1705, (C=C) 1640 cm^{-1}; ^1H NMR (in $CDCl_3$) one vinylic proton at 5.55, one methyl group at 1.98 for the aceto group, two methyl groups at 1.35, and other protons signals that were difficult to be assigned due to the overlapping and complex coupling pattern at the used field strength]. The absolute configuration of the suggested structure could not be determined with the available data.

Several compounds containing the azulene nuleus and possessing biological activities, i.e., antifungal, antibiotic, antineoplastic, and ichthyotoxic, have been isolated from algae (Howard and Fenical, 1981; Sun et al., 1981; Moore, 1979), from soft corals (Izac et al., 1981; Fusetani et al., 1981; Kashman et al., 1982) and from desert plants (Shafizadeh and Bhadane, 1972; Burnett and Jones, 1978; Tressl et al. 1983).

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Registry No. 1-(1,3,4,5,6,7-Hexahydro-4-hydroxy-3,8-dimethyl-5-azulenyl)ethanone, 55683-15-3.

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A Study of the Absorption, Excretion, Metabolism, and Residues in Tissues in Rats Fed Carbon-14-Labeled Sulfamethazine

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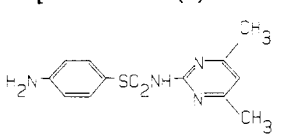
Sprague-Dawley male and female rats received medicated feeds containing 10, 40, 160, 640, 1280, and 2560 ppm of [*phenyl*-U-^{14}C]sulfamethazine for 7 days. Urinary radioactivity accounted for 51–68% of the dose in females and 31–45% in males. Sulfamethazine and N^4-acetylsulfamethazine accounted for 76–84% of the radioactivity in females and 22–63% in males. The N^4-glucose conjugate of sulfamethazine and two other sulfamethazine conjugates, on average, accounted for 73% of the urinary metabolites in males at drug levels below 640 ppm. At drug levels above 640 ppm, the concentration of these conjugates decreased in urine to suggest that there was a saturable process(es) in the male. Females also exhibited consistently higher ^{14}C residues in tissues and blood to further indicate there were apparent sex-related differences in the metabolism of sulfamethazine in rats. Sulfamethazine and N^4-acetylsulfamethazine were identified in the liver. No strain-related differences were noted in sulfamethazine metabolism in a study with Fischer-344 male rats.

Sulfamethazine [4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)benzenesulfonamide] is widely used in combination with antibiotics as feed supplements to promote growth and prevent disease in swine and cattle. Current regulations allow a tolerance of 100 ppb in uncooked edible tissues. Within the past few years sulfamethazine has come under close scrutiny because of the high rate of violative residues above the established tolerance (Trabosh, 1978). The major causes of the violative residue problem have been attributed to the cross contamination of nonmedicated feeds, improper drug withdrawal procedures, and the coprophagic nature of swine.



SULFAMETHAZINE

A request to increase the tolerance levels of sulfamethazine residues in feed and swine tissue so that the residue problem would be eased was reviewed by the Food and Drug Administration, and they decided that until new information became available that would allow modification of the established tolerances for tissues and feed, the current regulations would be retained. Accordingly, they initiated life-time feeding studies in two rodent species at

Metabolism Laboratories, Agricultural Research Division, American Cyanamid Company, Princeton, New Jersey 08540.

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the National Center for Toxicological Research to provide additional data to support any changes in the regulations affecting the tolerance level in swine tissues.

It is not known if the feeding of high levels of sulfamethazine for a long term will alter the metabolic pattern and tissue residue profiles in the rat. Therefore, a preliminary rat metabolism study was conducted in our laboratories to provide some basic information concerning the fate of sulfamethazine when high levels of the drug are given in the diet.

EXPERIMENTAL SECTION

Radiolabeled Sulfamethazine. The metabolism experiments were conducted using [*phenyl*-U-C^{14}]sulfamethazine. For the study with Sprague-Dawley rats (Table I), the radiochemical (supplied by Metabolism and Radiation Research Laboratories, State University, Fargo, ND) had a radiopurity of 98% as determined by two TLC systems [silica gel F_{254} plates (Brinkmann Instruments, Westbury, NY)]. System 1 was chloroform–acetone, 50:50 v/v (Bevill et al., 1977) and system 2 was diethyl ether–2-propanol, 40:10 v/v. The specific activity was 26.8 μCi/mg based on the TLC and HPLC analysis (Partisil-10 ODS-2 column, mobile phase methanol–water, 25:75 v/v, flow rate 2.0 mL/min). For the study with the Fischer-344 rats (Table II), the radiochemical (supplied by Wizard Laboratories, Davis, CA) had a radiopurity of 96.5% with no radioimpurity greater than 2.5% as determined by TLC analysis. The specific activity was 43.1 μCi/mg. Both radiotracers were used without further purification.

Animal Handling. Sprague-Dawley and Fischer-344 rats were obtained from Charles River Breeding Laboratories, Wilmington, MA. Upon delivery to the laboratory, all rats were held in quarantine for a period of 1 week after which time more than the required number of rats needed to conduct each experiment was acclimated to stainless steel metabolism cages (Acme Metal Products Inc., Chicago, IL) for several days before receiving medicated feed. Certified Rodent Laboratory Chow Animal diet no. 5002 (Ralston Purina Co., St. Louis, MO; hereafter referred to as feed) and water were provided during the pretreatment period. Prior to treatment with medicated feed, the rats were weighed and those most uniform in weight were selected and divided into groups.

