Environmental Degradation of the Insect Growth Regulator Methoprene. X. Chicken Metabolism

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Treatment of Leghorn chickens with a single oral dose of [5-^{14}C]methoprene [isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate] resulted in residual radioactivity in tissues and eggs. The chemical nature of the residual radiolabel in tissue (muscle, fat, liver), eggs, and excrement was thoroughly examined at several doses (0.6 to 77 mg/kg). Although a high initial dose (59 mg/kg) resulted in methoprene residues in muscle (0.01 ppm), fat (2.13 ppm), and egg yolk (8.03 ppm), these residues of methoprene represented only 39 and 2% of the total ^{14}C label in fat and egg yolk, respectively. Radiolabeled natural products from extensive degradation of methoprene were by far the most important ^{14}C residues in tissues and eggs, particularly at the lower dose of 0.6 mg/kg where [^{14}C]cholesterol and normal [^{14}C]fatty acids (as triglyceride) contributed 8 and 71% of the total radiolabel in egg yolk. Novel minor metabolites of methoprene were observed in lipid depots, resulting from saturation of the dienoate system. These minor metabolites were conjugated to glycerol and/or cholesterol.

Methoprene [1, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, ZR-515, trademark Altosid] is an example of a class of dienoate insect growth regulators with juvenile hormone activity (Henrick et al., 1973, 1975; Staal, 1975). Methoprene is particularly active toward dipteran larvae, and has been proposed as a feed additive for control of fecal-breeding houseflies in poultry rearing (Breeden et al., 1975; Morgan et al., 1975). In order to determine the safety of using methoprene in a "feed-through" application, a detailed chicken metabolism study has been performed, and we now report the chemical identity of ^{14}C residues from [5-^{14}C]methoprene which appeared in excrement, tissues, and eggs. This work is complementary to balance studies performed by Davison (1976).

EXPERIMENTAL SECTION

Mature white Leghorn hens were dosed orally with [5-^{14}C]methoprene (Schooley et al., 1975) at 0.6–77 mg/kg by Davison (1976). After collection of excrement for 2 days, certain chickens were sacrificed for analysis of ^{14}C residues in tissues; eggs were collected from other chickens for 2 weeks. Freeze-dried samples and frozen whole egg yolks were shipped in dry ice to Zoecon Corp. for analysis of the chemical identity of radioactive constituents.

Radioassay and Chromatography. Radioactivity was determined by liquid scintillation counting and total combustion as described previously (Quistad et al., 1974a). Thin-layer chromatography (TLC) plates were precoated (silica gel GF and alumina GF, Analtech). Analytical high-resolution liquid chromatographic (HRLC) separations were performed with a high-pressure pump (M-6000, Waters Associates), a loop injector (Valco CV-6-UHPa), and ultraviolet absorbance detector (LDC Model 1205). Dienoic metabolites were mixed with authentic standards (ca. 5 μg) for chromatography (Zorbax-ODS, DuPont; 25 × 0.21 cm, eluted with 75% methanol in water). Authentic dienoic standards **1**–**4** were prepared by methods similar to those published (Henrick et al., 1973, 1975) while **6** and **7** were synthesized by air oxidation of commercially available aldehydes (e.g. **5**). 11-Methoxy-3,7,11-trimethyldodecanoic acid (MTDA, **8**) and 11-hydroxy-3,7,11-trimethyldodecanoic acid (HTDA, **9**) were prepared by catalytic hydrogenation of the corresponding dienoic acids **4** and **3**. The cholesteryl ester **10**, triglyceride **11**, and diglyceride **12** containing MTDA were prepared by adding cholesterol, distearin, and monoolein respectively to the acid chloride of MTDA. MTDA, HTDA, and the cholesteryl ester **10** isolated from chickens were purified by preparative HRLC using a pneumatic amplifier pump (Haskel Engineering), a loop injector (Valco), an ultraviolet absorbance detector (Chromatronix Model 230), and a Zorbax-SIL column (22 × 0.79 cm, DuPont). Methyl esters of compounds **8** and **9** and metabolite **10** were eluted at 600 psi in 6.5, 25, and 5% ether in pentane, respectively. The methodology for gas–liquid chromatographic/mass spectrometric (GLC/MS) analysis of methyl esters of **8** and **9** was as previously described (Schooley et al., 1975) whereas problems in transfer across the instrument interface necessitated direct-inlet introduction of sample for **10**.

