Metabolism of Fenvalerate by a Chicken Liver Enzyme Preparation

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Metabolism of α-cyano-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate (fenvalerate) has been studied in a chicken liver enzyme preparation by using two forms of ^{14}C labeling (carbonyl and phenoxy ring). The metabolism of fenvalerate was due to cleavage of the ester bond. Microsomal oxidation processes were not involved to any great extent in the metabolism of the compound. The major metabolites identified included 3-phenoxybenzaldehyde, 3-phenoxybenzoic acid, 2-(4-chlorophenyl)-3-methylbutyric acid, and 3-(4-hydroxyphenoxy)benzoic acid. GC methods for analyses of various compounds are detailed.

Fenvalerate is classified as a synthetic pyrethroid even though it does not contain a cyclopropyl ring, a common feature of pyrethroids. Its chemical structure has been established as (*S,R*)-α-cyano-3-phenoxybenzyl (*S,R*)-2-(4-chlorophenyl)-3-methylbutyrate. The compound is marketed under trade names of Sumicidine, Belmark, Pydrin, S5602, WL43775, and SD 43775. It has a high insecticidal activity against a broad spectrum of insects, moderate mammalian toxicity, and adequate stability in the field (Ohno et al., 1977).

Fenvalerate effectively controls northern fowl mites, an important external parasite of poultry in Canada and the United States (Hall et al., 1978; Loomis et al., 1979). Its usage in ear tags of cattle greatly reduces the population of face fly, horn fly, and ticks (Harvey and Brethour, 1981; Knapp and Herald, 1981; Davey et al., 1980).

The metabolic fate of fenvalerate in rats (Ohkawa et al., 1979; Lee et al., 1982) and a comparative study with rats and mice (Kaneko et al., 1980) have been reported. In vitro studies with subcellular fraction from rat liver homogenate [Boyer, Shell Development Co., personal communication, 1976, as cited in *FAO Plant Prod. Prot. Pap., Suppl.* (1980)] and mouse liver microsomes (Soderlund and Casida, 1977) have been carried out.

Although fenvalerate is gaining acceptance in agricultural usage, very little has been reported in the literature on the metabolism of this compound in poultry and farm animals. Wszolek et al. (1980, 1981) detected the residues of fenvalerate in milk, urine, and feces of dairy cows fed grain spiked with the insecticide. As part of the ongoing research on the metabolism of synthetic pyrethroids in farm animals, the present studies were undertaken to obtain information on the metabolism of fenvalerate by a chicken liver homogenate.

MATERIALS AND METHODS

Chemicals. Pesticide-grade solvents were used as received. Radiolabeled [*carbonyl*-^{14}C]fenvalerate was prepared by the procedures detailed here. Condensation of 4-chlorobenzyl chloride with potassium cyanide-^{14}C gave (4-chlorophenyl)acetonitrile-*1*-^{14}C. A reaction of the nitrile with 2-chloropropane in the presence of NaH produced 2-(4-chlorophenyl)-3-methylbutyronitrile-*1*-^{14}C, which on treatment with 64% sulfuric acid at reflux gave 2-(4-chlorophenyl)-3-methylbutyric-*1*-^{14}C acid. Coupling of the acid chloride with α-cyano-3-phenoxybenzyl alcohol (3-phenoxybenzaldehyde cyanohydrin) in the presence of pyridine afforded [*carbonyl*-^{14}C]fenvalerate (I). Similarly, a reaction between the acid chloride of 2-(4-chlorophenyl)-3-methylbutyric acid (II) and α-cyano-3-[phenoxy-U-^{14}C]phenoxybenzyl alcohol yielded [*phenoxy*-U-^{14}C]fenvalerate (phenoxy ring labeled fenvalerate). α-Cyano-3-[*phenoxy*-U-^{14}C]phenoxybenzyl alcohol was prepared from a reaction of (*phenoxy*-U-^{14}C)phenol with 3-bromobenzaldehyde followed by treatment with HCN in situ. Unlabeled fenvalerate was obtained from esterification of the II with α-cyano-3-phenoxybenzyl alcohol (III). 3-Phenoxybenzaldehyde (IV), 3-phenoxybenzoic acid (V), and III were obtained from Aldrich Chemical Co., New York. 3-(4-Hydroxyphenoxy)benzoic acid (VI) and the methyl ester of 4′-hydroxyfenvalerate were synthesized by the methods of Unai and Casida (1977).

