posed to the chromatogram obtained immediately following reconstitution of lyophilized fraction 11, the chromatogram of stored reconstituted fraction 11 contained a single radioactive spot (R_f = 0.59), far removed from a D-glucose standard (R_f = 0.24). These data suggested degradation of the sugar with storage at a slightly alkaline pH.

Since the metabolites of $^{14}CF_3$-labeled Preforan, both in the cells and culture medium, appeared to represent ^{14}C incorporation into natural products, fractions 13, 14, 15, 19, and 20 were not further investigated. ^{14}C incorporation into natural products has been reported in studies of $^{14}CF_3$-labeled trifluralin and benefin metabolism in plants (Kearney and Kaufman, 1969). Further studies of the fate of the 2-nitro-4-(trifluoromethyl)phenyl moiety of the Preforan molecule will necessitate the use of Preforan ring-labeled with ^{14}C in that moiety.

A simplified scheme for the metabolism of Preforan by tobacco cells in culture is presented in Figure 3. The metabolites produced from $^{14}C_1$- and $^{14}CF_3$-labeled Preforan by this rapid *in vitro* system are in good agreement with metabolites reported in whole plant studies (Geissbühler *et al.*, 1969; Kearney and Kaufman, 1969; Rogers, 1971). For this reason, the use of tobacco cells in suspension culture may prove a useful tool in studies of pesticide metabolism by plant tissue.

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Isopropyl Carbanilate (Propham) Metabolism in the Chicken: Balance Studies and Isolation and Identification of Excreted Metabolites

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Leghorn hens were given a single oral dose of either isopropyl-(2-^{14}C) carbanilate (A) or isopropyl carbanilate-[phenyl-^{14}C(U)] (B). Total carbon-14 excreted in the urine accounted for 79.8% of the activity given as A and 87.0% of the activity given as B during the 48-hr collection period; the fecal elimination accounted for 6.4 and 7.0% of carbon-14 given as A and B, respectively. When A was given, 6.7% of the carbon-14 was expired, whereas only trace amounts of expiratory carbon-14 were detected when B was given. Carbon-14 remaining in the hens 48 hr after dosing accounted for 1.2 and 1.5% of the activity given as B and A, respectively. Eggs collected for 12 days after dosing contained 0.075% of the carbon-14 given as a single oral dose of B. Urinary metabolites were identified as the glucuronide conjugate of isopropyl 4-hydroxycarbanilate (I), *p*-aminophenyl sulfate, the sulfate ester of isopropyl 3-methoxy-4-hydroxycarbanilate, the sulfate ester of isopropyl 4-hydroxycarbanilate (VI), the 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate (VII), an incompletely identified conjugated form of isopropyl 3,4-dihydroxycarbanilate, and the sulfate ester of isopropyl 3-hydroxycarbanilate. The feces contained I, VI, VII, and isopropyl 4-hydroxycarbanilate.

Isopropyl carbanilate (propham) is used extensively as a selective preemergent and postemergent herbicide. The effect of this compound on the growth of plants (Templeman and Sexton, 1945) and its effect at the cellular level in plants (Ennis, 1948) and animals (Timson, 1970) have been reported. The toxicology of propham in several animals has been investigated (FAO/WHO, 1964). More recently, Holder and Ryan (1968) reported that rats given unlabeled propham intraperitoneally excreted the sulfate ester of isopropyl 4-hydroxycarbanilate in the urine.

Since propham is widely used as a pesticide it seems likely that chickens and other farm animals may become exposed to this compound either directly or indirectly through feedstuffs.

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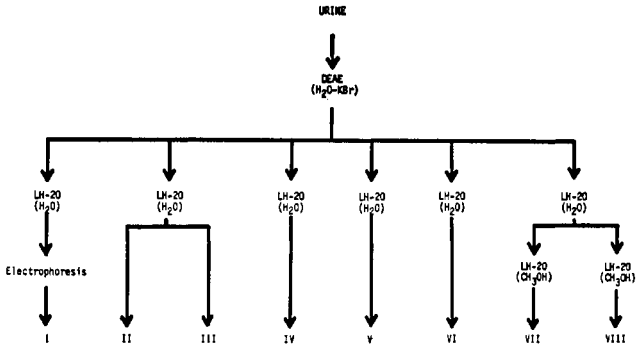


Figure 1. Separation and purification of radio-labeled metabolites in chicken urine after dosing with isopropyl carbanilate-[phenyl-^{14}C(U)]

The purposes of this study were: (1) to determine the rates and routes of elimination of propham or its metabolites by the chicken; (2) to determine the levels of tissue and egg residues; and (3) to isolate and identify propham metabolites in the feces and urine of the chicken.

EXPERIMENTAL

Chemicals and Supplies. The source of chemicals and supplies was as follows: guaiacol carbonate, polyethylene glycol 400, Matheson Coleman and Bell; *p*-nitrophenyl β-D-glucuronide, Pierce Chemical Co.; 10% palladium on carbon, Sargent-Welch Scientific Co.; DEAE Sephadex, Sephadex LH-20, Sephadex G-10, Pharmacia Fine Chemicals, Inc.; β-glucuronidase-aryl sulfatase (*Helix pomatia*), Calbiochem; OV-1, Gas Chrom Q, Chromosorb W, Applied Science Laboratories; Dexsil, Analabs Inc.; and SS 2043B electrophoresis paper, Camag Inc. Isopropyl carbanilate-[phenyl-^{14}C(U)], isopropyl-(2-^{14}C) carbanilate, and unlabeled isopropyl carbanilate were supplied by PPG Industries, Inc. The purity of the carbon-14-labeled compounds was greater than 99% as determined by comparing their 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 radio-labeled compounds were acetylated (Sullivan *et al.*, 1967); in both cases the product migrated as a single radioactive component in the gas chromatograph. The infrared spectra of the acetylated products were identical with the spectrum of authentic isopropyl *N*-acetylcarbanilate.

Treatment of Birds. Mature white Leghorn hens were surgically modified to facilitate separate collection of feces and urine (Paulson, 1969) and in all cases were fed a commercial 16% protein laying mash *ad libitum*. In experiment I, each of three hens was given a single oral dose of isopropyl-(2-^{14}C) carbanilate and each of three hens was given isopropyl carbanilate-[phenyl-^{14}C(U)]. The doses were dissolved in polyethylene glycol 400 and given in a gelatin capsule. Each dose contained from 9.5 to 11.4 μCi of carbon-14 and was made to supply 10 mg of isopropyl carbanilate per kg of body weight by the addition of the unlabeled compound. Feces, urine, and expiratory gases were collected (Paulson, 1969) at 6, 12, 24, 36, and 48 hr after the dose was given; the hens were sacrificed after 48 hr and tissues were removed. The expiratory gases, urine, 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; and the $^{14}CO_2$ was measured (Packard Technical Bulletin Number 18). Under the conditions used, the limit of detectability was approximately 0.0005% of the dose per g of dry matter.