For experiment 1 only Sprague-Dawley rats were used (Table I). There were seven groups (one female and two males per group). The average body weight of females (10 weeks old) was 205 g and the average body weight of the males (10 weeks old) was 272 g. For experiment 2, only Fischer-344 and Sprague-Dawley males were used (Table II). For the three groups, there was one Sprague-Dawley and two Fischer-344 rats per group. The average body weight of the Fischer-344 rats (11 weeks old) was 214 g and the average body weight of the Sprague-Dawley rats (9 weeks old) was 270 g.

To prepare the 10-ppm medicated feed, the radiolabeled drug (6.0 mg, 1.64 μCi) was used without dilution. The drug was dissolved in methanol and added to 600 g of the Certified rodent feed. The other feed samples (Table I) were prepared by mixing the radiolabeled drug (6.0 mg, 1.64 μCi) with the appropriate amount of nonlabeled drug in methanol before addition to 600 g of the rodent feed. After the feeds had air-dried, the samples were sieved and thoroughly mixed. Two 5.0-g aliquots of each feed sample were analyzed for homogeneity of mixing and verification of the drug level by solvent extraction, liquid scintillation, and HPLC analysis. Medicated feeds containing only 10, 160, and 2560 ppm [^{14}C]sulfamethazine were prepared separately for experiment 2 (Table II).

In each experiment, each rat was given only 25 g of the medicated feed daily for 7 days. A group of control rats (one female and two males) was given nonmedicated feed throughout experiment 1 only. Feed spillage was collected and weighed back to determine feed consumption and drug intake of each rat. Urine and feces were collected daily during the medication period. The urine samples were refrigerated and the feces samples were frozen. After 7 days, the rats were weighed and sacrificed for collection of blood and selected tissue samples for radioanalysis.

Analysis of Samples. Urine samples were mixed with Aquasol-2 scintillator solution (New England Nuclear Corp., Boston, MA), and the radioactivity was determined by liquid scintillation counting (Table III). The liver, kidney, and muscle samples were extracted with methanol (3×) and with 1% hydrochloric acid in methanol (1×) in a tissue homogenizer using 5 mL of solvent/g of tissue. Fat was extracted with hexane (3×) and then methanol (1×). Extracts were counted in Aquasol-2. The ^{14}C in blood and unextracted ^{14}C in tissues were determined by combustion. Feces were not analyzed.

Urine samples were stored for 1–2 weeks in the refrigerator before TLC analysis. For TLC, 25-μL aliquots of the urine collected on day 2, day 4, and day 6 (Table V) were spotted directly on silica gel plates. Extracts of liver (methanol and acid–methanol) were concentrated separately prior to TLC. The following TLC systems were used for one-dimensional solvent development: (CA) chloroform–acetone, 100:100 v/v, and (CMAH) chloroform–methanol–ammonium hydroxide, 100:100:1 v/v/v. The solvent was allowed to migrate exactly 15 cm from the spotting origin. For two-dimensional TLC analyses of urine samples, plates were developed in the first dimension with system CMAH and then in the second dimension with system CA. The distance of solvent migration was limited to 15 cm in each direction. Unlabeled standards, considered to be potential metabolites of sulfamethazine, were cochromatographed with the radioactive sample and were detected with UV light (254 nM). The radioactive compounds were detected by autoradiography on SB-5 blue-sensitive single-coated X-ray film (Eastman Kodak, Rochester, NY).

The individual radioactive silica gel regions were scraped from the plate and transferred to liquid scintillation counting vials. To each vial was added 4.0 mL of distilled water; the vials were placed in an ultrasonic clearer bath to disperse the gel before addition of the scintillator solution (Aquasol-2). The radioactive regions were quantitated by liquid scintillation counting.

Isolation of Metabolites. Urine from rats receiving 2560 ppm of sulfamethazine (Table II) was chromatographed on silica gel plates in system CA. The radioactive gel zones, detected by autoradiography, were scraped from the plate and extracted with methanol. The TLC isolates were further examined by two-dimensional TLC (CMAH vs. CA) before proceeding with further cleanup by reverse-phase HPLC on a Model 7000 HPLC (Micromeritics Instrument Co., Norcross, GA) fitted with a Partisil-10 ODS-2 column (Whatman Co., Clifton, NJ). The mobile phase for the HPLC cleanup was methanol–water (25:75 v/v) at a flow rate of 2.0 mL/min. In preliminary runs, HPLC assay of TLC isolates was performed by UV detection (254 nM) of the column eluate followed by collection of the column eluate using an LKB 2112 RediRac (LKB Instruments, Inc., Rockville, MD) and liquid scintillation counting. The HPLC fraction representing the major UV peak with associated radioactivity was examined by two-dimensional TLC in system CMAH vs. CA to es-

