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Received for review November 10, 1989. Revised manuscript received February 5, 1990. Accepted February 13, 1990.

Fate of 3-Phenoxybenzaldehyde: Diphenyl Ether Cleavage, a Major Metabolic Route in Chicken†

M. Humayoun Akhtar

Animal Research Centre, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

The fate of 3-phenoxybenzaldehyde was investigated in chicken after oral administration of the unlabeled compound for 3 days, followed by a single dose of ald-^{14}C-labeled material. Approximately 98% of the administered radiocarbon was eliminated within 24 h. Radiocarbon metabolites were excreted free as well as conjugated. Free metabolites identified in excreta were 3-hydroxybenzoic acid (8%), 3-phenoxybenzoic acid (5%), and 2′- and/or 4′-hydroxy-3-phenoxybenzoic acid (traces, <1%). Only a small amount (8%) of 3-hydroxybenzoic acid was eliminated as glucuronic acid and sulfate conjugates. Hydrolysis of the polar metabolites with 2 N HCl produced 37–42% 3-hydroxybenzoic acid. Similarly, 3-hydroxybenzoic acid (both free and conjugates) was also detected in the excreta of hens fed deltamethrin or fenvalerate. The diphenyl ether cleavage of 3-phenoxybenzaldehyde is a major route, probably via 3-phenoxybenzoic acid, in chickens.

3-Phenoxybenzaldehyde (3-PBald) is a transitory metabolite of several pyrethroids including the cyano-substituted derivatives (Miyamoto et al., 1981). In the latter case, formation of 3-PBald is a two-step process that involves (i) generation of the cyanohydrin by the ester cleavage and (ii) rapid elimination of a molecule of HCN from the cyanohydrin to produce 3-PBald. The aldehyde oxidizes rapidly into 3-phenoxybenzoic acid (3-PBacid). Comparative metabolism studies of 3-PBacid, including that generated during metabolism of pyrethroids, have reported considerable species variation. For example, the acid undergoes glutamic acid conjugation in the cow (Gaughan et al., 1978a), glycine conjugation in the goat (Ivie and Hunt, 1980), glycylvaline dipeptide conjugation in the mallard duck (Huckle et al., 1981b, and sulfate conjugation of 3-(4-hydroxyphenoxy)benzoic acid in the rat (Huckle et al., 1981a). In the chicken, Huckle et al. (1982) identified α-N-acetyl-δ-*N*-(3-phenoxybenzoyl)-L-ornithine as the principal excretory metabolite. These workers observed rapid and quantitative elimination of radiocarbon in the excreta of chickens given a single dose of 3-phenoxy[*ring*-U-^{14}C]benzoic acid. Although 63.5% of the excreted radioactivity was extractable in methanol, only about half of the labeled material was identifiable (Huckle et al., 1982). Similarly, very little radiocarbon from [*alcohol*-^{14}C]permethrin

† Contribution No. 1563.

0021-8561/90/1438-1417$02.50/0 Published 1990 by the American Chemical Society

was accounted for in the excreta of hens (Gaughan et al., 1978b). Akhtar et al. (1985) identified about 50% of the radioactivity in the excreta of laying hens dosed with [*benzyl*-^{14}C]deltamethrin.

The metabolic fate of 3-PBald has been investigated in chickens to provide a better understanding of the metabolism of 3-PBald and its oxidized product 3-PBacid. Further, extracts of excreta from laying hens given [*benzyl*-^{14}C]deltamethrin (Akhtar et al., 1985) or fenvalerate (Akhtar et al., 1989) were analyzed for the major metabolite derived from 3-PBald study to determine if this was formed also from these insecticides. Recently, Quistad et al. (1988) reported O-dephenylation of 3-phenoxybenzoic acid in chickens, followed by conjugation with benzoylornithine. These workers observed O-dephenylation to be an important metabolic path (15%) in chickens.

MATERIALS AND METHODS

Chemicals. Solvents used were distilled in glass or were of HPLC grade (Caledon Laboratories, Georgetown, ON, Canada). ^{14}C-Labeled 3-PBald (sp act. 55 mCi/mol) and disodium salt of 4′-[(hydroxysulfonyl)oxy]-3-phenoxybenzoic acid [disodium salt of 4′-(OSO_3H)-3-PBacid] were gifts from Hoechst Canada through Roussel Uclaf of France. The radiochemical purity of 3-PBald (^{14}C) was >99%, as indicated by thin-layer chromatography followed by autoradiography. On standing at room temperature and on contact with air, 3-PBald readily converted to 3-PBacid.

