability of the official diazo colorimetric method has been questioned.

In view of this issue, a controlled [^{14}C]sulfamethazine balance study was undertaken to provide information concerning the excretion and tissue depletion of the drug and to characterize and identify major components of the residue in tissue and excreta.

MATERIALS AND METHODS

Dosing of Animals and Sample Collection. [^{14}C]-Sulfamethazine, uniformly labeled in the benzene ring, was synthesized in the Lilly Research Laboratories. Evaluation of purity by TLC showed that 97% of the radioactivity was associated with sulfamethazine. Gelatin capsules each containing 220 mg of [^{14}C]sulfamethazine (specific activity 2.06 μCi/mg) were prepared.

Department of Agricultural Biochemistry, Lilly Resarch Laboratories, Greenfield, Indiana 46140.

Three crossbred barrows (Yorkshire × Yorkshire × Hampshire) weighing approximately 57 kg each were acclimated to stainless steel metabolism crates and established on a feeding regimen of 1000 g of ration containing approximately 110 ppm of tylosin fed twice a day. After a 7-day acclimation period, capsules containing [^{14}C]-sulfamethazine were administered orally with 100 mL of water with a dosing syringe. Animal A was dosed with a capsule containing 220 mg of [^{14}C]sulfamethazine (equivalent to 110 ppm in the diet) on 2 consecutive days and slaughtered 24 h following the last dose. Animal B was dosed with a capsule containing 220 mg of [^{14}C]sulfamethazine on 4 consecutive days and slaughtered 15 days after the last dose. Animal C, the experimental control, was also slaughtered. Tissue samples from the three animals were collected. Daily urine and feces samples were collected from 2 days before dosing until the slaughter.

Preparation of Samples for ^{14}C Assay. Urine samples were mixed with Aquasol scintillator solution (New England Nuclear Corp.) and the ^{14}C-labeled residue determined by liquid scintillation counting. Feces from the daily collections were blended with an equal weight of water in a Waring blender to form a smooth homogeneous paste, and portions of approximately 1 g were combusted for determination of ^{14}C-labeled residue. Muscle and fat tissue were ground several times in a Hamilton Beach grinder. Liver and kidney tissues were blended in a blender. Tissues were refrigerated before grinding, and

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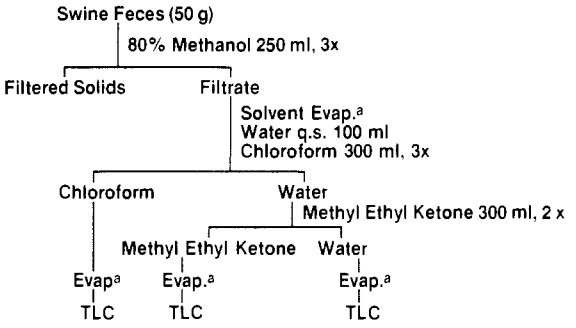

Figure 1. Procedure A for the fractionation of the [^{14}C]sulfamethazine residue in swine feces. The superscript a denotes vacuum evaporation at 40–50 °C.

the processed tissues were frozen (−20 °C) within 4 h of the time of slaughter. Small portions (0.3–0.5 g) of the processed tissues were combusted for determination of total ^{14}C-labeled residue.

^{14}C-Labeled Residue in Urine. Urine was evaporated in a heating block under an air stream (30–40 °C), and the residue was redissolved in methanol. Urine residues insoluble in methanol contained no significant amount of ^{14}C-labeled residue (<1%). The distribution of urinary ^{14}C-labeled residue was characterized by thin-layer chromatography (TLC) on silica gel 60 F-254 plates (E. Merck) developed with benzene–methyl ethyl ketone–ethanol–water, 30:30:30:10. The ^{14}C-labeled residues separated by TLC were visualized by radioautography, scraped from the TLC plates, and eluted from the silica gel with liquid scintillator solution. The ^{14}C-labeled residue was quantitated by liquid scintillation counting.

