feed-through insect larvicide. The detection of only small residues of [$^{14}$C]diflubenzuron in the tissues also lends confidence to the use of this compound for this purpose.

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# In Vivo and Liver Microsomal Metabolism of Diflubenzuron by Two Breeds of Chickens

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The metabolism and fate of [$^{14}$C]diflubenzuron, *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide, in White Leghorn (WL) and Rhode Island Red/Barred Plymouth Rock (RIR/BPR) chickens were studied after an oral dose of 5 mg/kg. Quantitation of [$^{14}$C]diflubenzuron equivalents confirmed a breed-related 2-fold higher egg residue level in WL chickens. Biotransformation of only small amounts of the administered compound with rapid excretion of unaltered diflubenzuron demonstrated its resistance to in vivo metabolism. WL chickens were shown to produce a higher percentage and greater number of metabolites in the excreta, but the breed-related egg residue difference was not completely a result of different in vivo metabolic rates. In vitro incubations of diflubenzuron using WL and RIR/BPR liver microsomal preparations showed less than 10% conversion to metabolites. Neither induction nor inhibition of mixed-function oxidase (MFO) activity altered diflubenzuron metabolism. The breed-related egg residue difference was thus not the result of MFO activity.

Diflubenzuron, *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide, is a potent, broad spectrum insecticide that inhibits the formation of insect cuticle (VanDaalen et al., 1972). Diflubenzuron has been registered as Dimilin for use against the gypsy moth and conditionally registered to control the boll weevil. It has been demonstrated that diflubenzuron will control fly larvae breeding in the manure of chickens and cattle when added to their diet (Miller, 1974; Miller et al., 1975, 1976; Wright, 1974, 1975; Wright and Spates, 1976). In the course of these "feed-through" investigations Miller et al. (1975), pointed out a 2-fold higher diflubenzuron egg residue level in White Leghorn (egg production type) chickens than in New Hampshire (meat production type) hens after 3 weeks of 50 ppm of diflubenzuron in the diet. Further investigation (Miller et al., 1976) indicated that diflubenzuron residue levels were higher in White Leghorn (WL) fat tissues than crossed Rhode Island Red/Barred Plymouth Rock (RIR/BPR) hen tissues after receiving 10 ppm of diflubenzuron in the diet for 15 weeks. The present study was undertaken to describe the in vivo metabolism and liver microsomal metabolism of [$^{14}$C]diflubenzuron in these two types of chickens as its relates to the demonstrated 2-fold egg residue level difference.

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## MATERIALS AND METHODS

**Chemicals.** [$^{14}$C]Diflubenzuron, uniformly radiolabeled in both rings (specific activity 17.42 mCi/mmol), and technical diflubenzuron were supplied by the Thompson-Hayward Chemical Co., Kansas City, KS. Possible hydrolysis products, (4-chlorophenyl)urea, 4-chloroaniline, and 2,6-difluorobenzoic acid, as well as the hydroxylated materials, *N*-[[(4-chloro-2-hydroxyphenyl)amino]-carbonyl]-2,6-difluorobenzamide and *N*-[[(4-chloro-3-

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hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide, were also supplied by Thompson-Hayward. Possible metabolites, 2,6-difluorobenzamide, *N*-(4-chlorophenyl)-formamide, and *N,N'*-bis(4-chlorophenyl)urea, were synthesized in this laboratory as previously described (Opdycke et al., 1982). Sodium phenobarbital and glucose-6-phosphate dehydrogenase were obtained from Sigma Biochemicals, and SKF-525A was obtained from Smith Kline & French. Radioassay techniques used were also previously described (Opdycke et al., 1982).

**Preparation and Use of Microsomes.** Chickens were weighed and then killed by cervical dislocation. The livers were removed quickly, washed in cold distilled water, weighed, minced with scissors, and homogenized with ice-cold 0.25 M sucrose at 10 g/40 mL in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged in a Beckman L-2 ultracentrifuge at 600*g* for 10 min, the supernatent was centrifuged at 18000*g* for 30 min, and that supernatant was centrifuged at 105000*g* for 90 min. The 105000*g* microsomal pellet was resuspended in 0.15 M KCl and recentrifuged at 105000*g* for 60 min to obtain washed microsomes. The final preparation was resuspended in 0.1 M phosphate buffer. The protein concentration of the final microsomal suspension was determined according to the method of Lowry et al. (1951). Cytochrome P-450 was determined (Omura and Sato, 1964) with a Bausch & Lomb Model 505 recording spectrophotometer.

For assessment of the activity of the microsomal preparations, aldrin to dieldrin epoxidation assays were conducted as positive controls. Incubation mixtures consisted of 10 μg of aldrin in 20 μL of methyl-Cellosolve, 0.8 mL of 0.1 M phosphate buffer, pH 7.4, containing 1.8 μmol of NADP, 18 μmol of glucose 6-phosphate, 8 μmol of magnesium chloride, 0.1 mL of glucose-6-phosphate dehydrogenase (2 units), and 0.1 mL of 0.1 M phosphate buffer with 1 mL (1 mg/mL) of microsomal protein to make a 2-mL total incubation mixture. After a 5-min incubation at 37 °C aldrin and dieldrin were extracted with two 10-mL portions of hexane, a small amount of anhydrous sodium sulfate was added, and 1–5 μL of this extract was quantitated by gas chromatography.