In experiment II, three laying white Leghorn hens were each given a single oral dose of isopropyl carbanilate-[phenyl-^{14}C(U)]; each dose contained 10 mg of isopropyl carbanilate per kg of body weight (21.2 to 36.3 μCi of carbon-14). Eggs were collected for 12 days after dosing; the yolk and white were separated, freeze-dried, and analyzed for carbon-14 by the methods used for measuring carbon-14 in tissues.

In experiment III, four surgically modified hens were each given a single oral dose of isopropyl carbanilate-[phenyl-^{14}C-(U)] at the rate of 100 mg per kg of body weight (29.1 to 31.4 μCi of carbon-14). The urine and feces were collected for 6 hr after dosing and were used as the source of metabolites in characterization studies.

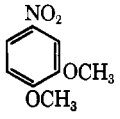
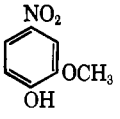
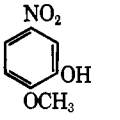
Column Chromatography. DEAE Sephadex was swelled in 1 *M* KBr and then poured to form a 2.5- × 25-cm column; the column was then washed with 1 to 2 l. of H_2O prior to use. The sample was applied in a small volume and eluted with a KBr gradient (1000 ml of H_2O in chamber 1 and 1000 ml of 1 *M* KBr in chamber 2, flow rate approximately 0.2 ml per min). Sephadex G-10 was swelled in H_2O and poured to form a 1- × 60-cm column; the sample was eluted with H_2O (flow rate approximately 0.1 ml per min). Sephadex LH-20 was swelled in either CH_3OH or H_2O and poured to form a 1- × 60-cm column; the sample was eluted with the solvent used to swell the gel (flow rate approximately 0.1 ml per min). 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 radio-labeled, the effluent was monitored with a Laboratory Data Control model 1103 Refractomonitor.

Gas–Liquid Chromatography. Gas–liquid chromatography (glc) was performed on a Barber-Colman Series 5000 gas chromatograph equipped with a hydrogen flame detector and a radioactivity monitoring system (column, 6 or 8 ft, 5-mm i.d.; support, Gas Chrom Q or Chromosorb W; liquid phase, 2% OV-1 or 5% Dexsil; helium flow rate, 55 ml per min; injection port temperature, 350°C; detector temperature, 350°C, temperature programmed from 120 to 325°C at 5°C per min). Capillary tubes were used for trapping compounds from the gas chromatograph.

Instrumental Analysis. Infrared spectra were taken with a model 337 Perkin-Elmer grating infrared spectrometer using the micro KBr technique (1.5-mm disk with a 4× beam condenser). Mass spectral data were obtained with a Varian M-66 mas spectrometer equipped with a V5500 interface control console. Nuclear magnetic resonance spectra were taken with a Varian A-60A nmr spectrometer.

Purification of Urinary Metabolites. The urine obtained in experiment III was concentrated under vacuum and placed on a DEAE Sephadex column. Six radioactive fractions were eluted from the DEAE column by the H_2O–KBr gradient; each fraction eluted from the DEAE column (Figure 1) was evaporated to dryness under vacuum. The radioactivity in the residue of each fraction was extracted with 25 to 50 ml of *n*-butanol; each butanol extract was then evaporated to dryness and further fractionated and purified on Sephadex LH-20 columns (Figure 1). Final purification of metabolite I was accomplished using the Camag high voltage electrophoresis system (Support, SS 2043B paper; buffer, pyridine–acetic acid–H_2O, 100:10:890, v/v/v; potential, 2000 V; current, 80 mA; electrophoresis time, 30 min). The paper was scanned

Table I. Nuclear Magnetic Resonance Spectral Data for 3,4-Dimethoxy-nitrobenzene, 3-Methoxy-4-hydroxy-nitrobenzene, and 3-Hydroxy-4-methoxy-nitrobenzene

Compound	Chemical shift in $CDCl_3$, ppm δ Proton				Upfield shift in benzene-d_6 ppm Proton			
	6	2	5	Methoxy	6	2	5	Methoxy
	7.91	7.73	6.98	3.98 3.97	0.23	0.21	0.83	0.78 0.77
	7.84	7.75	6.92	4.03	0.26	0.39	0.37	1.08
	7.87	7.77	6.97	3.98	0.11	0.21	0.99	1.00

with a Packard model 7200 radiochromatogram scanner to determine the location of the carbon-14; the radio-labeled compound was eluted from the paper with methanol.

Purification of Fecal Metabolites. The feces collected in experiment III were extracted 4× with 100 ml of methanol; the combined methanol extracts, which contained 64.5% of the activity originally in the fecal material, were evaporated to dryness under vacuum. The residue from the methanol extract was dissolved in 50 ml of H_2O, and the solution was extracted 3× with 50 ml of benzene. The benzene extract was concentrated to dryness under vacuum and acetylated and methylated as described in a later section. The acetoxy and methoxy derivatives were purified by glc and characterized by comparative infrared and mass spectrometry. The radioactive metabolites which remained in the aqueous phase after benzene extraction were separated and purified by DEAE and gel filtration chromatography, as shown in Figure 2.

Enzymatic Hydrolysis and Preparation of Derivatives of Metabolites. Metabolites were hydrolyzed with a mixture of β-glucuronidase and aryl sulfatase (*Helix pomatia*) as previously described (Paulson and Zehr, 1971). Acetylation of intact metabolites, methylated metabolites, and enzymatically hydrolyzed metabolites was performed by the method reported by Paulson and Portnoy (1970). The acetylated compounds were purified by glc. Methylation was accomplished by dissolving 1 to 3 mg of the metabolite or of the enzymatically hydrolyzed metabolite in approximately 0.5 ml of methanol and then adding a slight excess of ethereal diazomethane; the mixture was allowed to remain at room temperature for 15 min. The solvent was then evaporated under N_2 and the methyl derivative dissolved in benzene and purified by glc. When the methoxy derivatives were not soluble in benzene, they were dissolved in CH_3OH or H_2O and purified as described under "Purification of Polar Synthetic Compounds."

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 decline in the H_2 pressure, the mixture was filtered through Nuchar to remove the palladium–carbon, and the filtrate was evaporated to dryness under vacuum.

