Identification of *p*-Toluoyl Chloride Phenylhydrazone (TCPH) Metabolites in the Urine and Feces of Sheep

Metabolism of *p*-toluoyl chloride phenylhydrazone (TCPH) was studied in sheep from an oral dose of 50 mg/kg. About 75% of the dose was excreted in feces and ~15% in urine during the first week of treatment. Thin-layer chromatography of ethyl acetate extract of feces showed that ~12% of the dose was unchanged TCPH, and less than 2% was *p*-toluic acid phenylhydrazide (TAPH) and aniline. Major fecal metabolite (27% of dose) was characterized as 1-phenyl-1-acyl-2-*p*-toluoylhydrazine (where acyl is a mixture of stearyl, palmityl, myristyl, and lauryl groups). TCPH and TAPH were not found in urine. Small amounts (<1% of dose) of *p*-toluic acid, α-ketoglutaric acid phenylhydrazone, and pyruvic acid phenylhydrazone were also observed based on cochromatography with synthetic compounds. The major urinary radioactivity (~10% of the dose) was characterized as *p*-methylhippuric acid, indicating molecular cleavage of TCPH.

Recently, we (Jaglan et al., 1976, 1977) described the excretion of radioactivity following an oral dose of anthelmintic *p*-toluoyl chloride phenylhydrazone (TCPH, uniform ring label phenylhydrazine and carboxyl label) to sheep. To the author's knowledge, metabolism of acid chloride phenylhydrazone or phenylhydrazide except the phenylhydrazine, the parent compound of phenylhydrazone (Colvin, 1969; Juchau and Horita, 1972; McIsaac et al., 1958), has not been described. This is probably due to their high chemical reactivity and instability in the presence of air, moisture, and light. In this study only extractable fecal and urinary metabolites have been examined. Conjugated and polar metabolites, although undoubtedly useful to understand the mechanism of metabolism (Mallipudi et al., 1979) have not been examined so far.

MATERIALS AND METHODS

Compounds. The synthesis of [^{14}C]TCPH (either uniform ring label phenylhydrazine or carboxyl label) was described recently (Jaglan et al. 1976). *p*-Toluic acid, phenylhydrazine, aniline, and phenol were purchased from Eastman Kodak Co. TCPH, *p*-toluic acid phenylhydrazide (TAPH), α-ketoglutaric acid phenylhydrazone, and pyruvic acid phenylhydrazone were authentic known compounds. *p*-Methylhippuric acid was synthesized by reaction of glycine with *p*-toluoyl chloride in tetrahydrofuran containing triethylamine (Rector, 1972). A model metabolite, 1-phenyl-1-palmityl-2-*p*-toluoylhydrazine (PPTH) was synthesized as follows: Twenty-five millimoles of palmitic acid (6.4 g) was suspended in 100 mL of absolute ethanol, to which 25 mmol (2.8 g) of potassium *tert*-butoxide was added, and the solution was refluxed for 20 min. After cooling, 25 mmol of TCPH (6.1 g) was added and the solution was refluxed for 20 min. The sample was cooled, 150 mL of water was added, and the sample was extracted with 150 mL of methylene chloride twice. The methylene chloride extracts were evaporated to dryness, and an aliquot was purified by preparative TLC on 2-mm silica gel GF_{254} plate, using hexane/acetone (7:3) as the mobile phase. The plate was developed for 15 cm, and UV band at R_f 0.57 was scraped and eluted in acetone.

Treatment of Sheep. The sheep were treated with a single oral dose of 50 mg/kg of [^{14}C]TCPH (40.7 μCi/mmol) as previously described (Jaglan et al., 1976), and urine and feces were collected separately. Radiotracer techniques to count radioactivity in feces and urine were described before (Jaglan et al., 1976).

Characterization of Fecal Metabolites. Feces were extracted with 2 volumes of ethyl acetate. The ethyl acetate extracts were concentrated, and an aliquot was spotted 1.5 cm above the lower edge of 250 μ silica gel GF_{254} (Analtech, Inc.) plate. TCPH, TAPH, aniline, and phenylhydrazine were spotted as reference compounds. The plates were developed to 12 cm in hexane/acetone (7:3), dried and examined under UV light (254 nm). The silica gel from the plate was scraped in 1-cm increments and counted in 15 mL of diotol (Burdick & Jackson). Feces extracts were also radioautographed, which revealed a major fraction E at R_f 0.57 (Figure 1). This fraction was eluted with acetone, and infrared spectrum of it and PPTH as KBR pellet was obtained (Figure 2). Mass spectrum of KBR pellet was recorded via direct probe at 250 °C on a LKB 9000 mass spectrometer.

