strates the successful use of gas chromatography/mass spectrometry as a routine residue analysis tool.

Application of gas chromatography/mass spectrometry has allowed the quantitation of carbofuran phenols with a minimum amount of sample cleanup. This has reduced the per sample analysis time by half relative to the that of the published derivatization procedure.

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Registry No. Carbofuran, 1563-66-2; 7-phenol, 1563-38-8; 3-keto-7-phenol, 17781-16-7; 3-hydroxy-7-phenol, 17781-15-6.

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Metabolism of Pentachloronitrobenzene by Goats and Sheep

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The metabolic fate of a single oral dose of pentachloronitro[^{14}C]benzene ([^{14}C]PCNB) was examined in four goats and a sheep. Two general pathways of metabolism were observed. One involved the reduction of the nitro group to an amine and formation of secondary metabolites such as the *N*-glucuronide of pentachloroaniline (PCA), the sulfamate of PCA, and tetrachloroaminophenol. This was the principal pathway for animals receiving 2 mg of [^{14}C]PCNB/kg. Only [^{14}C]PCA and small amounts of [^{14}C]PCNB were identified in feces of these animals. The other pathway results in replacement of a nitro group with a sulfur-containing group such as a thiol, a methylthio, an *N*-acetylcysteine, and an *S*-glucuronide or in which both the nitro and a chlorine have been replaced to form compounds such as the *S*-glucuronide of tetrachloro(methylthio)thiophenol, bis(methylthio)tetrachlorobenzene) or *S*-[(methylthio)tetrachlorophenyl]-*N*-acetylcysteine. This type of metabolism was observed with animals receiving 30 mg or more of [^{14}C]PCNB/kg of body weight. In these animals, [^{14}C]PCNB was isolated from feces in amounts equal to or greater than those of [^{14}C]PCA.

Pentachloronitrobenzene (PCNB) is a soil fungicide that is relatively persistent in soil (Beck and Hansen, 1974; Murthy and Kaufman, 1978). Some information is available about the metabolic fate of PCNB in rabbits (Betts et al., 1955), cattle (St. John et al., 1965; Borzelleca et al., 1971), dogs and rats (Kuchar et al., 1969), and sheep (Avrahami and White, 1976). However, these studies did not employ radiotracers, and therefore, analysis was for suspected metabolites and parent compound. More recently, metabolism studies using radiotracer techniques have been reported. Kogel et al. (1979a–c) observed excretion of ^{14}C and the pattern of radiolabeled metabolites after oral administration of [^{14}C]PCNB to three rhesus monkeys (single dose of 2 and 91 mg/kg of body weight; 2 ppm in diet for 71 days). Excretion occurred via both feces and urine with slightly larger amounts in feces. The pattern of metabolites was similar in the excreta from the three monkeys and pentachloroaniline (PCA) was the predominant metabolite in both feces and urine. Other metabolites in excreta were pentachlorobenzene, bis(methylthio)tetrachlorobenzene, pentachlorothiophenol, tetrachloroaniline (TCA), and tetrachloroaminothioanisole. Pentachlorophenol, tetrachlorophenol, tetrachlorothioanisole, and tetrachlorophenyl methyl sulfoxide were found only in urine while PCNB was found only in feces. O'Grodnick et al. (1981) reported *S*-(pentachlorophenyl)-*N*-acetylcysteine as the predominant urinary metabolite from Osbourne-Mendel rats after a single oral dose of 5 mg of [^{14}C]PCNB/kg of body weight. PCA was the predominant metabolite in feces and also a major urinary metabolite. Small amounts of pentachlorothioanisole were recovered from urine and feces. PCNB was found only in feces. PCA was recovered from one unidentified chromatographic fraction (from urine) after acid hydrolysis. Bahig et al. (1981) observed the fate of [^{14}C]PCNB added to water that contained fish. The major products were conjugates of pentachlorophenol and pentachlorothiophenol. We have investigated the metabolic fate of PCNB in goats and a sheep with [^{14}C]PCNB, and the results are presented in this report.

MATERIALS AND METHODS

Chemicals. [^{14}C]PCNB used in this study was synthesized in two batches from uniformly labeled [^{14}C]-nitrobenzene (Mallinckrodt, Amersham); one as described by Kadunce and Lamoureux (1976) and the other by a modification of that procedure and the procedure of Sandrock et al. (1978). Because a high yield of PCNB was not obtained when a Dewar condenser was used as in the method of Kadunce and Lamoureux (1976) or when chlorine was bubbled into the reaction mixture as in the

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method of Sandrock et al. (1978), an efficient condenser was fabricated from a 1 × 60 cm tube and a polystyrene container that was filled with dry ice. Two Vigreaux indentations were positioned to provide drop points for return of the condensed chlorine. This condenser held a charge of chlorine for at least 12 h. The progress of the reaction was monitored by gas chromatography (GC) on OV-1. After $3^1/_2$ h at 25 °C, a mixture of PCNB (72%) and two tetrachloronitrobenzene isomers (19 and 9%) was obtained. After $12^1/_2$ h, a mixture of PCNB (94.3%), hexachlorobenzene (1.5%), and two tetrachloronitrobenzene isomers (2.6 and 1.5%) was obtained. Chlorination for longer times and at higher temperatures gave predominantly hexachlorobenzene (i.e., after 5 days at 45 °C, 3% PCNB and 97% hexachlorobenzene). Purification was accomplished by recrystallization from ether–hexane or by chromatography on silica gel with hexane–methylene chloride (9:1). The tetrachloro isomers were readily removed by recrystallization but were difficult to remove by chromatography, whereas hexachlorobenzene was easily removed by chromatography but was difficult to remove by recrystallization. Radiopurity was estimated to be at least 99.5% by GC and thin-layer chromatography (TLC). The desired specific activities were achieved by mixing nonradioactive PCNB (K & K Laboratories, 98% purity) with the synthesized [^{14}C]PCNB.

[^{14}C]PCA was prepared from [^{14}C]PCNB by reducing with palladium on charcoal. Purification was accomplished by chromatography on silica with hexane as the solvent. *N*-Hydroxyanaline and *N*-hydroxypentachloroaniline (*N*-hydroxy-PCA) were synthesized by methods described by Weisburger and Weisburger (1973).

Animals, Animal Treatments, and Sample Handling. The goats were females of mixed dairy breeding (44–67 kg) and the sheep was a yearling ewe of mixed breeding (32 kg). Before an experiment was initiated, all animals had been on the premises for a minimum of 30 days and had been acclimated to the collection stalls. The dose was prepared by mixing a small amount of ground feed with the desired amount of PCNB in a mortar and pestle. This mixture was transferred to gelatin capsules and the solution of [^{14}C]PCNB in dichloromethane was pipetted onto the feed. The PCA dose was prepared similarly. Solvent was allowed to evaporate from the open capsules for a minimum of 2 h. A balling gun was used to administer the capsules. One goat was dosed intravenously (iv) with [^{14}C]PCNB dissolved in approximately 3 mL of dimethyl sulfoxide. The dose was injected over a period of 5 min via a polyethylene catheter inserted into the jugular vein. Details of the dosing of each animal are given in Table I.