Aniline and aniline derivatives were reacted with isopropyl chloroformate (ICF) to produce the corresponding isopropyl carbanilates. A mixture of 0.01 mol of the amino compound and 0.01 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 I, V, and VII in Table VI) was cooled in an ice bath; 0.01 mol of ICF was added slowly so that the temperature did not exceed 15°C. If the desired 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 desired product was purified by glc as previously described; if the desired product was H_2O-soluble, it was purified by column chromatography as described in a later section.

Phenols and carbamates were converted to their acetoxy and *N*-acetyl derivatives by a previously described procedure (Paulson and Portnoy, 1970). Phenols were converted to their methoxy derivative by the procedure described under "Enzymatic Hydrolysis and Preparation of Derivatives of Metabolites."

The sulfate ester of several phenols was prepared by reacting the phenol with $H_2S^{35}O_4$ and dicyclohexylcarbodiimide (DCC), as previously described by Hoiberg and Mumma (1969). A few sulfate esters (Table VI) were synthesized by reacting the phenol with chlorosulfonic acid (Feigenbaum and Neuberg, 1941).

The methods of Pollecoff and Robinson (1918) were used for the synthesis of 3-methoxy-4-hydroxynitrobenzene from 3,4-dimethoxynitrobenzene and for the synthesis of 3-hydroxy-4-methoxynitrobenzene from guaiacol carbonate. The identity of these products was confirmed by observing the nmr spectra of these two compounds and 3,4-dimethoxynitrobenzene using deuterochloroform as the solvent and then comparing them with the spectra obtained when deuterobenzene was used as the solvent. The upfield shift for the ring protons and the methoxy protons in deuterobenzene is shown in Table I. As expected, there was a strong upfield shift in the absorption of the methoxy protons when deuterobenzene was the solvent (Fales and Warren, 1967). The two monomethoxy isomers had distinctly different aromatic absorption frequencies when measured in deuterobenzene. The assignment of the isomers (Table I) is compatible with the nmr spectra if the amount of solvent-induced upfield shift associated with a particular aromatic proton is assumed to result from its proximity to a methoxy group.

4-Acetoxynitrobenzene, prepared by acetylation of 4-hy-

Table II. Cumulative Elimination of Carbon-14 in Feces, Urine, and Expiratory Gases from the Chicken after a Single Oral Dose of Radio-Labeled Isopropyl Carbanilate (Experiment I)

Time, hours	Isopropyl-(2-)^{14}C carbanilate			Isopropyl carbanilate-[phenyl-^{14}C(U)]		
	Feces % dose	Urine % dose	Expiratory gases % dose	Feces % dose	Urine % dose	Expiratory gases % dose
6	2.8 ± 1.1[a]	37.3 ± 12.7	1.7 ± 1.1	3.2 ± 1.7	57.9 ± 4.4	0.0 ± 0.0
12	3.8 ± 1.0	54.5 ± 11.3	3.3 ± 2.3	5.8 ± 3.0	73.5 ± 2.4	0.1 ± 0.0
24	4.9 ± 0.8	74.3 ± 4.6	5.5 ± 4.2	6.4 ± 3.2	84.8 ± 2.2	0.1 ± 0.1
36	5.9 ± 0.5	78.2 ± 4.3	6.4 ± 5.0	6.9 ± 2.9	86.7 ± 3.7	0.1 ± 0.1
48	6.4 ± 0.5	79.8 ± 4.4	6.7 ± 5.2	7.0 ± 2.9	87.0 ± 3.8	0.2 ± 0.1

[a] Mean ± standard error.

Table III. Carbon-14 in Tissues from the Chicken 48 hr after a Single Oral Dose of Radio-Labeled Isopropyl Carbanilate (Experiment I)

Fraction	Isopropyl carbanilate-[phenyl-^{14}C(U)]		Isopropyl-(2-^{14}C) carbanilate	
	% dose/g of dry matter	% dose in total fraction	% dose/g of dry matter	% dose in total fraction
GI contents	0.112 ± 0.034	0.346 ± 0.114	0.064 ± 0.019	0.265 ± 0.094
Liver	0.025 ± 0.003	0.145 ± 0.021	0.020 ± 0.001	0.154 ± 0.003
Kidney	0.016 ± 0.005	0.020 ± 0.008	0.010 ± 0.001	0.024 ± 0.002
Blood	0.009 ± 0.005	0.051 ± 0.020	0.008 ± 0.004	0.044 ± 0.022
Lungs	0.005 ± 0.003	0.007 ± 0.004	0.012 ± 0.003	0.016 ± 0.003
Intestine	0.003 ± 0.001	0.030 ± 0.005	0.003 ± 0.000	0.031 ± 0.003
Gizzard	0.002 ± 0.001	0.015 ± 0.008	0.003 ± 0.001	0.024 ± 0.002
Remainder of carcass	0.002 ± 0.001	0.608 ± 0.307	0.002 ± 0.000	0.924 ± 0.131
Heart	0.002 ± 0.001	0.002 ± 0.001	0.003 ± 0.000	0.004 ± 0.000
Fat	0.001 ± 0.000		0.001 ± 0.000	
Total		1.234		1.486

droxynitrobenzene, was reduced to give 4-acetoxyaniline. The 4-acetoxyaniline was reacted with ICF to give isopropyl 4-acetoxycarbanilate; this product was 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 and isopropyl 3-acetoxy-*N*-acetylcarbanilate from 3,4-dihydroxynitrobenzene and 3-hydroxynitrobenzene, respectively. Acetylation of 4-hydroxy aniline gave 4-acetoxy-*N*,*N*-diacetylaniline.

Isopropyl 3-methoxycarbanilate, isopropyl 4-methoxycarbanilate, and isopropyl 3,4-dimethoxycarbanilate were prepared by reacting ICF with the appropriate methoxy aniline; the products were purified by glc.

3-Methoxy-4-hydroxynitrobenzene was acetylated and then reduced to give 3-methoxy-4-acetoxyaniline; the latter compound was reacted with ICF to give isopropyl 3-methoxy-4-acetoxycarbanilate. The isopropyl 3-methoxy-4-acetoxycarbanilate was acetylated to give isopropyl 3-methoxy-4-acetoxy-*N*-acetylcarbanilate. The same sequence of reactions was used to synthesize isopropyl 3-acetoxy-4-methoxy-*N*-acetylcarbanilate from 3-hydroxy-4-methoxynitrobenzene; the final products were purified by glc.

Isopropyl 4-hydroxycarbanilate and isopropyl 3-hydroxycarbanilate were prepared by hydrolysis of isopropyl 4-acetoxycarbanilate and isopropyl 3-acetoxycarbanilate, respectively (50 to 100 mg of the acetoxycarbanilate, 1 g of $NaHCO_3$, 100 ml of CH_3OH, and 1 ml of H_2O at room temperature for 24 hr). The products were identified by infrared analysis and by preparation of acetoxy derivatives.