Diflubenzuron incubations were carried out in the same manner as those with aldrin. Each flask contained the NADPH-generating system, 1 mL of enzyme preparation (equivalent to 0.25 g of liver, ranging in protein concentration from 4.91 to 3.35 mg/mL), and 6.4 μg, 20000 dpm, of diflubenzuron in 20 μL of acetone. Incubations were carried out for 90 min at 37 °C and stopped with 15 mL of ethyl acetate. Diflubenzuron and metabolites were extracted with two additional 15 mL of ethyl acetate portions. The volume of these organic extracts was reduced. A sample of the extract was radioassayed, and the remaining portion was chromatographed on TLC followed by autoradiography of the plates. Radioactive spots were scraped from plates and counted for metabolite quantitation. When appropriate, SKF-525A preincubations were for 5 min at 6 or 12 μM (Gillette, 1976).

**Animal Treatment.** Eight adult hens, four WL (36 weeks old) and four RIR/BPR (46 weeks old), were obtained commercially. The chickens were housed in cages (1 × 1 × 1.5 ft) in a 20 × 20 × 12 ft room that was well ventilated and lighted naturally. The chickens were fed 16% laying mash ad libitum and always had access to fresh water. They were allowed to acclimate to the room for 20 and 14 days, respectively, during which time individual weights, feed intake, and egg production were monitored for comparison with the treatment period. The chickens were given approximately 5 mg/kg [$^{14}$C]diflubenzuron (25 μCi to WL and 5 μCi to RIR/BPR hens), which is about

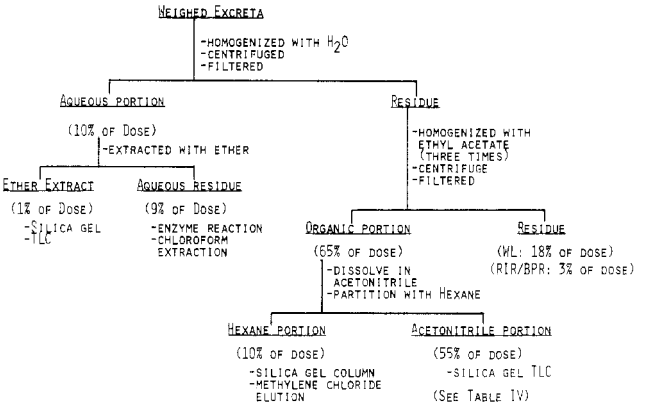


**Figure 1.** Extraction scheme for chicken excreta.

10 times the dose needed for control of housefly larvae in the excreta. Administration of the radiolabeled diflubenzuron was achieved by dissolving the appropriate amounts of radiolabeled and technical material into 4 mL of acetone and adding 1 mL to each of four gelatin capsules containing feed. The acetone was allowed to evaporate overnight, and the capsules were assembled the morning of administration. The capsules were administered orally by placing them into the chicken's crops with forceps. Mineral oil was used to facilitate administration.

Chickens used for liver microsomal investigations were obtained and housed as described by Opdycke and Menzer (1982). Chickens intended as donors for the preparation of liver microsomes were pretreated with sodium phenobarbital at 10 g/3 L in their drinking water for 4 days prior to sacrifice. All hens were held without food for 24 h before microsome preparation.

**Excreta Collection, Extraction, and Quantitation.** Individual chicken excreta were collected at 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 120, 192, and 288 or 312 h (for WL and RIR/BPR final collection, respectively) after the administration of [$^{14}$C]diflubenzuron. The excreta were allowed to fall to aluminum foil sheets beneath the individual cages, collected at the indicated intervals, weighed, and stored at −20 °C until extracted. The WL excreta were extracted by blending the excreta 3 times in a Waring blender with an equal volume of ethyl acetate each time. Centrifugation (2.5*g* for 10 min) was done to separate the homogenate into an organic (ethyl acetate) portion, an aqueous portion if present, and a residue. Aliquots of each phase were taken for radioassay. Extraction of RIR/BPR chicken excreta was modified by first blending the excreta with water (1 mL/g of excreta), filtering with suction to separate the aqueous phase, and then blending the residue 3 times with ethyl acetate to yield the organic and residue portions. This extraction scheme is illustrated in Figure 1.

**Chromatography Procedures.** Thin-layer chromatography was on 20 × 20 × 0.25 cm precoated silica gel 60 F-254 plates (E. Merck). The solvent systems used were the following: (1) chloroform–ethanol–acetic acid, 85:10:5; (2) chloroform–methanol, 90:10; (3) benzene–dioxane–acetic acid, 90:30:1; (4) chloroform–methanol–water, 65:25:4; (5) benzene–methanol–diethylamine, 100:20:10. Gas chromatography was on a Hewlett-Packard 5840 instrument using $^{63}$Ni electron capture detection, with an OV-101 column at 250 °C with methane–argon (95:5) carrier gas. High-pressure liquid chromatography was on a Du Pont 830 liquid chromatograph with previously described conditions (Opdycke et al., 1982).