Methyl esters were obtained by treating the acids (II, V, and VI) with freshly prepared diazomethane. The methylating agent, diazomethane, was prepared by a reaction of cold aqueous 50% potassium hydroxide with *N*-nitroso-*N*-methylurea (Schultz et al., 1971). **Caution**: Extra precaution must be taken in handling diazomethane due to its carcinogenic properties. Boron trichloride–2-chloroethanol (10% BCl_3, v/v) was purchased from Applied Science, Pennsylvania. Sigma Chemical Co. (Missouri) was the supplier of the NADPH-generating system.

Enzyme Preparation. Chicken liver homogenate was prepared in 0.134 M phosphate, pH 7.4 (8 g/40 mL). The homogenate was centrifuged at 1740*g* for 5 min at 4 °C. The precipitate was discarded and the supernatent was centrifuged at 11950*g* for 10 min at 4 °C. The precipitate was discarded and the supernatant (soluble plus microsomes) was used in the incubation. A portion of the supernatant was further centrifuged at 40000 rpm (105000*g*) for 30 min at 4 °C to obtain the soluble fraction.

All enzyme preparations were used on the same day they were prepared.

In Vitro Incubations. Incubations were carried out in Erlenmeyer flasks (50 mL) at 37.5 °C for 3 h. Each incubation mixture consisted of 302 μg of [^{14}CO]I [302 μg = 3.2×10^5 dpm] or 338 μg of [$^{14}C_6H_5O$]I [338 μg = 6.2×10^3 dpm] and 20 mL of enzyme preparation, equivalent to 4 g wet weight of chicken liver. Types of incubations carried out were (a) in buffer, (b) in boiled enzyme, (c) in the soluble fraction under N_2, and (d) in the soluble plus microsomes (11950*g*) without the additional NADPH-generating system and (e) with the NADPH-generating system. The enzymatic reaction was terminated by the addition of acetone (20 mL).

Extraction and Isolation of Products. The mixture was treated with 2 mL of 1 N HCl, mixed thoroughly, and filtered, and the residue was washed with acetone. Extracts were concentrated to 5–7 mL on a rotary evaporator and quantitatively transferred into a centrifuge tube. The volume of the extract was reduced to 3–4 mL under N_2 and then extracted with ether (5 × 5 mL). The ether extract was passed over Na_2SO_4, filtered, and evaporated to dryness. The residue was redissolved in methanol and the final volume was adjusted to 5 mL. A total of 0.5 mL of

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Table I. Gas Chromatographic Conditions[a] for Identification of Fenvalerate and Its Metabolites

compound	temperature, °C injector	oven	retention time, min
I, methoxy VII	275	225	2.17 (I),[b] 4.16 (VII)
	275	240	1.12 (I)
methyl ester of II and V	225	140	1.15 (II), 3.98 (V)
methyl ester of VI	200	100	2.57
2-chloroethyl ester of II and V	225	170	1.82 (II), 5.63 (V)

[a] Under all operating conditions the detector temperature was maintained at 400 °C and the flow rate of the carrier gas (5% methane in argon) was 30 mL/min. Note: A satisfactory GC condition for the analysis of *m*-phenoxybenzaldehyde (IV) was not obtained on a 3% SE-30 column. [b] The number in parentheses refers to the compound. See Figure 1.

methanol solution, in duplicate, was used for the radioactivity measurement. The remaining solution was reduced to 0.1 mL and applied on a TLC plate.