Fraction E was refluxed in 2 N sodium hydroxide for 3 h, pH adjusted to 2.0 with hydrochloric acid (6 N), and extracted with ether. The ether layer was dried and the residue methylated using diazomethane (Jaglan et al., 1976). An aliquot was analyzed by a Varian CH 7, GLC mass spectrometer (Figure 3). Mass spectrum of each GLC peak was recorded and the structure of the compound in each GLC peak was deduced from molecular ion and fragmentation pattern.

Characterization of Urinary Radioactivity. Aliquots of urine were extracted with two volumes of ether at pH 2 and 7 and chromatographed on 250 μ silica gel GF_{254} plates. TCPH, TAPH, α-ketoglutaric acid phenylhydrazone, pyruvic acid phenylhydrazone, toluic acid, and *p*-methylhippuric acid were spotted as reference compounds, and the plates were developed to 12 cm in either hexane/acetone (7:3) or benzene/dioxane/acetic acid (90:25:4). The silica gel from plates was scraped in 1-cm increments and counted similarly to feces. Because most of the radioactivity was present in the acidic fraction, this fraction was methylated as described before (Jaglan et al., 1976) and purified by chromatography on 250 μ silica gel GF_{254} plate in hexane/acetone (7:3). The area corresponding to *p*-methylhippuric acid methyl ester was eluted with acetone. GLC–mass spectrometry of an aliquot was carried out on 5% OV-225 coated Gas-Chrom Q 80/100 at 200 °C in 180 × 4 mm column.

RESULTS AND DISCUSSION

Fecal Metabolites. About 75% of the dose was excreted through feces, out of which about 40% was extracted in ethyl acetate. Radioautography (Figure 1) following TLC revealed that R_f of fractions F, E, D, and C was similar to TCPH, phenylhydrazine, aniline, and TAPH, respectively. Counting of the radioactivity in each zone showed a major portion of radioactivity was present in fraction F (~12% of dose) or in fraction E (27% of dose). Other spots (fractions A, B, C, D) showed less than 2% of the radioactivity and were not investigated further. Although the R_f of fraction E was identical with phenyl-

0021-8561/80/1428-0682$01.00/0

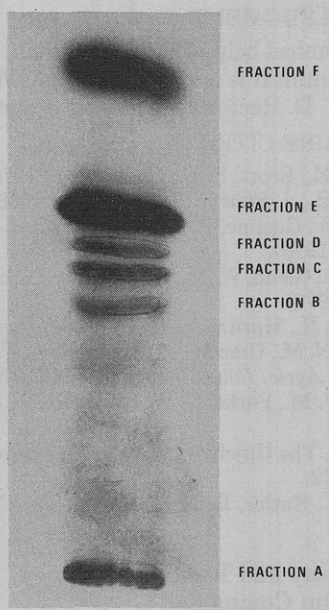

Figure 1. Radioautograph of ethyl acetate extract of feces chromatographed on 250 μ silica gel GF_{254} plate in hexane/acetone (7:3).

