Isolation and Identification of Propham (Isopropyl Carbanilate) Metabolites from the Rat and the Goat

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Rats and a goat were each given a single dose (100 mg/kg body wt) of isopropyl carbanilate-[phenyl-$^{14}C(U)$]. The primary route of elimination of ^{14}C was *via* the urine, with smaller amounts appearing in the feces and milk (goat). Goat urinary metabolites included the sulfate ester of isopropyl 4-hydroxycarbanilate, the glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate, another conjugated form of isopropyl 4-hydroxycarbanilate, a conjugate of isopropyl 3,4-dihydroxycarbanilate, the sulfate ester of isopropyl 2-hydroxycarbanilate, a conjugate of 4-hydroxyaniline, the sulfate ester of 2-hydroxyaniline, another conjugated form of 2-hydroxyaniline, the glucuronic acid conjugate of 4-hydroxyacetanilide, a conjugate of (2-hydroxyisopropyl)4-hydroxycarbanilate, and several other minor unidentified radiolabeled compounds. Rat urinary metabolites included the sulfate ester of isopropyl 4-hydroxycarbanilate, the glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate, the sulfate ester of 4-hydroxyacetanilide, and several other minor unidentified radiolabeled compounds.

Isopropyl carbanilate (propham, IPC) is a widely used pre- and postemergence herbicide. Therefore, it is likely that animals may be exposed to this compound or its metabolites either directly or indirectly through feedstuffs, and knowledge concerning its fate in animals is of environmental importance. Holder and Ryan (1968) and Bend *et al.* (1971) have isolated and identified one propham metabolite from the urine of rats. More recent work in our laboratory led to the isolation and identification of eight different metabolites of propham in the excreta of the chicken (Paulson *et al.*, 1972). Thus, it became apparent that either propham is metabolized quite differently by various animal species or that the metabolism of this compound is generally more complex than originally reported. The purpose of these studies was to investigate the metabolism of propham in the ruminant and to further investigate its metabolism in the monogastric animal.

EXPERIMENTAL SECTION

Chemicals and Supplies. The sources of chemicals and supplies were as follows: 2-hydroxynitrobenzene and 2-hydroxyacetanilide, Eastman Kodak Co.; Porapak Type Q, Waters Associates Inc.; prepacked "Permaphase ETH" columns, E. I. Du Pont de Nemours & Co., Instrument Products Division; isopropyl carbanilate and isopropyl carbanilate-[phenyl-$^{14}C(U)$], PPG Industries Inc. The chemical purity of the carbon-14-labeled compound was greater than 99%, as determined by comparison of its chromatographic behavior with that of authentic isopropyl carbanilate on silica gel thin-layer plates developed in hexane–ether (9:1, v/v). To further verify purity, the radiolabeled compound was acetylated (Sullivan *et al.*, 1967); the product migrated as a single radioactive component in the gas chromatograph. The infrared spectrum of the acetylated product was identical with the spectrum of authentic isopropyl *N*-acetylcarbanilate.

All other chemicals and supplies were obtained as described earlier (Paulson *et al.*, 1972).

Treatment of Animals. Six male Sprague–Dawley rats (weight 220–274 g) were given a single oral dose of isopropyl carbanilate-[phenyl-$^{14}C(U)$]. The doses were dissolved in approximately 0.59 g of polyethylene glycol 400 (average molecular weight 380 to 420) and administered by stomach tube. The dose contained from 6.78 to 8.64 μCi of carbon-14 and was made to supply 100 mg of propham/kg of body weight by the addition of an appropriate amount of the unlabeled compound. Water and feed (Purina Laboratory Chow) were provided *ad libitum*. Feces and urine were collected separately (Bakke *et al.*, 1967) at 6, 24, and 48 hr after dosing.

A lactating goat (weight 48 kg) was given a single dose of isopropyl carbanilate-[phenyl-$^{14}C(U)$]. The dose was dissolved in approximately 19 g of polyethylene glycol 400 and was given by rumen puncture. The dose contained 37.2 μCi of carbon-14 and was made to supply 100 mg of propham/kg of body weight by the addition of an appropriate amount of the unlabeled compound. Water, alfalfa hay, and oats were provided *ad libitum*. Feces, urine (*via* indwelling bladder catheter), and milk were collected at 6, 24, and 48 hr after dosing.

The urine, milk, and feces were analyzed for carbon-14 as previously described (Paulson and Feil, 1969). Tissue samples were freeze-dried and then combusted (100 to 200 mg of dry matter) in a model 305 Packard Tri-Carb sample oxidizer; the $^{14}CO_2$ was trapped and measured (Packard Technical Bulletin Number 18, 1969). The limit of detectability was defined as 2× background (approximately 2×10^{-5} % of the dose/g of dry goat tissue and approximately 2×10^{-3} % of the dose/g of dry rat tissue for the conditions used).

Chromatography. DEAE-Sephadex, Sephadex G-10, and Sephadex LH-20 columns were prepared and eluted as previously described (Paulson *et al.*, 1972). Porapak Q was swelled in H_2O and poured to form a 1 × 30 cm column; the sample was eluted with H_2O (flow rate approximately 0.1–0.2 ml/min). In one case (metabolite 1 from rat urine), the Porapak column was first washed with H_2O and then the metabolite was eluted with CH_3OH. The system used to monitor the column effluent for radioactivity and the fraction collection system were as previously described (Paulson *et al.*, 1970). The radioactivity in all column effluents was quantitated by liquid scintillation techniques (Paulson *et al.*, 1970). When the compound(s) placed on the column was not radiolabeled, the effluent was monitored with a Laboratory Data Control Model 1103 Refractomonitor.

High-pressure liquid chromatography (hplc) was performed with a DuPont Model 830 liquid chromatograph equipped with a uv photometer and a refractometer. Columns and conditions used will be outlined in later sections.

Gas–liquid chromatography (glc) was performed on a Barber-Colman series 5000 gas chromatograph using previously described columns and conditions (Paulson *et al.*, 1972).