Pure I, II, V, VI, and incubation extracts were spotted on a TLC plate and developed in one dimension in a chloroform–acetic acid (98.5:1.5 v/v) solvent system and viewed under UV light (243 nm). Incubation extracts with or without the substrate exhibited a large number of spots (up to 10). Plates were exposed to X-ray films (autoradiography) for 2 weeks to identify the radioactive regions (two to three spots) which were scraped off the plates and extracted with methanol. The volume of the extract was adjusted, and the radioactivity content was determined. Nonradioactive regions were also extracted. The region corresponding to I was rechromatographed by developing in a toluene–hexane (80:20 v/v) system. Standard I and IV were also applied beside the extract. The TLC plates were viewed under UV light and the visible regions were scrapped off the plates and extracted. After appropriate volume adjustment the radioactivity content of each fraction was determined. A portion of the extracts after appropriate dilution was analyzed by GC. The extracts representing regions of polar metabolites (e.g., II, V, and VI) were reduced in volume and treated separately with diazomethane and BCl_3–2-chloroethanol. The resultant methyl or 2-chloroethyl derivatives were taken up in hexane and analyzed on a gas chromatograph.

Measurement of Radioactivity. Radioactivity was determined by using a Beckman scintillation system, Model LS-238. Counting effeciency was calculated by using the channel ratio method.

Thin-Layer Chromatography (TLC). Fenvalerate and its metabolites were analyzed on precoated (250 μm) TLC plates. Incubation mixtures were analyzed on silica gel linear-K preabsorbent (Whatman) plates and developed in a chloroform and acetic acid (98.5:1.5 v/v) system. The R_f values for I, II, IV, V, and VI were 0.81, 0.47, 0.75, 0.39, and 0.28, respectively. In a toluene–hexane (80:20 v/v) solvent system, the R_f values for I, IV, and the methyl derivatives for II, V, VI, and VII were 0.55, 0.45, 0.57, 0.49, 0.39, and 0.47, respectively.

Gas Chromatography (GC). Hexane extracts were analyzed on a Perkin-Elmer Sigma 1 gas chromatograph equipped with a ^{63}Ni electron-capture detector. The column used was a glass tube [1 m × 4 mm (i.d.)] packed with 3% SE-30 on 80–100-mesh Chromosorb WHP. A single operating condition was not suitable for identification of all the compounds. The compounds were analyzed under different operating conditions as shown in Table I.

Identification of Metabolites. Positive identification of fenvalerate, its metabolites, and derivatives were carried out on a gas chromatography–mass spectrometry (GC–MS) system consisting of a Finnigan Model 9500 gas chromatograph connected to a Finnigan Model 3100D mass spectrometer. A 1.52 m × 4 mm (i.d.) glass column packed with 3% SE-30 on 80–100-mesh Chromosorb WHP was used for chromatographic separation. The GC was run at various temperatures as shown in Table I and the helium flow rate was 30 mL/min. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

The data in Table II show the extent of metabolism of fenvalerate (I) by a chicken liver enzyme system. It is clear that only a fraction of I (<10%) was degraded under the conditions used in this study. The soluble fraction metabolized 6.9–7.2% of I and the degradation followed entirely the hydrolytic route. Incubation with the soluble plus microsomal fraction metabolized 8.2–8.8% of I, at least 96.6% of which was by hydrolysis. Addition of a NADPH-generating system to the incubating media consisting of soluble plus microsome enzymes seem to increase degradation of I somewhat (8.8–9.2%), but again the predominant (89%) route was by the hydrolytic cleavage of the ester link. Although an increase in the rate of degradation of I was indicated in microsome plus soluble enzyme systems, the product distribution failed to show initial participation of an oxidative mechanism in the degradation of I as would be evidenced by the presence of VII or VIII. Most of the radioactivity of the metabolites was associated with hydrolytic products depending on the position of ^{14}C. One of the expected major oxidation products of I, namely, 4′-hydroxyfenvalerate (VII), was not isolated in any significant quantity but was detected only in trace amounts by GC as a methyl derivative. Another expected oxidative metabolite of I would be 3-hydroxyfenvalerate (VIII), the presence or absence of which could not be demonstrated due to lack of an authentic standard. In any case, it could not have been produced in a significant quantity since only 0.1–0.3% of the extractable radioactivity was unaccounted for (Table II). Thus, the