General Extraction Procedure. Freeze-dried samples of feces, muscle, fat, liver, and nonlyophilized eggs (whites and yolks) were homogenized with acetonitrile. The filtered acetonitrile (400 ml) was diluted with water (1.4 l.) then sodium chloride (25 g) was added, and the solution was acidified to pH 2. Extraction of the aqueous acetonitrile with ethyl acetate afforded a metabolite extract (i.e. methoprene and primary metabolites **2**–**7**, cf. Miller et al., 1975). The ethyl acetate was evaporated under reduced pressure and primary metabolites were characterized by TLC, TLC of methyl esters (where applicable), and HRLC of esters. In cases of high lipid content (e.g. fat), a low-temperature precipitation of the lipid in the acetonitrile was necessary prior to dilution with water or extraction with ethyl acetate (Miller et al., 1975).

The solids (Celite + tissue) from filtration of the initial acetonitrile extract were extracted exhaustively with $CHCl_3$ and methanol. Residual tissue solids were then combusted to quantitate radioactivity.

Urine was filtered to remove precipitated uric acid. The solid was thoroughly washed with water and then purified to constant specific activity by alternate dissolution in alkali followed by precipitation upon addition of acid. The isolated uric acid (1150 dpm/mg) showed a satisfactory ultraviolet spectrum when compared to authentic standard [λ_{max} 293 nm (ϵ 8500, water), 239 (6300)]. The combined aqueous phases after removal of uric acid were extracted with an equal volume of ether. A portion of the aqueous urine (pH 4) was treated with β-glucuronidase and sul-

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fatase (10 mg each, from *H. pomatia*, Sigma Chemical Co.) for 16 h at 37 °C to cleave conjugated primary metabolites (Quistad et al., 1974a). A second portion was treated with 0.1 M HCl overnight and then extracted with $CHCl_3$.

Whole blood (28 ml) was added to a saturated solution of magnesium sulfate in 2.5% H_2SO_4. After addition of Celite (10 g) the mixture was extracted with ethyl acetate (200 ml) by homogenizing in a Waring blender. The ethyl acetate extract (1.7% total ^{14}C in blood) was examined by TLC on silica gel (benzene–EtOAc–HOAc, 100:30:3). Although neither methoprene nor metabolites **2–7** were detectable (<0.01 ppm), the major radioactive components were cholesterol and cholesteryl esters (1.2 and 0.1% of total blood radiolabel). For verification of structural identity, [^{14}C]cholesterol was derivatized to cholesteryl benzoate and the [^{14}C]cholesteryl esters were saponified to free cholesterol (Quistad et al., 1975a).

Characterization of MTDA, HTDA, and the Cholesteryl Ester of MTDA. Triglycerides containing MTDA and HTDA were isolated from the acetonitrile and chloroform extracts of egg yolk by preparative TLC on silica gel (hexane–ethyl acetate, 4:1). These anomalous triglycerides were separable from natural triglycerides by virtue of their increased polarity (R_f 0.51 for natural, 0.34 for MTDA, 0.21 for HTDA). Saponification of natural triglycerides gave only radiolabeled natural fatty acids while saponification of triglycerides containing MTDA and HTDA gave only MTDA and HTDA respectively as *radiolabeled* acids. The methyl esters of MTDA and HTDA were purified by HRLC as described. The mass spectrum of the methyl ester of MTDA was identical with an authentic sample: *m/e* (relative intensity), 271 (1), 109 (3), 95 (2), 81 (2), 73 (100), and 69 (7). The mass spectrum of the methyl ester of HTDA also matched an authentic standard: 257 (1), 225 (3), 222 (2), 207 (2), 171 (10), 125 (10), 101 (100), 97 (11), 69 (24), and 59 (74). Likewise diglycerides containing MTDA and HTDA were saponified to release free acids which were converted to methyl esters for structure verification by TLC and HRLC. Synthetic standards of glycerol-2-(11-methoxy-3,7,11-trimethyldodecanoate)-1,3-distearate (**11**) and *rac*-glycerol-1-(11-methoxy-3,7,11-trimethyldodecanoate)-3-oleate (**12**) migrated on TLC in the same regions as the respective tri- and diglycerides containing MTDA from egg yolk.

