CONCLUSION

These HPLC determinations are direct, requiring no extraction or derivatization. The microliter-sized samples can be withdrawn with negligible disturbance to the dynamics of a controlled-release experiment, and analysis time for both herbicides is less than 7 min. Good sensitivity is provided by UV detectors, especially the variable-wavelength type. Should other applications require the determination of concentrations of 2,4-D or dichlobenil lower than those studied here, it is suggested that the HPLC methods be optimized by using the guidelines and procedures given by Kirkland (1974) and Macy and Loh (1980).

LITERATURE CITED

Cardarelli, N. "Controlled Release Pesticides Formulations"; CRC Press: Cleveland, OH, 1976; pp 93–119.
Edwards, R. W.; Nonnemaker, K. A.; Cotter, R. L. *NBS Spec. Publ. (U.S.)* **1979**, *No. 519*, 87–94.
Eichner, M.; Renner, R. *Z. Lebensm.-Unters. -Forsch.* **1980**, *170*, 1–4.
Kirkland, J. J. *Analyst (London)* **1974**, *99*, 859–885.
Macy, T. D.; Loh. A. *Anal. Chem.* **1980**, *52*, 1381–1383.
Van Rossum, A.; de Wilde, P. C.; de Boer, F. G.; Korver, P. K. In "Analytical Methods for Pesticides and Plant Growth Regulators, Vol. X, New and Updated Methods"; Zweig, G.; Sherma, J., Eds.; Academic Press: New York, 1978; pp 311–320.

Received for review July 13, 1981. Revised manuscript received November 5, 1981. Accepted November 5, 1981. Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

Isolation and Identification of a Polar Sulfamethazine "Metabolite" from Swine Tissue

Deborah D. Giera,* Riaz F. Abdulla, John L. Occolowitz, Douglas E. Dorman, James L. Mertz, and Robert F. Sieck

The metabolism of orally administered [^{14}C]sulfamethazine was studied in swine. A major "metabolite" was isolated from liver and muscle and identified as N^4-glucopyranosylsulfamethazine by mass spectral and nuclear magnetic resonance analysis. However, the genesis of the glucose conjugate became suspect when subsequent investigation revealed the drug's spontaneous reaction with endogenous components in control swine tissue extracts in vitro. The glucose adduct and a number of other minor metabolites were formed in vitro by spiking control liver extracts with [^{14}C]sulfamethazine.

As sulfonamide animal products come under more intense U.S. Department of Agriculture scrutiny because of the high incidence of violative residues (Trabosh, 1978), additional residue studies can be anticipated. Giera et al. (1982) described the excretion and tissue residue distribution when [^{14}C]sulfamethazine was orally administered to swine. Essentially all of the administered drug was excreted 15 days postmedication, depleting total ^{14}C residues in tissue to levels well below the 100-ppb sulfamethazine tolerance. In this study, the isolation and spectral identification of a major sulfamethazine derivative, N^4-glucopyranosylsulfamethazine, in liver and muscle tissue extracts from 1 day postmedicated swine are described.

Further investigation revealed, however, that the analysis of 1 day drug withdrawal tissue samples were complicated by the parent compound's spontaneous derivatization in vitro. The glucose conjugate might have arisen totally or in part from an in vitro reaction between sulfamethazine and an endogenous tissue extract component.