Table II. Metabolism of Carbonyl-^{14}C- or Phenoxy-^{14}C-Labeled Fenvalerate under Various Incubation Conditions

incubation conditions	% extracted radioactivity[a] ^{14}CO I	II	V	VI	phenoxy-^{14}C I	IV	V	VI
I[b] + buffer	99.7	0.3			99.2		0.8	
I + boiled enzyme	99.5	0.5			99.1		0.9	
I + enzyme[c]	91.8	8.0 (97.6)[d]			91.2		8.5 (96.6)	0.2 (2.3)
I + enzyme + NADPH	91.2	8.5 (96.6)			90.8		8.2 (89.1)	0.9 (9.8)
I + soluble fraction	93.1	6.9 (100)			92.8	3.0 (41.7)	4.1 (56.9)	

[a] Accumulated data from four incubations. [b] Radiopurity of I was 99.8%, purified by TLC 3 times prior to use. [c] Enzyme refers to soluble plus microsomal enzymes (11950*g*). [d] Values in parentheses refer to the percentage of the radioactivity of the metabolites.

predominant route of degradation of fenvalerate by chicken liver enzymens) is the hydrolytic cleavage of the ester linkage. The recovery of I was between 82 and 87%.

In the incubation extracts from the soluble fraction (105000*g*), 3-phenoxybenzaldehyde (IV) was also found in sufficient quantity in addition to 3-phenoxybenzoic acid (V). The formation of IV may be due to fast elimination of HCN from α-cyano-3 phenoxybenzyl alcohol (III), a hydrolytic product. Partial formation of 3-phenoxybenzoic acid (V) could have been due to either action of residual microsomes in the soluble fraction or air oxidation during workup. In this regard it should be mentioned that IV was totally converted into V when allowed to stand at room temperature.

Compounds II and V were the major metabolites in soluble plus microsomal systems. Only traces of IV were detected. Quantifiable amounts of oxidation product VI were also produced, the concentration of which increased in media fortified with a NADPH-generating system. It has been postulated that in vitro the hydrolysis of VII, an initial oxidation product of I, gives rise to VI (Ohkawa et al., 1979). However, in the present study only trace amounts of VII were found, although not fully authenticated. This tends to suggest that either VII is rapidly converted to VI and hence unstable in the media or VII is not the precursor. Enzymatic oxidation of V appears to be a viable alternative route for VI.

The identity of products in the radiolabeled regions was authenticated by cochromatography with authentic standards and GC–MS analyses. Various GC operating conditions were used and are listed in Table I.

Extracts of the TLC region of R_f 0.75–0.85 showed a single peak on gas chromatograms with retention time of 2.17 min, identical with that for fenvalerate. However, on a GC–MS (1.52 m; 3% SE-30 column), it appeared as two peaks with retention time 5.3 and 5.8 min due to two disastereoisomers. Mass spectra due to these peaks were identical and exhibited peaks at m/e 419 ($M^{+\cdot}$, weak), 225, 209, 197, 181, 167, 152, 141, and a base peak at 125. An identical GC–MS was obtained for pure I.

The radioactive region with R_f 0.35–0.5 correspond to that of for II and V, which were analyzed on GC as methyl derivatives. Two peaks were found with retention time of 1.15 and 3.98 min. The mass spectrum of the peak at 1.15 min exhibited a molecular ion peak ($M^{+\cdot}$ at m/e 226, while the peak with retention time of 3.98 min had a molecular ion peak at m/e 228. The GC retention time and spectral data agreed with those recorded for the pure methyl esters of II and V.

The identity of the two acids in the TLC region of R_f 0.35–0.5 was further confirmed by reaction with BCl_3–2-chloroethanol. The reaction products were analyzed by GC at 170 °C. Again, two peaks with retention times of 1.82 and 5.62 min were observed, which corresponded to the 2-chloroethyl esters of II and V, respectively. The MS of the peak with a retention time of 1.82 min showed a molecular ion peak at m/e 274 ($M^{+\cdot}$) and had an isotope pattern for two chlorine atoms. Other major ions associated with this compound were at m/e 232, 167, 152, and 125. The spectrum was identical with that of the authentic 2-chloroethyl ester of II. The compound with a retention time of 5.62 min exhibited a molecular ion peak at m/e (276, $M^{+\cdot}$) and had an isotope pattern for a molecule containing one chlorine atom. Furthermore, the spectrum was consistent with that of a reference compound, the 2-chloroethyl ester of V.