Table I. Weights and Feed Intake of Sprague-Dawley Rats Treated with [^{14}C]Sulfamethazine (Experiment 1)

treatment level, ppm	rat no.	sex	body weight, g			daily feed intake, g, for day							total feed	mg kg^{-1} day^{-1} [a]
			start	sacrifice	change	1	2	3	4	5	6	7		
10	4	F	201	220	+19	18.7	18.3	17.6	16.4	14.1	16.7	20.4	122.2	0.83
10	9	M	267	300	+33	24.9	24.9	24.9	24.9	24.9	24.9	24.9	174.3	0.88
10	16	M	266	305	+39	24.3	23.8	24.0	24.4	24.0	22.7	23.4	166.6	0.83
40	6	F	200	217	+17	13.6	17.6	17.2	17.2	16.9	13.9	17.8	114.2	3.12
40	11	M	282	326	+44	24.5	24.8	24.8	24.9	24.5	24.7	24.9	173.1	3.26
40	13	M	268	305	+37	24.7	24.1	24.1	24.3	24.4	22.8	24.4	168.8	3.41
160	3	F	212	218	+6	20.3	16.2	16.7	14.5	19.1	14.9	15.8	117.5	12.5
160	18	M	276	320	+44	24.8	24.7	24.8	24.9	24.8	24.8	24.8	173.6	13.3
160	23	M	280	311	+31	24.5	24.4	24.5	24.4	24.3	23.3	24.8	170.2	13.2
640	7	F	199	208	+9	15.1	12.9	15.4	17.6	17.8	13.3	17.3	109.4	49.1
640	19	M	276	299	+23	24.5	24.5	24.4	24.4	24.3	24.4	24.6	171.1	54.4
640	28	M	270	290	+20	23.5	23.3	23.9	23.9	24.0	23.8	24.2	166.6	54.4
1280	8	F	218	231	+13	17.0	12.8	16.6	18.2	19.3	17.1	17.4	118.4	96.4
1280	17	M	268	304	+36	24.6	24.7	24.5	24.7	24.6	24.7	24.5	172.3	101.5
1280	24	M	267	297	+30	24.7	24.7	24.7	24.8	24.8	24.7	24.4	172.8	112.1
2560	1	F	197	208	+11	14.1	16.8	13.3	18.2	19.5	19.1	14.4	115.4	207.7
2560	10	M	265	296	+31	23.4	23.9	23.9	24.3	24.5	24.7	24.5	169.2	220.7
2560	15	M	267	305	+38	24.9	24.9	24.9	24.9	24.9	24.9	24.9	174.3	223.0
control	25	F	212	232	+20	18.5	21.1	21.3	14.1	22.0	21.8	20.4	139.3	
control	14	M	266	307	+41	24.2	24.6	23.8	24.4	24.2	24.5	24.0	169.7	
control	20	M	287	329	+42	24.8	24.8	24.9	24.9	24.9	24.9	24.8	174.0	

[a] Based on the average body weight of the rat and the total amount of feed consumed. The specific activity of the radiolabeled sulfamethazine for each treatment level was as follows: 10 ppm = 26.8 μCi/mg, 40 ppm = 6.829 μCi/mg, 160 ppm = 1.707 μCi/mg, 640 ppm = 0.426 μCi/mg, 1280 ppm = 0.213 μCi/mg, and 2560 ppm = 0.107 μCi/mg.

Table II. Weights and Feed Intake of Sprague-Dawley and Fischer-344 Male Rats Treated with [^{14}C]Sulfamethazine (Experiment 2)

treatment level, ppm	rat no.	sex	strain	body weight, g			daily feed intake, g, for day							total feed	mg kg^{-1} day^{-1} [a]
				start	sacrifice	change	1	2	3	4	5	6	7		
10	3	M	Sprague-Dawley	257	289	+32	23.1	23.6	23.8	23.9	23.9	23.9	23.6	165.5	0.97
10	9	M	Fischer-344	214	197	−17	8.3	8.1	3.9	5.5	10.3	14.1	14.0	64.2	0.44
10	15	M	Fischer-344	218	224	+6	14.8	16.1	15.3	14.9	13.7	16.1	14.9	105.8	0.68
160	4	M	Sprague-Dawley	278	313	+35	23.7	24.3	23.4	23.3	23.9	24.3	24.4	167.3	12.9
160	11	M	Fischer-344	208	216	+8	16.9	16.5	15.6	16.4	15.6	16.3	17.6	114.9	12.1
160	16	M	Fischer-344	215	233	+18	12.6	18.0	18.9	19.5	17.7	19.1	19.5	125.3	12.8
2560	7	M	Sprague-Dawley	274	314	+40	23.6	22.6	22.3	22.6	23.7	23.6	24.4	162.8	202.4
2560	14	M	Fischer-344	216	165	−51	2.0	2.6	2.4	3.1	2.3	3.1	21.8	37.3	71.7
2560	17	M	Fischer-344	212	226	+14	16.4	14.9	16.2	16.1	16.6	15.9	16.5	112.6	188.1

[a] Based on the average body weight of the rat and the total amount of feed consumed. The specific activity of the radiolabeled sulfamethazine for each treatment level was as follows: 10 ppm = 43.16 μCi/mg, 160 ppm = 2.693 μCi/mg, and 2560 ppm = 0.168 μCi/mg.

tablish the radiopurity and its relation to the silica gel isolate. A number of subsequent preparative runs were performed on each gel isolate to collect the material that was used for spectral analysis on a Model 4000C Finnegan automated EI–CI mass spectrometer system.

The [^{14}C]sulfamethazine and selected TLC metabolite isolates were also hydrolyzed with 1 N hydrochloric acid for 1 h at 130 °C (Manuel and Steller, 1981) and the degradation products analyzed by two-dimensional TLC.