Instrumental Analysis. Infrared (ir) spectra were recorded with a Model 337 Perkin-Elmer grating ir spectro-

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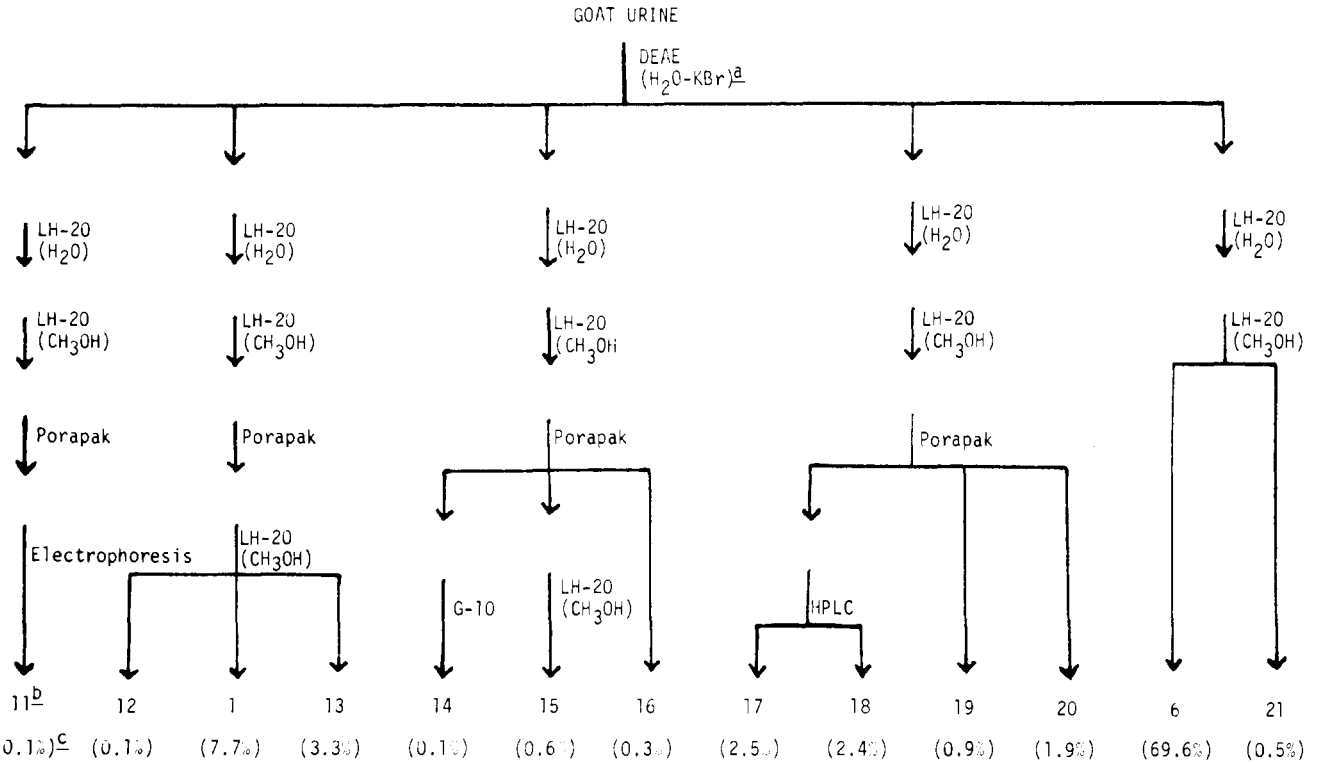


Figure 1. Separation and purification of radiolabeled metabolites in goat urine after dosing with isopropyl carbanilate (phenyl-^{14}C). [a] Fractions were eluted with increasing KBr concentrations as shown from left to right. [b] Code number for fraction. Fractions shown to be the same as those identified in rat or chicken urine (Paulson *et al.*, 1972) were given the same number. All unidentified fractions were given a different number. [c] Percent of the carbon-14 activity recovered.

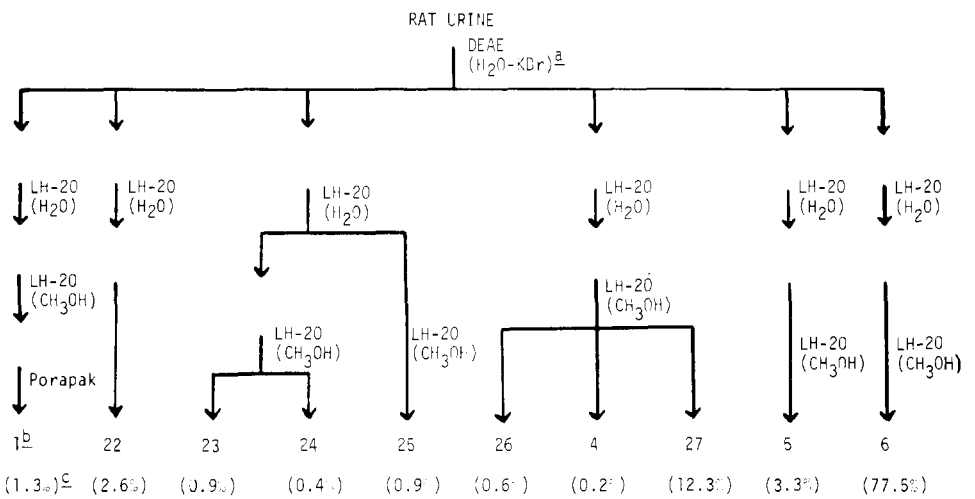


Figure 2. Separation and purification of radiolabeled metabolites in rat urine after dosing with isopropyl carbanilate (phenyl-^{14}C). [a] Fractions were eluted with increasing KBr concentration as shown from left to right. [b] Code number for fraction. Fractions shown to be the same as those identified in goat or chicken urine (Paulson *et al.*, 1972) were given the same number. All unidentified fractions were given a different number. [c] Percent of the carbon-14 activity recovered.

photometer using the micro KBr technique (1.5-mm disk with a 4× beam condenser). Mass spectral data were obtained with a Varian M-66 mass spectrometer equipped with a V5500 interface control console. Nuclear magnetic resonance (nmr) spectra were recorded with a Varian A-60A nmr spectrometer equipped with a Digilab FTS NMR-3 Fourier system.

Purification of Urinary Metabolites. Preliminary studies (ether extraction) indicated that there were no propham or relatively nonpolar metabolites in the urine. Aliquots of the urine (75 ml or less) collected from 0 to 6 hr after dosing were concentrated to a small volume under vacuum and placed on a DEAE-Sephadex column. Five radioactive fractions were eluted from the DEAE column by the H_2O-KBr gradient when goat urine was placed on the column (Figure 1), and six radioactive fractions were obtained when rat urine was placed on the column (Figure 2). Each radioactive fraction eluted from the DEAE column was evaporated to dryness under vacuum and then extracted with 25 to 50 ml of 1-butanol. Each butanol extract was then evaporated to dryness and further fractionated and purified on Sephadex LH-20 columns, Sephadex G-10 columns, and Porapak columns (Figures 1 and 2). Metabolites 17 and 18 in goat urine were separated by hplc using a 1-m "Permaphase ETH" column (mobile phase, hexane–ethanol 3:1, v/v; column temperature, ambient; column pressure, 600 psi; flow rate, 0.8 ml/min). Final purification of goat urinary metabolite 11 was accomplished using the Camag high voltage electrophoresis system (support, SS 2043B paper; buffer, pyridine–acetic

acid–H_2O, 1:1:89, v/v/v; potential, 3500 V; current, 107 mA; electrophoresis time, 25 min). The paper was scanned with a Packard model 7200 radio chromatogram scanner to determine the location of the carbon-14; the radiolabeled compound was eluted from the paper with CH_3OH. The average recovery was 89% for each of the various steps in the purification scheme; the values shown in Table IV show the percent distribution of the activity which was recovered.