Unlabeled 3-PBald (95% pure), 3-PBacid, 3- or 4-hydroxybenzoic acid (3- or 4-HO-Bacid) were purchased from Aldrich Chemical Co., Milwaukee, WI. (2′- and 4′-hydroxyphenoxy)-benzoic acids (2′- and 4′-HO-3-PBacid) were available from a previous study (Akhtar et al., 1985). Diazomethane was prepared by the reaction of *N*-methyl-*N*-nitrosourea with 50% KOH.

Enzymes. Glucurase (β-glucuronidase from bovine liver, 5000 Sigma units/mL) and sulfatase (Type VII from abalone entrails, 30–50 units/mg of solid) were purchased from Sigma Chemical Co., St. Louis, MO. Incubations were carried out in 0.5 M acetate buffer (pH 4.5) at 37 °C for 18 h.

Animal Treatment. Four adult White Leghorn hens having an average body weight of 1.5 kg, and at 75% egg production, were acclimatized in individual cages for 14 days. For 3 consecutive days, the hens were administered treated feed containing 10 mg of unlabeled 3-PBald/day by using the precision feeding technique described by Sibbald (1976). Twenty-four hours after the third dosing with unlabeled 3-PBald, each bird was given a single dose of feed that contained 10 mg of [*ald*-^{14}C]-3-PBald (4.95 μCi). Treated feed was prepared by adsorbing labeled and unlabeled 3-PBald onto the feed particles as detailed previously (Akhtar et al., 1985). Excreta was collected on trays covered with polyethylene film for (i) 0–24, (ii) 24–96, and (iii) 96–144 h after dosing with ^{14}C material, and stored at −20 °C until analyzed.

Experimental details for deltamethrin and fenvalerate with chickens were reported previously (Akhtar et al., 1985, 1989).

Measurement of Radioactivity. This was carried out by liquid scintillation counting (LSC) of extracts and combusted material as described previously (Akhtar et al., 1985).

Thin-Layer Chromatography (TLC). Extracts were analyzed on Whatman plates precoated with silica gel F254. Solvents used were (A) ethyl acetate/formic acid/water (35:2:2), (B) hexane/toluene/acetic acid (3:15:2), and (C) toluene/diethyl ether/acetic acid (75:25:1) (all proportions v/v). The R_f values of reference compounds in various solvent systems are listed in Table I. Radioactive compounds were detected by autoradiography on a Berthold Beta camera LB 292.

High-Performance Liquid Chromatography (HPLC). This was performed on a Waters Associates HPLC instrument equipped with a Model U6K loop injector and a Model 450 variable-wavelength UV detector connected to a Berthold HPLC radioactivity monitor LB 504. Polar metabolites were chromatographed on a 300 × 3.9 mm μBondapak C18 liquid chromatography column protected by a 70 × 2.1 mm Whatman "guard column" packed with C18. Operating conditions included the following: ambient temperature; (A) acetonitrile buffer (NaH_2PO_4, 0.05 M, pH 4.5), 1:1 (v/v), flow rate 0.6 mL/min; (B) 0.1 M ammonium acetate in methanol/water (35:65 v/v); flow rate 0.5 or 1.5 mL/min. The completely methylated derivatives were analyzed on a 300 × 3.9 mm μporasil silica gel column protected by a guard column packed with silica gel. The column was operated at ambient temperature and eluted with (C) hexane/tetrahydrofuran (975:25 v/v) at 0.6 mL/min. Under all operating conditions, the detector wavelength was set at 243 nm with sensitivity at 0.4 aufs. The retention times of various compounds are listed in Table II. The sensitivity of the radioactivity monitor was fixed at 500 dpm full-scale deflection with provision to reject peaks at or under 50 dpm since the background was at approximately 45 dpm. For quantification, the peaks were collected, rechecked by HPLC for purity, and quantitated by LSC followed by mass spectral analysis.

Table I. Relative Retention (R_f) of Reference Compounds on Thin-Layer Chromatography[a]

	solvent system[c]				
compound[b]	A	B	B[d]	C	C[d]
3-PBald	0.9				
3-PBacid	0.36	0.38	0.63	0.58	0.87
2′-HO-3-PBacid	0.85	0.11	0.20		
4′-HO-3-PBacid	0.84	0.13	0.23	0.20	0.38
2-HO-Bacid	0.87	0.34	0.54		
3-HO-Bacid	0.83	0.14	0.23	0.20	
4-HO-Bacid	0.83	0.13			
2′-HO-3-PBacid-Me		0.33	0.55	0.49	
4′-HO-3-PBacid-Me		0.21	0.36	0.37	
2-HO-Bacid-Me		0.52	0.81		
3-HO-Bacid-Me		0.18	0.33	0.37	0.67
4-HO-Bacid		0.14	0.27		

[a] The R_f exhibited variation with plate batches, temperature, and degree of saturation, but relative order was the same. [b] See text for structures. [c] Solvent systems: A, ethyl acetate/formic acid/water (35:2:2 v/v/v); B, hexane/toluene/acetic acid (3:15:2 v/v/v); C, toluene/diethyl ether/acetic acid (75:25:1 v/v/v). [d] Developed twice in the same solvent system.