**Egg Extraction and Quantitation.** Eggs were collected at 12-h intervals, weighed, and extracted according

Table I. Recovery of Excreted Radioactivity following Treatment of Chickens with [$^{14}$C]Diflubenzuron[a]

| time after treatment, h | aqueous | | organic | | residue | | total | |
|---|---|---|---|---|---|---|---|---|
| | WL | RIR/BPR | WL | RIR/BPR | WL | RIR/BPR | WL | RIR/BPR |
| 4 | 2.23 | 0.38 | 14.66 | 0.09 | 0.38 | 0.07 | 17.27 | 0.54 |
| 8 | *b* | 1.60 | 41.03 | 39.18 | 6.63 | 1.31 | 47.66 | 42.09 |
| 12 | *b* | 1.56 | 4.52 | 15.40 | 1.22 | 0.53 | 5.74 | 17.49 |
| 24 | 0.91 | 2.63 | 2.84 | 4.58 | 3.53 | 0.46 | 7.28 | 7.67 |
| 36 | 0.88 | 1.49 | 1.11 | 6.73 | 1.79 | 0.66 | 3.78 | 8.88 |
| 48 | 0.50 | 0.81 | 0.66 | 0.15 | 0.36 | 0.22 | 1.52 | 1.18 |
| 60 | 0.36 | 0.66 | 0.24 | 0.22 | 0.75 | 0.50 | 1.35 | 1.38 |
| 72 | 0.18 | 0.42 | 0.21 | 0.05 | 0.49 | 0.09 | 0.88 | 0.56 |
| 84 | 0.11 | 0.28 | 0.13 | 0.06 | 1.26 | 0.08 | 1.50 | 0.42 |
| 96 | 0.09 | 0.21 | 0.10 | 0.05 | 0.44 | 0.06 | 0.63 | 0.32 |
| 120 | *c* | 0.17 | *c* | 0.08 | *c* | 0.11 | *c* | 0.36 |
| 192 | 1.98 | 0.57 | 0.19 | 0.15 | 0.06 | 0.03 | 2.23 | 0.75 |
| 288 (WL) | | | | | | | | |
| 312 (RIR/BPR) | 0.59 | 0.17 | 0.03 | 0.13 | 0.11 | 0.06 | 0.73 | 0.36 |
| totals | 7.83 | 10.95 | 65.72 | 66.87 | 17.02 | 4.18 | 90.57 | 82.00 |

[a] Results are expressed as percent of the administered dose (average of four replications). [b] No aqueous phase was developed in these samples. [c] No excreta were collected at this time.

Table II. Residual Radioactivity in Eggs Based on Three to Four Eggs per Day following Treatment of Chickens with [$^{14}$C]Diflubenzuron

| days after administration | ppm ± SD | |
|---|---|---|
| | WL | RIR/BPR |
| 1 | 0.083 ± 0.025 | 0.012 ± 0.011 |
| 2 | 0.214 ± 0.139 | 0.061 ± 0.049 |
| 3 | 0.248 ± 0.133 | 0.090 ± 0.030 |
| 4 | 0.233 ± 0.049 | 0.144 ± 0.019 |
| 5 | 0.121 ± 0.036 | 0.119 ± 0.030 |
| 6 | 0.159 ± 0.011 | 0.161 ± 0.019 |
| 7 | 0.115 ± 0.044 | 0.127 ± 0.022 |
| 8 | 0.098 ± 0.010 | 0.097 ± 0.022 |
| 9 | 0.026 ± 0.010 | 0.048[a] |
| 10 | 0.013 ± 0.004 | 0.043 ± 0.010 |
| 11 | 0.010 ± 0.001 | *b* |

[a] Single egg. [b] No eggs collected.

Table III. Residual Radioactivity in Tissues following Treatment of Chickens with [$^{14}$C]Diflubenzuron

| tissue | ppm[a] | |
|---|---|---|
| | WL | RIR/BPR |
| fat | 0.01 | 0.04 |
| liver | 0.06 | 0.15 |
| kidney | 0.19 | 0.14 |
| gizzard | 0.01 | 0.04 |
| ovary with internal eggs | 0.16 | 0.09 |
| breast muscle | 0.01 | 0.03 |
| egg shells | 0.40 | ND |
| brain | ND[b] | 0.25 |
| heart | 0.01 | ND |
| intestine and contents | 0.01 | ND |

[a] Limit of detectability was considered to be the mean of the individual background counts plus twice the standard deviation of the background counts. [b] ND = none detected.

to the method of Miller et al. (1975), blending individual eggs with 150 mL of ethyl acetate and 25 g of anhydrous sodium sulfate, followed by suction filtration. The residual radioactivity in the filtrate was determined and related to the original egg weight for calculation of ppm of diflubenzuron.