The urinary metabolites with R_f values ranging from 0.4 to 0.8 were localized by TLC radioautography and isolated by scraping the silica gel from the TLC plate in the area in which they had migrated. The silica gel was suspended and sonicated in methanol to elute the ^{14}C-labeled residue. Following filtration of the silica gel and evaporation of the methanol filtrate, the radioactive residue was subjected to enzymatic hydrolysis with sulfatase (Type V, Sigma Chemical Co.) in 0.01 M acetate buffer, pH 5.0, and glucuronidase (Type VIII, Sigma Chemical Co.) in 0.5 M phosphate buffer, pH 6.8. After incubation at 37 °C overnight, the hydrolytic products were extracted from the reaction mixtures with methyl ethyl ketone and chromatographed on silica gel TLC plates as described above.

^{14}C-Labeled Residue in Feces. The ^{14}C-labeled residue in feces was fractionated according to the procedure A illustrated in Figure 1. The distribution of the ^{14}C-labeled residue in the chloroform, methyl ethyl ketone, and spent aqueous feces extracts was characterized by TLC on silica gel 60 F-254 plates developed with chloroform–acetone, 50:50, and quantitated as described above for urine.

^{14}C-Labeled Residue in Tissue. The ^{14}C-labeled residue in swine tissue (liver, kidney, muscle) was fractionated according to the procedure B illustrated in Figure 2. The methyl ethyl ketone and spent aqueous extracts were chromatographed with a 30–100% methanol gradient on 30% methanol equilibrated Porapak Q (Waters Associates, Inc.) columns prior to TLC. The ^{14}C-labeled residue in fat tissue was fractionated according to the procedure C illustrated in Figure 3. The spent aqueous extract from fat tissue was passed through a C_{18} SepPak Cartridge (Waters Associates, Inc.) and eluted with 0–100% methanol gradient prior to TLC. The distribution of the ^{14}C-labeled residue in the tissue extracts thus fractionated was characterized and quantitated by TLC as described above for the ^{14}C-labeled residue in urine.

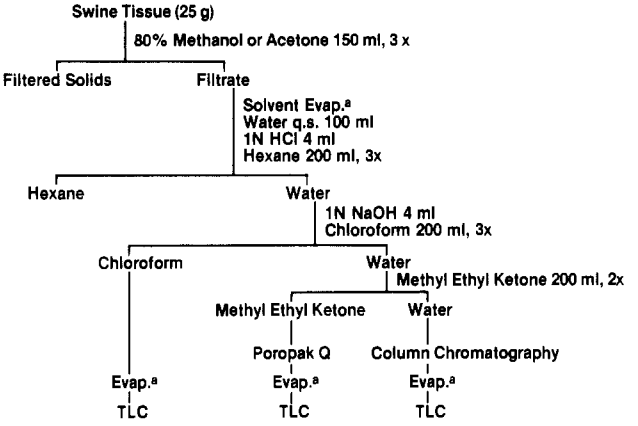

Figure 2. Procedure B for the fractionation of the [^{14}C]sulfamethazine residue in swine liver, kidney, and muscle tissue. The superscript a denotes vacuum evaporation at 40–50 °C.

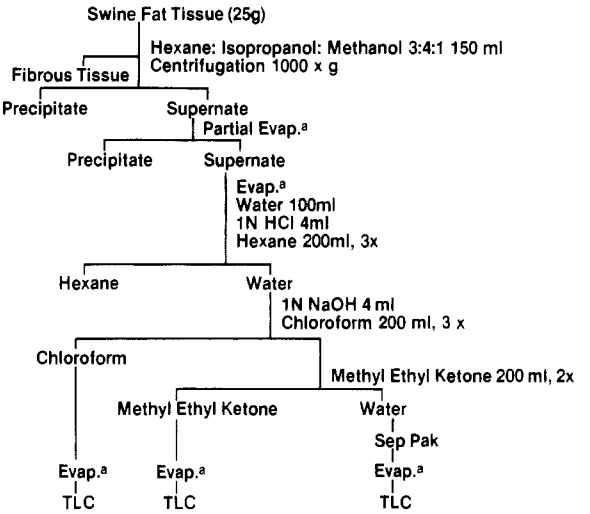

Figure 3. Procedure C for the fractionation of the [^{14}C]sulfamethazine residue in swine fat tissue. The superscript a denotes vacuum evaporation at 40–50 °C.