Animals were held in metabolism cages described by Robbins and Bakke (1967). The expired air of goat 68 was monitored for $^{14}CO_2$ for 48 h after the oral dose of [^{14}C]-PCNB (Robbins and Bakke, 1967). Urine collections were made with indwelling urinary bladder catheters. Excreta was allowed to collect for 12-h intervals during the first 48 h after dosing and for 24 h during the remainder of the collection period. The urine was cooled during collection by packing ice around the collection vessel and was then held at −15 °C until analyzed for metabolites. Each feces sample was mixed thoroughly by using a large food mixer (Hobart), and a sample of approximately 100 g was freeze-dried for radioassay. The remainder was held at −15 °C until extracted. At the end of the collection period, animals were sacrificed (except after the oral dose for goat 68), and samples of tissues and organs were obtained. After these samples were ground with a small meat grinder (Hobart), a small sample was homogenized in a Waring-type blender and freeze-dried for radioassay. The entire eviscerated carcass was ground in a large grinder (Autio Co., Astoria, OR) and mixed thoroughly in a large food mixer, and an approximately 100-g sample was homogenized and freeze-dried for radioassays. Fat and milk samples were held at −15 °C until analyzed for metabolites.

Table I. Balance Trial Protocols

animal	dose[a]	duration of excreta[b] collection period, h
goat 68[c]	2 mg/kg [^{14}C]PCNB; sp act. = 1008 dpm/μg	144
goat 68[c]	1.5 mg/kg [^{14}C]PCNB, IV; sp act. = 933 dpm/μg	96
goat 71	2 mg/kg [^{14}C]PCNB; sp act. = 833 dpm/μg	72
goat 80	30 mg/kg [^{14}C]PCNB; sp act. = 81 dpm/μg	96
sheep 321	30 mg/kg [^{14}C]PCNB; sp act. = 63 dpm/μg	72
goat 82	30 mg/kg [^{14}C]PCA; sp act. = 64 dpm/μg	96
goat 93	100 mg/kg [^{14}C]PCNB; sp act. = 21 dpm/μg	72

[a] All doses were given orally except for the second dose to goat 68. [b] All animals were sacrificed after the collection period except when goat 68 was dosed orally. [c] Goat 68 was used in two experiments approximately 5 months apart.

Radioassays. Quantitation of radioactivity was done by liquid scintillation techniques using an external standard for quench correction. For radioassay of urine and blood plasma, 0.5 mL of sample was added to 15 mL of Insta-gel (Packard Instrument Co.). Approximately 250 mg of freeze-dried samples was combusted and CO_2 was trapped for ^{14}C assay (Model 306 sample oxidizer, Packard Instrument Co.). Combustions of samples were done in triplicate. When the variation was more than ±10% of the average, the analysis was repeated and these values were used regardless of variation. Recovery of ^{14}C in the combustion system was over 97%; no corrections for recovery were made.

Chromatographic Procedures. Various types of chromatography were employed. Unless indicated otherwise, the eluate of all liquid chromatogrphic columns was monitored for radioactivity with a liquid flow monitor (Packard Instrument Co., Model 320E) in which the detector cell was packed with glass bead scintillator. Radioactive bands on TLC plates were located with a Packard Model 7200 scanner. Porapak Q (120–200 mesh, Waters Associates Inc.) was packed in glass columns as an aqueous slurry and washed with several column volumes of water, followed by methanol. Immediately before use, the column was washed with several column volumes of water and the sample applied to the column in an aqueous solution. As much as 250 mL of urine was applied to a 2.4 × 20 cm column. After application of the sample, the column was washed with water (until the eluate was nearly clear with urine samples) and then with a stepwise gradient of 30, 50, 80, and 100% methanol–water. For milk samples, Amberlite XAD-2 (Rohm and Haas) was used in place of Porapak Q. The Porapak Q column was also used to remove salts introduced by other procedures. In these cases, the elution sequence was water, 80% methanol, and 100% methanol.

LH-20 (24–100 μm, Pharmacia) was slurried in NH_4HCO_3 solution (1 mg/mL) and poured in 1 × 50 cm columns.

Table II. TLC Systems and R_f Values

compounds	R_f silica solvent 1[a]	R_f silica solvent 2[a]	R_f avicel[b]
PCNB	0.7		
PCA	0.5	1.0	1.0
2,3,4,5-TCA	1.0	0.7	
TCAP[c]	1.0	0.3	
PCA sulfamate[c]			0.4
glucuronides[c]			0.0

[a] Solvent 1: hexane–chloroform, 9:1. Solvent 2: benzene–methanol, 70:30. [b] Solvent: ethyl acetate–58% NH_4OH–acetone, 10:10:8 (upper phase). [c] R_f values are for isolated metabolites. Synthesized standards not available.

Samples were applied in aqueous solutions (1–2 mL), and the column was eluted with the NH_4HCO_3 solution followed by water and then methanol.

DEAE-Sephadex (A-25, 40–120 μm, Pharmacia) was poured in 1 × 25 cm columns as an aqueous slurry and washed with several column volumes of water. The sample was applied in water (1–2 mL) and the column eluted with an approximately linear gradient from water to 1 M KBr (total volume = 200 mL).

Aluminum oxide (Woelm basic, Allupharm Chemicals) was poured in 1 × 16 cm columns in a hexane slurry. The samples were applied in hexane and the column was eluted with approximately 100 mL of hexane followed by benzene. Radioactivity in the eluate of the alumina column was not monitored by a continuous flow system but was located by assays of various fractions.

High-performance liquid chromatography (HPLC) was accomplished with an Altex Model 332 system using a 3.2 × 250 mm C-18 LiChrosorb column and a water–acetonitrile gradient. With some samples, preliminary cleanup was accomplished with a C-18 Sep-PAK using water and methanol (Waters Associates, Inc.).

GC was performed with a Barber-Colman series 5000 instrument equipped so that the effluent from the column could be split between a hydrogen flame detector and a radioactive monitor or between a hydrogen flame detector and a heated exit port where samples could be trapped in glass capillary tubes for spectral analysis. The usual GC column for analyses of PCA, TCA, and tetrachloroaminophenol (TCAP) was a 2 m by 2 mm i.d. 4% OV-101 on Gas-Chrom Q. A typical program was 125–225 °C at 10 °C/min. The GC column used for analysis of glucuronides was a 1 m by 2 mm i.d. 2% OV-101 on Gas-Chrom Q. A typical program was 150–275 °C at 10 °C/min, and glucuronides eluted from 245 to 260 °C.

TLC was accomplished on either precoated 5 × 20 cm silica gel plates (25 mm, Brinkman silica gel 60 without indicator) or with 5 × 20 cm cellulose-coated plates (25 mm, Avicel, Analtech Inc.). Table II lists solvent systems used and R_f values observed with various compounds.

