Isolation and Identification of Propham (Isopropyl Carbanilate) Metabolites from Animal Tissues and Milk

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Chickens and a goat were given a single dose (100 mg/kg of body weight) of isopropyl carbanilate-*phenyl-U*-^{14}C. Radiolabeled metabolites in goat milk, goat tissue, and chicken tissue were isolated by solvent extraction and column chromatography and then identified by comparative spectrometry. Goat milk and goat tissue contained the sulfate ester of isopropyl 4-hydroxycarbanilate (A) and two minor unidentified ^{14}C-labeled fractions. Chicken tissue contained A, the glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate, isopropyl carbanilate, and two minor unidentified ^{14}C-labeled fractions.

Propham (isopropyl carbanilate) is an effective preemergent and postemergent herbicide. The effect of this compound on plants (Templeman and Sexton, 1945; Ennis, 1948) and animals (Timson, 1970) has been described. A large number of propham metabolites have been identified in the feces and urine of chickens (Paulson *et al.*, 1972), in rat urine (Bend *et al.*, 1971; Holder and Ryan, 1968; Paulson *et al.*, 1973), and in goat urine (Paulson *et al.*, 1973). Carbon-14 residues were observed in milk, eggs, and tissues after dosing animals with ring-labeled propham (Paulson *et al.*, 1972, 1973). The studies reported here were initiated to isolate and identify the propham metabolites in tissues and milk.

EXPERIMENTAL SECTION

Chemicals and Supplies. Isopropyl carbanilate and isopropyl carbanilate-*phenyl-U*-^{14}C were supplied by PPG Industries, Inc. The chromatographic behavior (Brinkman silica gel 254 thin-layer plates; solvent, hexane–ether (8:1, v/v); R_f 0.76) of the ^{14}C-labeled compound indicated that its radiochemical purity was more than 99%. The purity of the radiolabeled compound was tested further by acetylation (Sullivan *et al.*, 1967) followed by gas chromatography (Paulson *et al.*, 1972). The acetylation product migrated as a single radiolabeled component in the chromatograph, and its infrared spectrum was identical with that of authentic isopropyl *N*-acetylcarbanilate. The sources of other chemicals and supplies and the synthesis of propham metabolites and related derivatives have been described (Paulson *et al.*, 1972, 1973).

Treatment of Animals. Three mature Leghorn hens, each weighing from 1.63 to 1.71 kg, were each given a single oral dose of isopropyl carbanilate-*phenyl-U*-^{14}C. The doses were dissolved in polyethylene glycol 400 and given in a gelatin capsule. Each dose contained from 30.5 to 31.4 μCi of carbon-14 and was made to supply 100 mg of isopropyl carbanilate/kg of body weight by the addition of the appropriate amount of the unlabeled compound. The birds were fed a commercial 16% protein laying mash and H_2O *ad libitum* before and during the experimental period. The birds were sacrificed by decapitation 6 hr after dosing, and the feathers and all internal organs were removed. The remaining carcass was ground and stored at −6° until metabolite isolation studies were initiated.

A single dose of isopropyl carbanilate-*phenyl-U*-^{14}C dissolved in polyethylene glycol 400 was given by rumen puncture to a 48-kg lactating goat. The dose contained 37.2 μCi of carbon-14 and supplied 100 mg of propham/kg of body weight. The handling of the animal and collection of milk and tissue samples have been described (Paulson *et al.*, 1973). Milk samples were analyzed for carbon-14 by methods previously outlined (Paulson and Feil, 1969). Tissue samples were freeze-dried and analyzed for carbon-14 with a Model 305 Packard Tri-Carb sample oxidizer (Paulson *et al.*, 1973).

Instrumentation. Gas–liquid chromatography (glc) was done with a Barber-Colman Series 5000 gas chromatograph with previously described conditions (Paulson *et al.*, 1972). Infrared (ir) spectra were prepared with a Model 337 Perkin-Elmer grating ir spectrophotometer (micro-KBr technique; 1.5-mm disk with a 4× beam condenser). Instruments used to monitor column effluents for radioactivity and the fraction collection system have been described (Paulson *et al.*, 1970).