RESULTS AND DISCUSSION

Drug Intake. Table I summarizes the quantity of feed ingested by each rat, their body weights before and after treatment, and the total amount of drug administered, expressed as mg kg^{-1} day^{-1} for experiment 1. The increasing concentration of sulfamethazine in the diet did not have an adverse impact on the amount of feed ingested by the Sprague-Dawley rats. Both female and male rats showed body weight gains very similar to those of the control rats. Female rats ingested less feed than male rats; however, on a mg/kg basis, the drug intake by the female was similar to the drug intake by males at each level. Animals in experiment 2 (Table II) appeared healthy and normal at the start; however, during the experiment, feed consumption by two Fischer-344 rats (no. 9 and 14) on different levels of drug in the diet was considerably lower than that of the companion rats of this strain. These two rats were subsequently excluded from analysis. The reduced feed intake was not related to the drug or its level in the feed but may have been due to an incurrent infection.

Table III. Recovery of Radioactivity in Urine of Rats Treated with [^{14}C]Sulfamethazine, Expressed as Percent of Dose

level of sulfamethazine in diet, ppm	expt 1, Sprague-Dawley		expt 2	
	female	male	Sprague-Dawley male	Fischer-344 male
10	55.3	31–44	44.3	44.1[a]
40	59.8	46–48		
160	51.5	42–52	51.5	42–54
640	60.2	49–52		
1280	68.4	47–50		
2560	67.2	44–54	50.1	46.0[a]

[a] One rat only.

Analysis of Urine, Tissues, and Blood. The results in Table III show the comparison of the recovery of radioactivity in the urine for one female vs. two males from experiment 1. Clearly, the female rat was able to consistently absorb and excrete a greater percentage of the ingested radioactivity in the urine than the male at each diet level tested. Apparent differences in the metabolism of sulfamethazine between female and male rats were also noted in the ^{14}C-residue levels found in selected tissues and blood (Table IV) wherein the female exhibited consistently

Table IV. Residue Levels in Tissues and Blood, Expressed as ppm Equivalents of [^{14}C]Sulfamethazine

	expt 1												expt 2					
	Sprague-Dawley female						Sprague-Dawley male[a]						Fischer-344 male			Sprague-Dawley male		
	4[c]	6	3	7	8	1	9 & 16	11 & 13	18 & 23	19 & 28	17 & 24	10 & 15	11 & 15	16[a]	17	3	4	7
liver																		
methanol extract	0.52	0.85	4.84	15.50	21.20	36.0	0.14	0.51	1.83	3.21	10.56	23.65	0.18	3.34	48.84	0.17	2.58	58.75
acid–methanol extract	0.02	0.06	0.27	1.24	2.10	5.89	0.08	0.24	0.88	2.97	4.39	6.39	0.07	0.90	7.05	0.11	0.81	5.63
marc	0.01	0.06	0.22	1.03	2.00	4.45	0.09	0.23	0.99	3.22	5.13	6.57	0.07	1.24	7.97	0.13	1.39	9.98
total	0.55	0.97	5.33	17.77	25.30	46.34	0.31	0.98	3.70	9.40	20.08	36.61	0.32	5.48	63.86	0.41	4.78	74.4
kidney																		
methanol extract	0.65	2.25	4.81	15.1	39.5	51.3	0.26	0.89	2.39	5.58	14.75	36.45	0.21	3.78	48.13	0.26	3.93	89.24
acid–methanol extract	0.04	0.13	0.66	0.70	0.04	2.18	0.03	0.09	0.39	0.63	1.21	2.71	0.02	0.25	2.25	0.02	0.25	2.32
marc	<0.01	0.03	0.10	0.25	0.52	0.81	0.01	0.04	0.14	0.40	0.74	1.24	0.04	0.56	1.61	0.05	0.55	1.68
total	0.69	2.41	5.57	16.05	40.06	54.29	0.30	1.02	2.92	6.61	16.70	40.40	0.27	4.59	51.99	0.33	4.73	93.2
muscle																		
methanol extract	0.30	0.70	1.87	6.67	14.9	29.10	0.07	0.23	0.62	1.47	5.89[b]	10.04	0.04	0.90	15.7	0.06	0.91	26.8
acid–methanol extract	<0.01	0.01	0.03	0.11	0.44	0.42	<0.01	<0.01	0.03	0.08	0.30	0.48	<0.01	0.02	0.57	0.03	0.02	0.61
marc	<0.01	0.01	0.04	0.09	0.37	0.39	<0.01	0.01	0.05	0.10	0.18	0.73	<0.01	0.04	0.75	<0.01	0.03	0.35
total	0.30	0.72	1.94	6.87	15.71	29.91	0.07	0.24	0.70	1.65	6.37	11.25	0.04	0.96	17.02	0.09	0.96	27.8
fat																		
hexane extract	<0.01	0.02	0.01	0.13	0.45	0.33	<0.01	0.03	0.02	0.04	0.07	0.35	<0.01	0.02	0.84	<0.01	0.04	0.80
methanol extract	0.14	0.42	0.76	2.39	6.72	7.79	0.03	0.07	0.25	0.56	1.96	4.42	0.01	0.78	4.28	0.03	0.38	10.2
marc	<0.01	0.04	0.04	0.11	0.37	0.31	<0.01	<0.01	0.03	0.04	0.18	0.36	<0.01	0.03	0.44	<0.01	0.02	<0.01
total	0.14	0.48	0.81	2.63	7.54	8.43	0.03	0.10	0.30	0.64	2.21	5.13	0.01	0.83	5.56	0.03	0.44	11.0
blood	1.26	5.05	8.05	22.3	68.6	88.8	0.38	1.64	3.64	6.87	24.8	47.9	0.24	5.42	63.4	0.32	4.67	101.6

[a] Average of two rats. [b] Rat no. 24 only. [c] Rat number.