Enzymatic Hydrolysis and Preparation of Derivatives of Metabolites. Metabolites were incubated with a mixture of β-glucuronidase and aryl sulfatase (*Helix pomatia*). Approximately 1 mg of metabolite was dissolved in 1 to 20 ml of 0.05 *M* Na acetate buffer (pH 4.75), and then 0.1 ml of enzyme (11,000 units of β-glucuronidase activity and 11,500 units of aryl sulfatase activity) was added. The mixture was incubated 16 to 18 hr at 38°; the reaction mixture was then extracted 3× with equal volumes of benzene. The benzene phase was dried over Na_2SO_4, evaporated to dryness, and derivatized.

Acetylation of intact metabolites, except metabolites 1, 11, and 20, and enzymatically hydrolyzed metabolites was performed by a previously described method (Paulson and Portnoy, 1970). Approximately 1.5 mg of metabolite 20, 2 ml of acetic anhydride, and 0.05 ml of methane-sulfonic acid were heated at 135° for 3 hr. The reaction mixture was poured into ice water and extracted with an equal volume of benzene; the benzene extract was washed with 1% $NaHCO_3$ and dried over Na_2SO_4. The acetylation products, unless otherwise stated, were purified by glc.

The acetylation, methylation, and subsequent characterization of aryl glucuronide conjugates by mass spectral analysis (metabolites 1 and 11) was accomplished by a previously described technique (Paulson *et al.*, 1973).

Synthesis of Chemicals. Several aromatic nitro compounds were reduced to the corresponding amino compounds. One gram of 10% palladium on carbon was added to a flask containing 1 to 3 g of the nitro compound in 150 ml of ethanol. The flask was purged with nitrogen, and then the H_2 (35–50 psi) was added at room temperature. When there was no further decrease in the H_2 pressure, the mixture was filtered through Nuchar to remove the palladium–carbon; the filtrate was evaporated to dryness under vacuum.

Anilines were reacted with isopropyl chloroformate (ICF) to produce the corresponding isopropyl carbanilates. A mixture of 0.01 mol of the amino compound and 0.1 mol of triethylamine in 20 ml of toluene (methanol was used to dissolve the potassium salt of compounds conjugated with glucuronic acid or with sulfate as shown for the synthesis of metabolites 1 and 20 in Table III) was cooled in an ice bath; 0.01 mol of ICF was added slowly so that the temperature did not exceed 15°. If the product was nonpolar, the mixture was transferred to a separatory funnel and washed several times with H_2O. The organic phase was then concentrated (usually to an oily residue), and the product was purified by glc. If the product was H_2O soluble, it was purified by column chromatography as described in a later section.

Unless otherwise stated (see Table III), phenols, anilines, and carbanilates were converted to their acetoxy and *N*-acetyl derivatives by a previously described procedure (Paulson and Portnoy, 1970).

The sulfate ester of isopropyl 4-hydroxycarbanilate (Table III) was prepared by reacting the phenol with $H_2S^{35}O_4$ and dicyclohexylcarbodiimide (DCC) (Hoiberg and Mumma, 1969). Other sulfate esters (Table III) were synthesized by reacting the phenol with chlorosulfonic acid (Feigenbaum and Neuberg, 1941).

4-Hydroxynitrobenzene was acetylated and then reduced to give 4-acetoxyaniline. The latter was reacted with ICF and then acetylated to give isopropyl 4-acetoxy-*N*-acetylcarbanilate. The final product was purified by glc. The same general synthetic scheme and purification technique was used to prepare isopropyl 3,4-diacetoxy-*N*-acetylcarbanilate from 3,4-dihydroxynitrobenzene.

Table I. Cumulative Elimination of Carbon-14 in the Feces, Urine, and Milk from the Rat and Goat after a Single Dose[a] of Isopropyl Carbanilate[Phenyl-^{14}C(U)]

	Rat		Goat		
Time, hr	Urine, % dose	Feces, % dose	Urine, % dose	Feces, % dose	Milk, % dose
6	47.33 ± 4.63[b]	0.23 ± 0.19	65.20	0.24	0.20
24	94.68 ± 0.98	1.98 ± 0.71	89.33	2.52	0.39
48	95.80 ± 0.82	2.33 ± 0.90	90.21	3.30	0.45

[a] Dose, 100 mg/kg of body weight; rats were dosed by stomach tube, and the goat was dosed by rumen puncture. [b] Mean ± standard error.

Isopropyl 2-acetoxy-*N*-acetylcarbanilate (uncorrected mp = 55.5°) was prepared by the following sequence of reactions: 2-acetoxynitrobenzene was reduced, then reacted with ICF, and finally acetylated (as described for metabolite 20). The mass, nmr, and ir data were in agreement with the assigned structure, isopropyl 2-acetoxy-*N*-acetylcarbanilate [*m/e* at 279, 237, 195, 153, and 109; (CCl_4) δ 1.15 (d, *J* = 6 Hz, 6 H), 2.2 (s, 3 H), 2.5 (s, 3 H), 4.6–5.2 (m, 1 H), 7.1–7.3 (m, 4 H); strong ir absorption at 1710, 1730, and 1170 cm^{-1}]. Further confirmatory evidence for the assigned structure was the observation that its ir spectrum was identical with that observed for the acetylation product (Paulson and Portnoy, 1970) of the sulfate ester of isopropyl 2-hydroxycarbanilate [see Table III for synthesis scheme of the latter compound and note the carbonyl absorption at 1730 cm^{-1} (Figure 3), which is in agreement with the assigned carbanilate structure, rather than indicating the presence of a carbonate linkage (Colthup *et al.*, 1964)]. It should be noted that subsequent comparative ir studies showed that the reduction product of 2-acetoxynitrobenzene was 2-hydroxyacetanilide; thus, it is uncertain whether the next intermediate in the synthesis scheme was isopropyl 2-hydroxy-*N*-acetylcarbanilate or isopropyl 2-acetoxycarbanilate. The rearrangement of 2-acetoxynitrobenzene to 2-hydroxyacetanilide under reducing conditions has been previously reported (LeGuyader and Peltier, 1965).