Table II. Retention Times of Reference Compounds on High-Performance Liquid Chromatography

	retention time[b] (min) for solvent system[c]		
compound[a]	A	B	C
3-PBacid	11.8	14.9 (~60)	
2′-HO-3-PBacid	7.6	5.8 (19.3)	
4′-HO-3-PBacid	7.2	5.9 (19.5)	
2-HO-Bacid	4.7	–	
3-HO-Bacid	5.2	2.2 (6.4)	
4-HO-Bacid	5.5	2.1 (6.2)	
2′-HO-3-PBacid-Me	16.6	–	
4′-HO-3-PBacid-Me	15.4	–	
2-HO-Bacid-Me	16.4	–	
3-HO-Bacid-Me	8.4	10.4 (29.7)	
4-HO-Bacid-Me	8.3	10.4 (30.2)	
3-PBacid-Me			9.3
2′-OCH_3-3-PBacid-Me			16.4
4′-OCH_3-3-PBacid-Me			13.8
2-OCH_3-Bacid-Me			25.3
3-OCH_3-Bacid-Me			10.5
4-OCH_3-Bacid-Me[d]			12.3

[a] See text for structures. [b] Retention times are highly susceptible to temperature and source of solvents; the relative order was maintained. [c] (A) Acetonitrile buffer (NaH_2PO_4 0.05 M, pH 4.5; 1:1 v/v), flow rate 0.6 mL/min, μBondapak C18 column. (B) 0.1 M ammonium acetate in methanol/water (35:65 v/v), flow rate 1.5 mL, μBondapak C18 column. Values in parentheses are for flow rate 0.5 mL/min. (C) Hexane/tetrahydrofuran (975:25 v/v), flow rate 0.6 mL/min, μporasil silica gel column. [d] 1.8–1.9 times more UV sensitive than 3-OCH_3-Bacid-Me.

Gas Chromatography–Mass Spectrometry (GC–MS). This was performed on a Finnigan MAT-312 mass spectrometer system connected to a Varian gas chromatograph fitted with either a 15M or 20M DB5-J column. The column was operated between 100 and 260 °C with an increment of 15 °C min^{-1}. Spectra were recorded in electron impact (EI) mode at 70 eV.

Extraction and Analysis of Metabolites in Excreta. Daily excreta (85–100 g) from individual birds were extracted with methanol (2.5 mL/g of wet excreta) as described previously (Akhtar et al., 1985, 1989). Radioactivity in methanol and residues were measured. Techniques employed to isolate, quantitate, and characterize the radioactive products from methanol extracts are detailed below.

(*i*) *Partitioning.* A known volume of the methanol extract was evaporated to almost dryness in a fumehood at room temperature. The residue was dissolved in water, filtered to remove particles, and radioassayed. A portion of the aqueous solution was extracted four times with the same volume of ether. The ether phase was dried over Na_2SO_4, filtered, and evaporated to dryness, the residues were dissolved in methanol, and the radioactivity was measured. The aqueous phase was concentrated, and the radioactivity was determined.

(*ii*) *Hydrolysis.* A portion of the aqueous phase containing the polar metabolites was treated with 50 mL of 2 N HCl and heated at 80–90 °C for 3 h. When cooled, the reaction mixture was extracted with ether (4 × 50 mL). The ether layer was passed through Na_2SO_4, filtered and concentrated, and the radiocarbon content was measured. A portion of aqueous phase was also incubated with (i) buffer, (ii) β-glucuronidase, and (iii) sulfatase. The radioactive aglycons were removed from the reaction mixture with ether as detailed above, and radioactivity was measured. In all cases, ether extract residues were taken up in methanol for radioactivity measurement.

The methanol extract of excreta, after removal of methanol on a rotary evaporator, was treated with 2 N HCl to cleave all conjugating bonds to release ^{14}C compounds. The released ^{14}C compounds were removed from the reaction mixture following the procedures detailed above. Radioactivity was measured in the ether extract, and the nature of the radioactive components was ascertained by TLC, HPLC, and GC–MS.