The chloroform extract of fat (23.2 g) contained a large mass of lipid (>20 g) which was saponified with methanolic KOH (100 ml, 3 M) at 25 °C for 16 h. The saponification products were acidified and then extracted with $CHCl_3$ (2 × 200 ml). [^{14}C]Cholesterol was precipitated by treating an aliquot (ca. 1 g) with digitonin. Radiolabeled cholesterol in the precipitate was verified by cleaving the digitonide with glacial HOAc (Quistad et al., 1975a). A portion of the mixture of free acids from saponification (128 mg, 820 dpm) was resolved into normal fatty acids (2.9 dpm/mg) and MTDA by preparative TLC on silica gel (eight 20 × 20 cm plates, benzene–ethyl acetate–acetic acid, 100:30:3).

Extraction of liver with acetonitrile gave a major metabolite zone slightly less polar than normal triglycerides after TLC. Saponification of this zone gave radioactive MTDA (identified by TLC and HRLC of its methyl ester) and nonradioactive cholesterol (identified by TLC). The metabolite was purified by HRLC and was characterized as cholesteryl 11-methoxy-3,7,11-trimethyldodecanoate (**10**) by mass spectroscopy: *m/e* (relative intensity), 369 (42), 368 (100), 353 (8), 260 (16), 255 (10), 247 (14), 147 (8), and 73 (63).

Simplified Analysis for Methoprene, MTDA, and HTDA. Egg yolks (10–16 g) and various lyophilized tissue homogenates (3–50 g) were extracted with $CHCl_3$ (vide supra) and one-tenth of the sample was evacuated to dryness. After transfer of the extract in dry benzene (10 ml) to a centrifuge tube, methanolic sodium methoxide (20 ml, 0.5 M, stored under nitrogen) was added and the solution was heated at 50 °C for 10 min. The mixture of methyl esters was acidified, diluted with water, and extracted with hexane. An aliquot of the hexane extract was purified by preparative TLC on silica with development in hexane–ethyl acetate (100:15). Addition of standard **1** prior to development allowed two distinct TLC zones to be scraped: zone 1, more mobile than methoprene (fatty acid methyl esters); zone 2, methoprene and remainder of plate excluding origin (includes methyl ester of **4** and cholesterol). Zone 2 was eluted from the silica, then dissolved in methanol–water (90:10) for structure verification by HRLC on Zorbax-ODS. Although trace amounts of **1**, HTDA, and MTDA were easily soluble in aqueous methanol, most of the mass in zone 2 was insoluble and was characterized as cholesterol by TLC. Since HTDA and MTDA are nonchromophoric at 254 nm, their elution was determined relative to added authentic standards of the methyl esters of **3** and **4**. Both HTDA and MTDA methyl esters eluted slightly after the 2*E* isomers of methyl esters of **3** and **4**, respectively (as noted previously for HTDA and MTDA isolated from yolk triglyceride and characterized by GLC–MS). Under the conditions of transesterification, **1** was converted to the methyl ester of **4** in ca. 80% yield, but since **4** was not detectable (either free or conjugated) as a metabolite in yolk or tissue homogenates, the total residue of **1** was merely the sum of radioactivity in regions corresponding to **1** and the methyl ester of **4**.

Table I. Radiolabeled Constituents in Chicken Feces, Urine, and Blood of Chicken Dosed with Methoprene at 59–64 mg/kg

	% total ^{14}C
	In Feces
Feces (0–24 h)	
Methoprene (**1**)	38.9
Hydroxy ester **2**	1.9
Hydroxy acid **3**	2.3
Methoxy acid **4**	9.1
Total identified	52.5
	In Urine
Urine (0–24 h)	
Methoprene	<0.1
Unconjugated **2–7**	<0.1
Conjugated **3**	4.0
Conjugated **4**	5.3
Uric acid	5.3
Aqueous soluble unknowns	84.8
	In Blood
Blood (48 h)	
Methoprene + **2–7**	<0.1
Cholesterol	1.2
Cholesteryl esters (natural)	0.1
Precipitated solids	96.5

RESULTS AND DISCUSSION

Excrement. Since the principle of feed-through fly control by methoprene (**1**) is based on transport of unmetabolized **1** through the chicken and into fecal droppings, it was not surprising that **1** was the major ^{14}C residue in feces (Table I). Feces collected at 0–24 h from a colostomized hen dosed at 59 mg/kg with unformulated **1** contained 6.4% of the applied dose as unmetabolized methoprene. Excreted methoprene was largely unisomerized (Table II) which is an important aspect of its metabolic fate considering the greatly reduced biological activity of the 2*Z* isomer of **1** (Henrick et al., 1975). It