**Tissue Quantitation.** The WL and RIR/BPR hens were killed after 12 and 13 days, respectively, with samples of fat, liver, kidney, gizzard, ovary with internal eggs, breast muscle, heart, brain, and intestine taken for [$^{14}$C]diflubenzuron residue quantitation. Samples from individual birds of fat, liver, kidney, muscle, gizzard, and ovary with internal eggs were each extracted in a Soxhlet apparatus for 8 h with ethyl acetate. Unextracted residues were determined by combusting in the sample oxidizer. For analysis of intestines and content (about 15 cm from each hen), hearts, brains, and egg shells, the four replications were combined for each breed of chicken and Soxhlet extracted for 16 h with ethyl acetate. Unextracted residues were combusted for radioassay. All results of chicken [$^{14}$C]diflubenzuron residues were converted to ppm based on the original wet organ or tissue weight. The results obtained from four replications for each tissue were judged significant if they were higher than the background plus twice the standard deviation of the background.

**Metabolite Isolation and Characterization.** The ethyl acetate extracts of the chicken excreta were concentrated by using a rotary evaporator at 50 °C. The residues were taken up in acetonitrile and washed 3 times with hexane to remove lipoidal materials. This extract was then applied to thin-layer plates and developed with solvent system 1. Radiolabeled metabolites, as indicated by radioautography, were scraped from the plates for radioassay. Organosoluble metabolites from chicken excreta were isolated by preparative TLC using successive elutions of solvent system 1 and ether. Final purification of certain isolated metabolites was accomplished on Sephadex LH-20 columns eluted with methylene chloride. TLC cochromatography with known reference compounds and HPLC characterization with authentic reference compounds were used for metabolite characterization.

The aqueous fractions remaining after extraction of chicken excreta with ethyl acetate were adsorbed on Amberlite XAD-2 columns and eluted with water and methanol. The resulting water and methanol fractions from the columns were concentrated on a rotary evaporator at 50 °C and applied to TLC plates. Characterization of metabolites was accomplished by cochromatography with known reference compounds in the solvent systems described. The material remaining at the origin of the TLC plates from the water fractions was scraped from the plates, incubated with either β-glucuronidase for 24 h at 37 °C or 1 N HCl for 1 h at 50 °C, and extracted with chloroform. Appropriate controls were used for enzyme and acid treatments of the water fractions.

## RESULTS

**Chicken Metabolism of [$^{14}$C]Diflubenzuron.** Chickens showed no outward signs of physiological stress

Table IV. Quantitation and Thin-Layer Chromatographic Characterization of [$^{14}$C]Diflubenzuron and Metabolites in Organic Fractions of Chicken Excreta

| metabolite | % of dose | | $R_f$ values in solvent systems[a] | | | |
|---|---|---|---|---|---|---|
| | WL | RIR/BPR | 1 | 2 | 3 | 4 |
| I, diflubenzuron | 49.90 | 63.39 | 0.87 | 0.85 | 0.75 | 0.99 |
| II, unknown | 1.21 | | 0.78 | 0.67 | 0.59 | 0.84 |
| III, unknown (2-hydroxy-4-chloro-) | 1.22 | 0.50 | 0.73 | 0.56 | 0.51 | 0.80 |
| IV, unknown (3-hydroxy-4-chloro-) | 1.02 | 0.51 | 0.69 | 0.53 | 0.72 | |
| V, 4-chloroaniline | 0.44 | 0.58 | 0.62 | 0.51 | 0.38 | 0.71 |
| VI, 2,6-difluorobenzamide | 1.98 | | 0.46 | | 0.45 | 0.68 |
| VII, unknown [*N*-(4-chlorophenyl)formamide] | 0.35 | 0.32 | 0.42 | 0.46 | 0.22 | 0.56 |
| VIII, (4-chlorophenyl)urea | 3.14 | 0.38 | 0.38 | 0.28 | 0.10 | 0.43 |
| IX, unknown | 0.48 | | 0.32 | | 0.04 | |
| X, unknown | 0.48 | 0.35 | 0.26 | 0.28 | 0.05 | 0.47 |
| XI, 2,6-difluorobenzoic acid | 1.35 | 0.22 | 0.06 | 0.11 | origin | 0.32 |
| XII, origin | 4.31 | 0.56 | origin | origin | | 0.28 |

[a] Solvent systems: 1, chloroform–ethanol–acetic acid (85:10:5); 2, chloroform–methanol (90:10); 3, benzene–dioxane–acetic acid (90:30:1); 4, chloroform–methanol–water (65:25:4).