Acetylation of 4-hydroxyaniline and 2-hydroxyaniline gave 4-acetoxy *N,N*-diacetylaniline and 2-acetoxy *N,N*-diacetylaniline, respectively.

Isopropyl 4-hydroxycarbanilate was prepared by hydrolysis of isopropyl 4-acetoxycarbanilate (50 to 100 mg of the acetoxycarbanilate, 1 g of $NaHCO_3$, 100 ml of CH_3OH, and 1 ml of H_2O were held at room temperature for 24 hr).

4-Aminophenyl-β-glucuronide, prepared by the reduction of 4-nitrophenyl-β-D-glucuronide, was reacted with ICF to prepare the glucuronide conjugate of isopropyl 4-hydroxycarbanilate. The glucuronide conjugate of isopropyl 4-hydroxycarbanilate was acetylated and then methylated (Paulson *et al.*, 1973) to prepare the 6-methyl ester 2,3,4-tri-*O*-acetyl *N*-acetyl derivative; the latter was purified by glc.

4-Aminophenyl-β-D-glucuronide was acetylated and methylated (Paulson *et al.*, 1973) to prepare the 6-methyl ester 2,3,4-tri-*O*-acetyl *N*-acetyl derivative. The latter was purified by recrystallization from H_2O–CH_3OH, followed by LH-20-methanol chromatography and then glc.

The synthetic schemes for the preparation of a variety of polar compounds are outlined in Table III; the conditions used for the various reactions were as previously described.

Purification of Polar Synthetic Compounds. The six polar compounds synthesized (Table III) to confirm the structure of propham metabolites isolated from the goat and rat urine were all contaminated to varying degrees

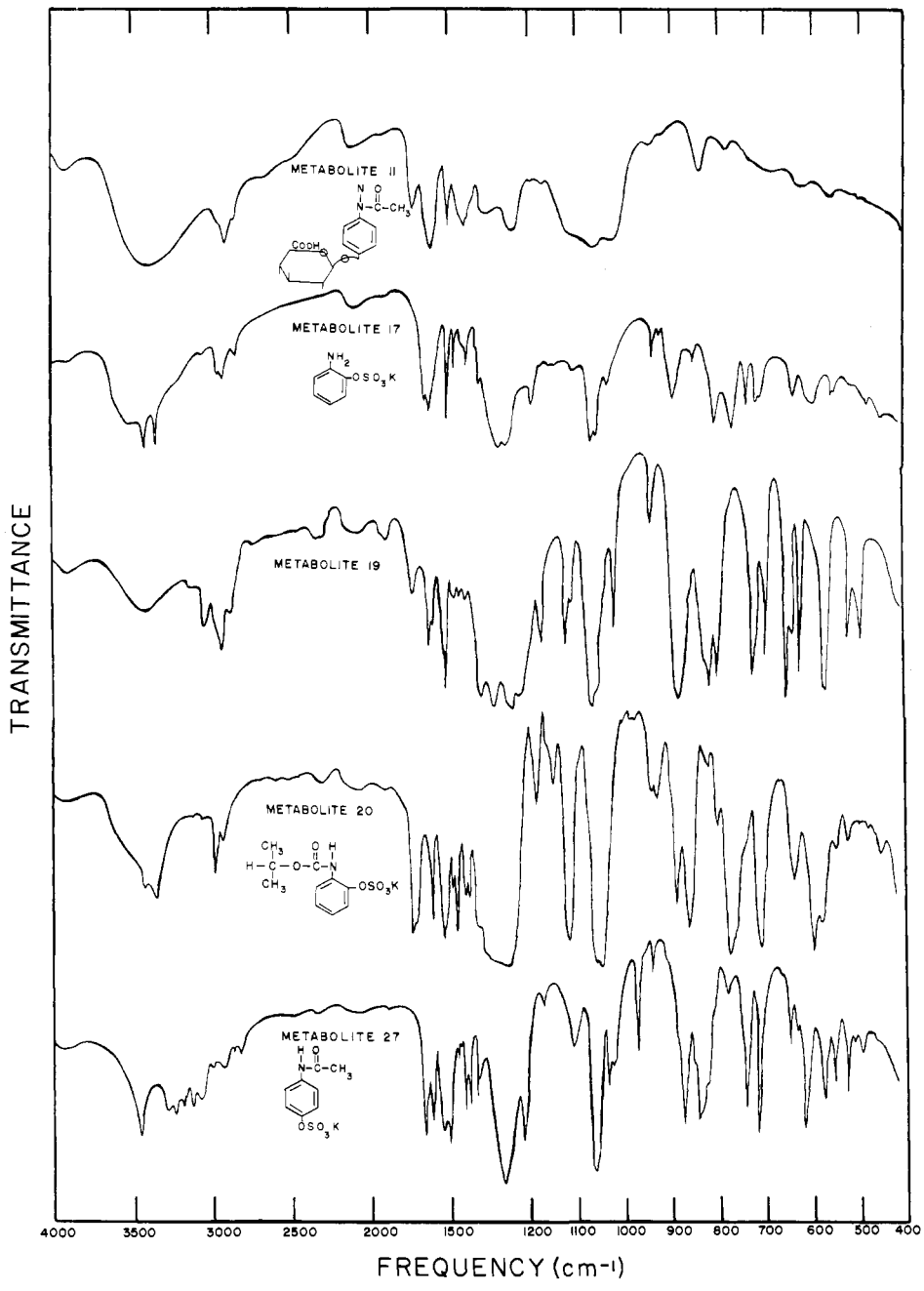


Figure 3. Infrared spectra for isopropyl carbanilate metabolites 11, 17, 19, 20, and 27 isolated from rat and goat urine.

and, therefore, required purification before being used as reference compounds. Isopropyl 4-hydroxycarbanilate-*O*-glucuronide (1), the glucuronide conjugate of 4-hydroxyacetanilide (11), and the sulfate ester of 4-hydroxyacetanilide (27) were purified on a 1 × 60 cm Sephadex LH-20 column eluted with methanol. The sulfate ester of isopropyl 4-hydroxycarbanilate (6) and the sulfate ester of isopropyl 2-hydroxycarbanilate (20) were purified by passing them through a Sephadex G-10 column eluted with H_2O. The sulfate ester of 2-hydroxyaniline (17) was purified by hplc using the conditions developed for final purification of metabolite 17 from goat urine.