MATERIALS AND METHODS

Extraction and Purification of Polar Metabolite. Twenty-five grams of liver tissue from swine dosed orally with [^{14}C]sulfamethazine (Giera et al., 1982) was extracted with 80% methanol or acetone (3 × 150 mL) in a blender. Following each extraction, the sample was filtered (Whatman No. 1 paper), and the pooled extracts were evaporated, under vacuum at 40–50 °C, to remove organic solvent. The volume of the aqueous sample was adjusted with water to 100 mL and acidified with 4 mL of 1 N HCl. The aqueous sample was extracted with hexane (3 × 200 mL) which was discarded. The aqueous phase was neutralized with 1 N NaOH and extracted with $CHCl_3$ (3 × 200 mL) and then methyl ethyl ketone (MEK; 2 × 200 mL). The $CHCl_3$, MEK, and spent aqueous extracts were separately taken to dryness under vacuum at 40–50 °C. All extractions were done in a separatory funnel. The MEK and aqueous extracts were separately chromatographed on a Porapak Q (Waters Associates, Inc., Milford, MA), 80–100-mesh, column (2.8 × 20 cm). The column material was soaked overnight in methanol and slurry packed. The column was sequentially prewashed with 250-mL portions of methanol, acetone, methanol, water, and 30% methanol prior to sample loading (30% methanol). The sample was eluted from the column with a methanol gradient under gravity pressure (Figure 1). Twenty-milliliter fractions were collected.

Fractions (12–35), which contained the polar metabolite, were pooled and evaporated under vacuum at 40–50 °C, and the residue was redissolved in methanol and streaked on silica gel 60F-254, 0.25-mm TLC plates (EM Laboratories, Darmstadt, Germany). The plates were developed in benzene–MEK–ethanol–water, 30:30:30:10. The me-

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

0021-8561/82/1430-0260$01.25/0

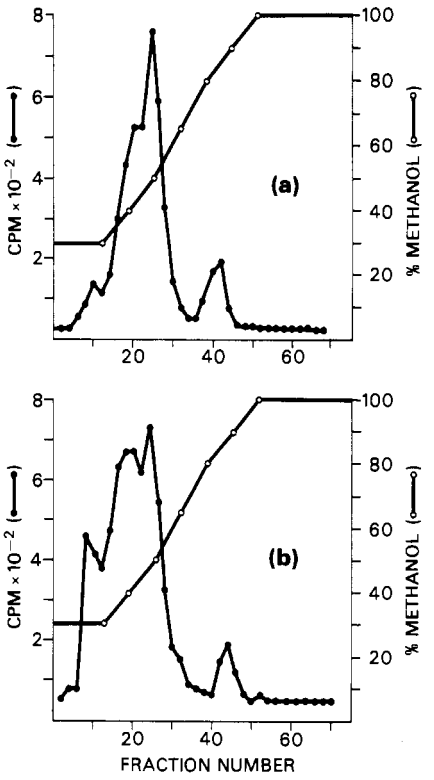

Figure 1. Radioactive eluate profile off the Porapak Q column. (a) Methyl ethyl ketone liver extract. (b) Spent aqueous liver extract.

tabolite zone was detected by radioautography or visualized by spark chamber. The hepatic polar metabolite was eluted from the silica with methanol and further purified 3 times with HPLC using a μBondapak C_{18} column, 7.8 mm i.d. × 30 cm (Waters Associates, Inc.). The eluate monitoring wavelength was 250 nm, and the flow rate was 2 mL/min. Three consecutive developments of the compound through the column were required to obtain final purification. Solvents used, in order, were methanol–water, 5:95; acetonitrile–2-propanol–water, 5:0.5:94.5; and methanol–water, 30:70. The purified compound was subjected to field desorption ionization mass spectrometry with a Varian-Mat, Model 731, mass spectrometer (Varian Associates, Inc., Palo Alto, CA) and nuclear magnetic resonance spectrometry with a Bruker, Model WH-360, nuclear magnetic resonance spectrometer (USA Bruker Instruments Inc., Billerica, MA).

Hydrolysis Studies. The polar metabolite was subjected separately to enzymatic hydrolysis with sulfatase (Type V, Sigma Chemical Co., St. Louis, MO) in 0.1 M acetate buffer, pH 5.0; glusulase (Endo Laboratories, Garden City, NY) in 0.1 M acetate buffer, pH 5.0; and β-glucuronidase (Type VII, Sigma Chemical Co.) in 0.1 M phosphate buffer, pH 6.8. After incubation at 37 °C overnight, the hydrolytic products were extracted from the reaction mixtures with MEK and chromatographed on silica gel 60F-254, 0.25-mm TLC plates (EM Laboratories, Darmstadt, Germany) with benzene–MEK–ethanol–water, 30:30:30:10.