Table V. Liver to Body Weight Ratios and Cytochrome P-450 Content of White Leghorn and Rhode Island Red/Barred Plymouth Rock Chicken Liver Microsomes with and without Sodium Phenobarbital Pretreatment

| | WL | | RIR/BPR | |
|---|---|---|---|---|
| | control | phenobarbital | control | phenobarbital |
| | Liver/Body Weight (Ratio) | | | |
| unwashed[a] | 0.014 ± 0.001 | 0.019 ± 0.004 | 0.011 ± 0.001 | 0.015 ± 0.004 |
| washed[b] | 0.014 ± 0.002 | 0.016 ± 0.001 | 0.012 ± 0.001 | 0.017 ± 0.004 |
| | P-450 Content (Nanomoles per Milligram of Protein) | | | |
| unwashed[a] | 0.030 ± 0.018 | 0.157 ± 0.051 | 0.068 ± 0.038 | 0.134 ± 0.004 |
| washed[b] | 0.00118 | 0.213 ± 0.183 | 0.079 ± 0.102 | 0.204 ± 0.237 |

[a] 13-, 14-, and 16-week-old chickens; mean ± SD. One chicken each week. [b] 17- and 19-week-old chickens; mean of two ± SD.

from the 5 mg/kg single oral dose and only negligible changes in body weight and feed intake. Egg production decreased slightly from 0.96 to 0.83 egg/day in the WL hens and from 0.82 to 0.76 egg/day in the RIR/BPR hens.

Table I shows the quantitation of [$^{14}$C]diflubenzuron equivalents in chicken excreta as they were partitioned according to the scheme illustrated in Figure 1. In excess of 90% of the administered dose was accounted for in the WL excreta. Rapid elimination of 65 and 43% of the dose within the first 8 h after administration suggests similar excretion patterns for the WL and RIR/BPR chickens. Partitioning of the respective excreta showed similar percentages for the aqueous and organic fractions but not for the residue (Table I). The difference between 17% of administered radioactivity in the residue of WL chicken excreta and 4% in the RIR/BPR chickens may result from the fact that WL chicken excreta contained considerably more water than excreta from RIR/BPR chickens, which was quite dry. Most of the radioactivity excreted by both strains of chickens was extractable with ethyl acetate.

[$^{14}$C]Diflubenzuron equivalents secreted into the eggs were 0.79 and 0.30% of the administered dose for WL and RIR/BPR chickens, respectively. Table II shows average residues in parts per million, indicating peak levels of 0.248 ppm on the third day in WL eggs and 0.161 ppm on the sixth day in RIR/BPR eggs. WL egg residues were consistently higher than RIR/BPR egg residues for 4 days posttreatment but seemed to peak in about half the time. Secretion of diflubenzuron equivalents in eggs was faster in WL chickens while the residues in RIR/BPR chicken eggs were higher at later time intervals. TLC and HPLC analysis of the egg ethyl acetate extracts revealed only unchanged diflubenzuron.

Table III reports [$^{14}$C]diflubenzuron equivalents in chicken tissues, showing that in general higher residues were present in the RIR/BPR tissues. The highest residue was 0.25 ppm in the brain tissue of the RIR/BPR hens and 0.19 ppm in the kidney tissue of the WL birds. Because of the great difference in bird size, RIR/BPR being about twice as heavy as WL, the higher residues found in the RIR/BPR hens seem to account for the smaller percentage of the administered dose present in their excreta. Levels of radioactivity in the chicken tissues were too low for further characterization.

Table IV shows the percentage of administered dose for each of the metabolites isolated from the organic phase of the chicken excreta. WL chickens metabolized a greater percentage of the [$^{14}$C]diflubenzuron, and a larger number of compounds was detected. In WL chickens, 16% of the administered dose was transformed to $^{14}$C-labeled metabolites, while RIR/BPR hens transformed only 3.4% of the dose. In both cases the major product excreted was unchanged diflubenzuron.

The various compounds separated on TLC plates are designated I–XII in Table IV, where I is the parent compound and higher numbers designate compounds with progressively lower $R_f$ values. Characterization of metabolites was accomplished by comparing $R_f$ values in four solvent systems with those reported by other workers, cochromatography on these systems with known reference compounds, diazomethane derivatization and cochromatography in some cases, and HPLC cochromatography with standards in some cases. The compounds that could be identified are named in the table. Tentative identifications are noted in cases where both HPLC characterization and cochromatography with known compounds could not be accomplished.

Positively identified metabolites included 4-chloroaniline, 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, and (4-chlorophenyl)urea. Tentatively identified metabolites were *N*-(4-chlorophenyl)formamide, *N*-[[(4-chloro-2-hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide,