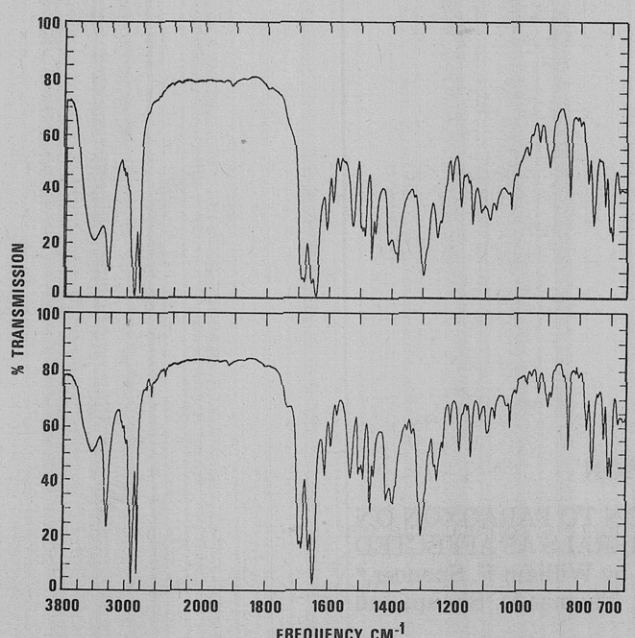

Figure 2. IR spectrum of fraction E (upper) and PPTH (lower) obtained using Digilab Model 14D Fourier transform Spectrophotometer equipped with Perkin Elmer sixfold reflective microsampling unit.

hydrazine, partitioning of radioactivity between ether and 1 N hydrochloric acid, 2% sodium bicarbonate, and 5% sodium hydroxide precluded the presence of phenylhydrazine. Fraction E could not be gas chromatographed, which indicated a metabolite of high molecular weight or high polarity. The mass spectrum of it by direct probe at 250 °C gave major ions at m/e 226, 119, 105, and 91. These values were identical with TAPH, but the metabolite could not be TAPH since it was well resolved on TLC from the metabolite.

The infrared spectra shown in Figure 2 indicated that fraction E and PPTH were identical. The infrared data confirmed the following structural features in both figures: 3430 (H_2O and O–H stretch), 3240 (amide N–H stretch), 3000 (aromatic C–H stretch), 2920 and 2850 (aliphatic C–H stretch), 1695 and 1685 (nonconjugated amide C=O stretch), 1665, 1650 (toluoyl C=O stretch), 1610, 1593, 1575, 1505, and 1490 (aromatic rings C–C stretch), 1530 (secondary amide), 1465, 1415, 1395 (aliphatic C–H deformation), 1305 (amide C–N stretch), 835 (para-substituted phenyl ring, C–H deformation). It should also be mentioned that IR is of limited use in distinguishing homologues of the fatty acid PATH (A = acyl); therefore further experiments were conducted to deduce the identity of PATH.

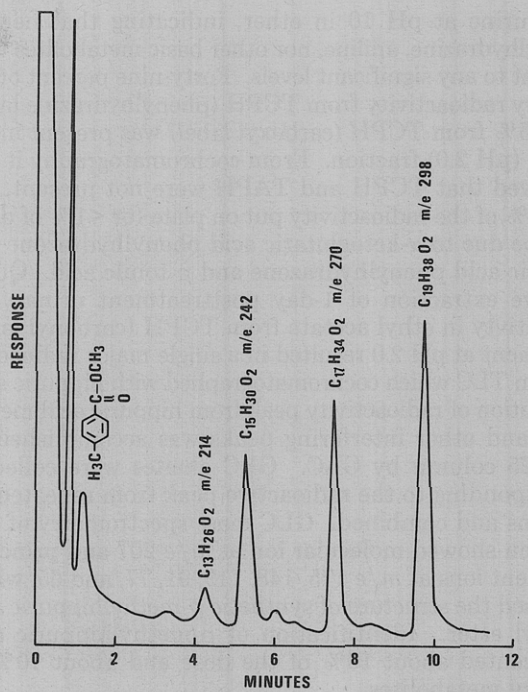

Figure 3. Chromatogram of fraction E hydrolysate after methylation. Borosilicate glass column 1200 × 3 mm i.d., filled with 1% OV-1 coated on 100/120 mesh Gas-Chrom Q. Helium, 40 mL/min. Column, 120 °C. Temperature programmed at the rate of 10 °C/min to 250 °C.

The detailed mass spectrum of fraction E showed a molecular ion at m/e 464 and a 492, which suggested the existence of PPTH and PSTH (S = stearyl), respectively. The spectrum indicated a loss of water, giving rise to ions at m/e 474 and 446. The molecules further fragment by losing 133 amu, probably by expulsion of toluoyl isocyanate, to give rise to ions at m/e 359 and 331. In both cases, the mass spectra was dominated by the aromatic part of the molecule, giving ions at m/e 226, 119, 105, and 91, which were also found in mass spectrum of TAPH and PPTH.