Purification of Polar Synthetic Compounds. Six polar compounds wree synthesized, as outlined in Table VI, to confirm the structure of IPC metabolites isolated from the chicken urine. All of the synthetic products were contaminated to varying degrees; in each case they were purified by column chromatography. Isopropyl 4-hydroxycarbanilate *O*-glucuronide (I) was purified on 1- × 60-cm Sephadex LH-20 column eluted with methanol. The sulfate ester of isopropyl 3-methoxy-4-hydroxycarbanilate (V) was purified by passing it through the following sequence of columns: Sephadex LH-20 eluted with H_2O; Sephadex LH-20 eluted with methanol; Sephadex G-10 eluted with H_2O. The sulfate ester of isopropyl 3-hydroxy-4-methoxycarbanilate was purified by passing it through the following sequence of columns: Sephadex LH-20 eluted with methanol; Sephadex LH-20 eluted with H_2O; Sephadex LH-20 eluted with methanol; Sephadex G-10 eluted with H_2O. *p*-Amino phenylsulfate (IV) was purified with a Sephadex LH-20 column eluted with methanol. The sulfate ester of isopropyl 3-hydroxycarbanilate (VIII) and the sulfate ester of isopropyl 4-hydroxycarbanilate (VI) were purified by passing them through a Sephadex G-10 column eluted with H_2O.

RESULTS AND DISCUSSION

Carbon-14 from both the phenyl-labeled and isopropyl-labeled propham was rapidly excreted in the urine during the first 6 hr after the dose was given (experiment I, Table II); during subsequent collection periods there were significant, but progressively smaller, amounts in the urine. The cumulative 48-hr urinary excretions of carbon-14 from the isopropyl-labeled and phenyl-labeled compounds were 79.8 and 87.0% of the dose, respectively. The cumulative fecal excretion during the 48-hr collection period accounted for 6.4 and 7.0% of the carbon-14 given as the isopropyl- and phenyl-labeled compounds, respectively. Approximately 7% of the radioactivity given as the isopropyl-labeled compound was eliminated in the expiratory gases during the 48-hr collection period, whereas only trace amounts of radioactivity from the

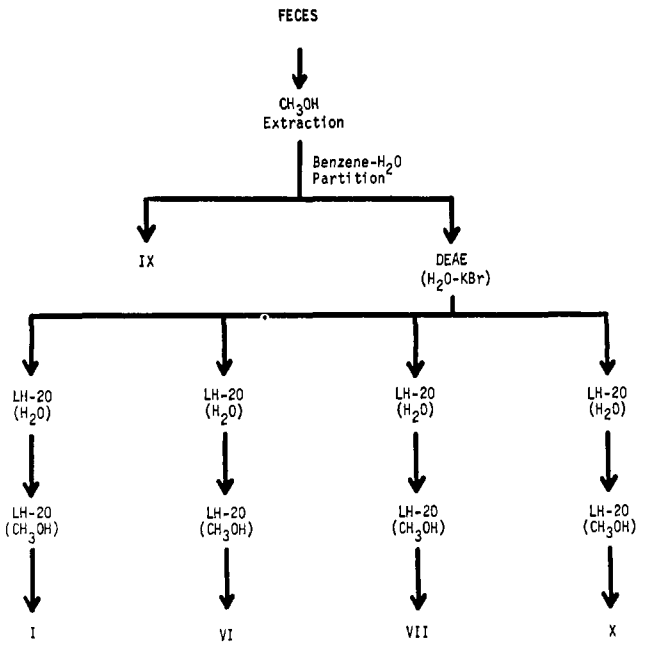


Figure 2. Separation and purification of radio-labeled metabolites in chicken feces after dosing with isopropyl carbanilate-[phenyl-^{14}C(U)]

phenyl-labeled compound were detected in that fraction. Thus, this experiment indicated that the metabolism of at least 6.5% of the propham involved cleavage of the isopropyl side chain.

The specific activity and the percentage of the dose in various tissues 48 hr after the dose was given are shown in Table III (experiment I). Approximately 1.2 and 1.5% of the carbon-14 given as the phenyl-labeled and isopropyl-labeled compound, respectively, remained in the hens 48 hr after the dose was given. The specific activity was highest in the liver, kidney, and blood.

Less than 0.1% of the carbon given as the phenyl-labeled compound was present in eggs collected for 12 days after dosing (experiment II, Table IV). The specific activity in the yolk was highest 4 days after the dose was given and then declined slowly. The specific activity in the egg white was highest on the first day after dosing and then rapidly declined to trace amounts. The differences in the rate of accumulation and decline of residues in the yolk and white probably reflected the different site and time of formation of these two parts of the egg.

In experiment III, the average urinary excretion of carbon-14 during the 6-hr collection period was 49% of the administered dose. Preliminary investigations showed that no appreciable amounts of radioactivity were removed from the urine by benzene or ether extraction. Eight radio-labeled urinary metabolites were separated and purified by column chromatography and electrophoresis (Figure 1). The recovery of radioactivity applied to the various columns ranged from 89 to 105%. The percent of the total carbon-14 in the urine which each of the eight metabolites accounted for is shown in Table VII.

The average fecal elimination of carbon-14 during the 0- to 6-hr collection period in experiment III was 0.6% of the administered dose; methanol extraction removed 64.5% of the radioactivity from the feces. One fecal metabolite in the CH_3OH extract was benzene-soluble; the water-soluble metabolites in the methanol extracts were separated and purified by DEAE and gel filtration columns (Figure 2). The recovery of radioactivity applied to the various columns ranged from 79 to 94%. The percent of the total carbon-14 in the feces methanol extract which each of the five metabolites accounted for is shown in Table VII.