Derivatization Procedures. Trimethylsilyl (Me_3Si) derivatives were formed by reacting the samples with an excess of bis(trimethylsilyl)trifluoroacetamide (Regisil, Regis Chemical Co.). Usually the Regisil was added directly to the dry sample, but sometimes the sample was first dissolved in dry acetone. This reaction mixture was heated to 100 °C for 1 h or allowed to stand overnight at ambient temperature. (Note: Chloroanilines formed isopropylidines when acetone was used as a solvent in silyation reactions.) Acetylation was accomplished with acetic anhydride or, in some cases, with trifluoroacetic anhydride. Butyl esters were formed by reacting with 3 N HCl in anhydrous butanol for 20 min at 90 °C.

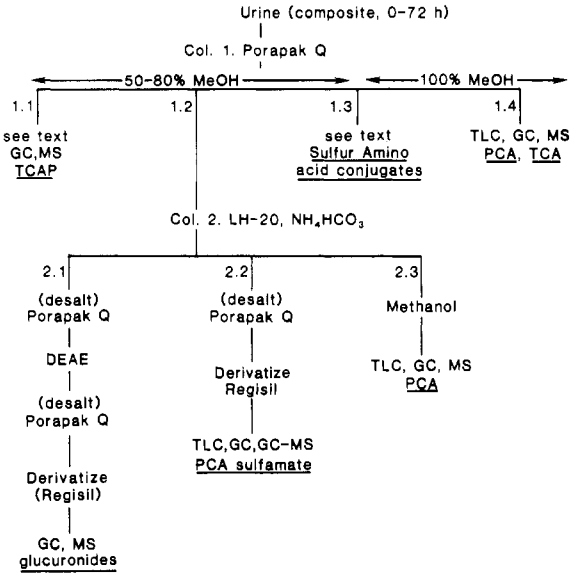

Figure 1. Flow diagram for isolation of urinary metabolites.

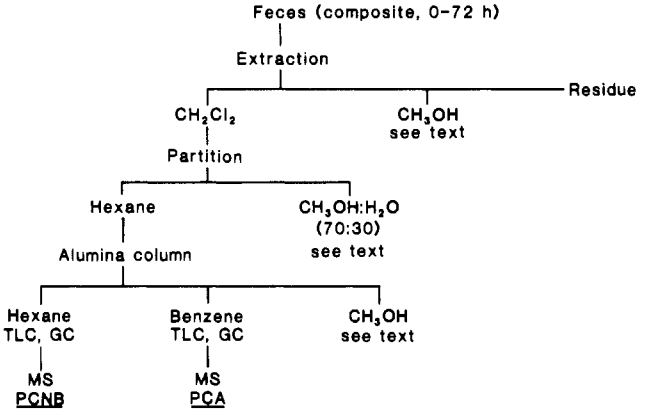

Figure 2. Flow diagram for isolation of fecal metabolites.

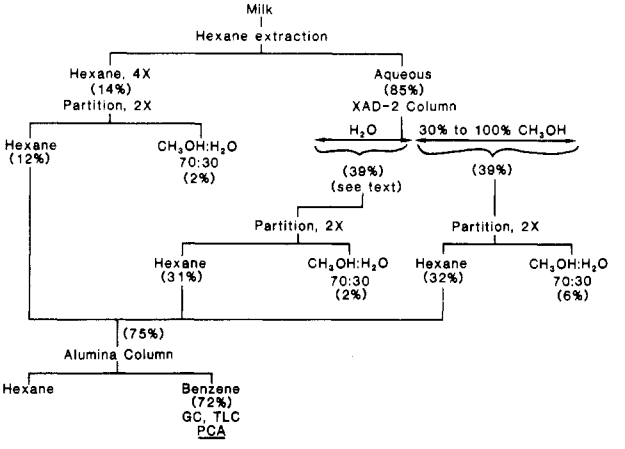

Figure 3. Flow diagram for isolation of metabolites from milk.

Mass Spectra. A Varian MAT-5 DF mass spectrometer with either a SS-100 or SS-200 data system was used to obtain most spectra. Samples were introduced with either a solid sample probe or via a GC interfaced with the spectrometer by a Watson-Bieman-type separator. Spectra were obtained at 70 eV and exact masses were determined by the peak matching technique. A Hewlett-Packard 5992A GC–MS and a Varian MAT-MS-112 were used to obtain some spectra.

Isolation of Metabolites. Figures 1, 2, and 3 give the general procedures for isolation of metabolites from urine,

Table III. Distribution of ^{14}C after Dosing with [^{14}C]PCNB

	% of dose					
	goat					sheep
recovered	68a[a]	68b[b]	71[c]	80[d]	93[e]	321[f]
urine	78.6	44.5	81.8	39.6	37.0	64.6
feces	14.0	39.0	9.7	42.9	51.3	25.4
milk[g]	1.6	0.3	0.7	0.2	0.1	
GI tract and contents		0.6	1.2	0.8	1.7	1.0
eviscerated carcass		3.0	2.4	4.7	3.1	4.0
total	94.2	87.9	95.8	88.2	93.2	95.0

[a] 144-h collection period after an oral dose of 2 mg/kg. [b] Slaughtered 96 h after an iv dose of 1.5 mg/kg. [c] Slaughtered 72 h after an oral dose of 2 mg/kg. [d] Slaughtered 96 h after an oral dose of 30 mg/kg. [e] Slaughtered 72 h after an oral dose of 100 mg/kg. [f] Slaughtered 72 h after an oral dose of 30 mg/kg. [g] Average milk production per day (kg): goat 68a, 2.1; goat 68b, 1.7; goat 71, 1.1; goat 80, 0.7; goat 93, 0.5.

Table IV. Concentrations of ^{14}C in Selected Tissues after Dosing with [^{14}C]PCNB

	ppm[f]				
	goats				sheep
tissue	71[a]	80[b]	93[c]	68b[d]	321[e]
liver	0.2	3.1	8.4	0.2	2.5
bile	0.3	2.5	33.8	0.4	2.4
kidney	0.1	1.3	6.1	0.1	1.9
fat (dermal)	0.6	1.9	13.5	0.2	5.1
fat (visceral)	0.4	4.1	14.5	0.3	6.0
adrenals	0.1	0.9	2.2	0.5	0.6
lung	<0.1	0.3	1.3	<0.1	0.6
spleen	<0.1	0.3	0.7	<0.1	0.4
muscle (round)	<0.1	0.2	0.8	<0.1	0.4
heart	<0.1	0.3	2.0	<0.1	1.5
ground carcass (eviscerated)	<0.1	2.1	4.9	0.1	1.7
blood plasma	<0.1	0.5	2.4	<0.1	0.5

[a] Slaughtered 72 h after an oral dose of 2 mg/kg. [b] Slaughtered 96 h after an oral dose of 30 mg/kg. [c] Slaughtered 72 h after an oral dose of 100 mg/kg. [d] Slaughtered 96 h after an iv dose of 1.5 mg/kg. [e] Slaughtered 72 h after an oral dose of 30 mg/kg. [f] Expressed as ppm equiv of PCNB in fresh tissue.

feces, and milk, respectively. No control experiments were conducted to determine the rate of recovery or stability of the PCNB or metabolites when subjected to these procedures. Metabolites were recovered from fat by the method of Kuchar et al. (1969). Cleanup was accomplished with Florisil columns as described by Johnson (1965), and final purification was done by GC and GC–MS.