To determine which fatty acids were present, fraction E was hydrolyzed, extracted in ether, and methylated. The GLC/mass spectrum of methylated products by temperature programming is given in Figure 3. These data show that stearic, palmitic, myristic, and lauric acid were components of the mixture.

It appears that TCPH reacts with fatty acid anions by the scheme postulated by Scott and Butler (1966) who established from UV, IR, and NMR data of several synthetic analogues, that acyl groups attach to the hydrazino nitrogen rather than the hydrazide nitrogen. IR absorption of nonconjugated amide C=O stretch at 1695 and 1685 and toluoyl C=O stretch at 1665 and 1650, and secondary amide at 1530 cm^{-1} of fraction E in Figure 2 shows the acyl group was attached to hydrazino nitrogen and not to hydrazide nitrogen of 1-phenyl-1-acyl-2-*p*-toluoylhydrazides which paralleled the work of Butler and Scott (1970).

Urinary Metabolites. About 15% of the dose was excreted via urine. Very little radioactivity was extracted

from urine at pH 10 in ether, indicating that neither phenylhydrazine, aniline, nor other basic metabolites were present to any significant levels. Forty-nine percent of the urinary radioactivity from TCPH (phenylhydrazine label) and 75% from TCPH (carboxyl label) was present in the acidic (pH 2.0) fraction. From cochromatography it was observed that TCPH and TAPH were not present, but 10–20% of the radioactivity put on plate (or <1% of dose) may be due to α-ketoglutaric acid phenylhydrazone and pyruvic acid phenylhydrazone and *p*-toluic acid. Quantitative extraction of 1-day posttreatment urinary radioacitivty in ethyl acetate from TCPH (carbonyl label) treatment at pH 2.0 resulted in a single major radioactive spot on TLC which cochromatographed with hippuric acid. Resolution of radioactivity peak from hippuric acid methyl ester and other interfering peaks was accomplished on OV-225 column by GLC. GLC eluates were collected corresponding to the radioactive peak from repeated injections and combined. GLC mass spectrometry of this fraction showed molecular ion at m/e 207 and principal fragment ions at m/e 175, 148, 119, 91, 77, and 65, which matched the structure of synthetic *p*-methylhippuric acid methyl ester. Identification of *p*-methylhippuric acid represented about 10% of the dose and about 70% of urinary metabolites.

ACKNOWLEDGMENT

The technical help of P. A. Meulman, L. Baczynski, and L. F. Krzeminski is acknowledged. A. W. Neff supervised the study. D. Rector synthesized *p*-methylhippuric acid.

LITERATURE CITED

Butler, R. N., Scott, F. L., *Chem. Ind.*, 1216 (1970).
Colvin, L. B., *J. Pharm. Sci.* **58**, 1433 (1969).
Jaglan, P. S., Gosline, R. E., Neff, A. W., *J. Agric. Food Chem.* **24**, 659 (1976).
Jaglan, P. S., Glenn, W. M., Neff, A. W., *J. Toxicol. Anim. Health* **2**, 815 (1977).
Juchau, M. R., Horita, A., *Drug Metab. Rev.* 1, 71 (1972).
Mallipudi, N. M., Umetsu, N., Tonia, R. F., Talcot, R. E., Fukuto, T. R., *J. Agric. Food Chem.* **27**, 463 (1979).
McIsaac, W. M., Parke, D. W., Willia, R. R., *J. Biochem.* **70**, 688 (1958).
Reactor, D., The Upjohn Company, Kalamazoo, MI, unpublished data, 1972.
Scott, F. R., Butler, R. N., *J. Chem. Soc. C*, 1202 (1966).

Prem S. Jaglan

Biochemistry and Residue Analysis
The Upjohn Company
Kalamazoo, Michigan 49001

Received for review October 22, 1979. Accepted January 28, 1980.

CORRECTION

CONVERSION OF PARATHION TO PARAOXON ON SOIL DUSTS AND CLAY MINERALS AS AFFECTED BY OZONE AND UV LIGHT, by William F. Spencer,* James D. Adams, Ron E. Hess, Thomas D. Shoup, and Robert C. Spear
J. Agric. Food Chem. **1980**, *28*, 366.

Ron E. Hess was inadvertently omitted from the list of authors.