The hepatic polar metabolite found in the methyl ethyl ketone and spent aqueous liver extracts was isolated after elution from TLC plates by high-performance liquid chromatography on a C_{18} μBondapak prep column (Waters Associates, Inc.). The structure of the isolated compound was determined by field desorption ionization mass spectrometry with a Varian Mat, Model 731, mass spectrometer (Varian Associates, Inc.), and nuclear magnetic resonance spectrometry was assessed with a Bruker, Model WH-360, nuclear magnetic resonance spectrometer (USA Bruker Instruments, Inc.).

^{14}C Assays. ^{14}C radioactivity was measured by liquid scintillation counting. Two liquid scintillation counters, Tri-Carb, Models 3380 and 3385 (Packard Instrument Co.), were used. Counting efficiencies were determined by internal standardizations. Scintillator solution containing 200 g of naphthalene, 400 mL of water, 2 L of dioxane, 14 g of PPO (Packard Instrument Co.), and 0.6 g of Me_2-POPOP (Packard Instrument Co.) was used for elution and counting of the ^{14}C-labeled residue in TLC silica gel scrapings. Scintillator solution containing 500 mL of toluene, 1.5 L of dioxane, 1.5 L of 2-methoxyethanol, 280 g of naphthalene, 35 g of PPO, and 1.75 g of Me_2-POPOP was used for all other samples except for raw urine samples, for which Aquasol was used.

Samples containing radioactive residues were dissolved directly in scintillator solution for counting whenever possible. Radioactive residues in tissue and feces homo-

Table I. Net [^{14}C]Sulfamethazine Residue, as Daily and Cumulative Percentage of Total Dose,[a] in Urine and Feces of an Orally Dosed Pig

	urine		feces		urine and feces	
	daily %	cum. %	daily %	cum. %	daily %	cum. %
dose 1						
2	7.9	7.9	0.1	0.1	8.0	8.0
3	13.4	21.3	0.3	0.4	13.7	21.7
4	17.1	38.4	1.7	2.1	18.8	40.5
day 1	18.2	56.6	3.6	5.7	21.8	62.3
2	10.7	67.3	1.7	7.4	12.4	74.7
3	6.2	73.5	1.8	9.2	8.0	82.7
4	2.8	76.3	1.5	10.7	4.3	87.0
5	3.3	79.6	1.8	12.5	5.1	92.1
6	1.7	81.3	1.1	13.6	2.8	94.9
7	1.3	82.6	0.6	14.2	1.9	96.8
8	0.7	83.3	0.6	14.8	1.3	98.1
9	0.3	83.6	0.4	15.2	0.7	98.8
10	0.2	83.8	0.1	15.3	0.3	99.1
11	0.2	84.0	0.2	15.5	0.4	99.5
12	0.1	84.1	0.1	15.6	0.2	99.7
13	0.1	84.2	0.1	15.7	0.2	99.9
14	0.1	84.3	0	15.7	0.1	100.0
15	0	84.3	0	15.7	0	100.0

[a] Total dose was 1839.7 μCi given in four equal portions.