Table VI. Aldrin Epoxidation in Chicken Microsomes

| | WL | | | | RIR/BPR | | | |
|---|---|---|---|---|---|---|---|---|
| | control | | phenobarbital induced | | control | | phenobarbital induced | |
| time, weeks | % conversion[a] | nM/mg, 5 min[a] | % conversion[a] | nM/mg, 5 min[a] | % conversion[a] | nM/mg, 5 min[a] | % conversion[a] | nM/mg, 5 min[a] |
| 11 | 6.9 ± 0.2 | 1.84 ± 0.08 | 4.2 ± 0.4 | 1.71 ± 0.05 | | | | |
| 13 | 5.7 ± 0.2 | 0.57 ± 0.06 | 21.3 ± 0.9 | 1.83 ± 0.25 | 2.5 ± 0.1 | 0.34 ± 0.02 | 9.8 ± 0.4 | 1.34 ± 0.14 |
| 14 | 9.3 ± 0.4 | 1.41 ± 0.05 | 67.7 ± 0.9 | 14.38 ± 0.63 | 42.8 ± 1.2 | 6.9 ± 0.17 | 63.5 ± 0.6 | 11.3 ± 0.1 |
| 16 | 51.6 ± 1.7 | 13.54 ± 0.45 | 94.4 ± 0.1 | 24.79 ± 0.06 | 62.7 ± 0.1 | 16.45 ± 0.08 | 64.6 ± 1.6 | 17.1 ± 0.51 |
| 17 | 17.9 ± 0.7 | 3.72 ± 0.16 | 74.7 ± 1.5 | 17.95 ± 0.39 | 29.2 ± 1.9 | 6.19 ± 0.35 | 62.0 ± 1.9 | 15.49 ± 0.50 |
| 19 | 17.5 ± 0.8 | 1.84 ± 0.04 | 58.8 ± 0.7 | 8.02 ± 0.68 | 23.1 ± 1.5 | 2.57 ± 0.06 | 61.3 ± 3.2 | 6.22 ± 0.54 |
| unwashed[b] | 22.20 ± 25.52 | 5.17 ± 7.26 | 61.13 ± 36.90 | 13.66 ± 11.47 | 36.00 ± 30.67 | 7.89 ± 8.10 | 45.96 ± 31.3 | 9.92 ± 7.95 |
| washed[c] | 17.70 ± 0.28 | 2.78 ± 1.32 | 66.75 ± 11.21 | 12.98 ± 7.09 | 26.15 ± 9.31 | 4.57 ± 2.56 | 6.165 ± 0.49 | 10.85 ± 6.55 |

[a] Percent conversion of aldrin to dieldrin; nanomoles of dieldrin formed per milligram of protein in the incubation mixture during 5 min of incubation (mean of triplicate incubations ± SEM). [b] Average values from 13-, 14-, and 16-week-old chickens. [c] Average values from 17- and 19-week-old chickens.

and *N*-[[(4-chloro-3-hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide. Tentative identification of *N*-(4-chlorophenyl)formamide is based on HPLC cochromatography with standards.

**In Vitro Metabolism of [$^{14}$C]Diflubenzuron.** Preliminary experiments established that the microsomal fraction was more active in metabolizing diflubenzuron than the soluble fraction or the S9 fraction. Furthermore, it was established that optimum conditions for chicken liver microsomal incubations were pH 7.4, 37 °C, and 90-min incubation times. The ability of the preparations to metabolize aldrin to dieldrin was monitored to serve as a positive control for the activity of microsomes.

Microsomes were prepared from chicken liver homogenates at intervals from 11 to 19 weeks, and comparisons were made between the WL and RIR/BPR chickens both with pretreatment and without pretreatment with sodium phenobarbital. The results of the pretreatment with sodium phenobarbital, expressed as liver/body weight ratios and cytochrome P-450 content, are presented in Table V. The microsomes from 13-, 14-, and 16-week-old chickens were not subjected to a final resuspension and centrifugation after preparation and are termed "unwashed". Microsomes from 17- and 19-week-old chickens were washed prior to incubation with substrates. Phenobarbital pretreatment resulted in significantly higher liver/body weight ratios and cytochrome P-450 levels in both WL and RIR/BPR chickens than in controls. There seems to be no difference between WL and RIR/BPR microsomes.

The results of incubations to ascertain aldrin epoxidation activity in microsomes are presented in Table VI. Phenobarbital pretreatment significantly induced aldrin epoxidation in both WL and RIR/BPR chickens. There seems to be no difference between washed and unwashed microsomes with respect to aldrin epoxidation. Preincubation of microsomes with SKF-525A inhibited all formation of dieldrin.

The extent of diflubenzuron metabolism in chicken liver microsomes is expressed in terms of the change in the extractability of [$^{14}$C]diflubenzuron equivalents by ethyl acetate following incubation (Table VII). There were no differences between recoveries of [$^{14}$C]diflubenzuron equivalents in either WL or RIR/BPR control or phenobarbital-induced microsomal incubations.

Ethyl acetate extracts were chromatographed on TLC using solvent system 5. Table VIII reports the percent recoveries of each spot compared to the total applied radioactivity following their removal from the plates separately for radioassay. The results indicate the resistance of diflubenzuron to metabolism in that 72–99% of the organic fraction was recovered as the intact parent compound ($R_f$ 0.65). There were additional chromatographic spots in both WL and RIR/BPR microsomes. These spots behave chromatographically like the cleavage metabolites 2,6-difluorobenzamide, (4-chlorophenyl)urea, 2,6-difluorobenzoic acid, and 4-chloroaniline.