RESULTS AND DISCUSSION

Radioactivity was rapidly eliminated in the urine of both the rat and the goat during the first 6 hr after the dose was given; during subsequent collection periods there were significant, but progressively smaller, amounts in the urine (Table I). The cumulative 48-hr excretion of carbon-14, expressed as a percent of the dose given, was 95.8 and 90.2% in the urine and 2.3 and 3.3% in the feces of the rat and goat, respectively (Table I). These results are similar to those obtained when rats (Bend *et al.*, 1971) and chickens (Paulson *et al.*, 1972) were dosed with radiolabeled propham.

The secretion of carbon-14 in the milk of the goat was greatest during the first 6 hr after dosing (Table I); during subsequent collection periods, the specific activity of the milk rapidly declined. The cumulative 48-hr secretion of carbon-14 in the goat's milk was 0.45% of the dose. The nature of the radioactive compound(s) in the milk is being studied in this laboratory at the present time.

The specific activity and the percentage of the dose in various body components 48 hr after dosing are shown in Table II. The liver was the tissue with the highest specific activity (expressed as percent of the dose/g of dry matter) in both the rat and goat; however, the relative specific activity of the heart, kidney, and intestine was quite different for the monogastric and ruminant animals. The specific activity of the gastrointestinal contents was higher

Table II. Carbon-14 in Tissues of the Rat and Goat 48 Hr after a Single Dose[a] of Isopropyl Carbanilate[Phenyl-^{14}C(U)]

Fraction	Goat		Rat	
	% dose/g of dry matter × 10^{-5}	% dose in total fraction	% dose/g of dry matter × 10^{-3}	% dose in total fraction
Liver	12	0.036	16 ± 4[b]	0.038 ± 0.009
Heart	<2[c]		6 ± 2	0.003 ± 0.002
Kidney	3	0.001	8 ± 2	0.005 ± 0.001
Intestine	2	0.007	12 ± 5	0.015 ± 0.003
GI contents	14	0.077	71 ± 17	0.074 ± 0.035
Remainder of carcass	6	0.407	7 ± 3	0.388 ± 0.162
Total		0.528		0.543 ± 0.174

[a] Dose, 100 mg/kg of body weight; the rats were dosed by stomach tube, and the goat was dosed by rumen puncture. [b] Mean ± standard error. [c] The limit of detectability was defined as 2× background (approximately 2×10^{-5} % of the dose per g of dry goat tissue and approximately 2×10^{-3} % of the dose per g of dry rat tissue for the conditions used).

than any of the measured body tissues on a dry weight basis; this may have resulted, at least in part, from biliary secretion of propham metabolite(s) into the GI tract (Bend *et al.*, 1971).

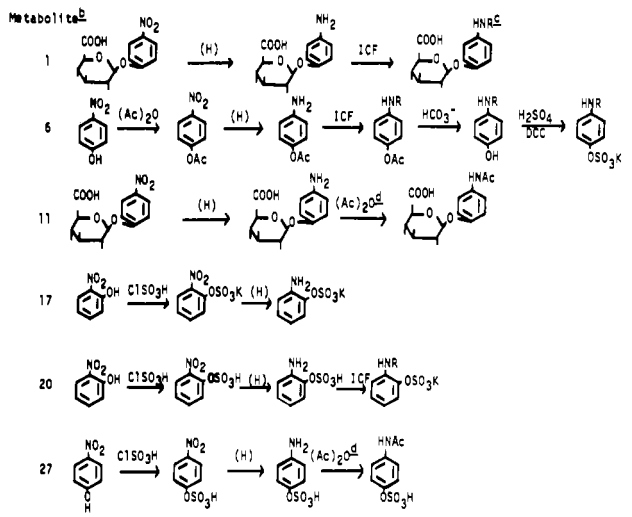
Thirteen radiolabeled metabolites were separated and purified from goat urine (Figure 1) and ten radiolabeled metabolites were obtained from rat urine (Figure 2). The same number was assigned to propham metabolites which were common to goat, rat, and chicken (Paulson *et al.*, 1972) urine. All unidentified fractions were assigned different numbers; it is quite possible, however, that one or more of the unidentified fractions from an animal may have been the same as one of the unidentified fractions from the other animals.

Metabolite 1, when subjected to enzyme hydrolysis followed by acetylation of the nonpolar hydrolysis product, yielded isopropyl 4-acetoxy-*N*-acetylcarbanilate. When intact metabolite 1 was acetylated and then methylated, the mass spectrum of the product showed intense peaks at *m/e* 317, 257, 215, 197, 155, and 127, which strongly indicated that derivatized glucuronic acid was a part of the molecule (Paulson *et al.*, 1973). Thus, these data indicated that metabolite 1 was the glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate; final confirmation of structure was by synthesis (Table III) and comparative ir spectrometry.

Metabolite 6, when enzymatically hydrolyzed and then acetylated or when acetylated directly, yielded isopropyl 4-acetoxy-*N*-acetylcarbanilate. The ir spectrum of intact metabolite 6 suggested the presence of a sulfate ester (strong absorption bands at 1000 to 1060 cm^{-1} and 1200 to 1300 cm^{-1}). These data indicated that metabolite 6 was the sulfate ester of isopropyl 4-hydroxycarbanilate; this structure was confirmed by synthesis (Table III) and comparative ir spectrometry.

Attempts to hydrolyze metabolite 11 enzymatically were unsuccessful. However, its chromatographic behavior and ir spectrum (Figure 3) indicated that it was probably a glucuronic acid conjugate (broad bands at 1010 to 1130 cm^{-1} and 1220 to 1300 cm^{-1}); thus, metabolite 11 was acetylated and then methylated, purified, and finally characterized by mass spectral analysis. The mass spectrum of the derivative showed intense peaks at *m/e* 317, 257, 215, 197, 155, and 127, which indicated that the conjugating group was glucuronic acid (Paulson *et al.*, 1973). The mass spectrum also showed intense peaks at *m/e* 109 and 151, which indicated that the metabolite contained a monosubstituted aniline; a detailed description of the characteristic fragmentation patterns of acetylated isopropyl carbanilate and related compounds has been reported

Table III. Confirmation of Structures of Metabolites by Synthesis



[a] See methods section for detailed description of synthetic reactions used. [b] Numbers were assigned to show their relationship to urinary metabolites. [c] R = O C - O - C - H(CH3)2 [d] The acetylation was accomplished by reacting the amino compound with acetic anhydride (1:1) for 1 hr at room temperature.