RESULTS AND DISCUSSION

Excretion and Distribution of ^{14}C from [^{14}C]PCNB. The distribution of ^{14}C after oral dosing of goats and a sheep with [^{14}C]PCNB is shown in Table III. Caution is necessary in interpretation of apparent variation among animals receiving different levels of PCNB because adequate estimates of animal variation are not available. Also, the low level of milk production of goats dosed with 30 or 100 mg/kg further confounds any significance of the amounts of ^{14}C secreted with milk. There does appear to be a tendency for a greater proportion of ^{14}C to be excreted in the feces of animals receiving larger doses of PCNB.

Total recoveries of ^{14}C ranged from 88 to 96%. No $^{14}CO_2$ was found in the expired air of the one goat that was monitored after an oral dose of 2 mg of [^{14}C]PCNB/kg of body weight. Some ^{14}C was found in the condensate after freeze-drying feces samples (<1–5%). No correction was made for this loss and no attempt was made to characterize the ^{14}C in the condensate.

The concentrations of ^{14}C (as PCNB equivalents) in various tissues are given in Table IV. Bile contained the highest concentrations followed by fat, liver, and kidney. Concentrations in tissues appeared to be proportional to the size of the dose. Concentrations in the goat dosed intravenously were not different from one given a similar dose orally.

Urinary Metabolites of PCNB. Radioactive compounds isolated from urine are listed in Table V. The quantities are estimates because several factors made accurate measurements difficult, as will be discussed later. In the first chromatographic step (column 1, Figure 1), from 2 to 5% of the ^{14}C was not retained on the column or was eluted with the initial water elution. No effort was made to characterize the radioactive compounds in this fraction. The radioactive fraction that eluted with 100% methanol (1.4) contained PCA and small amounts of TCA. This fraction was analyzed directly by GC or GC–MS. Positive identifications were made by comparison of mass spectra of the isolated compounds with a standard. The amounts of PCA present were estimated from the first chromatographic step (Figure 1) where urine was applied directly to a Porapak Q column. The occurrence of PCA (and TCA) in urine as excreted may be in question because of the presence of easily hydrolyzable conjugates. Throughout further purification steps, free PCA and TCA were often observed when previous chromatographic

Table V. Radiolabeled Compounds Isolated from Urine after Oral [^{14}C]PCNB

	% of ^{14}C in urine				
	goats				sheep
	68 (2)[a]	71 (2)	80 (30)	93 (30)	321 (100)
PCA	19.5	15.3	9.9	15	10
TCA	+[b]	+	+	+	+
TCAP	2.6	3.0	1.9	+	+
PCA sulfamate	24.1	36.3	18.6	?	5[c]
glucuronide conjugates	26.2	23	20.5[d]	50[d]	40[d]
N-acetylcysteine conjugates			5.5		9.0
pentachlorothiophenol			+		+
pentachlorothioanisole			+		+
bis(methylthio)tetrachlorobenzene			+		
tetrachloro(methylthio)thiophenol			?	+	+
total	72.4	56.9	54.6	65	64

[a] Numbers in parentheses indicate the dose level as mg of PCNB/kg of body weight. [b] (+) indicates trace. [c] Not rigorously identified; see the text. [d] Includes both *N*- and *S*-glucuronides.

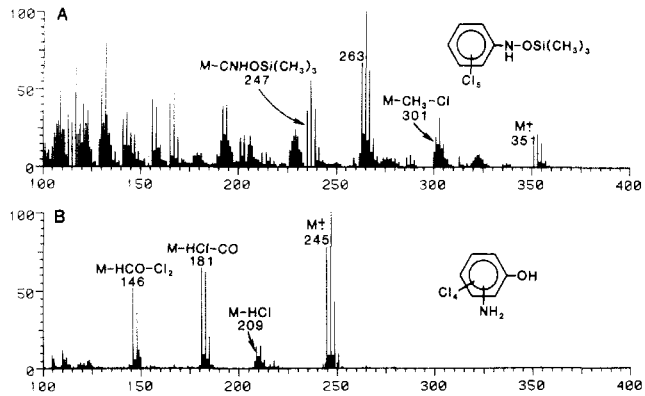

Figure 4. Mass spectra of metabolites identified as *N*-hydroxypentachloroaniline, Me_3Si derivative (A), and tetrachloroaminophenol (B).

properties of the ^{14}C in that fraction indicated a much more polar compound. When further purification of a relatively polar fraction yielded a single hydrolyzable conjugate of PCA and free PCA, the total amount of ^{14}C in that fraction was used as an estimate of the amount of the conjugate. However, when the fraction yielded more than one conjugate, no adjustments were made for the free PCA that was recovered in that step.

With sheep 321, fraction 1.4 contained two incompletely resolved radioactive peaks. The latter of these two unresolved peaks contained only PCA. TLC analysis of the other unresolved peak (6% of ^{14}C in urine) confirmed that the ^{14}C was not PCA (R_f = 0; silica with hexane–chloroform, 9:1). However, when this peak was anlzyed by GC using the usual conditions for PCA, only one radioactive peak with a retention time similar to PCA was observed. GC–MS confirmed that the radioactive peak was PCA. If the sample was derivatized with Regisil before GC, a small radioactive peak was observed in addition to the peaks corresponding to PCA and the silyl derivative of PCA. This suggested a labile metabolite was breaking down to PCA. GC–MS analysis of the derivatized sample produced spectra of PCA and of the silyl derivative of PCA and another spectrum shown in Figure 4a. The apparent molecular ion at m/z 351 with a five-chlorine cluster suggested that the compound could have been the silyl derivative of *N*-hydroxy-PCA. *N*-Hydroxy-PCA was synthesized and the mass spectrum of the silyl derivative of the synthetic material was very similar to the mass spectrum of the derivatized metabolite. The major difference was in the shape of the cluster at m/z 263, which was a typical five-chlorine cluster in the synthetic material but was distorted for some unknown reason in the spectrum of the metabolite. The ion at m/z 263 involves an unusual fragmentation. To form the ion, $OSi(CH_3)_3$ would have to be cleaved from the molecular ion and a hydrogen transferred to the remainder of the molecule to form PCA. This hydrogen did not come from the silyl group that was cleaved because the corresponding ion did not shift by the expected 1 mass unit in the mass spectrum of the deuteriotrimethylsilyl derivative of *N*-hydroxyaniline. The silyl derivative of *N*-hydroxy-PCA was unstable and also considerable breakdown apparently occurred in conventional GC injection systems. *N*-Hydroxy-PCA was observed only in the urine of sheep 321. However, its presence in some other urine samples may have been obscured by the presence of larger amounts of PCA (from which it was poorly separated in our isolation procedure).

Fraction 1.1 eluted with 50% methanol as a broad radioactive peak. After further purification on a LH-20 column eluted with 0.1% NH_4HCO_3, the radioactive component was identified by mass spectrometry as TCAP. The spectrum of a sample trapped from a GC is shown in Figure 4b. Small amounts of TCAP were also observed in fractions eluted from column 1 with 100% methanol.