The polar metabolite was subjected to mild acid hydrolysis with 0.5 and 1.0 N HCl at 40 °C. The acid reaction mixtures were neutralized with NaOH and extracted with MEK. The hydrolytic products were chromatographed on silica gel TLC plates as described above for the enzymatic hydrolysis.

Compound Synthesis. Anhydrous cupric sulfate (0.5 g) was added to an equimolar portion of sulfamethazine

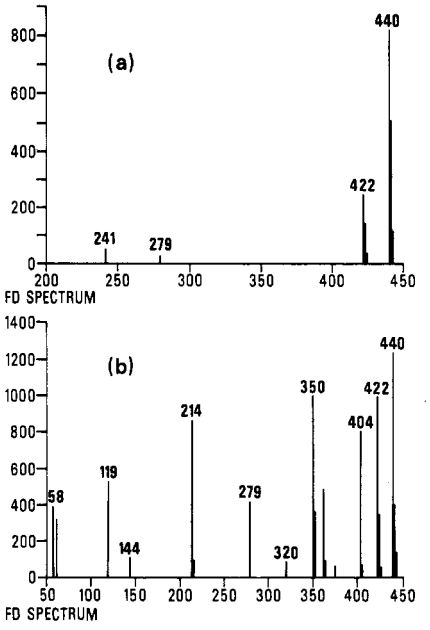

Figure 2. Field desorption mass spectra of N^4-glucopyranosylsulfamethazine. (a) HPLC-purified metabolite from swine liver. (b) Authentic N^4-glucopyranosylsulfamethazine.

(0.28 g) and D-glucose (0.18 g) dissolved in 20 mL of dioxane and stirred at room temperature. The appearance of the glucosyl conjugate was monitored by TLC. At the end of 2 weeks, the content of the reaction flask was centrifuged and the supernatant solution evaporated in vacuo at 40–50 °C. The residue was dissolved in water, and the unreacted sulfamethazine was removed by extraction with chloroform. The aqueous sample was chromatographed on a Sephadex G-10 column (Pharmacia Inc., Piscataway, NJ) with water eluent to remove the cupric sulfate from the compound. The compound was further purified via TLC and HPLC (described under Hydrolysis Studies).

^{14}C Detection on TLC Plates. Areas containing radioactivity on TLC plates were localized by radioautography with BB-5 X-ray film (Eastman Kodak Co., Rochester, NY) or with a Birchover radiochromatogram spark chamber, Model 986-010 (Baird Atomic, Bedford, MA).

RESULTS AND DISCUSSION

Early Tissue Fractionation and Enzymatic Studies. Following oral administration of [^{14}C]sulfamethazine to swine (Giera et al., 1982), a polar sulfamethazine metabolite which comprised approximately 14% of the total ^{14}C-labeled residue in liver and muscle was observed in 1 day postmedicated swine tissue extracts. Efforts to isolate the derivative, which was resorcinol positive on the TLC plate, were hampered by its unremitting breakdown to sulfamethazine especially in the presence of silica gel for periods longer than a few hours. The derivative was hydrolyzed enzymatically when incubated with Glusulase or chemically with dilute HCl. However, it was not cleaved when incubated individually with either sulfatase of β-glucuronidase. Additionally, hydrolysis results were puzzling because although sulfamethazine appeared as the major product, N^4-acetylsulfamethazine also appeared as a minor hydrolytic product. The conjugate was finally purified with high-pressure liquid chromatography in sufficient amounts for ^{1}H NMR, FD, and CI mass spectral analysis.