To record their stability in HCl, 3-PBacid, 3-PBacid-glycine, 2′- and 4′-HO-3-PBacid, the disodium salt of 4′-(OSO_3H)-3-PBacid were heated with 2 or 3 N HCl. The reaction mixtures were extracted with ether and analyzed directly by HPLC (UV detector).

(*iii*) *Analysis.* For analysis, the volume of methanol of various extracts were reduced and centrifuged, and the clear layer was applied on TLC plates. The plates were developed in solvent B (two developments) and autoradiographed by the Beta camera. The radioactive zones were scraped off the plates and extracted with a mixture of methanol/ether (1:1), and the radioactivity was measured. Extracts of various radioactive regions were then analyzed by HPLC on a C18 column (solvents A and B).

Radioactive compounds eluting from the column were collected individually, and their radioactivity content was measured by LSC. Structural identity of the isolated radioactive compounds was established by comparison of GC–MS fragmentation patterns with those of known standards before and after methylation with excess diazomethane.

Deltamethrin. The methanol extract of excreta from hens given [*benzyl*-^{14}C]deltamethrin (Akhtar et al., 1985) was refluxed with 3 N HCl for 5 h. The liberated radioactivity was removed with ether and analyzed by TLC. The radioactive region corresponding to 3- and 4-HO-Bacids was removed from plate and the extract analyzed by HPLC, before and after methylation.

Fenvalerate. The methanol extract of excreta, after removal of methanol, from hens given [*carbonyl*-^{14}C]fenvalerate (Akhtar et al., 1989) was hydrolyzed with 2 N HCl, and the free compounds were extracted with ether. The ether extract along with pure 3- and 4-HO-Bacids was applied on TLC plates. Plates were developed twice in solvent B. Silica gel from regions having R_f similar to pure 3- and 4-HO-Bacids was scraped off the plates and extracted with methanol. This methanol extract was analyzed by HPLC for 3- and 4-HO-Bacids before and after methylation with diazomethane.

RESULTS

Elimination of Radioactivity in Excreta. Radioactivity from [*ald*-^{14}C]-3-PBald was rapidly eliminated in the chicken excreta. About 98% of the administered radioactivity was voided in excreta within 24 h, an additional 1–2% in the next 48 h, and only traces in the final 72 h.

Nature of Excreted Radiocarbon. Chickens treated with [*ald*-^{14}C]-3-PBald eliminated a wide range of ^{14}C-labeled compounds. Approximately 84% of the radioactivity in the excreta (i.e., 82% of the administered dose) was extracted with methanol. The methanol extract, when analyzed by TLC in solvent A, exhibited six distinct radioactive regions. Analyses of the extracts of the radioactive zones indicated the presence of 3-PBacid, 2′- and/or 4′-HO-3-PBacid, and 3- and/or 4-HO-Bacid. These compounds accounted for 13–16% of the total radioactivity in the methanol extract. The major portion (65–73%) of the radioactivity was associated with water-soluble polar compounds for which reference materials were not available. These radioactive compounds had short retention times on a C18 column when eluted with solvent system A or B.

To positively identify and quantify the less polar metabolites, methanol extracts from two birds were pooled, and the radioactive compounds in methanol were partitioned between ether and water as detailed above and reported (Akhtar et al., 1985, 1989). The ether extract was analyzed by TLC in solvent B (two developments), and the autoradiograph showed three distinct radioactive regions. Since the R_f values for 3-HO-Bacid and 4′-HO-3-PBacid were similar in the solvent system employed, identification and quantification of these compounds were done mainly by HPLC because of vast differences in their HPLC retention times. Radioactive compounds eluting from HPLC column were collected and quantitated, and their structures were determined by GC–MS before and after methylation with excess diazomethane.

Zone 1 (R_f 0.65, 5.1% of the total dose) was tentatively assigned as 3-PBacid, because of its TLC and HPLC retention times. Further structural confirmation was obtained by GC–MS of its methyl derivative, which was identical with that recorded for the pure methyl ester of 3-PBacid (3-PBacid-Me).

Compounds in zone 2 (R_f 0.28, 9.8% of the total dose) when analyzed by HPLC on a C18 column eluted with solvent systems A and B indicated the major radioactivity (90%) to be associated with compounds that had retention times similar to that of 3- and 4-HO-Bacid. The direct probe mass spectrum of the major radioactive compound, collected by preparative HPLC, exhibited peaks at m/z 138, 121 (base peak), 93, and 65, which were identical with those obtained for 3- and 4-HO-Bacid. In addition, this compound was readily converted to a less polar compound when allowed to stand in methanol with a trace amount of HCl. The mass spectra of the methylated compound consisted of major peaks at m/z 152 ($M^{\bullet+}$), 121 (base peak), 93, and 65. The spectrum was in excellent agreement with the spectra obtained for 3-HO-Bacid-Me or 4-HO-Bacid-Me (methyl 3- or 4-hydroxybenzoate). This suggested that the R_f 0.65 zone contained 3- and/or 4-HO-Bacid.