Table II. Mean ^{14}C-Labeled Tissue Residue from Swine Dosed Orally with [^{14}C]Sulfamethazine

	residue, net ppm[a]	
tissues	animal A[b]	animal B[c]
muscle	2.22	NDR[d]
liver	4.29	0.033
fat	1.29	NDR[d]
kidney	4.56	0.013

[a] Values measured as sulfamethazine are means for four tissue samples and are corrected for mean control values. [b] One-day posttreatment withdrawal period. Total dose was 437.9 mg (2.0 μCi/mg). [c] Fifteen-day posttreatment withdrawal period. Total dose was 884.5 mg (2.08 μCi/mg). [d] No detectable residue. Values did not differ ($P \leq 0.05$) from control means.

genates and filtered solids from the fractionation procedures were released in the form of $^{14}CO_2$ by combustion in a three-section electrically heated furnace (Sola Basic Industries, Watertown, WI). Combustion products were absored in 10 mL of 30% 2-aminoethanol in 2-methoxyethanol, which was then combined with scintillator solution for counting. Combustion recovery values for tissue and feces samples were adjusted for combustion recovery values by combustion of a standard, [^{14}C]methyl methacrylate (New England Nuclear Corp.), with a portion of the respective control sample.

Sulfamethazine Standards. Sulfamethazine, U.S.P. grade, was obtained from Syntetic (Brabrand, Denmark). N^4-Acetylsulfamethazine and N^4-glucopyranosylsulfamethazine were synthesized from the above sulfamethazine in the Lilly Research Laboratories (Giera et al., 1982).

RESULTS AND DISCUSSION

[^{14}C]Sulfamethazine Depletion. ^{14}C excretion data on animal B are summarized in Table I. Essentially all of the ^{14}C dose was excreted during the treatment and 15-day drug withdrawal period. [^{14}C]Sulfamethazine tissue residue data for animals A and B are summarized in Table II. After a 15-day drug withdrawal period, tissue levels were depleted to levels well below the 100-ppb established tolerance for sulfamethazine.

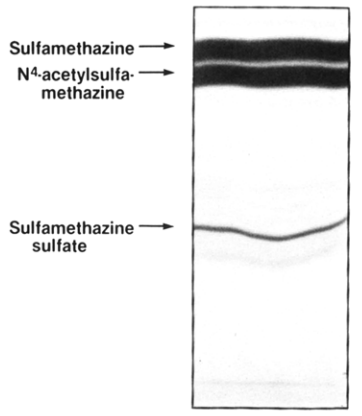

Figure 4. [^{14}C]Sulfamethazine residue in swine urine.

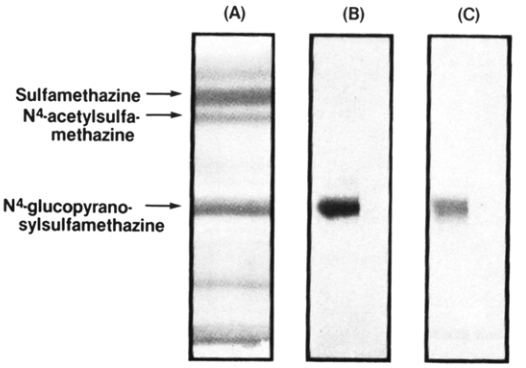

Figure 5. [^{14}C]Sulfamethazine residue in swine liver tissue: (A) chloroform extract; (B) methyl ethyl ketone Porapak Q eluate; (C) spent aqueous Porapak Q eluate.

Urine and Feces. The distribution of urinary ^{14}C-labeled residue at the peak of its excretion (24 h following last of four doses) is shown in the TLC radioautogram in Figure 4. The two main metabolities which comprised 39 and 45% of the total urine residue cochromatographed with sulfamethazine and N^4-acetylsulfamethazine. Subsequent isolation and electron impact mass spectrometry verified their identity. The third most abundant urinary component was identified as a sulfamethazine sulfate conjugate from enzymatic hydrolysis experiments. It accounted for 5% of the total urinary sulfamethazine residue. The distribution of ^{14}C-labeled material in this peak sample was typical of the profiles found in other daily urine collection samples from animal A and animal B. Thus, the metabolism of sulfamethazine in swine with the formation of the N^4-acetylsulfamethazine derivative and the sulfate conjugate were similar to sulfonamide metabolism in other species (Williams and Park, 1964).