Purification of Metabolites. Goat milk (492 ml, collected 6 hr after dosing; contained 9.35 mg of propham equivalents) was evaporated to dryness, and the residue was extracted with three 400-ml aliquots of methanol. The methanol extracts were combined, concentrated to 100 ml under vacuum, and then held at 0° until a precipitate formed. The precipitate was then removed by filtration. Carbon-14 in the dried milk was quantitatively extracted by this procedure, and there was no loss of activity in the precipitate that was filtered off. The methanol solution was evaporated to dryness, and the residue was dissolved in 100 ml of water. The aqueous solution was then extracted three times with an equal volume of benzene, which removed 1.1% of the carbon-14. The benzene solution is shown as fraction C in Table I. The H_2O phase was concentrated to a small volume and placed on a 2.5 × 20 cm Sephadex DEAE column (DEAE was swelled in 1 *M* KBr, poured into the column, and then washed with 1–2 l. of water before use). The radioactivity was eluted with a KBr gradient (1000 ml of water in chamber 1 and 1000 ml of 1 *M* KBr in chamber 2; flow rate, approximately 0.2 ml/min; recovery of carbon-14, 90%). The radioactive fraction from the DEAE column was evaporated to dryness, and the carbon-14 was extracted from the residue with several small volumes of butanol (95% of carbon-14 extracted). The butanol extracts were combined and evaporated to dryness under vacuum. The residue was dissolved in a small amount of methanol, applied to a 1 × 60 cm LH-20 column, and eluted with methanol (LH-20, swelled and poured in methanol; flow rate, approximately 0.1 ml/min; recovery of carbon-14, 86%). Two radiolabeled fractions, A and D as shown in Table I, were eluted from the LH-20 column.

Ground goat carcass (11.2 kg, containing 7.1 mg of propham equivalents) was extracted with approximately 10 l. of methanol; 90% of the carbon-14 was extracted in this manner. The methanol extract was concentrated to a small volume, and then diethyl ether was added until a precipitate formed. The precipitate was removed by filtra-

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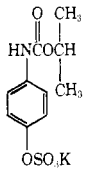
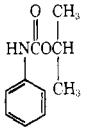
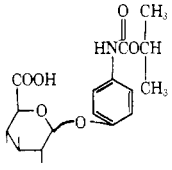
Table I. Radiolabeled Compounds in Goat Milk, Goat Carcass, and Chicken Carcass after Dosing the Goat and Chicken with Isopropyl Carbanilate-*phenyl*-*U*-^{14}C

Fraction(s)	Structure	Goat milk, %[a]	Goat carcass, %	Chicken carcass, %
A		98	77	15
E		ND[b]	ND	54
G		ND	ND	5
B, C, D, F, H, I[c]	Unknown(s)	2	23	26

[a] The values shown in this table give the percentage distribution of the carbon-14 in the milk or tissue which was recovered after completion of the separation and purification procedure. See the Experimental Section for percentage recovery in each step of the purification procedure. The milk (collected 6 hr after dosing) contained 0.196% of the administered carbon-14. The goat carcass contained 0.388% of the administered carbon-14 48 hr after dosing. The chicken carcass contained 7.9% of the administered carbon-14 6 hr after dosing. [b] ND, not detected. [c] Fraction B, goat carcass; fractions C and D, goat milk; fractions F, H, and I, chicken carcass, as described in the Experimental Section.

tion (77% of the carbon-14 in the extract remained in the filtrate). The filtrate was evaporated to dryness, and the residue was dissolved in a small volume of water and then applied to a 1 × 60 cm Sephadex G-10 column. The radioactivity was eluted with water (Sephadex G-10 was swelled and poured in water; flow rate, approximately 0.1 ml/min; recovery of carbon-14, 88%). The radioactive fraction from the Sephadex G-10 column was then further purified on a Sephadex LH-20 column (eluting solvent, methanol; flow rate, approximately 0.1 ml/min; recovery of carbon-14, 98%) and a Sephadex DEAE column (H_2O–KBr gradient as previously described; flow rate, approximately 0.2 ml/min; recovery of carbon-14, 88%). A Sephadex LH-20 column (eluting solvent, methanol; flow rate, approximately 0.1 ml/min; recovery of carbon-14, 93%) was used for final purification and separation of the radioactivity into two components (fractions A and B as shown in Table I).