A μporasil silica gel column separated 3-PBacid-Me, 2′- and 4′-OCH_3-3-PBacid-Me (methyl 2′- or 4′-methoxy-3-phenoxybenzoate), and 3- and 4-OCH_3-Bacid-Me (methyl 3- or 4-methoxybenzoate) with good resolution (Figure 1A). To provide further evidence in support of the above structures (3- and 4′-HO-Bacid), the extract was treated

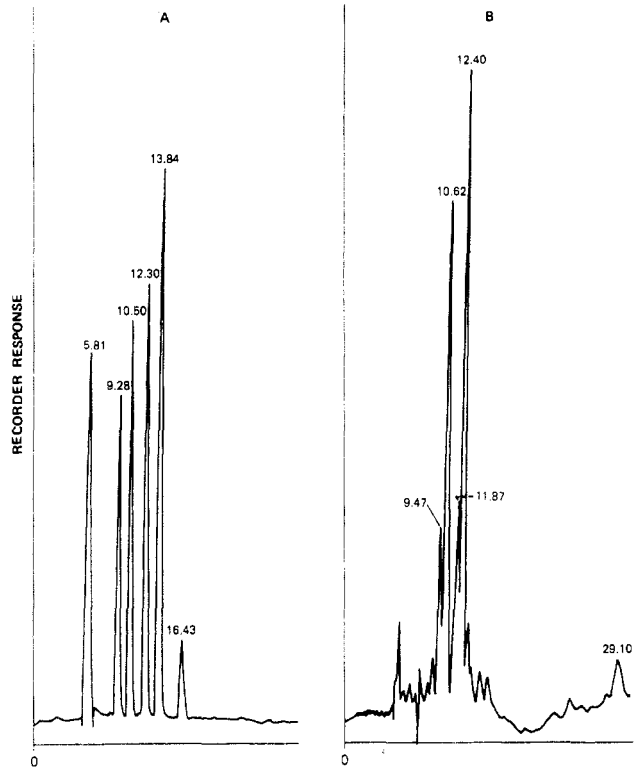

Figure 1. (A) HPLC chromatogram of a mixture of various authentic methyl derivatives, 3-PBacid-Me (9.29 min); 3-OCH_3-Bacid-Me (10.50 min); 4-OCH_3-Bacid-Me (12.30 min); 4′-OCH_3-3-PBacid-Me (13.84 min); 2′-OCH_3-3-PBacid-Me (16.43 min). (B) HPLC chromatogram of methylated derivative of radioactive compound(s) with R_f 0.64 in solvent C. Compound with retention time 10.62 min contained >85% of the radioactivity; peak with retention time 12.40 min was due to nonradioactive 4-OCH_3-Bacid-Me. HPLC operating conditions: solvent, hexane/tetrahydrofuran (975:25 v/v); flow rate, 0.6 mL/min; aufs, 0.4; UV detector at 243 nm. Under these conditions retention time for 2-OCH_3-Bacid-Me was 25.26 min.

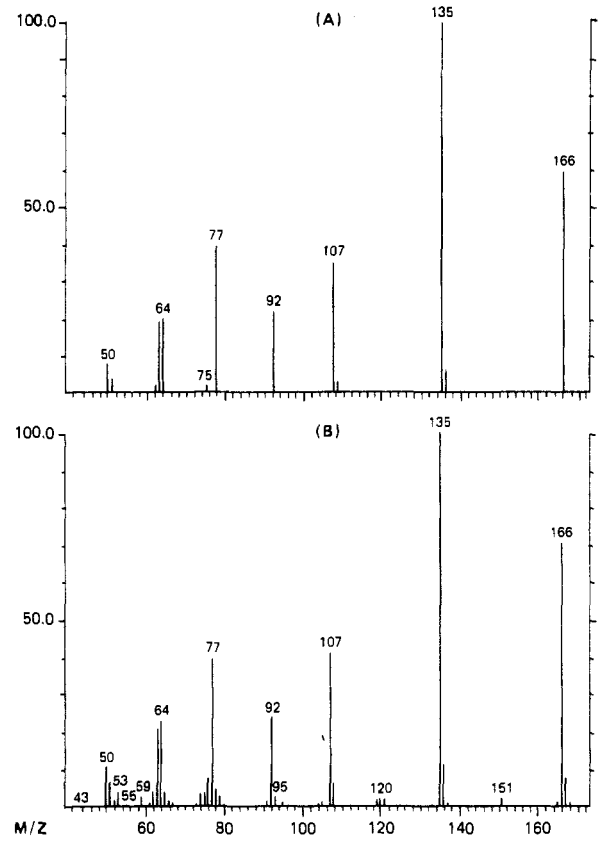

Figure 2. Mass spectra of (A) the methyl derivative isolated by HPLC (retention time 10.50 min) and (B) authentic 3-OCH_3-Bacid-Me.