Heat-inactivated microsomes were the only treatment in which no metabolites were seen. There did not appear to be any significant differences in the patterns of any of the other treatments or between either type of chicken microsomes. The fact that TLC patterns were similar in the presence and absence of mixed-function oxidase cofactors indicated that diflubenzuron was not metabolized by these enzymes. This conclusion is further supported by the facts that sodium phenobarbital induction and preincubation with SKF-525A did not affect the results. However, since the heat-inactivated preparation resulted in only unmetabolized diflubenzuron while the other three preparations were active, this shows that the metabolites

Table VII. Percentage of [$^{14}$C]Diflubenzuron Equivalents Partitioned into Organic Fraction after Incubation with Chicken Liver Microsomes

| | WL | | RIR/BPR | |
|---|---|---|---|---|
| | control | phenobarbital induced | control | phenobarbital induced |
| unwashed microsomes[a] | 55.6 ± 4.8 | 54.0 ± 0.5 | 53.6 ± 1.4 | 54.1 ± 5.2 |
| washed microsomes[b] | 79.2 ± 15.0 | 78.9 ± 2.0 | 64.3 ± 1.7 | 54.2 ± 5.3 |
| SKF-525A preincubated | 81.0 ± 14.0 | 72.1 ± 15.1 | 57.6 ± 2.9 | 59.9 ± 7.8 |

[a] Means ± SD of one hen each from 13-, 14-, and 16-week-old chickens. [b] Means ± SD of one hen each of 17- and 19-week-old chickens.

Table VIII. Fractionation of Organic Solvent Extracts of Chicken Liver Microsomal Incubations with [$^{14}$C]Diflubenzuron[a]

| | boiled microsomes | | | | no cofactors | | | | complete incubation | | | | SKF-525A preincubation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WL | | RIR/BPR | | WL | | RIR/BPR | | WL | | RIR/BPR | | WL | | RIR/BPR | |
| $R_f$[b] of metabolite | control | induced | control | induced | control | induced | control | induced | control | induced | control | induced | control | induced | control | induced |
| 0.65 | 92.6 | 98.0 | 98.7 | 98.0 | 85.9 | 71.9 | 88.4 | 77.8 | 92.5 | 84.7 | 86.3 | 90.9 | 93.8 | 86.6 | 93.0 | 88.9 |
| 0.53 | | | | | 3.8 | 6.8 | 2.6 | 5.6 | 1.7 | 3.7 | 4.3 | 2.7 | 2.3 | 4.0 | 1.6 | 2.8 |
| 0.48-0.40 | | | | | 4.3 | 5.0 | 1.8 | 4.3 | 3.4 | 4.7 | 3.3 | 1.8 | 0.7 | 3.0 | 2.1 | 2.0 |
| 0.30 | | | | | 3.7 | 6.0 | 3.0 | 5.2 | 1.0 | 2.6 | 3.5 | 1.8 | 1.3 | 2.9 | 0.8 | 1.3 |
| 0.22 | | | | | 1.5 | 6.9 | 3.2 | 4.5 | 0.7 | 1.7 | 2.4 | 1.3 | 1.1 | 1.6 | 1.2 | 1.4 |
| origin | 7.3 | 1.7 | 1.2 | 1.6 | 1.4 | 3.1 | 1.2 | 2.3 | 0.1 | 1.3 | 1.7 | 1.2 | 0.5 | 1.4 | 1.0 | 1.5 |

[a] Expressed as percentage of recovered radioactivity. [b] Thin-layer plates were developed with benzene–methanol–diethylamine (100:20:10), and spots in the indicated zones were scraped from the plates for radioassay.

formed were from some other enzymatic system present in the microsomal preparation.

## DISCUSSION

Excretion patterns of diflubenzuron in the two breeds of chickens are similar, with most of the [$^{14}$C]diflubenzuron passing through the digestive tract unchanged within 12 h after administration. RIR/BPR chickens metabolized only 3.4% of the administered dose while WL chickens converted 16% of the administered dose to metabolites. In the WL chickens (4-chlorophenyl)urea was the principal metabolite, indicating cleavage of the urea bridge between the benzoyl carbon and the N′ urea nitrogen. Relatively high quantities of 2,6-difluorobenzoic acid were also present to support this interpretation. WL chickens metabolized diflubenzuron to a greater extent than did RIR/BPR chickens, on a body weight basis. This may be explained by a generally higher rate of metabolism in the WL chicken (Opdycke and Menzer, 1982). Metabolite characterization of RIR/BPR excreta suggest that they metabolize diflubenzuron to a lesser extent than WL chickens. It is difficult to identify a primary pathway of metabolism due to the lower quantities of metabolites, but 4-chloroaniline and 2,6-difluorobenzoic acid are present.

The presence of up to five unidentified metabolites makes further metabolism pathway proposals difficult. Other workers (Metcalf et al., 1975; Ivie, 1978; Willems et al., 1980) have all reported identification of hydroxylated diflubenzuron metabolites in sheep, cattle, and rats. Tentative identification of metabolites III and/or IV as hydroxylated products based on TLC chromatography in some solvent systems suggests that chickens are able to hydroxylate diflubenzuron.