Preparation and identification of metabolite derivatives, as outlined in Table V, was very important in the elucidation

Table IV. Carbon-14 in the Egg after a Single Oral Dose of Isopropyl Carbanilate-[phenyl-^{14}C(U)] (Experiment II)

Day	Yolk, % of dose in total fraction	White, % of dose in total fraction
1	0.004	0.006
3	0.012	0.001
4	0.015	0.001
6	0.012	<0.001
7	0.003	<0.001
8	0.005	<0.001
9	0.005	<0.001
10	0.003	<0.001
11	0.005	<0.001
12	0.003	<0.001
Total	0.067	0.008

Table V. Characterization of Metabolites of Isopropyl Carbanilate by Preparation of Derivatives[a]

Metabolite	Derivatization procedure: Direct acetylation	Enzyme hydrolysis followed by acetylation	Enzyme hydrolysis followed by methylation	Methylation followed by acetylation
I		AcNR[b], OAc		
II	AcNR, OAc, OAc	AcNR, OAc, OAc	HNR, OCH_3, OCH_3	
IV	AcNAc, OAc			
V	AcNR, OCH_3, OAc	AcNR, OCH_3, OAc	HNR, OCH_3, OCH_3	
VI	AcNR, OAc	AcNR, OAc	HNR, OCH_3	
VII	AcNR, OAc, OAc	AcNR, OAc, OAc	HNR, OCH_3, OCH_3	AcNR, OAc, OCH_3
VIII	AcNR, OAc		HNR, OCH_3	

[a] The structure of all derivatives was confirmed by comparison of ir and mass spectra with those of the authentic compound (see methods section for synthesis of compounds shown here). [b] R = $COOCH(CH_3)_2$.

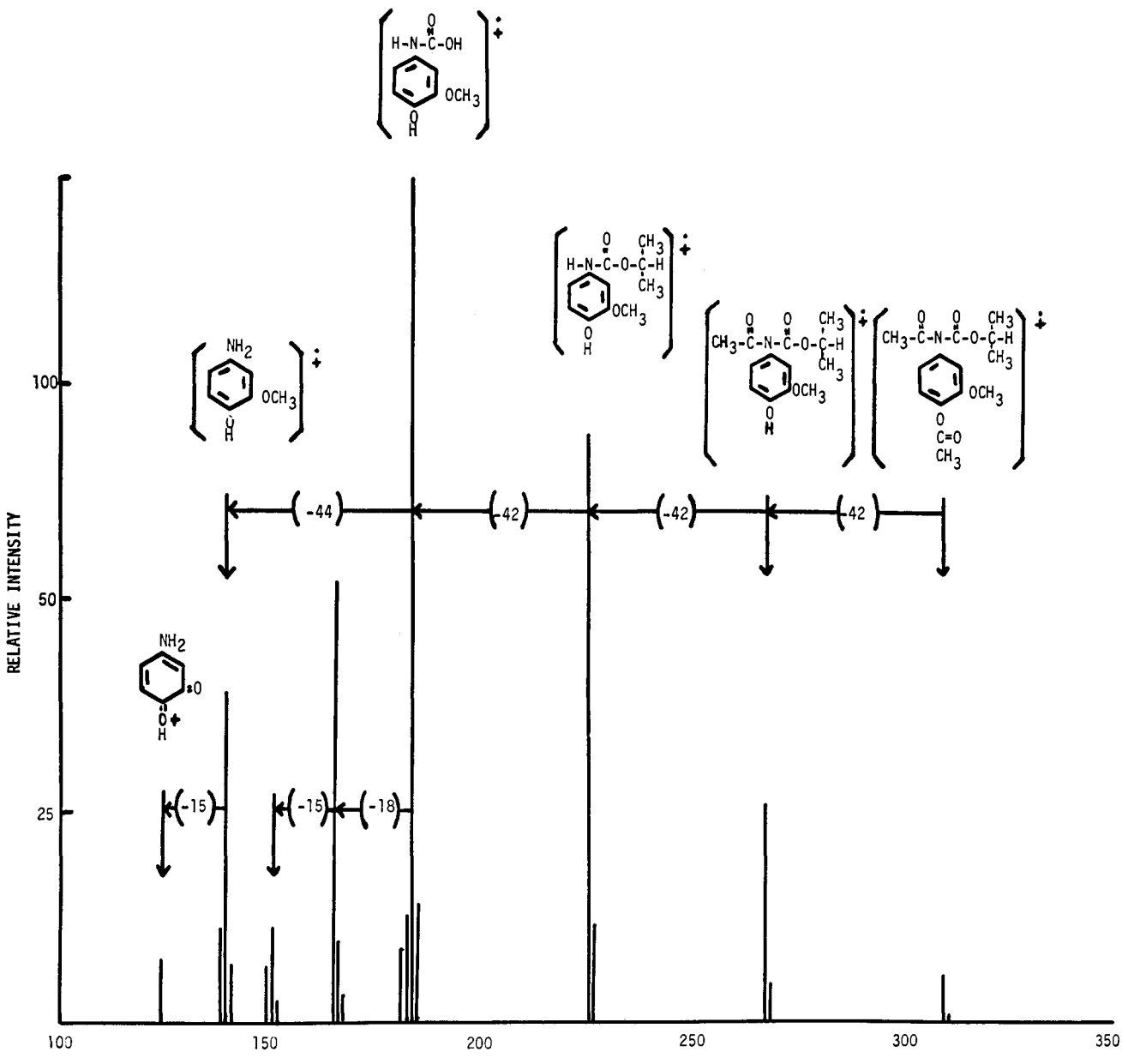


Figure 3. Mass spectrum of isopropyl methoxy-4-acetoxy *N*-acetylcarbanilate

of the structures of the metabolites (the complete structures of all metabolites ultimately identified in this study are shown in Table VII). In most cases, the sulfate ester or glucuronic acid conjugates were removed by enzymatic hydrolysis (mixture of β-glucuronidase and aryl sulfatase), and the hydrolysis product was methylated or acetylated. In some cases the sulfate ester conjugates were replaced with an acetyl group by direct acetylation (Paulson and Portnoy, 1970). Another very useful procedure was methylation followed by direct acetylation of the metabolites. These studies demonstrated that the methylation procedure converted free hydroxyl groups to methoxy groups but did not alter sulfate ester conjugates. Thus, when the metabolites containing a sulfate ester were methylated and then acetylated, the position of the methoxy group in the derivative indicated the position of the free hydroxyl group in the original metabolite, and the position of the acetoxy group in the derivatives indicated the position of the sulfate ester conjugate in the original metabolite. All of the methoxy, methoxy–acetoxy, and acetoxy derivatives were relatively nonpolar and were effectively purified by glc.