(Paulson *et al.*, 1972). The ir spectrum of metabolite 11 also indicated that the isopropyl side chain had been cleaved (absence of carbonyl absorption at 1700 to 1720 cm^{-1}). When metabolite 11 was acetylated with perdeuterioacetic anhydride and then methylated, the product gave a mass spectrum in which the peaks, due to the glucuronic acid moiety, were shifted as predicted (Paulson *et al.*, 1973); however, the peaks at *m/e* 109 and 151 were not shifted in the spectrum of the deuterium-substituted compound, which indicated that the *N*-acetyl group was of biological origin and that metabolite 11 was probably the glucuronic acid conjugate of 4-hydroxyacetanilide. This was confirmed by the synthesis of that compound (Table III) and comparison of its ir spectrum with that of metabolite 11 isolated from goat urine.

Metabolite 13 was enzymatically hydrolyzed and then acetylated and shown to yield isopropyl 4-acetoxy-*N*-acetylcarbanilate by comparative ir and mass spectrometry; thus, metabolite 13 contained the intact side chain and was substituted in the 4 position. The ir spectrum of the intact metabolite 13 was very similar to that of metabolite 1; however, these two metabolites were readily separated on a Sephadex LH-20 column eluted with methanol. Further studies to characterize the nature of the conjugating group in metabolite 13 were not successful.

Metabolite 14 yielded 2-acetoxy *N,N*-diacetylaniline when acetylated; acetylation with perdeuterioacetic anhydride, followed by purification and mass spectral analysis, clearly demonstrated that neither the acetyl groups nor the acetoxy group were of biological origin. Further attempts to characterize this metabolite were not successful.

When metabolite 17 was acetylated, the product was identified as 2-acetoxy *N,N*-diacetylaniline by comparing its ir spectrum with that of an authentic compound. These data and the presence of strong infrared absorption bands from 1050 to 1060 cm^{-1} and 1230 to 1300 cm^{-1} (Figure 3) indicated that metabolite 17 was the sulfate ester of 2-hydroxyaniline; this was confirmed by synthesis (Table III) and comparative ir spectrometry.

Metabolite 18 was converted to 4-acetoxy *N,N*-diacetylaniline by acetylation, which indicated that the isopropyl

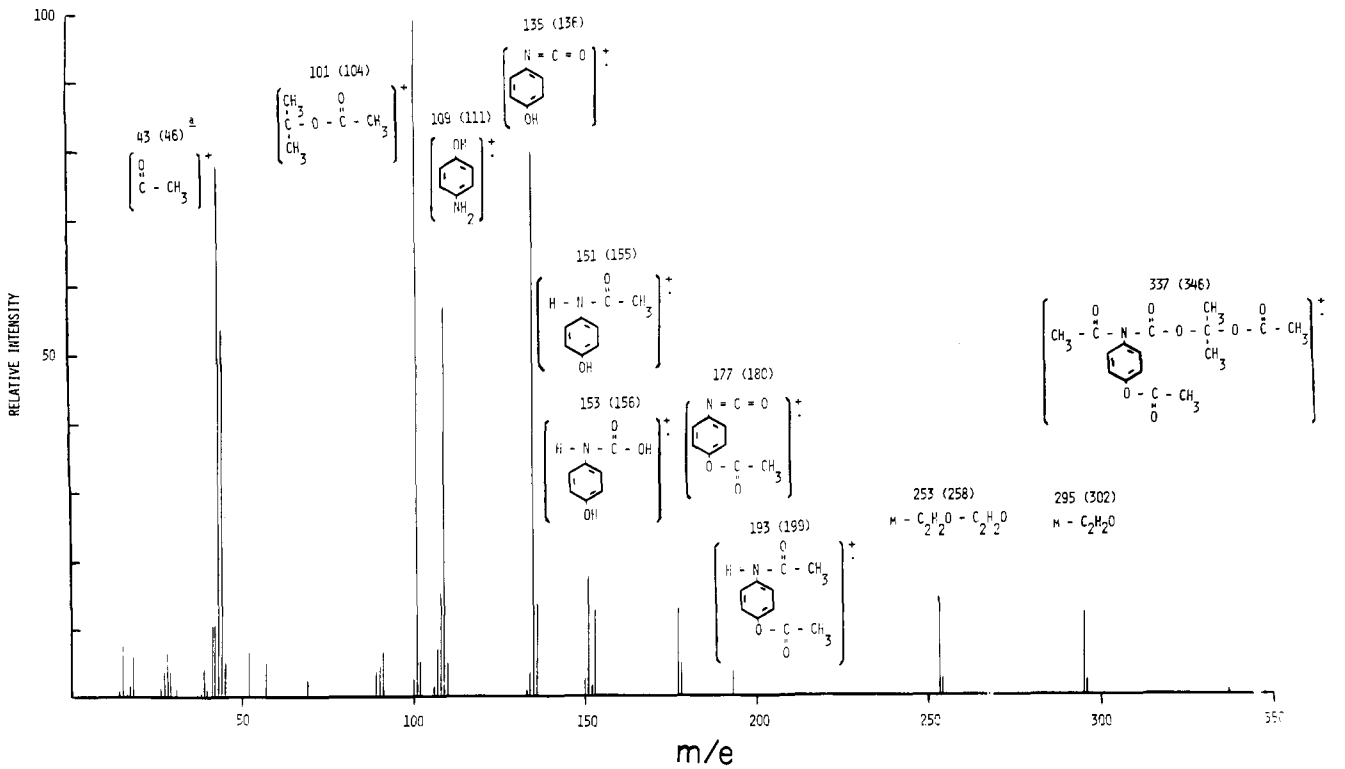


Figure 4. The mass spectrum and proposed fragments of acetylated metabolite 19. [a] The first value shows the mass (m/e) of the fragment from the acetylated compound and the value in parentheses shows the mass (m/e) of the fragment from the metabolite derivatized with perdeuterioacetic anhydride.

group had been cleaved and that there was hydroxylation in the 4 position. The infrared spectrum of the intact metabolite indicated that there was a sulfate ester present in the molecule; thus, it seems likely that metabolite 18 in goat urine may have been either the sulfate ester of 4-hydroxyaniline or the sulfate ester of 4-hydroxyacetanilide. However, additional attempts to further purify and identify this metabolite were without success.