Physical and Chemical Characteristics. The FD mass spectrum of the isolated polar metabolite indicated a molecular weight of 440 (Figure 2) which would conform to a glycoside formed from sulfamethazine and D-glucose.

Table I. ^{1}H NMR Spectrum of N^4-Glucopyranosylsulfamethazine

proton	multiplicity[a]	δ	coupling constants, Hz
1′	d	4.40	9
2′	t	3.14	9
3′	t	3.25	9.5
4′	t	3.08	9
5′	?	~3.25	b
6′a	dd	3.38	13, 6
6′b	dd	3.60	13, ~2

[a] Multiplicity after deuterium exchange. [b] Overlapped.

Also consistent with such a structure was a reproducible M − 18 peak of 422 representing the loss of water from the sugar.

The ^{1}H NMR spectrum (Me_2SO-d_6) of the polar metabolite showed that the pyrimidine ring was unchanged relative to sulfamethazine. The presence of the aminopyrimidine moiety was also supported by CI mass spectral data where a peak of m/e 124 (protonated (dimethylamino)pyrimidine) was obtained for both sulfamethazine and the polar metabolite. The only structural changes indicated by the NMR spectrum were in the region of the *p*-amino group; also, a series of resonances, chemical shifts from δ 3–5, suggested the presence of a sugar. Deuterium exchange removed resonances of at least four protons in this chemical shift range and left a much simpler spectrum (Table I).

Decoupling experiments showed that a doublet at δ 4.40 (J = 9 Hz) was coupled to a triplet at δ 3.14. Irradiation of the overlapping resonances (2 H) at δ 325 caused the resonances at δ 3.60 and 3.38 to collapse to an AB pattern (J = 13 Hz). These data strongly suggest the presence of a β anomeric linkage and a hydroxymethylene group, consistent with a β-D-glucosyl compound.

NMR and mass spectral comparisons (Figure 2) of the metabolite to a glucoside prepared by unambiguous synthesis support the above conclusion. Furthermore, chromatography results of the isolated polar metabolite and the synthesized compound were identical in the TLC and HPLC systems.

Acid Hydrolysis Studies. The acid hydrolysis studies in 0.5 and 1.0 N HCl were repeated in conjunction with metabolite degradation on the plate. Sulfamethazine and minor traces of the N^4-acetylsulfamethazine form via degradation on the TLC plate in the dark (24 °C; 168 h). Although still a minor reaction product, higher levels of the N^4-acetylsulfamethazine were formed during acid hydrolysis (40 °C; 12 h).

The origin of N^4-acetylsulfamethazine upon acidic hydrolysis can be rationalized according to the speculative steps delineated in Figure 3. The transformation of the hemiaminal in I to an amide in the final product involves an increase by 1 unit of the oxidation state of the glycoside carbonyl group. This may be achieved by the hydration step converting I to II. Acid-catalyzed dehydration affords III which undergoes keto–enol tautomerism to IV. Intermediate IV undergoes acidic cleavage to V (N^4-acetylsulfamethazine).

In Vitro Studies. Interim experiments which were initiated to investigate notable differences in the partitioning behavior of the [^{14}C]sulfamethazine residue from liver tissue stored in liquid nitrogen (−195 °C) and liver tissue stored in a standard freezer (−22 °C) revealed that the polar metabolite might simply be an in vitro artifact formed in the freezer and/or during the isolation procedure. When control liver tissue samples were spiked with