Swine metabolism of diflubenzuron (Opdycke et al., 1982) is comparable with chicken metabolism both quantitatively and qualitatively. Diflubenzuron seems to be cleaved at the urea bridge by all animals tested (Ivie, 1978; Metcalf et al., 1975; Williams et al., 1980; Opdycke et al., 1982), and the ability to hydroxylate diflubenzuron enhances further metabolism. Rats (Willems et al., 1980) metabolize all of orally administered diflubenzuron, and hydroxylation and conjugation metabolites compose 80% of the products, leaving cleavage metabolites as 20% of the products. Cattle (Ivie, 1978) hydroxylate about 30% of the administered diflubenzuron with 20% cleavage products and 50% unmetabolized. Sheep (Ivie, 1978) metabolize considerably less diflubenzuron with cleavage products as the main metabolites. Swine (Opdycke et al., 1982) and chickens seem to hydroxylate diflubenzuron only sparingly, producing mostly cleavage metabolites, and quantitatively are least effective in metabolizing diflubenzuron among the animals investigated.

Another possible explanation for the varying animal metabolism of diflubenzuron is absorption. Willems et al. (1980) have demonstrated that the absorption (and subsequent metabolism) of diflubenzuron by the rat is inversely related to the administered dose. Ivie (1978) has also indicated this trend with cannulated sheep. It may be that swine and chickens do not adsorb the diflubenzuron as efficiently as rats and cattle.

It is interesting to note the higher egg residual radioactivity secreted by the WL chickens, with the higher tissue residual radioactivity deposited in the RIR/BPR chickens. The residual radioactivity in the eggs was entirely from the parent compound; no metabolites were found in eggs. The difference in the total percent recoveries of [$^{14}$C]diflubenzuron equivalents from the different chickens might be explained by the higher tissue

radioactivity found in the heavier RIR/BPR chickens. Miller et al. (1976) reported higher tissue residue levels in the WL chickens. The delayed sampling of the tissues (14 days after a single oral dose) may explain this apparent conflict. Recoveries of [$^{14}$C]diflubenzuron in the eggs of the respective chickens confirm that WL chickens excrete about twice the amount of diflubenzuron in their eggs as RIR/BPR chickens. Higher metabolism rates by the WL chickens seemed to indicate this as a possible explanation. However, the presence of only unmetabolized diflubenzuron in the eggs makes any hypothesis as to an explanation based on metabolism difficult.

Comparison of the liver microsomal metabolism of diflubenzuron between these two types of chickens was made to further characterize the metabolic differences on a subcellular level. It was found that the microsomal metabolism differences were less than in vivo metabolism differences.

Chicken liver microsomal induction via phenobarbital has been demonstrated by Stephen et al. (1971) and Powis et al. (1976). The present study demonstrated liver microsomal induction after phenobarbital pretreatment, with increased liver to body weight ratios and increased amount of cytochrome P-450. All of these studies have found lower P-450 levels in chickens than those reported in rats. Induction of aldrin epoxidase levels in both WL and RIR/BPR chicken livers did not result in enhanced diflubenzuron metabolism.

Chicken liver microsomal metabolism of [$^{14}$C]diflubenzuron was very low in both types of hens, with or without phenobarbital pretreatment. Chromatographic characterization of organic extracts after microsomal incubations indicated some slight differences in WL and RIR/BPR metabolites. Both types of hens produced cleavage metabolites after microsomal incubation. Mixed-function oxidase enzymes do not seem to be primarily involved in diflubenzuron metabolism in chicken microsomes.

The results of this study of diflubenzuron metabolism by WL and RIR/BPR chickens indicate slight differences in metabolite formation. In no instance does the breed-related metabolism difference reach as much as 2-fold. Metabolism differences of WL and RIR/BPR chickens are not of the same order of magnitude as the egg residue level differences; therefore, they do not seem to account for the diflubenzuron egg residue level difference.

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# Pesticide Residues in Soil. 1. Gas Chromatographic Determination of Vinclozolin

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Vinclozolin [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyloxazolidine-2,4-dione] is a possible contaminant of soil as a result of fungicide treatments. It is taken up by benzene with pyrophosphate for analysis; the extracts are analyzed by gas chromatography with an electron-capture detector, without cleanup. For concentrations from 0.01 to 10 ppm, the recovery from soil is good (93 ± 2%). The detection limit is 0.001 ppm.

In fruit growing we cannot do without pesticides: most treatments are directed to the aerial part of plants, but it is not possible to avoid the fall of pesticides on soil, often also in large quantity. Moreover, rains, dews, and wind wash away and remove physically the residues from leaves and fruits to the soil, where both the active ingredient and some transformation products can accumulate. The presence of a pesticide can influence chemical and microbiological properties of the soil (Martin, 1963; Siegel, 1975; Wainwright, 1977), but the study of interactions between pesticides and soil is only possible if there are analytical methods able to assess the quantities of pesticides present.

It seemed to us interesting to take Vinclozolin into consideration: Vinclozolin is the active ingredient of the fungicide sold as Ronilan, with a fair gray-mold capability (Bolay et al., 1976; Piglionica and Ferrara, 1979; Di Giusto et al., 1980; Gullino et al., 1980; Carniel and Micolini, 1980), extensively used in the vineyard, in fruit and vegetable

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