Table II. Isomerization of Methoprene and Metabolites

	Z:E ratio at C-2
Feces (0-24 h)	
Methoprene 1	7:93
Hydroxy ester 2	38:62
Hydroxy acid 3	45:55
Methoxy acid 4	26:74
Fat	
1	16:84
2	14:86
Egg yolk (2 day posttreatment)[a]	
1	12:88
3	33:67
4	33:67
Urine	
Conjugated 3	29:71
Conjugated 4	36:64

[a] Chicken dosed at 77 mg/kg.

appears that the methoprene actually available in feces for insect control is rapidly excreted from the chicken since only 0.07% of the applied methoprene was found in feces from 24–48 h. The methoxy acid **4** was the most abundant primary metabolite in feces (9% total fecal ^{14}C) and it was considerably isomerized at C-2 as were metabolites **2** and **3** (Table II). The mechanism for isomerization of **1** and its metabolites is unknown.

Primary metabolites and **1** were not found in urine, but conjugated hydroxy acid **3** and methoxy acid **4** contributed 4 and 5% of the total urinary radiolabel (Table I). Extensive degradation of methoprene to natural biochemicals was evidenced by recovery of [^{14}C]uric acid (2% applied ^{14}C in 0–24 h); however, 85% of the radiolabel in urine was found as polar, nonextractable unknowns.

Eggs. Davison (1976) showed that up to 19% of the ^{14}C from a single oral dose of **1** was eliminated from the chicken in eggs. Radiolabeled residues in egg white maximized at 2% applied ^{14}C (total for eggs laid 0–48 h). Exhaustive extraction of egg white gave small amounts (<0.1 ppm) of methoprene and primary metabolites only when the chicken was dosed at an exaggerated rate of 77 mg/kg. Since 97% of the ^{14}C in egg white was unextractable with nonpolar or polar organic solvents, we presume by analogy with previous work (Quistad et al., 1975a) that most of the ^{14}C was associated with egg proteins.

Egg yolk was a considerably more important reservoir for excreted radiolabel (up to 17% applied dose for 12 eggs after 2 weeks). At doses of 0.6 to 77 mg/kg the major radiolabeled products were natural triglycerides. The radiolabeled fatty acids in these triglycerides undoubtedly arose from thorough degradation of [5-^{14}C]methoprene to [^{14}C]acetate with reincorporation into the normal complement of fatty acids (Quistad et al., 1974b, 1975b). For the range of doses tested, methoprene contributed only 1–2% of the total ^{14}C in yolk (Table III) and primary metabolites **2–7** were only detectable (<0.1 ppm) at the 77 mg/kg dose rate. [^{14}C]Cholesterol also contained up to 7% of the total ^{14}C in yolk indicating that [^{14}C]acetate was more readily converted to [^{14}C] fatty acids than to [^{14}C]cholesterol in contrast to results from steer metabolism of **1** (Quistad et al., 1975a).

Two unusual products from reductive metabolism of **1** were found at the exaggerated dose of 77 mg/kg. These metabolites (Table IV) were 11-methoxy-3,7,11-trimethyldodecanoic acid (MTDA, **8**) and 11-hydroxy-3,7,11-trimethyldodecanoic acid (HTDA, **9**). Although metabolites from the reduction of xenobiotics are less common, hydrogenation of double bonds has been reported for geraniol by rabbits and dogs (Kuhn et al., 1936) and a number of monocyclic terpenes (Parke, 1968). MTDA and HTDA in egg yolk were present almost solely in the conjugated form as glycerides (Table III). Since conjugation of metabolite acids as glycerides is apparently unprecedented for pesticides, we explored these unusual glycerides in greater detail. It is evident from the dose–response data in Table III that MTDA and HTDA glycerides were most important at the highest dose of 77 mg/kg. As the dose was lowered the relative abundance of these anomalous glycerides decreased more rapidly than the dosage rate. In fact, only 1–2% of the total ^{14}C in egg yolk was in the form of MTDA–HTDA glycerides at 0.6–31 mg/kg, whereas these glycerides contributed 37% of the total yolk ^{14}C at 77 mg/kg.