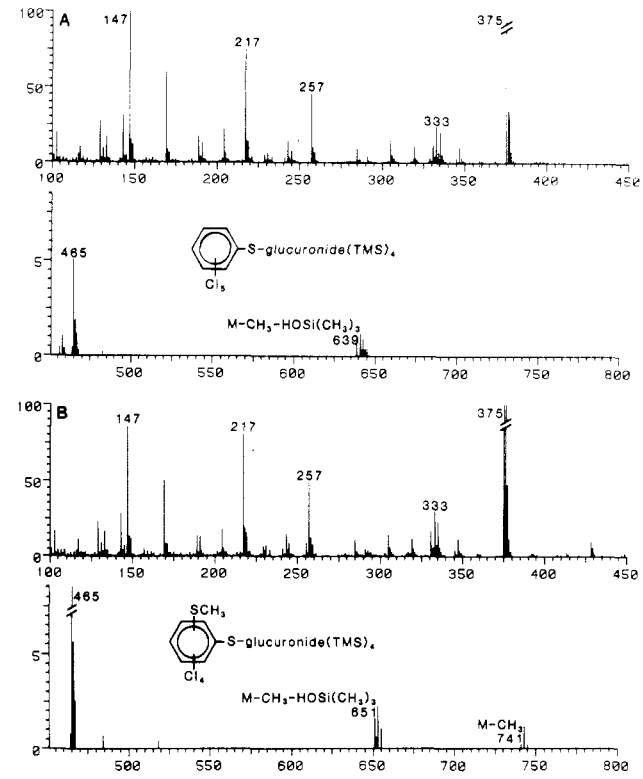

Figure 5. Mass spectra of metabolites identified as *S*-glucuronides of pentachlorothiophenol (A) and tetrachloro(methylthio)thiophenol (B), Me_3Si derivatives.

A major portion (65–75%) of the ^{14}C in urine was eluted from column 1 with 80% methanol (fraction 1.2). As shown in Figure 1, fraction 1.2 was resolved on column 2 into three fractions, 2.1, 2.2, and 2.3. The latter (2.3) required methanol for elution from column 2 and contained [^{14}C]PCA. Fraction 2.1 was desalted by using a Porapak Q column and further purified on a DEAE column. After being desalted again, the fraction was derivatized with Regisil. Samples from some animals contained only PCA glucuronide, which was identified by GC and GC–MS as trimethylsilyl derivatives. Mass spectra of silyl derivatives of *N*-glucuronide of PCA and their interpretation have been presented (Aschbacher et al., 1978). When fraction 2.1 from goats 80 and 93 and sheep 321 was treated similarly and analyzed by GC–MS, spectra were observed that contained fragment ions typical for per-tMS glucuronides (Bakke, 1976); however, fragments other than those expected from PCA *N*-glucuronide were present at higher m/z. Two compounds other than PCA *N*-glucuronide appeared to be present: one that was not separated from the PCA glucuronide by GC and another that had a slightly longer retention time. We concluded they were the *S*-glucuronides of pentachlorothiophenol and tetrachloro(methylthio)thiophenol. To obtain the spectra in Figure 5, *S*-glucuronides were separated from PCA glucuronide (fraction 2.1, goat 93) by HPLC before silylation and GC–MS. Appropriate molecular ions (m/z 744 and 756) were not visible, but typical clusters of chlorine containing fragments were present at M – 15 and/or M – 105. No other chlorine-containing fragments were observed. The fragments at m/z 331, 333, and 335 were not interpreted as a chlorine cluster because Mrochek and Rainey (1974) have reported similar clusters in spectra of Me_3Si derivatives of non-chlorine-containing glucuronides

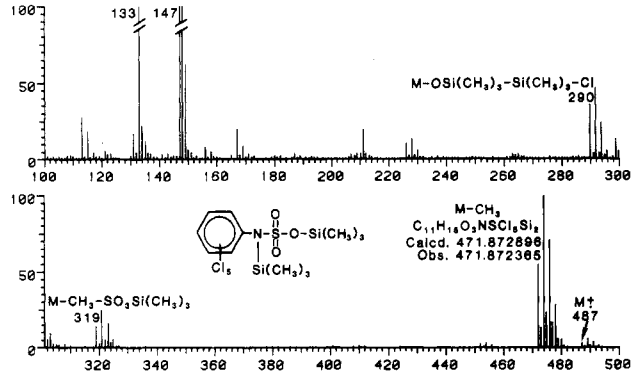

Figure 6. Mass spectrum of the metabolite identified as PCA sulfamate, Me_3Si derivative.

and also shown that the fragments at m/z 331 and 333 were derived from the glycon portion. The spectra in Figure 5 contain the glycon fragment ions typical of Me_3Si derivatives of aromatic *O*-glucuronides (Bakke, 1976); however, a fragment ion at M − 392 (loss of the glycon with a transfer of a Me_3Si to the aglycon), which is expected with *O*-glucuronides, was not observed. Estimates of the relative amounts of N- and S-glucuronides are only approximations because the glucuronides of PCA and pentachlorothiophenol were not separated by GC and *N*-glucuronides appear to be unstable under the HPLC conditions used. For goat 80 our approximation of the relative amounts of glucuronides of PCA, pentachlorothiophenol, and tetrachloro(methylthio)thiophenol are 73, 17, and 10% of the total glucuronide fraction. For goat 93, the *S*-glucuronides appeared to be present in amounts comparable to or larger than those of *N*-glucuronides. For sheep 321, the amounts of *S*-glucuronides were minor compared to *N*-glucuronides.

We concluded that radioactive compound in fraction 2.2 was the sulfamate of PCA based on the following information. After the final purification step, the ^{14}C in fraction 2.2 was converted quantitatively to PCA in a few hours if the pH was not maintained above 8. With time, breakdown to PCA occurred at pH 9. The possibility of a sulfamate was suspected because this is a known metabolite of aniline in rabbits (Parke, 1960). Attempts to synthesize PCA sulfamate were not successful. Synthetic routes that were effective for making sulfamates of aniline (aniline plus sulfur trioxide in chloroform followed by reaction with potassium hydroxide) were not effective for synthesis of PCA sulfamate. An IR spectrum of the metabolite was obtained after further purification on Avicel TLC; however, this evidence was inconclusive because no standard was available. After several attempts, a trimethylsilyl derivative was formed by reacting fraction 2.2 with Regisil. Upon GC (2 m by 2 mm i.d. 3% OV-17 on Gas-Chrom Q, 125–275 °C at 10 °C/min), most of the ^{14}C eluted with a retention time corresponding to the silyl derivative of PCA; however, a portion of the radioactivity emerged at approximately 225 °C. GC–MS analyses confirmed the identity of PCA and its silyl derivative but also produced the spectrum shown in Figure 6. The molecular ion at 487 with a five-chlorine cluster is compatible with the completely silyated sulfamate of PCA. Peak matching of the M − 15 fragment confirmed the proposed elemental composition (1.1 ppm from calculated). The mass spectral analyses were done on a sample isolated from goat 80 urine. Identification of PCA sulfamate in urine of other animals was based on column chromatographic and TLC properties. PCA sulfamate in sheep 321 was characterized by its typical elution from column 2. Several attempts to detect PCA sulfamate in urine from