Characterization of derivatives prepared by acetylation and/or methylation of metabolites by mass spectral analysis gave valuable information concerning the presence or absence of the isopropyl carbamate side chain, ring substitution, the presence or absence of a methoxy group in the original metabolite, and the number of free hydroxyl groups in the original metabolite. The spectrum of 4-acetoxy *N,N*-diacetylaniline showed three consecutive losses of 42 mass units (ketene) to yield an ion whose mass corresponded to that of 4-hydroxyaniline. The mass spectrum of isopropyl carbanilate showed an initial loss of 42 mass units (propylene) followed by the loss of 44 mass units (CO_2) to give an ion which corresponded to that of aniline. Isopropyl *N*-acetylcarbanilate showed two consecutive losses of 42 mass units (ketene loss from the *N*-acetyl group and propylene loss from the isopropyl group) followed by the loss of CO_2 to give aniline. The mass spectrum of isopropyl 4-acetoxy-*N*-acetylcarbanilate showed two consecutive losses of 42 mass units to yield an ion whose mass corresponded to the isopropyl 4-hydroxycarbanilate, as expected (Budzikiewicz *et al.*, 1967;

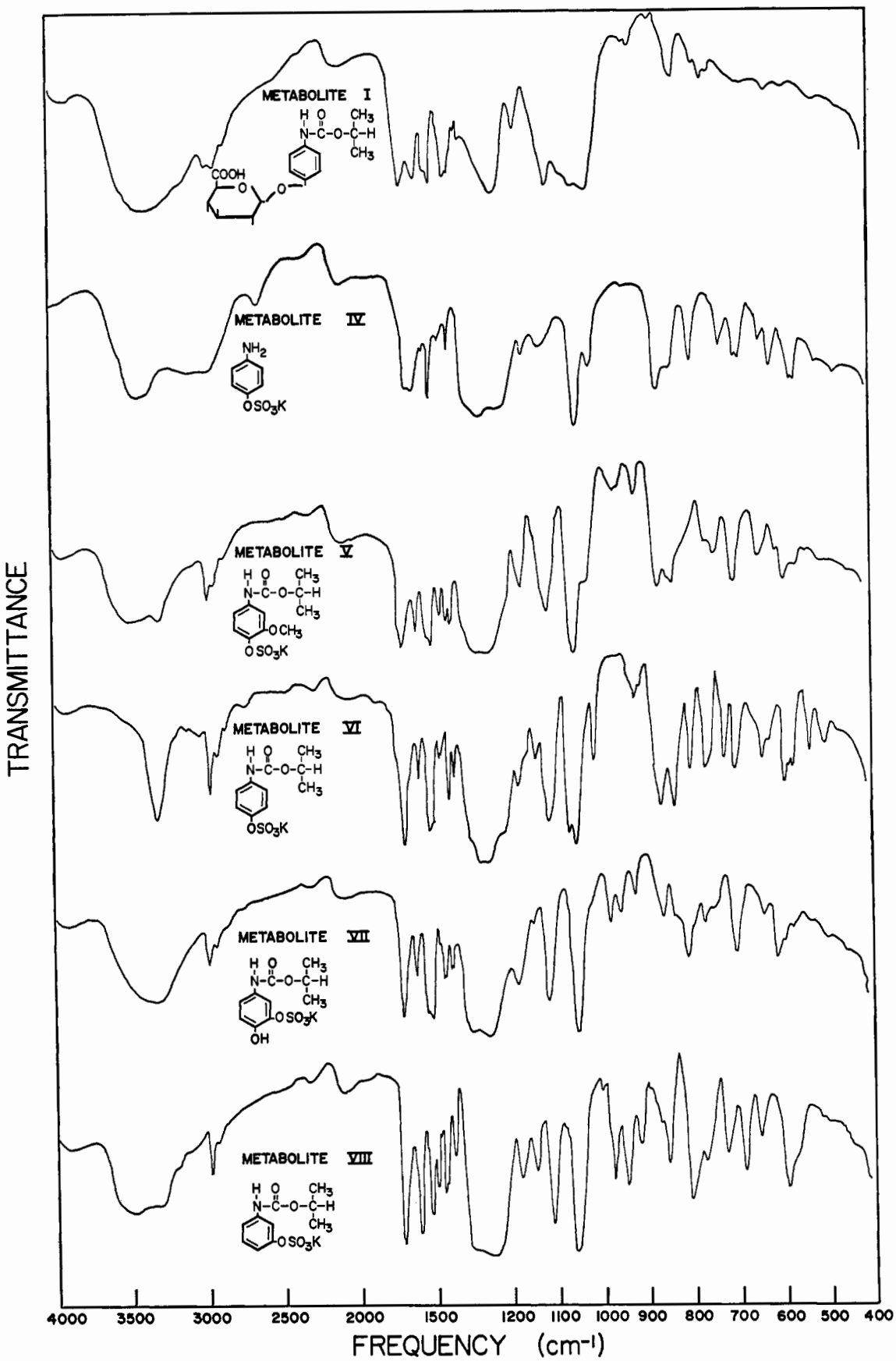
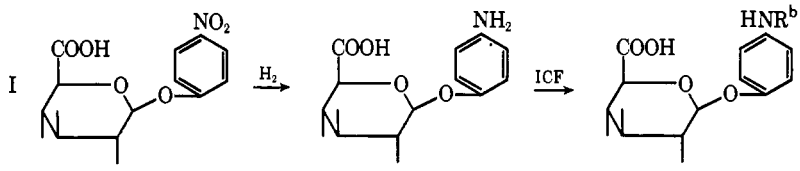
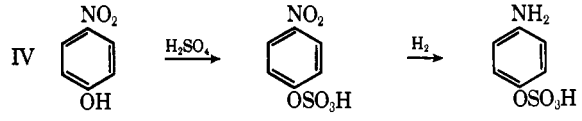
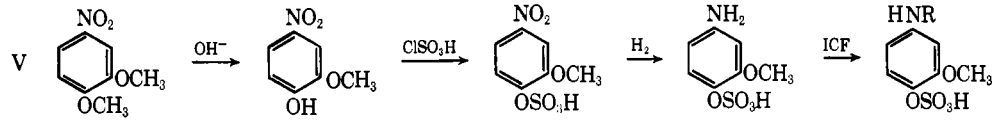
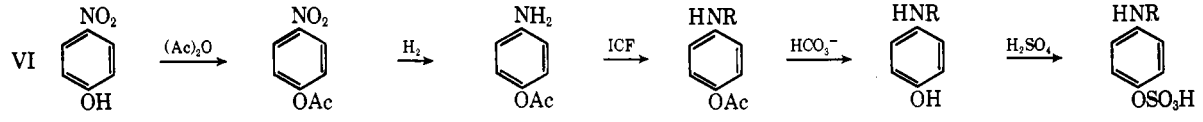
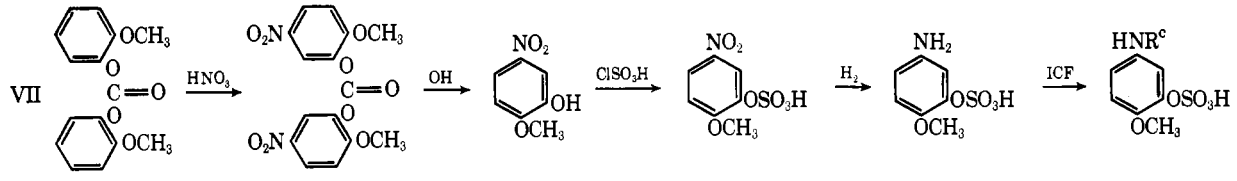
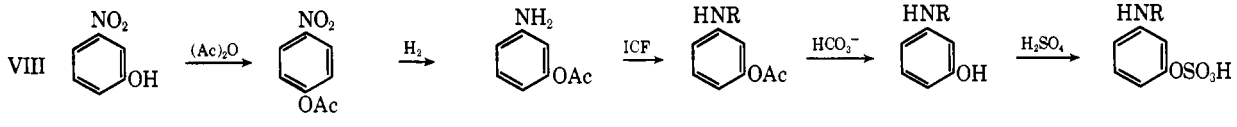