with excess concentrated diazomethane and analyzed by HPLC. Analysis of the methylated extract by HPLC on the μporasil silica column exhibited two major peaks with retention times of 10.6 and 12.4 min and containing >85% of the radioactivity (Figure 1B). The retention times of methylated metabolites at 10.6 and 12.6 min were in very good agreement with those recorded with authentic 3-OCH_3-Bacid-Me (10.5 min) and 4-OCH_3-Bacid-Me (12.30 min). In addition, the mass spectrum of the compound with a retention time of 10.5 min, isolated by preparative HPLC, was in excellent agreement with that of pure 3-OCH_3-Bacid-Me (Figure 2). Similarly, the mass spectra of pure 4-OCH_3-Bacid-Me and the compound with retention time of 12.6 min were identical. Thus, the identities of 3- and 4-HO-Bacid were confirmed. Note: Concentrated and large excess of diazomethane is required to methylate the hydroxy group of 3- and 4-HO-Bacid. Use of dilute diazomethane solution caused only partial methylation. 3-OCH_3-Bacid-Me is a highly volatile compound, and care must be taken to avoid losses.

To determine if one or both hydroxybenzoic acids were radioactive, the methylated derivatives were isolated by preparative HPLC on a μporasil silica gel column eluted with hexane/tetrahydrofuran (99:1). The use of hexane/tetrahydrofuran (975:25) resulted in carryover of some radioactivity from 3-OCH_3-Bacid-Me. Almost all radioactivity was associated with 3-OCH_3-Bacid-Me, with little or no radioactivity for 4-OCH_3-Bacid-Me. Thus, only 3-HO-Bacid was the metabolite of 3-PBald. The HPLC and GC–MS analysis of methylated extract also indicated the presence of 4′-OCH_3-3-PBacid-Me.

Zone 3 (R_f 0.0, 8.5% of the total dose) remains unidentified. However, when treated with 2 N HCl at 80–90 °C, it produced 3-PBacid (15%) and 3-HO-Bacid (33%).

Analysis of Water-Soluble Metabolites. The water-soluble metabolites on treatment with (i) buffer, (ii) enzymes, and (iii) 2 NHCl liberated radioactive compounds which were extracted with ether. Approximately 7% of the ^{14}C in the aqueous phase was released on treatment with buffer alone, while 16% was extractable when incubated with buffer and enzymes. About 65% of the aqueous radioactivity was extracted with ether when heated with 2 N HCl.

The nature of the liberated radioactivity from the above reactions was determined by a combination of TLC, HPLC, and GC–MS before and after methylation as detailed above. The identity of radioactive metabolite(s) from the buffer reaction could not be established. Only trace amounts of 3-PBacid and 3-HO-Bacid were detected in the ether extract. The extract from enzymatic reactions contained both 3-HO-Bacid and 3-PBacid in 3-PBacid in approximately 2:1 ratio and an unidentified polar metabolite similar to that obtained with buffer. It was estimated that about 8% of the total aqueous radioactivity was associated with glucuronides and sulfates of 3-HO-Bacid and 3-PBacid.

The ether extract of 2 N HCl hydrolysate consisted of 3-PBacid (11%), 3-HO-Bacid (70%), and unidentified polar metabolites (15%). These values are based on the total radioactivity in the ether extract. Trace amounts of 2′- and/or 4′-HO-3-PBacid were also detected in the extract.

Acid Hydrolysis of Methanol Extract of Excreta. The methanol extract, after removal of methanol, was

heated with 2 N HCl to liberate 3-HO-Bacid from conjugates and other adducts. The ether extract of the acid hydrolysate contained about 80% of the radioactivity before hydrolysis. The compounds identified by a combination of TLC, HPLC, and GC–MS in the ether extract were 3-PBacid (14%), 3-HO-Bacid (73%), small amounts of 4′-HO-3-PBacid, and unidentified products (13%).

Identification of 3-HO-Bacid in Excreta of Hens Fed [*benzyl*-^{14}C]Deltamethrin and [*carbonyl*-^{14}C]-Fenvalerate. (*a*) *Deltamethrin.* The ether extracts before and after hydrolysis of methanol extract contained ^{14}C-labeled 3-HO-Bacid. Identity of 3-HO-Bacid was confirmed by comparison of HPLC and GC–MS properties of its methyl derivative with those of pure 3-OCH_3-Bacid-Me. The amount of 3-HO-Bacid was not determined.