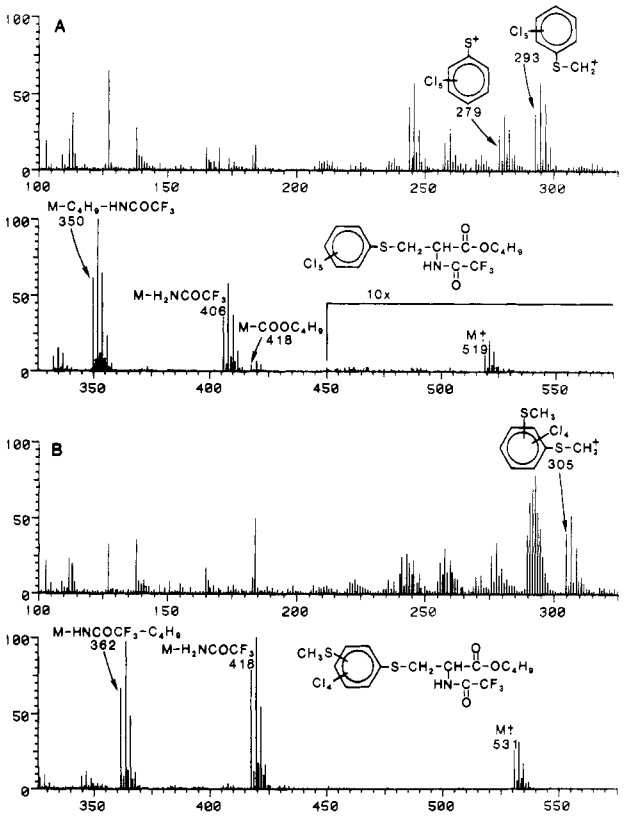

Figure 7. Mass spectra of metabolite derivatives identified as butyl esters of *S*-(pentachlorophenyl)-*N*-(trifluoroacetyl)cysteine (A) and *S*-[tetrachloro(methylthio)phenyl]-*N*-(trifluoroacetyl)-cysteine (B).

goat 93 were unsuccessful; however, acidification of the urine (pH 3.0; 3 h; 37 °C) increased the radioactivity eluted in the PCA-containing fraction of column 1 from 14 to 19.2% of the ^{14}C in the urine. PCA could arise from hydrolosis of the *N*-glucuronide of PCA as well as PCA sulfamate. We did not determine the effect of the acid hydrolosis procedure on the *N*-glucuronide.

Radioactivity corresponding to fraction 1.3 was observed only in urine from goat 80 and sheep 321. With urine from goat 80, fractions 1.2 and 1.3 were not fully resolved. Fraction 1.3 was chromtographed on a LH-20 with NH_4HCO_3 and the major radioactive fraction was then desalted with Poropak Q. After butylation and trifluoroacetylation, the spectra shown in Figure 7 were obtained from samples trapped from a GC and introduced into the mass spectrometer via a probe or from GC–MS of the derivatized sample. Molecular ions and fragmentation patterns consistent with the presence of the butyl ester of *S*-(pentachlorophenyl)-*N*-(trifluoroacetyl)cysteine and the butyl ester of *S*-[tetrachloro(methylthio)phenyl]-*N*-(trifluoroacetyl)cysteine were observed. These probably existed in the urine as *N*-acetylcysteine conjugates (with the acetyl group replaced by a trifluoroacetyl group during derivatization) because on other occasions when this fraction was derivatized with Regisil, the corresponding Me_3Si derivatives of the *N*-acetylcysteine conjugates were observed upon GC–MS. The pentachlorophenyl conjugate was present in a much larger amount relative to that of the tetrachloro(methylthio)phenyl conjugate. The GC–MS scans also contained spectra of trifluoroacetyl derivatives of pentachlorothiophenol and tetrachloro(methylthio)-thiophenol, which apparently formed during derivatization or chromatography.

When fraction 1.3 from sheep 321 was chromatographed on LH-20 with NH_4HCO_3, a small amount of radioactivity

Table VI. Radiolabeled Compounds Isolated from Feces after Oral [^{14}C]PCNB[a]

	goats				sheep
	68 (2)[b]	71 (2)	80 (30)	93 (100)	321 (30)
PCA	51	41	18	23	20
PCNB		1	38	39	20
unextractable	26	28	38	20	51
total	77	70	94	82	91

[a] Expressed as a percentage of the ^{14}C in feces. [b] Numbers in parentheses indicate dose level as mg of PCNB/kg of body weight.

eluted before the major peak containing the *N*-acetylcysteine conjugates. This fraction was subjected to GC–MS after derivatization with Regisil and several spectra with chlorine clusters were observed. One spectrum with an apparent molecular ion of m/z 363 and fragment ions at M – 15, M – 63, and M – 89 was compatible with the Me_3Si derivative of tetrachloroaminophenyl methyl sulfoxide, a proposed urinary metabolite of PCNB from rhesus monkeys (Kogel et al., 1979c).

Fecal Metabolites of PCNB. The ^{14}C-labeled compounds isolated and identified from feces are shown in Table VI. Identification was based on comparisons of chromatographic properties and mass spectra of isolated compounds with known compounds. The estimates of amounts of PCA and PCNB in feces are based on ^{14}C recovered in the final step of the isolation procedure. Unextractable ^{14}C was based on combustion analysis of the residue after extraction of feces with dichloromethane and methanol. After extraction with dichloromethane (Figure 2), an additional 10–20% of the ^{14}C was recovered by methanol extraction. This was either subjected to the same treatment as, or combined with, the dichloromethane extract. Except for small amounts of PCA, nothing was isolated from the 70% methanol phase (Figure 2), and this fraction contained most of the unidentified ^{14}C that was extractable. Most of the ^{14}C that eluted from the alumina column with methanol was demonstrated by rechromatographing on an alumina column to be either PCA or PCNB.

In some cases, the residue (after extraction with organic solvent) was suspended in 5 N sodium hydroxide at 60 °C for 3 h. Some additional ^{14}C was recovered in organic solvents after this treatment, but efforts to isolate a specific component were usually unsuccessful. The only exception was with feces from sheep 321. An additional 17.5% of the ^{14}C originally in the feces was recovered by extraction of the residue with methanol after base treatment. After some cleanup on a Porapak Q column, the sample was analyzed by GC–MS and several spectra were observed that contained clusters typical of ions with four or five chlorine atoms. Among those were spectra of PCNB, PCA, TCA, and tetrachlorothioanisole. Efforts to identify the other chlorine-containing compounds were limited because we observed them only in the feces of sheep 321.