Extracts of regions between R_f 0.25 and R_f 0.35 were methylated and analyzed by GC at 100 °C. At this temperature, the gas chromotograms exhibited a single peak with retention time of 2.57 min. The GC retention time and molecular ion peak agreed with that of a pure sample of methyl 3-(4-methoxyphenoxy)benzoate. Analysis of the methylated extracts at 140 °C indicated the presence of trace amounts of the methyl derivatives of II and V.

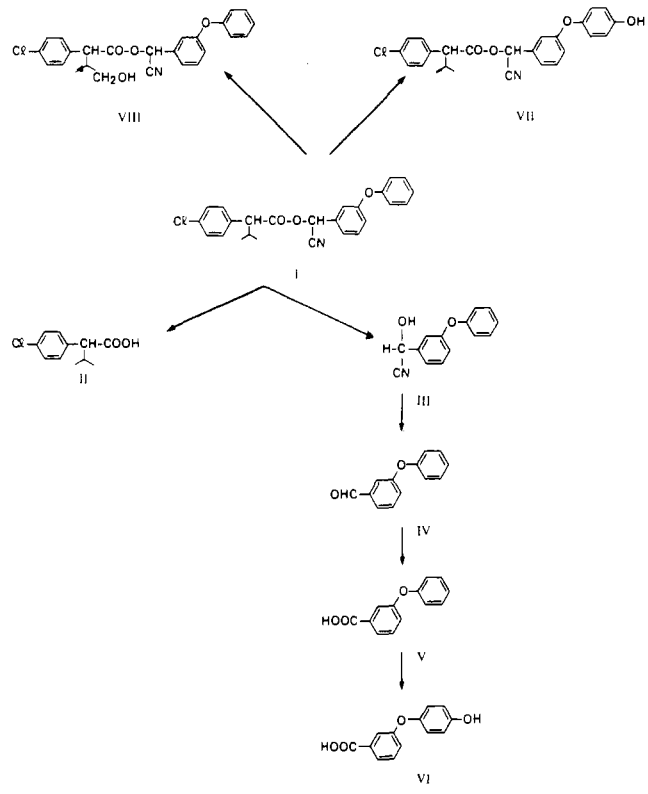

Figure 1. Metabolic pathways for fenvalerate in a chicken liver homogenate.

Methylation and GC analysis of extracts from R_f 0 to 0.25 at 225 °C showed the presence of traces of a compound that behaved like the methyl derivative of 4′-hydroxyfenvalerate (VII). No further information could be obtained from GC–MS analysis since the amount was not sufficient to record a mass spectrum.

The metabolic pathways of fenvalerate in a chicken liver homogenate are shown in Figure 1. Only a fraction (<10%) of the compound was metabolized, and the nature of the enzymes in the incubation media did not influence the rate and mode of degradation (Table II). The data suggest the presence of synthetic pyrethroid hydrolyzing enzyme(s) in both the soluble and soluble plus microsomal fractions of a chicken liver homogenate. Suzuki and Miyamoto (1978) have previously located pyrethroid carboxylesterase, which hydrolyzes the esters of chrysanthemic acid in the various fractions of rat liver homogenate. However, these workers observed greater hydrolyzing activity in the microsomal fraction than in the supernatant and mitochondria fractions.

A slow rate of degradation observed in the present study is in agreement with the earlier work by Soderlund and Casida (1977) with mouse liver microsomal systems. These workers observed that S-3206, fenvalerate, cypermethrin, and cyanophenothrin are very weakly susceptible to attack by hydrolysis and oxidation. They also showed that (2*R*,α*RS*)-fenvalerate was both hydrolyzed and oxidized by mouse liver microsomes; while the 2*S*,α*RS* diastereoisomer was predominantly degraded by oxidation. In general, the oxidative process was faster than hydrolysis. Boyer [Shell Development Co., personal communication, 1976, cited in *FAO Plant Prod. Prot. Pap., Suppl.* (1980)]

also observed degradation of fenvalerate by both oxidation and hydrolysis. However, other details were not available.