Table V. Percent Contribution of Urinary Radiometabolites from Rats Treated with [^{14}C]Sulfamethazine

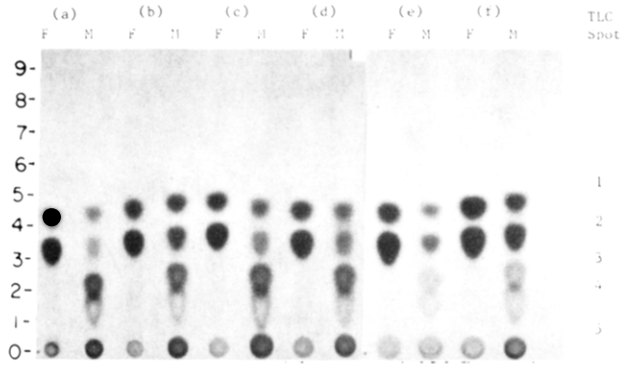
		expt 1												expt 2					
TLC spot		Sprague-Dawley female[a]						Sprague-Dawley male[a]						Fischer-344 male[a]			Sprague-Dawley male[a]		
no.	identity of TLC spot	4[b]	6	3	7	8	1	16	13	23	28	24	15	15	16	17	3	4	7
1	sulfamethazine	24.0	24.6	24.3	25.6	27.6	33.3	11.0	14.6	12.3	15.3	20.0	23.0	14.0	19.0	21.3	10.3	11.0	13.6
2	N^4-acetylsulfamethazine	57.3	53.3	59.6	55.3	56.3	42.6	11.6	18.0	14.0	14.6	26.6	40.0	12.6	41.0	35.3	10.6	11.0	16.6
3	sulfate or glucuronide conjugate of sulfamethazine	3.3	3.6	3.0	4.0	3.6	3.3	22.3	27.0	24.3	25.6	19.3	15.0	26.6	10.3	13.0	25.6	28.3	24.6
4	sulfate or glucuronide conjugate of N^4-acetylsulfamethazine	2.6	2.6	2.3	2.3	2.0	4.6	17.6	11.6	15.3	13.3	11.6	7.3	13.0	8.6	8.6	13.0	10.6	15.6
5	N^4-glucose conjugate of sulfamethazine	13.0	15.6	11.6	12.6	11.3	13.0	30.3	33.0	30.6	31.3	25.0	18.3	34.0	21.0	22.0	40.3	38.6	29.6

[a] Average of day 2, day 4, and day 6 urine for each treatment level (see Tables I and II and Figures 1 and 2). [b] Rat number.

higher residues. Liver and kidney were target tissues for sulfamethazine residues in the rat. A linear relationship of ^{14}C levels in blood vs. the mg/kg ingested daily was noted in both the female and the male. There were no apparent strain-related differences in the excretion of ^{14}C in urine and distribution of ^{14}C in tissues (experiment 2, Tables III and IV) between the Sprague-Dawley male rat and the Fischer-344 male rat.

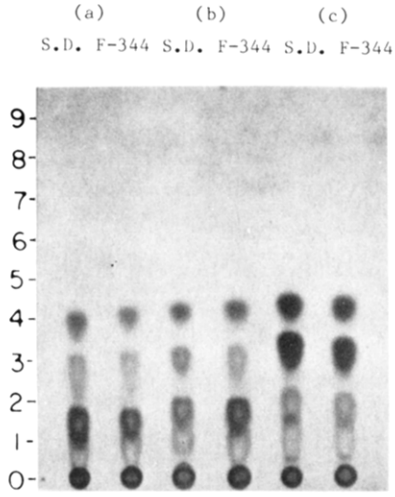
Identification of Metabolites in Urine. The results of TLC analysis in system CA of day 6 urine from the female rat and one male rat are shown in Figure 1. Chromatograms of day 2 and day 4 urine were similar qualitatively. Quantitation of the radiometabolites in the urine (Table V) showed there were three metabolites that were significant in the male. One metabolite was very significant in the female. There were no appreciable differences in metabolite composition within each treatment level between the day 2 and the day 6 urine profiles for the female and for the male, respectively. The results in Table V represent the average metabolite composition in urine based on the TLC of the day 2, day 4, and day 6 urine. No apparent strain differences in the metabolism of sulfamethazine between the Sprague-Dawley and Fischer-344 rats were noted (experiment 2, Table V and Figure 2).

It has been reported the *N*-glucuronide and *N*-glucose conjugates of sulfamethazine may be formed nonenzymatically in urine (Bridges and Williams, 1962) and swine tissue (Giera et al., 1982a). Therefore, control male and female rat urine samples were fortified with [^{14}C]sulfamethazine and refrigerated for 24 h prior to TLC analysis to determine if any of the urinary radiometabolites (Figure



(a) 10 ppm
(b) 40 ppm
(c) 160 ppm
(d) 640 ppm
(e) 1280 ppm
(f) 2560 ppm

Figure 1. One-dimensional TLC of day 6 urine in system CA from rats receiving [^{14}C]sulfamethazine: experiment 1.