Metabolites of PCNB in Milk and Fat. The concentrations of ^{14}C in milk were low, and attempts to isolate and identify this radioactivity were made only with milk secreted during first 24 h after an oral dose to goat 68. No satisfactory method was found to characterize the ^{14}C that remained in the aqueous phase of milk after extraction with hexane. However, a total of 72% of the ^{14}C in milk was recovered as PCA in procedures outlined in Figure 3. There was little evidence for chromatographic resolution with the XAD-2 column, which may be because milk is a complex suspension and not a true solution. The water eluate from the XAD-2 column formed a precipitate that contained most of the radioactivity in that fraction, and this ^{14}C was recovered by extraction with chloroform. As shown in Figure 3, all hexane fractions from partitionings were combined and chromatographed on an alumina column. Radioactivity was observed only in the benzene eluate and only PCA was observed upon TLC and GC. We concluded that the majority of ^{14}C in milk was associated with the PCA molecule, but the possibility exists that some (or all) of the [^{14}C]PCA is present as a labile conjugate such as the sulfamate. The ^{14}C recovered as PCA from the milk sample analyzed was the equivalent of approximately 0.4 ppm of PCA.

Extraction of visceral fat from goat 80 by the method of Kuchar (1969) recovered 63% of the ^{14}C in the sample. After additional cleanup on a Florisil column, a fraction that contained 43% of the ^{14}C in the fat sample was obtained. A single radioactive peak was observed upon GC, and mass spectral analysis confirmed the radioactive compound in this peak to be PCA. The ^{14}C recovered from the fat sample as PCA was the equivalent of approximately 1.6 ppm of PCA.

Metabolites Isolated after an iv Dose of PCNB. The methods outlined in Figure 1 were applied to the isolation of metabolites from urine of a goat dosed iv with PCNB. Elution patterns of radioactivity were quite different as compared to urine samples from a goat dosed orally. The only metabolites isolated and identified were the *S*-glucuronide conjugate of pentachlorothiophenol and TCA. PCA was not observed. Further efforts to isolate metabolites from a goat given [^{14}C]PCNB iv were not possible because the samples were lost as a result of a freezer failure.

Excretion of ^{14}C and ^{14}C Metabolites after Oral Dosing with [^{14}C]PCA. The nitro group of PCNB was reduced to an amine in all the metabolites isolated after an oral dose of 2 mg of PCNB/kg of body weight. In contrast, the limited data available indicated that the nitro group was not reduced when [^{14}C]PCNB was given iv. This suggested that the nitro group was reduced soon after ingestion, probalby in the rumen before absorption. A goat was dosed orally with [^{14}C]PCA (30 mg/kg of body weight) to see if metabolite patterns would be similar to those observed after administration of 2 mg of PCNB/kg of body weight. The distribution of ^{14}C 96 h after dosing was (as a percentage of the dose): urine, 66.5; feces, 10.3; GI tract, 2.0; eviscerated carcass, 14.7). The percentage of the dose excreted via the feces and urine was similar to that of goats dosed orally with 2 mg of PCNB/kg; however, the percentage of the dose remaining in the eviscerated carcass was 4–5 times greater than for any of the PCNB dose levels. Concentrations of ^{14}C in selected tissues were as followes (expressed as ppm equiv of PCA): liver, 3.4; bile, 10.2; visceral fat, 12; muscle, 1.2; eviscerated carcas, 5.6.

Metabolites were isolated as previously indicated for PCNB (Figures 1 and 2). Metabolites identified and the estimated amount (as a percentage of the ^{14}C in that sample) are as follows: for urine, PCA, 12, tetrachloroaminophenol, 4, PCA glucuronide, 20, and PCA sulfamate, 37; for feces, extractable, 87.5, unextractable, 13.5 (by assay of residue), and PCA, 80; for visceral fat, extractable, 71, and PCA, 56. The metabolites are similar qualitatively and quantitatively to those isolated after an oral dose of 2 mg of [^{14}C]PCNB/kg of body weight.

GENERAL DISCUSSION

Metabolism of PCNB by goats (and probably all ruminants) appears to follow two pathways that are shown in a proposed metabolic scheme in Figure 8. One involves

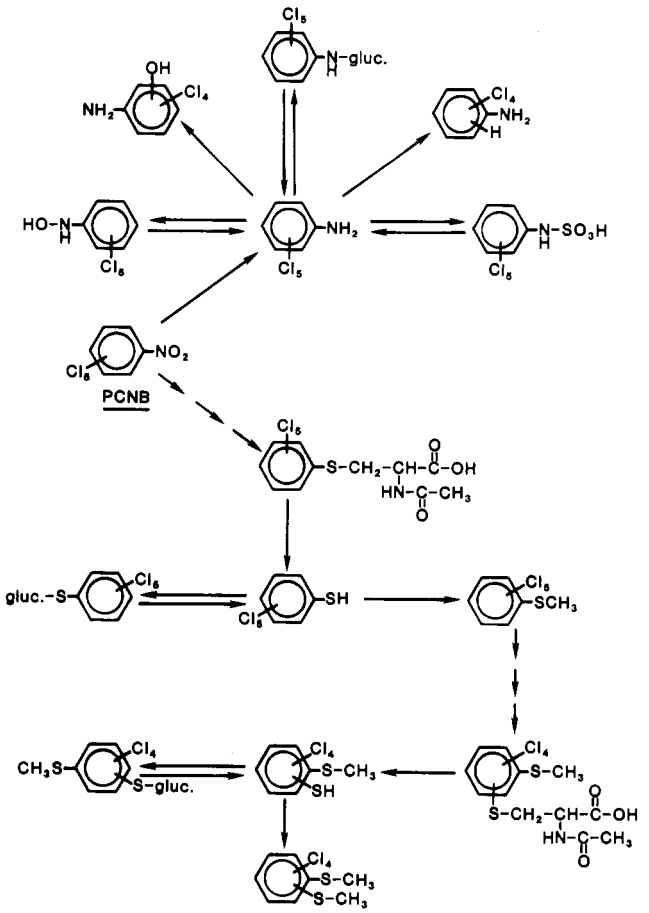

Figure 8. Proposed metabolic scheme for PCNB in ruminants.

the reduction of the nitro group to form PCA, which probably occurs in the rumen and throughout the gastrointestinal tract. After absorption of the PCA, the primary route is the formation of *N*-glucuronides and sulfamates of PCA that are excreted in the urine. A minor route of PCA metabolism is replacement of a chlorine with a hydroxyl to form TCAP, which is also excreted in the urine. Dechlorination to give TCA occurs to even a lesser extent. Goats receiving 2 mg of PCNB/kg of body weight metabolized PCNB primarily by reduction to PCA and formation of secondary products as described above. Only the metabolites discussed above and PCA were isolated from urine of these goats. PCA was the predominant metabolite isolated from feces along with small amounts of PCNB. Excretion patterns and metabolites isolated from goats dosed with 2 mg/kg PCNB were similar to those observed when a goat was dosed with 30 mg of [^{14}C]PCA/kg of body weight.

The second pathway results in metabolites in which the nitro group of PCNB is replaced by a sulfur-containing group such as a thiol, methylthio, *N*-acetylcysteine, and *S*-glucuronide or in which both the nitro and a chlorine have been replaced by these groups to form compounds such as the *S*-glucuronide of tetrachloro(methylthio)thiophenol, bis(methylthio)tetrachlorobenzene or *S*-[(methylthio)tetrachlorophenyl]-*N*-acetylcysteine. The data presented in this paper do not provide any evidence for the mechanisms involved in the formation of these metabolites in ruminants; however, there is evidence in rats that formation of these metabolites involves metabolism of mercapturic acid pathway metabolites by intestinal microflora and enterohepatic circulation (Bakke et al., 1981).