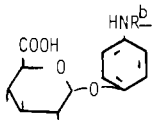
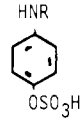
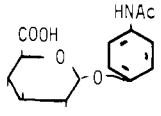
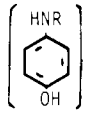
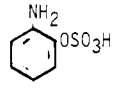
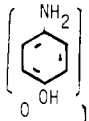
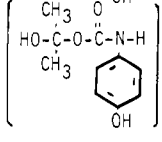
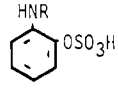
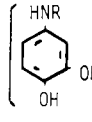
The nmr spectrum of metabolite 19 showed a singlet at δ 2.24 and a singlet at δ 7.05 (DMSO-d_6). The absence of a doublet in the δ 2–3 region indicated that the methine proton was absent. Metabolite 19 was then acetylated, purified by glc, and characterized by ir and mass spectral analyses. The mass spectrum of the acetylation product of metabolite 19, as well as the proposed structure and fragments, is shown in Figure 4. The base peak at m/e 101 strongly supported the previously described nmr data, which indicated that the methine proton was replaced. There were two losses of ketene to give peaks at 295 and 253, as would be predicted (Paulson *et al.*, 1972) for the proposed structure. The intense peak at m/e 109 was strong evidence that the aniline moiety had been monohydroxylated (Paulson *et al.*, 1972). The peaks at m/e 193, 177, 153, 151, and 135 were all compatible with the proposed structure (Figure 4) and further substantiated the conclusion that the molecule was a derivatized aminophenol. Additional evidence for the proposed structure was obtained by the acetylation of metabolite 19 with perdeuterioacetic anhydride, followed by purification and mass spectral analysis. The peaks for all of the proposed fragments were shifted, as would be required in the spectrum of the deuterium-substituted derivative (deuterium-substitution data are shown in Figure 4). The position of the acetoxy group on the aromatic ring was determined by comparing the ir spectrum of derivatized metabolite 19 with the spectra of isopropyl *N*-acetyl-2-acetoxycarbanilate (I), isopropyl *N*-acetyl-3-acetoxycarbanilate (II), and isopropyl *N*-acetyl-4-acetoxycarbanilate (III). The ir spectrum of derivatized metabolite 19 was nearly identical with that of III from 400 to 1200 cm^{-1}, as would be predicted for the proposed structure; in contrast, the ir spectra of both I and II were distinctly different in the 400 to 1200 cm^{-1} region. The ir spectrum of metabolite 19 (Figure 3) indicated that the conjugating group(s) was probably a sulfate ester (absorption from 1050 to 1070 cm^{-1} and 1200 to 1300 cm^{-1}); however, attempts to hydrolyze metabolite 19 with a mixture of glucuronidase and aryl sulfatase were not successful. Nevertheless, the evidence obtained indicated that metabolite 19 was a conjugated form of (2-hydroxyisopropyl)-4-hydroxycarbanilate, as shown in Table IV.

Metabolite 20 was acetylated and the product was identified as isopropyl 2-acetoxy-*N*-acetylcarbanilate by comparing its ir and mass spectra with those of the authentic compound. The ir spectrum of metabolite 20 (Figure 3) indicated that it contained a sulfate ester conjugate; thus, the sulfate ester of isopropyl 2-hydroxycarbanilate was synthesized as outlined in Table III. Comparison of the ir spectrum of metabolite 20 with that of the synthetic compound verified the assigned structure.

When metabolite 21 from goat urine was acetylated, the derivative was identified as isopropyl 3,4-diacetoxy *N*-acetylcarbanilate by comparative ir and mass spectrometry. The ir spectrum of the intact metabolite indicated the presence of a sulfate ester (strong absorption from 1050 to 1065 cm^{-1} and 1200 to 1300 cm^{-1}); thus, metabolite 21 may have been the mono- or disulfate ester of isopropyl 3,4-dihydroxycarbanilate. The 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate was identified in chicken urine (Paulson *et al.*, 1972). However, further efforts to completely purify and identify metabolite 21 in goat urine were not successful.

Metabolite 27 was acetylated and the product identified as 4-acetoxy *N,N*-diacetylaniline by comparative ir and mass spectrometry (peaks at m/e 235, 193, 151, and 109). The nmr spectrum of intact metabolite 27 indicated the presence of an *N*-acetyl group (D_2O), δ 2.1 (s, 3 H), 7.3–7.4 (m, 4 H). This was confirmed by acetylation of the metabolite with perdeuterioacetic anhydride followed by mass spectral analysis; the spectrum of the deuterium-

Table IV. Metabolites of Isopropyl Carbanilate in the Urine of the Rat and Goat

Metabolite	Structure	% of ^{14}C in urine[a] Rat	Goat
1		1.3	7.7
6		77.6	69.5
11		ND[c]	10.1
13	 conjugate	ND	3.3
14	 conjugate	ND	0.1
17		ND	2.5
18	 conjugate	ND	2.4
19	 conjugate	ND	0.9
20		ND	1.9
21	 conjugate	ND	0.5
27		12.3	ND

[a] The values show the percent distribution of the carbon-14 which was recovered. The average recovery was 89% for each of the various steps in the purification scheme (see Methods Section and Figures 1 and 2). [b] R = C(=O)OCH(CH$_3$)$_2$. [c] ND = not detected.

substituted compound was shifted as predicted (intense peaks at *m/e* 241, 197, 153, and 111). These data and the presence of strong ir absorption bands from 1030 to 1070 cm^{-1} and 1200 to 1300 cm^{-1} (Figure 3) indicated that metabolite 27 was the sulfate ester of 4-hydroxyacetanilide. This conclusion was verified by synthesis (Table III) and comparative ir spectrometry.

The ir spectrum of metabolite 5 from rat urine indicated that the isopropyl side chain was intact (strong absorption at 1710 cm^{-1}) and that a sulfate ester was present (strong absorption from 1050 to 1070 cm^{-1} and 1200 to 1300 cm^{-1}). The ir spectrum of this metabolite was very similar to that of the sulfate ester of isopropyl 3-methoxy-4-hydroxycarbanilate, which was previously observed in chicken urine. However, final purification and identification of metabolite 5 in rat urine was not accomplished.

These data and other recently reported information (Paulson *et al.*, 1972) clearly indicated that different animal species metabolize propham differently, and that its metabolism in the rat is more complex than previously reported (Bend *et al.*, 1972; Holder and Ryan, 1968). Hydroxylation at the 4 position of the aromatic ring was the primary route of metabolism of propham in the rat and goat (Table IV) and an important route for the metabolism of this compound in the chicken (Paulson *et al.*, 1972). Sulfate ester formation was the major type of conjugation of isopropyl 4-hydroxycarbanilate in all three species; however, the glucuronic acid conjugate of that compound was also of importance, especially for the chicken (Paulson *et al.*, 1972) and goat (Table IV).