(*b*) *Fenvalerate.* Unlabeled 3-hydroxybenzoic acid was detected in the ether extracts before and after acid hydrolysis. Again, identity of 3-HO-Bacid was confirmed by comparison of HPLC and GC–MS properties of its methylated product with those of pure 3-OCH_3-Bacid-Me. The amount of 3-HO-Bacid was not quantitated.

Analysis of Control Excreta for 3- and 4-HO-Bacids. Excreta from control chickens contained 4-HO-Bacid but no 3-HO-Bacid, as indicated by analysis of methanol extracts by the methods detailed above.

DISCUSSION

The data presented here identified diphenyl ether cleavage of 3-PBald, via 3-PBacid, as the major metabolic route in chickens. The cleavage product, 3-HO-Bacid, was excreted free as well as conjugated (glucuronide and sulfate) and as adducts of an unknown moiety(ies). About 45–52% of the applied ^{14}C in [*ald*-^{14}C]-3-PBald was associated with 3-HO-Bacid and its derivatives. Labeled and unlabeled 3-HO-Bacid and derivatives were voided in the excretion of hens fed [*benzyl*-^{14}C]deltamethrin and [*carbonyl*-^{14}C]fenvalerate, respectively. Quistad et al. (198) also identified 3-HO-Bacid in the excreta of chickens given [*benzyl*-^{14}C]fuluvalinate orally.

Enzymatic reactions liberated only a small amount of aglycons from water-soluble metabolites, while a considerably large amount (65%) was released with 2 N HCl. A similar observation was made with water-soluble excretory metabolites of [*benzyl*-^{14}C]deltamethrin in chickens (Akhtar et al., 1985). It is well documented that amide bonds are resistant to enzyme hydrolysis (β-glucuronidase and sulfatase) but are readily cleaved with strong acid (Guaghan et al., 1978a; Huckle et al., 1981a). Release of an aglycon(s) with 2 N HCl at 80–90 °C strongly suggested that the major portion of excretory metabolites were conjugates of amino acids. If true, then it appeared that phase I metabolites of 3-PBald formed considerably more amino acid conjugates than glucuronides and sulfates. Data by Huckle et al. (1981, 1982) provided support for this conclusion. These workers observed that both mallard duck and chickens excreted more amino acid conjugates than glucuronides and sulfates. For example, the mallard duck excreted 27% amino acid conjugate compared to only 11% glucuronides and sulfates of 3-PBacid (Huckle et al., 1981b). Similarly, in chickens these values were 19% and 7%, respectively (Huckle et al., 1982).

Although the present work did not identify the nature of the conjugating moieties, it determined the extent of the diphenyl ether cleavage route in chickens (45–52%). A 45% diphenyl ether cleavage is based on the total amount of 3-HO-Bacid produced on acid hydrolysis of the methanol extract of excreta. In this connection, it should be stated that no 3-HO-Bacid was produced when methanol extracts of excreta fortified with 3-PBacid (^{14}C) or 2′- or 4′-OH-3-PBacid were hydrolyzed with 2 N HCl at 80–90 °C for 3 h. Similarly, 3-HO-Bacid was not formed when 3-PBacid, 3-PBacid–glycine, 2′- or 4′-HO-PBacid, or disodium salt of 4′-(OSO_3H)-3-PBacid was heated with 2 N HCl at 80–90 °C for 3 h or refluxed with 3 N HCl for 6–12 h. Under these conditions, 3-PBacid–glycine and disodium salt of 4′-(OSO_3H)-3-PBacid gave mainly 3-PBacid and 4′-HO-3-PBacid, respectively.

About 16% of the dose was excreted as free and as glucuronide and sulfate conjugates of 3-HO-Bacid. The value of 16% is in excellent agreement with 15% reported recently by Quistad et al. (1988) for free and conjugates (known identities) of 3-HO-Bacid from 3-PBacid in chickens. The acid hydrolysis produced an additional 29% 3-HO-Bacid by splitting amide and other bonds that were formed on reactions between the cleavage product (phase I metabolite) and endogenous substances (phase II reaction). It was assumed that these polar metabolites were not precursors for 3-HO-Bacid in vivo.