Figure 4. Infrared spectra for isopropyl carbanilate metabolites I, IV, V, VI, VII, and VIII isolated from chicken urine

Table VI. Confirmation of Structures of Metabolites or Derivative by Synthesis

Metabolite	Methods used for chemical synthesis[a]
I	
II	
IV	
V	
VI	
VII	
VIII	

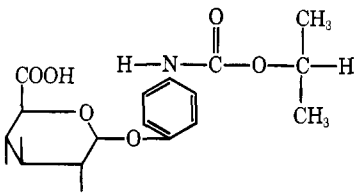
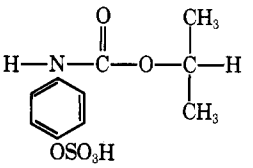
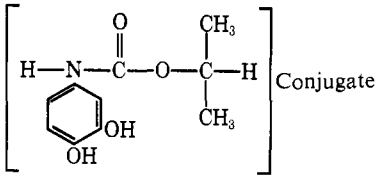
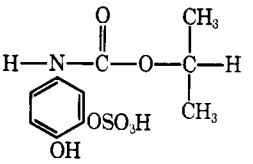
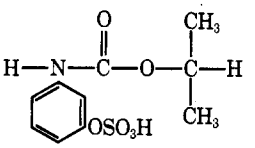
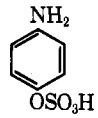
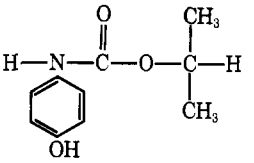
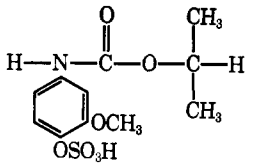
[a] See methods section for detailed description of synthetic reactions used. [b] R = $COOCH(CH_3)_2$. [c] When metabolite VII was methylated, the infrared spectrum of the product was identical to the infrared spectrum of the 3-sulfate ester of isopropyl 3-hydroxy-4-methoxycarbanilate.

Paulson *et al.*, 1970); isopropyl 3,4-diacetoxy *N*-acetylcarbanilate gave three consecutive losses of 42 mass units to yield an ion whose mass corresponded to isopropyl 3,4-dihydroxycarbanilate. The mass spectra of isopropyl 4-methoxycarbanilate and isopropyl 3,4-dimethoxycarbanilate showed consecutive losses of 42 and 44 mass units to give ions corresponding to those of 4-methoxyaniline and 3,4-dimethoxyaniline, respectively; the mass spectra also showed peaks which corresponded to the loss of a methyl radical from 4-methoxyaniline and 3,4-dimethoxyaniline, as expected (Budzikiewicz *et al.*, 1967). The mass spectra of methoxy–acetoxy–*N*-acetyl derivatives showed mass losses, which were expected on the basis of the previous discussion. For example, the mass spectrum of isopropyl 3-methoxy-4-acetoxy-*N*-acetylcarbanilate (Figure 3) showed *m/e* 309, 267, 225, 183, 139, and 124. To establish the fragmentation pathway, isopropyl 3-methoxy-4-trideuteroacetoxy-*N*-trideuteroacetylcarbanilate (A) and isopropyl 3-methoxy-4-acetoxy-*N*-trideuteroacetylcarbanilate (B) were synthesized by the use of deuteroacetic anhydride in one or both of the acetylation reactions used in the synthesis of this compound (see methods section). The mass spectrum of A showed *m/e* 315, 271, 227, 185, 141, and 126, which demonstrated that there were losses of ketene from the acetoxy and the acetyl groups prior to the loss of 42 mass units from the isopropyl group. The mass spectrum of B showed *m/e* 312, 270, 226, 184, 140, and 125, which demonstrated that there was a loss of ketene from the acetoxy group prior to the loss of ketene from the *N*-acetyl group, as shown in Figure 3.

Enzymatic hydrolysis and derivatization studies demonstrated that metabolites I and VI were conjugated forms of isopropyl 4-hydroxycarbanilate (Table V). The infrared spectrum of metabolite I (Figure 4) suggested the presence of a carbohydrate conjugate. Thus, isopropyl 4-hydroxycarbanilate-*O*-glucuronide was synthesized as shown in Table VI; the infrared spectrum of the synthetic compound was identical to that of metabolite I isolated from the urine. Metabolite VI was identified as the sulfate ester of isopropyl 4-hydroxycarbanilate by comparison of its infrared spectrum (Figure 4) with that of the synthetic compound prepared as outlined in Table VI; this compound was previously identified in the urine of rats dosed with propham (Holder and Ryan, 1968).

Metabolite IV was converted to 4-acetoxy *N,N*-diacetylaniline when hydrolyzed and acetylated or when acetylated directly (Table V). The infrared spectrum of metabolite IV,

Table VII. Metabolites of Isopropyl Carbanilate in the Feces and Urine of the Chicken

Metabolite	Structure	Urine (% of ^{14}C in urine)	Feces (% of ^{14}C in feces)[a]	Metabolite	Structure	Urine (% of ^{14}C in urine)	Feces (% of ^{14}C in feces)
I		16	9	VI		29	42
II	 Conjugate	3	ND[b]	VII		4	27
III	Not identified	6	ND	VIII		14	ND
IV		15	ND	IX		ND	14
V		13	ND	X	Not identified	ND	22

[a] The values shown for fecal metabolites are the percent distribution of the metabolites in the CH_3OH extract of the feces. The CH_3OH extract contained 65% of the activity originally in the feces. [b] Not detected.

as shown in Figure 4, suggested the presence of a sulfate ester (strong absorption bands at 1000 to 1060 cm^{-1} and 1200 to 1300 cm^{-1}). Therefore, *p*-aminophenyl sulfate was synthesized as shown in Table VI; the infrared spectrum of the synthetic compound was identical to that of metabolite IV (Figure 4).