Approximately 10 to 20% of the metabolism of propham involved cleavage of the carbamate side chain; most metabolites in which the side chain was cleaved were hydroxylated and conjugated at the 4 position, and in some cases also *N*-acetylated. It should be noted that all three animal species conjugated 4-hydroxyaniline differently. The chicken (Paulson *et al.*, 1972) excreted relatively large amounts of 4-aminophenyl sulfate, whereas this metabolite was not detected in the urine of the goat and rat. Instead, the sulfate ester of 4-hydroxyacetanilide was a major metabolite in rat urine, and the glucuronide conjugate of 4-hydroxyacetanilide was a metabolite in goat urine.

Propham was hydroxylated and conjugated at the 2 position by the goat; metabolites of this nature were not detected in rat and chicken urine. In contrast, hydroxylation and conjugation at the 3 position, a major route of metabolism of propham in the chicken, were not observed in the rat or goat.

Metabolites which contained the intact carbamate ester and which were substituted at both the 3 and 4 positions of the aromatic ring were of minor importance in the urine of the rat and goat (Table IV); however, metabolites of this nature were abundant in the urine of the chicken (Paulson *et al.*, 1972).

The unique metabolism of propham involving apparent hydroxylation of both the aromatic ring and the isopropyl group (metabolite 19) was observed only in the goat.

It is possible that the various modes of metabolism were operative in the different animals studied but were not detected (*i.e.*, the minor metabolites which were not identified may have been the end products of such metabolism). However, it is clear that, at least quantitatively, the metabolism of propham varied considerably in different animal species.

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Received for review April 9, 1973. Accepted July 9, 1973. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Metabolism of Lindane-^{14}C in the Rabbit: Ether-Soluble Urinary Metabolites

James C. Karapally, Jadu G. Saha,* and Young W. Lee

Uniformly labeled lindane-^{14}C (2.04 g) in gelatin capsules was fed to five rabbits over a period of 26 weeks. By the end of the feeding period, 54% of the administered radioactivity had been excreted in the urine and 13% in the feces. About 56% of the urinary metabolites were soluble in ether. From the ether-soluble urinary metabolites 2,3,5-, 2,4,5- and 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol were identified by comparing infrared spectra of the isolated chlorophenols and/or their anisole derivatives with those of the reference compounds. Three other metabolites, 2,3- and 2,4-dichlorophenol and 2,3,4,5-tetrachlorophenol, were identified by the gas chromatographic retention times and their mass spectra. Seven more chlorophenols and six chlorobenzenes were *tentatively* identified by their gas chromatographic retention times. These metabolites were: 2,5-, 2,6-, and 3,4-dichlorophenol, 2,3,4-, 2,3,6-, and 3,4,5-tetrachlorophenol, pentachlorophenol, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,4-, 1,2,4,5-, and/or 1,2,3,5-tetrachlorobenzene, and pentachlorobenzene.

Over the past two decades, there have been several reports on the degradation of lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) in soil, plants, insects, and animals. These studies have been reviewed by Menzie (1969). Lindane is slowly degraded by soil and 2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) has been the only product identified (Yule *et al.*, 1967). In plants, several metabolites of lindane have been indicated (Itokawa *et al.*, 1970) but only γ-PCCH has been identified (Bogdarina, 1957; San Antonio, 1959). Much work has been done on the metabolism of lindane in the housefly. While a significant portion of the metabolites still remains to be characterized, the following compounds have been identified: all the six isomers of dichlorothiophenol, 1,2,3- and 1,2,4-trichlorobenzene, 1,2,3,4- and 1,2,4,5-tetrachlorobenzene, pentachlorobenzene, γ-PCCH, and an isomer of PCCH (Menzie, 1969; Reed and Forgash, 1969, 1970).

Metabolism of lindane in mammals has been briefly studied with rats, rabbits, and dogs (van Asperen and Oppenoorth, 1954; Grover and Sims, 1965; Jondorf *et al.*, 1955; Koransky *et al.*, 1964; San Antonio, 1959). Although 1,2,4-trichlorobenzene, 2,4-dichlorophenylmercapturic acid, and 2,3,5- and 2,4,5-trichlorophenol have been identified, the characterization of all the metabolites is far from complete, as the identified products represented only a fraction of the total metabolites. After the present investigation was completed (Karapally *et al.*, 1971), Chadwick and Freal (1972) reported on the identification of six chlorophenols as urinary metabolites of lindane in the rat.

The object of the investigation reported here was to study the metabolism of orally administered lindane-^{14}C by rabbits. The results of the identification of ether-soluble urinary metabolites are reported here.

APPARATUS AND REAGENTS

A Nuclear Chicago Model Mark 1 liquid scintillation spectrometer was used to measure radioactivity. Infrared spectra were recorded on a Beckman 1R10 instrument equipped with beam attenuator and a beam condenser. The spectra were recorded in micro-KBr disks. Mass spectra were recorded with an AEI Model MS12 mass spectrometer.

Electron capture gas chromatography (ecgc) was done on an Aerograph Hi-Fy Model 600-D instrument. For thermal conductivity gas chromatography (tcgc), an Aerograph Model A90-P-3 gas chromatograph was used. The solid support for gas–liquid chromatography was 80–100-mesh acid-washed DMCS-treated Chromosorb W. All ecgc analyses were carried out with detector and injector temperatures of 210 and 190°, respectively, and a nitrogen flow rate of 30 ml/min. The following columns were used for gas chromatographic analyses: column a, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% SE-30 Chromosorb W; column b, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% QF-1 on Chromosorb W; column c, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% OV-17 on Chromosorb W; column d, 10 ft × ⅛ in. i.d. aluminum tube packed with a 5% QF-1 on Chromosorb W; and column e, 5 ft × 3/16 in. i.d. copper tube packed with 15% OV-17 on Chromosorb W. This column was used only for tcgc analysis with a detector temperature of 230° and He flow rate of 40 ml/min. All column temperatures are reported later at appropriate places.

All solvents were reagent grade and further purified by distillation. The following reference compounds were obtained from commercial sources and purified by distillation or recrystallization followed by tcgc: 1,2-, 1,3-, and 1,4-dichlorobenzene; 1,2,3-, 1,2,4-, and 1,3,5-trichlorobenzene; 1,2,3,4- and 1,2,4,5-tetrachlorobenzene; pentachlorobenzene; 2,4-, 2,5-, 2,3-, 2,6-, and 3,4-dichlorophenol; 2,4,5- and 2,4,6-trichlorophenol; 2,3,4,6-tetrachlorophenol, and pentachlorophenol (all from Canadian Laboratory Supplies Ltd., Winnipeg, Man.), and 2,3,6-trichlorophenol (Aldrich Chemical Co., Milwaukee, Wis.). All melting points are uncorrected.

1,2,3,5-Tetrachlorobenzene was prepared from 2,4,6-trichloroaniline by diazotization in dilute hydrochloric acid

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