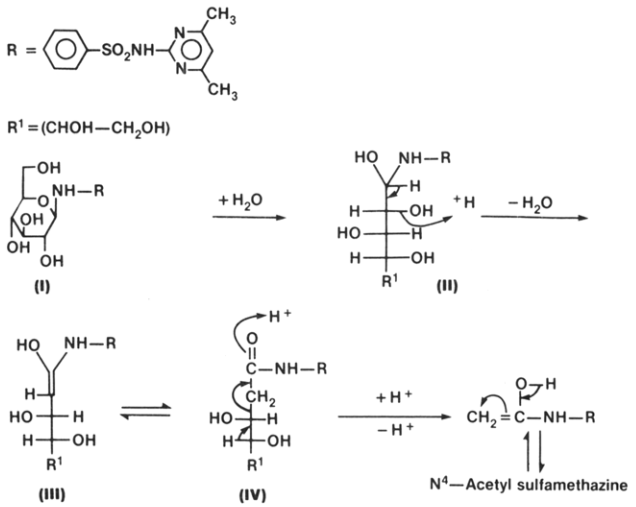

Figure 3. Acid hydrolysis; minor pathway of N^4-glucopyranosylsulfamethazine degradation to N^4-acetylsulfamethazine.

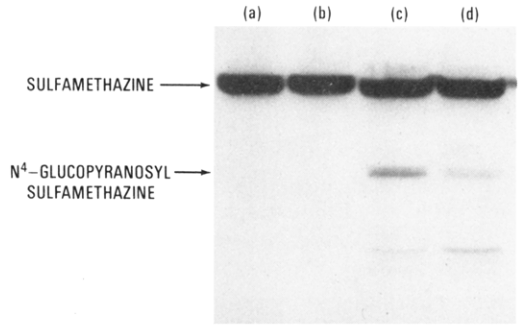

Figure 4. N^4-Glucopyranosylsulfamethazine formation in control liver tissue extracts. (a) Chloroform extract of methanol–water spiked with [^{14}C]sulfamethazine. (b) Chloroform extract of methanol–water spiked with [^{14}C]sulfamethazine and glucose. (c) Chloroform extract of control liver homogenate spiked with [^{14}C]sulfamethazine. (d) Chloroform extract of control liver homogenate spiked with [^{14}C]sulfamethazine and glucose.

[^{14}C]sulfamethazine, subsequent analysis revealed that there was a "component" in the methanol tissue extract that transferred a glucose molecule to the primary amino group of sulfamethazine. The production of the glucose adduct was not enhanced when the tissue extract was supplemented with glucose, suggesting that the simple sugar was not the glucose source. Boiling the hepatic extract prior to spiking did not decrease the sugar conjugate's synthesis, suggesting a nonenzymatic process. Incubation of glucose, UDP-glucose, and glucose 1-phosphate or glucose 6-phosphate with sulfamethazine at 37 °C in 80% methanol did not produce the metabolite, negating a purely chemical conjugation under the conditions of tissue extraction. The 40–50 °C rotary vacuum evaporation of the tissue extract during the ^{14}C-labeled residue fractionation and the length of time of freezer storage seemed to be two determinants that increased the propensity of the tissue extract to form the sugar adduct. A similar, nonenzymatic, spontaneous synthesis of *N*-glucuronides from aromatic amines in blood and urine has been reported by Bridges and Williams (1962).

Figure 4 illustrates a radioautograph obtained from samples and reagents spiked with equivalent amounts of [^{14}C]sulfamethazine, which were carried through the homogenization and fractionation procedure. Lanes a and b are respectively the partitioned $CHCl_3$ extracts of [^{14}C]sulfamethazine and of [^{14}C]sulfamethazine with glucose spiked into 80% methanol. No moieties other than

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.

LITERATURE CITED

Bridges, J. W.; Williams, R. T. *Biochem. J.* **1962**, *83*, 1–27.
Giera, D. D.; Sieck, R. F.; Graper, L. K.; Mertz, J. L.; Herberg, R. J. *J. Agric. Food Chem.* **1982**, following paper in this issue.
Trabosh, H. M. 1978, 12th Swine Sulfonamide Report, U.S. Department of Agriculture, Food Safety, and Quality Service.

Received for review September 2, 1980. Revised manuscript received October 5, 1981. Accepted October 20, 1981.

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

0021-8561/82/1430-0263$01.25/0