Our observations suggest that significant amounts of sulfur-containing metabolites are formed only when PCNB is absorbed intact, which may be dependent upon the level of intake. GC–MS of a fraction from sheep 321 urine (that contained primarily PCA) produced a spectrum similar to that reported by Kogel et al. (1979c) to be tetrachloroaminothioanisole, a metabolite of PCNB found in excreta of rhesus monkeys. As mentioned earlier, there was also evidence of tetrachloroaminophenyl methyl sulfoxide in urine of sheep 321. These were isolated in very small quantities. Reduction of the nitro group of PCNB to an amine could be a direct chemical reaction due to the low redox potential of the rumen. However, it is unlikely that a single dose of 30 mg of PCNB/kg of body weight would overwhelm the reductive capacity of the rumen. There is evidence for reduction of nitro groups on a phenyl ring by specific rumen microorganisms (Williams and Feil, 1971), and saturation of this type of a reaction would be more likely. Possibly the reduction observed in the present study could be an enzyme-catalyzed reaction mediated by specific microorganisms in the gastrointestinal tract.

Avrahami and White (1976) reported that PCNB (32 mg/kg of body weight) was excreted by sheep primarily as PCNB via the feces. In our study, a single sheep dosed at a similar rate with [^{14}C]PCNB excreted more than twice as much ^{14}C in the urine as the feces. The extractable ^{14}C in the feces was present as equal amounts of PCNB and PCA. The goat dosed at 30 mg/kg of body weight excreted approximately equal quantities of ^{14}C in urine and feces with about twice as much of the extractable ^{14}C in feces present as PCNB than as PCA. The results were similar for a goat dosed at 100 mg/kg of body weight. The apparent differences in results of Avrahami and White may be a reflection of the ease of detection of ^{14}C. Their procedures would not have detected many of the conjugates isolated from urine in our study. There is no ready explanation for the difference between the two studies in the relative amounts of PCA and PCNB in feces. The notable difference in procedures was that in our study the PCNB was mixed with a small amount of feed whereas Avrahami and White dosed with crystalline material in a gelatin capsule. The levels of PCA in fat were similar in the two studies.

The metabolism of PCNB by goats and sheep observed in this study is similar to that reported for nonruminants (Betts et al., 1955; Kuchar et al., 1969; Kogel et al., 1979a–c; O'Grodnick et al., 1981). One difference was that pentachlorophenol was a significant urinary metabolite in rhesus monkeys (Kogel et al., 1979c) and rats (O'Grodnick et al., 1981), but we found only traces in this study. Metabolites previously not identified by other workers include PCA sulfamate, *N*- and *S*-glucuronides, *N*-hydroxy-PCA, and TCAP; however, these are consistent with the general metabolic schemes previously proposed.

ACKNOWLEDGMENT

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Registry No. PCNB, 82-68-8; PCA *N*-glucuronide, 86823-19-0; PCA sulfamate, 86823-20-3; tetrachloroaminophenol, 86823-22-5; PCA, 527-20-8; pentachlorothiophenol, 133-49-3; pentachloro(methylthio)benzene, 1825-19-0; *S*-(pentachlorophenyl)-*N*-acetylcysteine, 68593-98-6; pentachlorothiophenol *S*-glucuronide, 86823-21-4; tetrachloro(methylthio)thiophenol *S*-glucuronide, 86823-24-7; bis(methylthio)tetrachlorobenzene, 36731-42-7; *S*-[(methylthio)tetrachlorophenyl]-*N*-acetylcysteine, 86823-23-6; TCA, 53014-40-7; *N*-hydroxy-PCA, 22876-48-8.

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2-Chlorotoluene Metabolism by Rats

Gary B. Quistad,* Kathleen M. Mulholland, and Gene C. Jamieson

When rats were given a single oral dose of 2-chloro[U-*ring*-^{14}C]toluene at 1 mg/kg, 85–92 and 5–8% of the applied ^{14}C were excreted in urine and feces, respectively. The major urinary metabolites were a glycine conjugate of 2-chlorobenzoic acid, a β-glucuronide of 2-chlorobenzyl alcohol, and a mercapturic acid, representing 20–23, 35–42, and 21–28% of the urinary ^{14}C, respectively. Within 4 days after dosage virtually all of the administered 2-chlorotoluene and its ^{14}C residues had been eliminated from the rats (<1% of applied dose in carcass). No significant metabolic differences were found between males and females.

Monochlorotoluenes are prepared by the catalyzed ring chlorination of toluene. The 2-chloro isomer is used extensively as a solvent and as a chemical intermediate in the synthesis of pesticides, dyes, and pharmaceuticals (Gelfand, 1979). Since workers are exposed occupationally to 2-chlorotoluene and environmental contamination could result in even greater human exposure, knowledge of the metabolic fate of 2-chlorotoluene could be useful in assessing its toxicity and environmental impact. Hence, we used the rat as a model for metabolic fate analysis.

Methyl group oxidation is the major metabolic pathway for toluene and xylenes, resulting in benzyl alcohol, benzoic acid, hippuric acid, benzoyl glucuronide, and their methylated analogues (van Doorn et al., 1981). Although mercapturic acids are minor metabolites of toluene, *m*-, and *p*-xylene (<2%), these thioethers contribute 21% of the applied dose for *o*-xylene which is isosteric with 2-chlorotoluene (van Doorn et al., 1981).

There are only a few limited studies on the metabolic fate of 2-chlorotoluene. This compound appears to be environmentally labile since bacteria have been isolated from a landfill waste site that are able to use 2-chlorotoluene as a sole carbon source (Vandenbergh et al., 1981). As early as 1903 Hildebrandt (1903) reported that 2-chlorotoluene is converted to 2-chlorohippuric and 2-chlorobenzoic acids by dogs and rabbits, respectively. Callow and Hele (1926) studied the likelihood that 2-chlorotoluene was metabolized by the mercapturic acid pathway in dogs by monitoring neutral sulfur excretion; they concluded that it was not involved in this pathway. More recently Wold and Emmerson (1974) reported on the metabolic fate of 2-chlorotoluene in rats given a relatively high dose (320 mg/kg). We initiated this work as an extensive examination of the metabolic fate of 2-chlorotoluene in rats in order to supplement and amplify on the data of Wold and Emmerson (1974), which is published in abstract form only with few details.

EXPERIMENTAL SECTION

Analytical Methods. Thin-layer chromatography (TLC) was performed with precoated, silica gel GF plates (Analtech), and radioactive metabolites were detected with a radiochromatogram scanner (Packard Model 7201). A Spectra-Physics 8000 instrument was used for reversed-phase liquid chromatography (LC): 10-μm LiChrosorb RP-8 column, 0.46 × 25 cm; ultraviolet detection at 254

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