(a) 10 ppm (b) 160 ppm (c) 2560 ppm

Figure 2. One-dimensional TLC of day 6 urine in system CA from rats receiving [^{14}C]sulfamethazine: experiment 2.

1) were in vitro artifacts. It was found that 8% of the radioactivity in the spiked urine samples remained at the spotting origin; 2% of the radioactivity in a methanol solution of sulfamethazine was also found at the TLC origin. It was concluded that a small percentage of the origin-bound radioactivity (Table V) could be due to an in vitro artifact whereas the remaining metabolites were due to sulfamethazine metabolism by the rat.

In urine, radiospots no. 1 and 2 cochromatographed with sulfamethazine and N^4-acetylsulfamethazine, respectively. Subsequent TLC isolation, HPLC, and chemical ionization mass spectrometry in methane in both positive ion (PICI) and negative ion (NICI) modes verified the identity of these two compounds. For radiospot no. 1, the PICI (CH_4) mode generated a molecular ion at $(M + H)^+$ at m/z 279$^+$ and a fragment ion at m/z 124$^+$ that characterized the 4,6-dimethyl-2-pyrimidinyl moiety. The m/z 155$^-$ ion in the NICI (CH_4) mode characterized the sulfonic acid moiety. The spectra for the isolate also agreed with those reported for sulfamethazine (Roach et al., 1980). For radiospot no. 2, the PICI (CH_4) mode generated a molecular $(M + H)^+$ ion at m/z 321$^+$ and a fragment ion at m/z 124$^+$. The m/z 197$^-$ ion in the NICI (CH_4) mode characterized the N-acetylated sulfonic acid moiety.

In the female, N^4-acetylsulfamethazine was the major component of the ^{14}C residue in urine followed by sulfamethazine (Table V). While the identification of sullfamethazine and N^4-acetylsulfamethazine in the urine of rats was anticipated in light of the earlier reports on the metabolism of sulfonamide drugs (Riemerdes and Thumim, 1970), it was of interest to find that the concentation of sulfamethazine and its N-acetyl conjugate in urine was apparently dose dependent in the male but not in the female.

Radiospot no. 5 was the third most abundant metabolite in the urine of the female and the male. TLC analysis revealed that the gel isolate had partially degraded to sulfamethazine, which suggested that this metabolite was a conjugate of sulfamethazine. After HPLC cleanup and removal of sulfamethazine, chemical ionization mass spectrometry of the metabolite gave an m/z 279$^+$ ion in the positive ion mode corresponding to the $(M + H)^+$ ion of sulfamethazine and an m/z 155$^-$ ion corresponding to the sulfonic acidy moiety and an m/z 178$^-$ ion that is the $(M - H)^+$ ion of glucosamine in the negative ion mode. These data show the metabolite is the N^4-glucose conjugate of sulfamethazine. The N^4-glucose conjugate has been reported as a metabolite of sulfamethazine in swine tissues and urine (Paulson et al., 1981; Giera et al., 1982b). The level of this metabolite in the female urine appeared to be dose independent (Table V).

The second predominant urinary metabolite in the male at drug levels below 1280 ppm was radiospot no. 3. It was a very minor urinary metabolite in the female at all drug levels (Table V). Chemical ionization mass spectrometry of the HPLC isolate of radiospot no. 3 gave an m/z 155$^-$ ion in the negative ion mode that was indicative of the sulfonic acid moiety. However, no ion was detected in the positive ion mode, which would serve to verify the structure of the metabolite.

Through further characterization of radiospot no. 3 by mild hydrolysis with 1 N hydrochloric acid (Manuel and Steller, 1981), it was observed that this metabolite yielded sulfamethazine, sulfanilic acid, and sulfonamide as radioactive hydrolysis products. Bray et al. (1951) have reported that sulfamethazine produced sulfonamide, sulfanilic acid, and 2-hydroxy-4,6-dimethylpyrimidine when heated with 5 N hydrochloric acid. 2-Hydroxy-4,6-dimethylpyrimidine was a secondary hydrolysis product of sulfamethazine and was formed from the 2-amino-4,6-dimethylpyrimidine, the primary hydrolysis product. When [^{14}C]sulfamethazine was heated in the presence of 1 N hydrochloric acid analogous to the metabolite, it was shown that the radiotracer was relatively stable; however, some radioactive sulfanilic acid and sulfonamide was produced under these conditions. Therefore, in the hydrolysis of radiospot no. 3, sulfamethazine was the primary hydrolysis product and sulfanilic acid and sulfonamide were the secondary hydrolysis products. The metabolite corresponding to radiospot no. 3 was most probably an N-glucuronide or an N-sulfate conjugate of sulfamethazine.