Ground chicken carcass (857 g, containing 10.12 mg of propham equivalents) was extracted 3× with 2 l. of methanol. The methanol extraction procedure removed 85% of the carbon-14. The methanol extracts were combined and concentrated to a small volume, diethyl ether was added, and the precipitate which formed was removed by filtration. The filtrate contained 79% of the carbon-14. The filtrate was evaporated to dryness, and the residue was dissolved in water (adjusted to pH 1 with HCl) and extracted 3× with ether. The ether phase contained 72% of the carbon-14 and the aqueous phase 28%. The ether phase was concentrated to a small volume and applied to a 1 × 60 cm silica gel column and eluted with ether (silica gel, Woelm; flow rate, approximately 0.1 ml/min; recovery of carbon-14, 85%). The radioactive fraction from the silica column was evaporated to dryness; the residue was dissolved in methanol and applied to a 1 × 20 cm Sephadex LH-20 column. Two radioactive fractions (shown as fractions E and F in Table I) were eluted with methanol (flow rate, approximately 0.1 ml/min; recovery of carbon-14, 84%). The aqueous phase from the water–ether partition, described above, was evaporated to a small volume and placed on a 2.5 × 20 cm Sephadex G-10 column. The radioactivity was eluted with water (flow rate, approximately 0.2 ml/min; recovery of carbon-14, 93%). The radioactive fraction from the Sephadex G-10 column was concentrated to a small volume and placed on a Sephadex DEAE column (1 × 20 cm column; H_2O–1 *M* KBr gradient; flow rate, approximately 1 ml/min; recovery of carbon-14, 92%). Two radioactive fractions were eluted from the Sephadex DEAE column, and one of the fractions was further purified on a Sephadex LH-20 column (1 × 60 cm column; eluted with methanol as previously described; recovery of radioactivity, 94%). This radioactive component is shown as fraction A in Table I. The other radiolabeled fraction from the Sephadex DEAE column described above was evaporated to dryness and placed on a 1 × 60 cm Sephadex LH-20 column, and the radioactivity was eluted with methanol as described above (recovery of carbon-14, 81%); three radioactive components, shown as fractions G, H, and I in Table I, were eluted from the LH-20 column. Each of these fractions was further purified on a Porapak Q column (sample applied to 1 × 10 cm column which was washed with H_2O, and then the carbon-14 was eluted with methanol; flow rate, approximately 0.1 ml/min).

RESULTS AND DISCUSSION

The milk collected 6 hr after the goat was dosed contained 0.196% of the administered carbon-14; three different radioactive components were observed in the milk. Fraction A accounted for 98% of the activity that was recovered by the purification procedures used (Table I); this component was identified as the sulfate ester of isopropyl 4-hydroxycarbanilate by comparing its ir spectrum with that of an authentic sample (Paulson *et al.*, 1972). Fraction D gave an infrared spectrum that suggested that it may have been a glucuronic acid conjugate (broad absorption bands from 1000 to 1120 cm^{-1}, from 1200 to 1300 cm^{-1}, and from 1600 to 1730 cm^{-1}); however, there was not sufficient sample for further purification and characterization studies. No attempts were made to identify the radiolabeled benzene-soluble fraction C in goat milk because of insufficient sample.

The goat carcass contained 0.388% of the administered carbon-14 when the animal was sacrificed 48 hr after dosing; the methanol extraction procedure removed 90% of the activity from the ground carcass. Two radioactive components, metabolites A and B (Table I), were observed in the methanol extract of goat carcass. The infrared spectrum of fraction A from the goat carcass was nearly identical with that of an authentic sample of the sulfate ester of isopropyl 4-hydroxycarbanilate; absorption bands from 1030 to 1070 cm^{-1} and from 1200 to 1300 cm^{-1}, typical of this sulfate ester conjugate (Paulson *et al.*, 1972), were intense. Further evidence of structure was obtained by acetylation (Paulson and Portnoy, 1970) of that metabolite, followed by glc purification and comparative ir spectrometry. The ir spectrum of the resulting derivative was identical with that of isopropyl *N*-acetyl-4-acetoxycarbanilate (Paulson *et al.*, 1972). Thus, we concluded that metabolite A in the goat carcass was the sulfate ester of isopropyl 4-hydroxycarbanilate. Efforts to purify and

identify metabolite(s) B from goat carcass were unsuccessful.