Preparation and identification of the methyl and acetoxy derivatives of metabolites II and VII (Table V) demonstrated that these two metabolites were conjugates of isopropyl 3,4-dihydroxycarbanilate. When metabolite VII was methylated and then acetylated, the product was identified as isopropyl 3-acetoxy-4-methoxy-*N*-acetylcarbanilate. The methylation procedure used converted free hydroxyl groups to methoxy groups but did not alter sulfate esters; however, the acetylation procedure used replaced sulfate esters with an acetoxy group. These data and the presence of strong infrared absorption bands from 1030 to 1070 cm^{-1} and 1200 to 1300 cm^{-1} (Figure 4) indicated that metabolite VII was the 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate. Attempts to synthesize this compound were not successful; however, when metabolite VII was methylated, the product was identified as the sulfate ester of isopropyl 3-hydroxy-4-methoxycarbanilate by comparison of its infrared spectrum with that of an authentic sample synthesized, as shown in Table VI. Thus, it was concluded that metabolite VII, as excreted by the chicken, was the 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate. The infrared spectrum of metabolite II suggested that it was a glucuronide conjugate of isopropyl 3,4-dihydroxycarbanilate (strong absorption band from 1000 to 1100 cm^{-1}); however, attempts to synthesize the possible glucuronide conjugates of this compound were not successful.

Acetylation of metabolite V, with or without prior enzyme hydrolysis, gave isopropyl 3-methoxy-4-acetoxy-*N*-acetylcarbanilate (Table V). When metabolite V was enzymatically hydrolyzed and then methylated, the product was identified as isopropyl 3,4-dimethoxycarbanilate. This information and the strong infrared absorption from 1010 to 1070 cm^{-1} and 1200 to 1300 cm^{-1} (Figure 4) indicated that metabolite V was the 4-sulfate ester of isopropyl 3-methoxy-4-hydroxycarbanilate. This was confirmed by comparng the infrared spectrum of this metabolite with the authentic sample synthesized, as shown in Table VI.

Derivatization procedures (Table V) demonstrated that metabolite VIII was a conjugated form of isopropyl 3-hydroxycarbanilate. This metabolite was identified as the sulfate ester of isopropyl 3-hydroxycarbanilate by comparing its infrared spectrum with that of the authentic compound synthesized as shown in Table VI.

Fecal metabolites included the glucuronide conjugate of isopropyl 4-hydroxycarbanilate, the sulfate ester of isopropyl 4-hydroxycarbanilate, the 3-sulfate ester of isopropyl 3,4-dihydroxycarbanilate, and the sulfate ester of isopropyl 3-hydroxycarbanilate (Table VII); these fecal metabolites were identified by the techniques used to identify urinary metabolites. The feces also contained isopropyl 4-hydroxycarbanilate. The structure of this metabolite was determined by

preparing its acetoxy and methoxy derivatives; the derivatives were identified by comparing their infrared spectra with those of authentic compounds.

Metabolite III, which accounted for 3% of the radioactivity in the urine, and metabolite X, which accounted for 14% of the radioactivity in the feces, were not identified (Table VII). However, preliminary investigations indicated that metabolite III contained an intact isopropyl carbamate side chain, and that the ring structure was substituted in at least two positions. Additional studies are being conducted to characterize these two metabolites and the radio-labeled compound(s) which remained in the tissues and eggs from the chicken. Further studies are also needed to evaluate the toxicological and pharmacological properties of the metabolites of isopropyl carbanilate and to determine the fate of these compounds in the environment.

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Tissue Residue Depletion of Sulfamerazine in Sheep

Herbert F. Righter,* John M. Worthington, and H. Dwight Mercer

Sulfamerazine was given orally to sheep at the recommended therapeutic dose of 132 mg per kg per day for 3 days. Sulfonamide residues were determined in muscle, liver, kidney, and fat at posttreatment days 0 (12 hr after the last dosing), 3, 5, 7, and 10. Residue depletion was most rapid between days 0 and 3 after the drug was withdrawn. Sulfonamide concentrations were below 0.1 ppm in liver, kidney, and fat by day 7, and in muscle by day 10. The data show that a 10-day withdrawal period is indicated for sulfamerazine in sheep given this therapeutic dosage.

Sulfamerazine is a chemotherapeutic agent which is of value in treating a number of infectious diseases of domestic animals. It is considered to be, along with sulfamethazine, the sulfonamide of choice in treating *Pasteurella* infections such as mastitis and pneumonia in sheep (Merck Veterinary Manual, 1967). Sulfamerazine is rapidly absorbed from the gastrointestinal tract and is excreted at a slower rate than its parent drug, sulfadiazine, thus allowing greater retention and prolongation of therapeutic action.

Although numerous studies have been conducted on sulfonamide concentrations in blood after therapy, tissue residue data have been essentially lacking. Sulfamethazine residues in edible tissues of swine at 7 days after treatment were less than 0.1 ppm when the drug, in combination with chlortetracycline and penicillin, was administered in the feed over a 14-week period (Messersmith *et al.*, 1967). In calf tissues, sulfamethazine residues were reduced to below 0.1 ppm by the eighth day after withdrawal when the drug was given as a drench at a dose of 99 mg per kg for 3 days; however, in tissues of breeder chickens, residues persisted at concentrations greater than 0.1 ppm at the tenth day after withdrawal, when the drug was given in the feed at 0.4% or in the drinking water at 0.1% (Righter *et al.*, 1971a). In swine treated with sulfathiazole at 330 mg per kg per day for 3 days, residues in edible tissues were depleted by 10 days withdrawal (Righter *et al.*, 1971b). A withdrawal period greater than 7 days was indicated in laying hens and cockerels administered sulfaquinoxaline at therapeutic (0.05%) or prophylactic (0.025%) doses (Righter *et al.*, 1970). In recent years, the sensitivity of methods for determining sulfonamides has been improved and smaller quantities of these drugs can be measured in body fluids and tissues. Because of increased concern over drug residues in edible tissues, more data are necessary to establish safe periods of drug withdrawal, in which tissue residues have reached what is considered a negligible amount, before treated animals are marketed. The purpose of this study was to determine residue levels of sulfamerazine in sheep tissues following a recommended dosage regimen.

MATERIALS AND METHODS

Thirteen 10-month-old lambs and one 22-month-old wether of mixed breeds were administered sulfamerazine orally at the recommended therapeutic dosage of 132 mg per kg of body weight per day (Merck Veterinary Manual, 1967). The drug was given in capsule form; a priming dose of 132 mg per kg was given initially and five consecutive doses of 66 mg per kg

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