Radiospot no. 4 was also a minor metabolite in the female and the least abundant metabolite in the male at all diet levels tested (Table V). The mass spectral analysis of the HPLC isolate gave ions at m/z 197$^-$ and m/z 155$^-$ in the negative ion mode. The m/z 197$^-$ ion suggested that the metabolite contained the N-acetylated sulfonic acid moiety; the m/z 155$^-$ ion could be a fragment ion from the metabolite or from a secondary sulfonamide in the sample. Again no ions were detected in the positive ion mode to help identify the structure of this metabolite. This me-

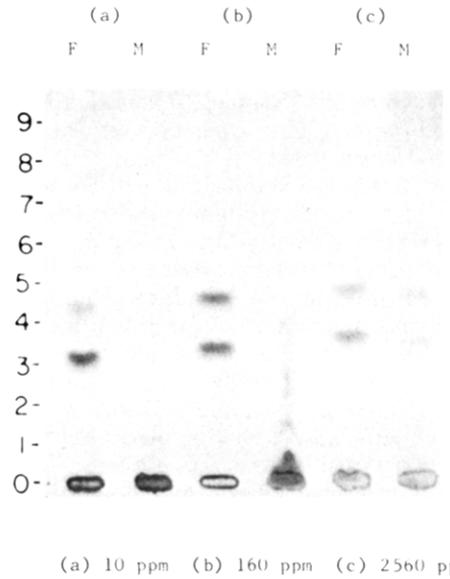


Figure 3. One-dimensional TLC in system CA of methanol extract of liver: experiment 1.

tabolite yielded the same hydrolysis products as radiospot no. 3. Thus, the metabolite corresponding to radiospot no. 4 is probably a secondary sulfate or glucoronide conjugate of N^4-acetylsulfamethazine. The sulfate and glucuronide conjugates of sulfamethazine have been identified in swine urine and tissues (Paulson et al., 1981; Giera et al., 1982b). Sulfanilic acid, sulfanilylguanidine, or sulfonamide was not found as sulfamethazine metabolites in rat urine based on two-dimensional thin-layer cochromatography of the urinary radioactivity with the reference compounds.

Whereas the concentration of N^4-glucose conjugate and the other two conjugates remained fairly constant in the female urine (Table V) and were not affected by the level of sulfamethazine in the feed, as the body burden of sulfamethazine was increased above 640 ppm in the male (Table V), the level of these conjugates decreased in urine. Sulfamethazine and its primary conjugate, N^4-acetylsulfamethazine, appeared as the predominant residues in the male urine at diet levels above 640 ppm. It has been reported (Levy et al., 1982) that major a pathway for acetaminophen metabolism in the rat is the formation of a glucuronide and a sulfate conjugate. As a consequence of feeding increasing doses of acetaminophen to the rats, the drug was cleared slowly from the rat's body and the rate of formation of acetaminophen sulfate decreased because the free (inorganic) sulfate, which was a cosubstrate of quantitative importance, was being depleted.

In our study with sulfamethazine and Sprague-Dawley male rats, a similar biological effect may be occurring in response to the increasing levels of the drug in the rat's diet. The endogeneous cosubstrates that are important in the metabolism of sulfamethazine and N^4-acetylsulfamethazine in the male rat may be depleted and the fraction of the dose excreted in the urine as unchanged sulfamethazine and its N^4-acetyl conjugate increased. This would suggest there is a saturable conjugation process(es) for the metabolism of sulfamethazine in the male rat.

Identification of Residues in Tissues. A major portion of the ^{14}C residue in the kidney, muscle, and fat was recovered in the methanol extract (Table IV) in both the female and the male rat. Between 80 and 95% of the ^{14}C tissue residue was extracted with methanol and very little ^{14}C residue was recovered in the acid–methanol extract or remained unextracted. There is an apparent sex-related difference in the extraction of the ^{14}C liver residue. For the female, 78–95% of the residue was extracted with methanol as compared to only 35–63% of the ^{14}C residue in the male. About half of the rest of ^{14}C residue in the male liver was recovered in the subsequent acid–methanol extract. TLC analysis in system CA showed a minimum of three compounds in the methanol extract (Figure 3) and one polar, origin-bound metabolite in the acid–methanol extract. Two of the ^{14}C components in the methanol extract were identified as sulfamethazine and N^4-acetylsulfamethazine by two-dimensional cochromatography with the reference compounds. The third component in the methanol extract was also polar and origin-bound like the ^{14}C component in the acid–methanol extract. This polar, origin-bound ^{14}C material could quite possibly be the N^4-glucose conjugate that had previously been identified as a urinary metabolite (Figure 1). No attempt was made to identify this component of the liver extract.

Table VI. Residue Level of Sulfamethazine, N^4-Acetylsulfamethazine, and Origin-Bound Radioactivity in Methanol Extract of Liver, Expressed as ppm Equivalents of Sulfamethazine

treatment level, ppm	sex	strain	sulfamethazine	N^4-acetylsulfamethazine	origin bound
10	F	Sprague-Dawley[a]	0.06	0.15	0.28
10	M	Sprague-Dawley[b]	0.01	0.01	0.13
10	M	Fischer-344[c]	0.01	0.01	0.13
160	F	Sprague-Dawley[d]	1.37	1.54	1.61
160	M	Sprague-Dawley[e]	0.12	0.18	2.05
160	M	Fischer-344[f]	0.22	0.32	2.37
2560	F	Sprague-Dawley[g]	8.6	9.9	14.9
2560	M	Sprague-Dawley[h]	5.3	7.8	41.4
2560	M	Fischer-344[i]	10.7	9.9	20.8

[a] Experiment 1, rat no. 4. [b] Experiment 2, rat no. 3. [c] Experiment 2, rat no. 15. [d] Experiment 1, rat no. 3. [e] Experiment 2, rat no. 4. [f] Experiment 2, rat no. 16. [g] Experiment 1, rat no. 1. [h] Experiment 2, rat no. 7. [i] Experiment 2, rat no. 17.