Chicken carcasses contained 7.9 ± 1.7% (mean ± standard error) of the administered carbon-14 6 hr after the dose was given. Fraction E (Table I), the major radiolabeled fraction in the chicken carcass, was identified as the parent compound, propham; this proof was obtained by comparing its ir spectrum with that of an authentic compound. The other radiolabeled component(s) in chicken carcass that was ether soluble (fraction F, as shown in Table I) was not identified. The major polar metabolite in the chicken carcass (metabolite A) was identified as the sulfate ester of isopropyl 4-hydroxycarbanilate; the ir sepctrum of this metabolite was identical with that of the authentic compound (Paulson *et al.*, 1972). Metabolite G was identified as the glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate by comparative ir spectrometry (Paulson *et al.*, 1972). The infrared spectra of metabolites H and I suggested that they may have been glucuronide conjugates (broad absorption bands at 1000–1100, 1200–1300, and 1500–1730 cm^{-1}). However, attempts to further purify and characterize metabolites H and I were not successful, primarily because of the small sample size.

These studies have shown that the parent compound, isopropyl carbanilate, was the major radioactive component in chicken carcass (Table I); however, this compound was not detected in the goat carcass. This difference could have been due to the fact that the chickens were sacrificed 6 hr after dosing and the goat was sacrificed 48 hr after dosing. In an attempt to answer this question, we dosed another group of chickens with isopropyl carbanilate-*phenyl-U-*^{14}C at the rate of 100 mg/kg of body weight and then sacrificed them 48 hr later. The carcass contained 0.5% of the administered carbon-14. In contrast to the successful methanol extraction of almost all of the carbon-14 from chicken carcasses 6 hr after dosing and from goat carcasses 48 hr after dosing, methanol extracted only trace amounts of carbon-14 from carcasses of chickens sacrificed 48 hr after dosing. Methanol was shown to be the solvent of choice for extracting propham and its metabolites from all other milk and tissue samples studied. When methanol failed to extract the radioactivity from the carcasses of chickens sacrificed 48 hr after dosing, successive extractions with ether, acetone, and butanol were attempted. However, these solvents also failed to remove more than trace amounts of carbon-14. Thus, these observations suggest that the carbon-14 in the chicken tissues 48 hr after dosing was present as a different compound(s), and that species variation in the metabolism of propham may be even greater than earlier studies indicated (Paulson *et al.*, 1972, 1973).

The present studies showed that the carbamate side chain was intact in the major propham metabolites which were present in goat milk and tissues of the chicken and goat (Table I). Previous studies on propham metabolites in the urine of the chicken (Paulson *et al.*, 1972), rat, and goat (Paulson *et al.*, 1973) indicated that only 10–20% of the metabolism of propham involved cleavage of the carbamate linkage. The sulfate ester of isopropyl 4-hydroxycarbanilate was the predominant metabolite in the urine of the goat (Paulson *et al.*, 1973), rat (Bend *et al.*, 1971; Paulson *et al.*, 1973), and chicken (Paulson *et al.*, 1972). The present studies showed that this compound was the predominant propham metabolite in goat and chicken tissues and in goat milk. The glucuronic acid conjugate of isopropyl 4-hydroxycarbanilate accounted for 16% of the propham metabolites in chicken urine (Paulson *et al.*, 1972) and 10% of the propham metabolites in goat urine (Paulson *et al.*, 1973). This metabolite was a minor component in the chicken tissue but was not identified in goat tissue or milk; however, it is possible that one of the unidentified components (fractions B, C, or D) in milk and goat tissue may have been that metabolite. Dihydroxy metabolites, which accounted for approximately 20% of the propham metabolites in chicken urine (Paulson *et al.*, 1972), were not observed in chicken tissues; however, such metabolites may have been a part of the unidentified fractions F, H, and I.

Carbon-14 was detected in eggs after laying hens were dosed with isopropyl carbanilate-*phenyl-U-*^{14}C (Paulson *et al.*, 1972). In the present study, attempts were made to identify propham metabolites in eggs produced by hens dosed with isopropyl carbanilate-*phenyl-U-*^{14}C at the rate of 100 mg/kg of body weight. Approximately 50% of the carbon-14 in the eggs was extracted with methanol. Solvents such as ether, acetone, and butanol extracted little or no additional carbon-14. The radiolabeled metabolite(s) in the egg-CH_3OH extract was eluted from a Sephadex G-10 column with water and from a Sephadex LH-20 column by methanol, indicating that it was polar in nature. Additional attempts to completely purify and identify this metabolite(s) have not been successful. This is an area of research worthy of further investigation.

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