Diphenyl ether cleavage of 3-PBacid, is a dominant route in chickens. Quistad et al. (1988) identified O-dephenylation of 3-PBacid as a major metabolic route (15%) in chickens. Further, they showed that 3-HO-Bacid conjugated with glucuronide, sulfate, and benzoylornithines. Diphenyl ether cleavage was reported in resistant Colorado potato beetles (Soderlund et al., 1987), soil (Ohkawa et al., 1979; Mikami et al., 1984), and houseflies (Kogiso et al., 1982). The mechanism of the cleavage is not understood. Soderlund et al. (1987) proposed 2′-hydroxyfenvalerate as an intermediate for the formation of dephenylfenvalerate and 3-HO-Bacid from fenvalerate. Attempts to identify dephenylfenvalerate and dephenyldeltamethrin, by mass spectral techniques, in the methanol extracts of excreta from chickens fed the respective compounds were not successful. It was thus believed that in chickens 3-HO-Bacid was produced from 3-PBacid and not from the hydrolysis of dephenyl derivatives. A similar reasoning was advanced by Quistad et al. (1988) for the formation of 3-HO-Bacid from fluvalinate in chickens.

By analogy to Soderlund et al. (1987), one can postulate 2′-hydroxy-3-PBacid as an intermediate for the formation of 3-HO-Bacid. However, presently there is no evidence to support or reject this view. Alternatively, enzymatic hydrolysis of a diphenyl ether linkage could also be considered a process leading to 3-HO-Bacid, but no report on the hydrolysis of diphenyl ether exists in the literature.

On this basis of the data presented here, it appeared that 3-PBald was rapidly converted to 3-PBacid, which in turn underwent diphenyl ether cleavage to produce 3-HO-Bacid. The cleavage product conjugated preferentially with amino acids and other endogenous substances before elimination from the body. Identification of 3-HO-Bacid in the excreta of chickens fed deltamethrin or fenvalerate strongly suggests that previous workers may have failed to identify this compound in chickens because both 3-HO-Bacid and 4′-HO-3-PBacid have similar R_f values in most of the commonly used solvent systems. To provide a better understanding of the complex metabolic pathways of 3-PBald and its oxidized product, 3-PBacid, in chickens, the identity of the conjugating moiety as well as the position of 2′-HO-3-PBacid needs to be established. Further, the fate of other 3-phenoxybenzyl-substituted pyrethroids needs to be reexamined in chickens.

ACKNOWLEDGMENT

I thank Claude Danis for his skilled technical assistance and Pierre LaFontaine for the mass spectral analysis.

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Received for review April 12, 1989. Revised manuscript received October 27, 1989. Accepted February 2, 1990.

Identification and Gas Chromatographic Determination of Some Carboxylic Acid Metabolites of *N,N*-Diethyl-*m*-toluamide in Rat Urine†

Wesley G. Taylor* and Roy W. Spooner

Agriculture Canada Research Station, P.O. Box 3000 Main, Lethbridge, Alberta, Canada T1J 4B1

Adult Wistar rats dosed intraperitoneally and topically (50 mg/kg) with *N,N*-diethyl-*m*-toluamide (**1**) excreted *m*-[(diethylamino)carbonyl]benzoic acid (**3**), *m*-[(ethylamino)carbonyl]benzoic acid (**6**), *m*-(aminocarbonyl)benzoic acid (**9**), and *m*-toluic acid (**10**) in their urine. These carboxylic acids were isolated by extraction of acidified urine samples with ethyl acetate and were detected as their methyl esters by GC and GC–MS. A quantitative analytical procedure, based on GC with a nitrogen–phosphorus detector, a capillary column of DB-1, and an internal standard of *m*-[(dipropylamino)carbonyl]benzoic acid (**13**), was developed to determine **3** and **6** in urine samples from treated rats. After 24 h, these major metabolites together accounted for 77% and 82% of the intraperitoneally administered dose in separate experiments with four male rats per experiment. In trials with **1** applied topically, **3** and **6** collectively represented 47% (males) and 49% (females) of the dose in 0–48-h urine collections. In the topical experiments, 5–6% of **1** was excreted unchanged.

N,N-Diethyl-*m*-toluamide (**1**), commonly known as deet, is used extensively as a topical insect repellent for protection against mosquitoes and other blood-sucking flies. It is applied ad libitum to the skin and clothing either neat or in alcohol-based sprays and other formulations. Despite a long history of use, published reports are scarce on the metabolism of **1** in man or animals [see the review by Robbins and Cherniack (1986)].

In experiments with rodents, the distribution and urinary excretion of radiolabeled **1** has been studied in mice (Blomquist et al., 1975; Blomquist and Thorsell, 1977; Lur'e et al., 1978) and rats (Lur'e et al., 1978; Moody et

† LRS Contribution No. 3878890.

0021-8561/90/1438-1422$02.50/0 Published 1990 by the American Chemical Society