The results in Table VI show a comparison of the ^{14}C residue level of sulfamethazine, N^4-sulfamethazine, and the origin-bound radioactivity in the female and male rat liver from selected treatment levels. Sulfamethazine and N^4-acetylsulfamethazine were the predominant residues in the liver of the female more so than in the male. The origin-bound radioactivity was more predominant in the male. The results of the analysis of the tissues from the Fischer-344 rats in experiment 2 are presented in Table VI and Figure 4.

The genesis of the N^4-glucose conjugate of sulfamethazine in swine liver has become suspect, based on an investigation by Giera et al. (1982a). They reported that by fortifying control swine liver extracts with [^{14}C]sulfamethazine, the glucose adduct and a number of other minor tissue metabolites of sulfamethazine could be formed in vitro. Parks (1984) has recently cited some evidence for the transformation of sulfamethazine to the N^4-glucose conjugate in swine liver during frozen storage.

In our study, the amount of polar, origin-bound radioactivity that was found when [^{14}C]sulfamethazine was added to dilute solutions of the methanol extracts of control liver from female and male rats after 24-h storage of the extracts at 10 °C was about 3%.

Because the methanol extracts of the liver from the medicated rats had been kept for several months before they were concentrated and chromatographed, the fortified tissue extracts were aged for a comparable time to determine if this would significantly increase the amount of origin-bound radioactivity in the sample. It was found that

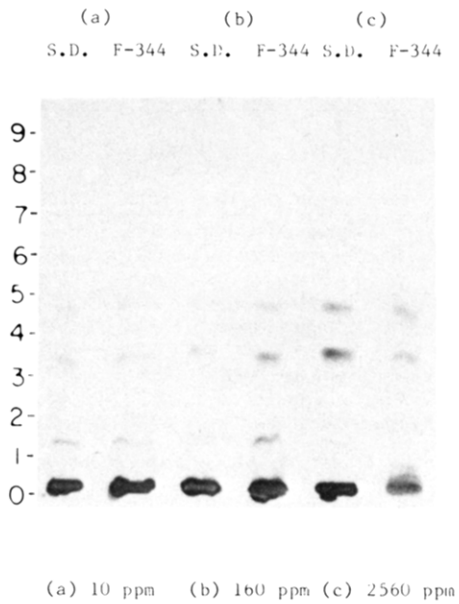


Figure 4. One-dimensional TLC in system CA of methanol extract of liver: experiment 2.

only 6% of the radioactivity was converted to polar, origin-bound radioactivity as a consequence of storage of the fortified extract as dilute solutions in methanol. Thus, the high levels of origin-bound radioactivity in the male liver (Table VI) were considered to be derived from the metabolism of the drug and not in vitro artifacts as a result of storage.

It was noted that when an aliquot of the fortified extract was concentrated and the concentrate aged for 24 h, the origin-bound radioactivity amounted to only 6%. On prolonged storage of the concentrate at 10 °C, the level of radioactivity that remained at the spotting origin on TLC had increased to 35% vs. only 6% for the dilute solution aged for a comparable length of time. These results show sulfamethazine can interact with endogeneous components in extracts of liver to give an in vitro artifact after extracts were concentrated and aged. The identity of this polar, origin-bound radioactivity in the fortified sample was not pursued.

Summary. Studies on the metabolism of sulfamethazine in cows (Nielsen, 1973), lambs (Bevill et al., 1977; Bourne et al., 1977), swine (Paulson et al., 1981; Giera, et al., 1982b), and the rabbit (Bray et al., 1951; Smith and Williams, 1948) and the metabolism of sulfonamides in general (Reimerdes and Thumim, 1970) show that N^4-acetylation, conjugation with sulfate, glucuronic acid, and glucose, and hydroxylation are routes for the metabolism of sulfa drugs. In vitro studies (Soulinna, 1980) showed that the parenchymal cells were the main sites for acetylation of sulfamethazine in rat and rabbit liver. Topham et al. (1972) have also investigated the biochemical changes in enzyme activity in rat liver in response to treatment with sulfamethazine for 14 days at a dose of 500 mg kg^{-1} day^{-1}.

In our preliminary studies, the major metabolites of sulfamethazine that were found in rats are identical with those found in swine. Some differences in urinary metabolite and tissue residue levels between the female and the male rat were noted. These apparent sex-related differences were in the number and concentration of metabolites. Also the ^{14}C residue in various tissues of the female rat was consistently higher than in male rats. In addition, the fate of sulfamethazine in the male rat was being altered in response to the increasing levels of the drug in the rat's diet. The biological significance of these changes in the metabolism of sulfamethazine in the rat, particularly in males receiving the drug above threshold levels, in a short-term study and their relationship to any toxicological effects that may be observed in long-term chronic feeding studies remains obscure. The results suggest that pharmacokinetic and dose–range finding studies should be conducted in order to arrive at the proper levels of drug to be administered when conducting long-term feeding studies with sulfamethazine and in the interpretation of the data obtained.

Registry No. Sulfamethazine, 57-68-1; N^4-acetylsulfamethazine, 100-90-3; sulfamethazine N^4-glucose conjugate, 55101-26-3.

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