Most of the radioactive residue in feces consisted of unidentified polar metabolities or residue that was not extractable into 80% methanol. Sulfamethazine and N^4-acetylsulfamethazine accounted for 7 and 10% of the total ^{14}C-labeled feces residue, respectively. The distribution of ^{14}C-labeled residue in feces from animal B was similar.

Liver. A TLC radioautograph which represents 96% of the total ^{14}C-labeled residue in liver from animal A is shown in Figure 5. After a 1-day drug withdrawal period, the predominant radioactive components present in the hepatic extracts cochromatographed with sulfamethazine and N^4-acetylsulfamethazine. These accounted for 55 and 3%, respectively, of the total hepatic residue from animal A. The predominant species present in the methyl ethyl ketone and spent aqueous fractions from animal A, "the hepatic polar metabolite", represented 14% of the total

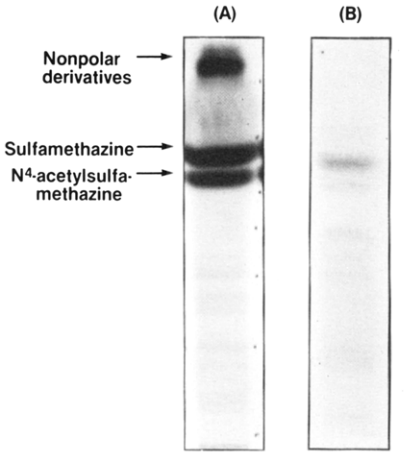

Figure 6. [^{14}C]Sulfamethazine residue in swine kidney tissue: (A) chloroform extract; (B) methyl ethyl ketone extract.

hepatic residue. The structure of the metabolite was determined to be N^4-glucopyranosylsulfamethazine from mass spectra and nuclear magnetic resonance spectra. The structural assignment was corroborated by spectra obtained from chemically synthesized N^4-glucopyranosylsulfamethazine (Giera et al., 1982).

After a 15-day drug withdrawal period, approximately half of the hepatic ^{14}C-labeled residue from animal B was unextractable into 80% acetone. The predominant radioactive species present in the extractable residue in both the chloroform and methyl ethyl ketone was the parent compound, sulfamethazine, which accounted for 29% of the total hepatic residue from animal B.

Kidney. A TLC radioautograph which represents 91% of the total ^{14}C-labeled residue in kidney from animal A is shown in Figure 6. The predominant radioactive species present in the renal chloroform and methyl ethyl ketone extracts after 1-day withdrawal were sulfamethazine and N^4-acetylsulfamethazine. These accounted for 33 and 25%, respectively, of the total kidney ^{14}C-labeled residue. The third most abundant radioactive components present in the renal residue from animal A were unidentified nonpolar metabolities, which accounted for 10% of the total radioactive residue. Similar nonpolar metabolites were reported by Paulson and Struble (1980), who identified one of them as deaminated sulfamethazine.

After a 15-day drug withdrawal period, 75% of the ^{14}C-labeled renal residue from animal B was not extractable into 80% methanol. Since the extractable residue represented only 2 ppb, the fractionation procedure was not attempted.

Muscle and Fat. The predominant radioactive species present in the extracts of muscle tissue from animal A cochromatographed with sulfamethazine and N^4-acetylsulfamethazine. These accounted for 59 and 6% of the total ^{14}C-labeled residue in muscle from animal A. The third most abundant radioactive component present in the muscle extracts, which accounted for 13% of the total ^{14}C-labeled residue present after 1-day withdrawal, cochromatographed with the glucose conjugate identified in liver. Nonpolar radioactive components resembling those found in kidney tissue extracts also composed 8% of the total ^{14}C-labeled residue in muscle from animal A. There was no detectable ^{14}C-labeled residue in muscle tissue from animal B.

The predominant radioactive species present in the fat tissue from animal A were sulfamethazine and N^4-acetylsulfamethazine, which accounted for 26 and 13%, respectively, of the total ^{14}C-labeled residue in fat tissue. There was no detectable ^{14}C-labeled residue in fat tissue from animal B.

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