Metabolism of fenvalerate in rats (Ohkawa et al., 1979) and mice and rats (Kaneko et al., 1981) predominantly followed the oxidation at the 4′-position of the alcohol moiety. Hydrolysis of ester linkage and oxidation at the 2′-position of the alcohol were also recognized as metabolic pathways.

In all these studies, oxidation was identified as the major route of degradation. However, in the present studies the only identifiable route was the hydrolysis of the ester bond to produce II and III. Compound III underwent fast elimination of HCN to produce IV, which was easily oxidized by air or enzymatically by oxidase to yield V. Metabolite VI was probably formed by the enzymatic oxidation of V.

The metabolism studies of fenvalerate with laying hens have been extended to include feeding the compound at levels of 50 and 100 ppm for 4 days. Preliminary data indicate that fenvalerate was efficiently metabolized, and the major metabolic route was the hydrolysis of the ester (Akhtar and Foster, 1982).

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Registry No. I, 51630-58-1; [*carbonyl*-^{14}C]I, 78387-52-7; [*phenoxy*-U-^{14}C]I, 86045-64-9; II, 2012-74-0; III, 39515-47-4; [*phenoxy*-U-^{14}C]III, 86045-63-8; IV, 39515-51-0; V, 3739-38-6; VI, 35065-12-4; VII, 67882-25-1; 4-chlorobenzyl chloride, 104-83-6; potassium cyanide-^{14}*C*, 5373-08-0; (4-chlorophenyl)acetonitrile-*1*-^{14}*C*, 78387-50-5; 2-chloropropane, 75-29-6; 2-(4-chlorophenyl)-3-methylbutyronitrile-*1*-^{14}*C*, 86045-60-5; 2-(4-chlorophenyl)-3-methylbutyric-*1*-^{14}*C* acid, 86045-61-6; 2-(4-chlorophenyl)-3-methylbutyric-*1*-^{14}*C* acid chloride, 86045-62-7; 2-(4-chlorophenyl)-3-methylbutyric acid chloride, 51631-50-6; [*phenoxy*-U-^{14}C]phenol, 73607-76-8; 3-bromobenzaldehyde, 3132-99-8; HCN, 74-90-8.

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Polynuclear Aromatic Hydrocarbons in Some Nigerian Preserved Freshwater Fish Species

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Major polynuclear aromatic hydrocarbon (PNAH) contents of traditionally preserved Nigerian freshwater fish species, *Clarias lazera*, *Sarotherodon niloticus*, *Sarotherodon galileus*, *Tilapia zilli*, and *Hemichromis fasciatus*, were determined. This composition was compared to the PNAH content of oven-dried (OD) and Ife solar (a University of Ife built and designed box-type solar dryer) dried (ISD) fish species. The result showed that ease of drying was dependent on the initial oil content of fish ($r = 0.98$), which was also species dependent. Traditionally smoked (TS) product always had a significantly higher PNAH content ($P < 0.01$) while OD products always had significantly lower values. Traditionally solar dried (TD) and ISD products had values greater than OD but lower than TS. The carcinogenic and mutagenic hydrocarbon concentrations in the smoked products were always 2–10 times higher than products from each other preservation method tested.

Traditional methods of fish preservation are widely used in Nigeria today, since the purchase and maintenance of freezing and/or chilling equipment are beyond the means of most fishermen. The fishes are preserved by two methods, smoking and solar drying. The traditional smoking method is widely used throughout most regions and involves exposing the fish directly to burning wood, which leaves heavy smoke deposits on the resulting product. The method as traditionally practiced in some states of northern Nigeria simply exposes the fish to open sunlight where it may be infested by insects or wind-borne sand. While the fisherman preserves his catch for economic reasons, the Nigerian consumer purchases this fish for its organoleptic qualities of odor, flavor, and appearance. Thus, smoked or solar-dried fish in modern Nigeria

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