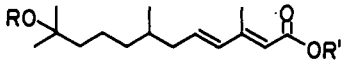
In order to explore further the possible relevance of MTDA–HTDA residues, hens were given a single oral dose at 0.6 mg/kg of **1** (approximating an anticipated field-use rate of 10 ppm of methoprene in feed) and eggs were collected for 2 weeks for subsequent analysis. In this time study, the cumulative abundance of HTDA (as glyceride) in egg yolk was negligible (0.03% applied ^{14}C). Yolk residues of MTDA (as glyceride) closely paralleled those of **1** (Figure 1), but never contributed more than 2–3% of the total ^{14}C in yolks (maximal for 2–6 days posttreatment). After 2 weeks the sum of all methoprene and MTDA (as glyceride) residues in yolk was only 0.22 and 0.26% of the applied dose. Normal glyceride [^{14}C]fatty acids were by far the most abundant radiolabeled fraction in egg yolk (Figure 2). An average of 71% of the total yolk

Table III. Chemical Identity of ^{14}C Residues in Egg Yolks 2 Days after a Single Oral Dose of Methoprene

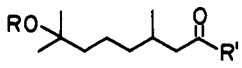
Product	% total ^{14}C (ppm) in yolk		
	77 mg/kg	31 mg/kg	0.60 mg/kg
Primary metabolites			
Methoprene (1)	2.4 (8.03)	1.2 (0.47)	2.0 (0.056)
Hydroxy acid 3	0.01 (0.024)		
Methoxy acid 4	0.02 (0.052)		
MTDA	0.01 (0.035)		
2, 5–7	<0.01 (<0.02)		
Natural products			
Triglycerides	42.3	70.8	75.8
Cholesterol	1.5	6.8	4.0
Metabolite glycerides			
Triglyceride with MTDA	31.4 (88.8)	1.3[a] (0.46)	2.2[a] (0.058)
Diglyceride with MTDA	2.3 (6.2)		
Triglyceride with HTDA	1.8 (4.5)	0.2[a] (0.080)	0.4[a] (0.0088)
Diglyceride with HTDA	1.2 (3.0)		
Total identified	82.9	80.3	84.4

[a] Includes diglyceride-conjugated aglycone.

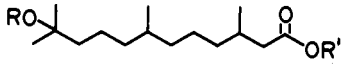
Table IV. Metabolic Derivatives of Methoprene and Model Compounds



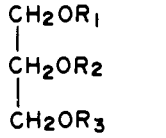
1, R = CH_3; R′ = $CH(CH_3)_2$
2, R = H; R′ = $CH(CH_3)_2$
3, R = R′ = H
4, R = CH_3; R′ = H



5, R = CH_3; R′ = H
6, R = CH_3; R′ = OH
7, R = H; R′ = OH



8, R = CH_3; R′ = H (MTDA)
9, R = R′ = H (HTDA)
10, R = CH_3; R′ = cholesteryl



11, R_1 = R_3 = stearate; R_2 = MTDA
12, R_1 = oleate; R_2 = H; R_3 = MTDA

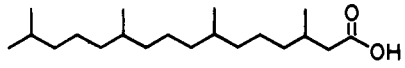


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Table V. Chemical Identity of ^{14}C Residues in Chicken Liver after Oral Dosage with Methoprene

	64 mg/kg		0.58 mg/kg	
Product	% total ^{14}C in liver	ppm	% total ^{14}C in liver	ppm
Primary metabolites				
Methoprene (1)	<0.01	<0.01	<0.01	<0.01
MTDA (8)	0.16	0.24	<0.01	<0.01
HTDA (9)	<0.02	<0.03	<0.01	<0.01
2-7	<0.01	<0.01	<0.01	<0.01
Natural products				
Triglyceride	0.3		18.8	
Cholesterol	1.9		10.3	
Metabolite glycerides				
Triglyceride with MTDA	2.0	3.0		
Triglyceride with HTDA	1.4	2.0	<0.1	<0.01
Cholesteryl MTDA (10)	14.6	22	1.0[a]	0.0024[a]
Total identified	20.4		30.1	

[a] Includes glyceride-conjugated MTDA.

Table VI. Radiolabeled Constituents in Fat and Muscle of Chicken Dosed Orally with Methoprene at 59 mg/kg

	% total ^{14}C	ppm
Fat	In Fat	
Primary metabolites		
Methoprene	39.3	2.13
Hydroxy ester 2	2.3	0.12
Hydroxy acid 3	0.3	0.013
4-7	<0.3	<0.01
Natural products		
Triglycerides	20.2	
Cholesterol	1.9	
Metabolite glycerides		
Triglycerides with MTDA	17.0	0.93
Total identified	81.0	
	In Muscle	
Muscle (breast)		
Methoprene	0.5	0.01
Primary metabolites (i.e. 2-7)	<0.5	<0.01
Residual solids	62.3	

^{14}C could be identified as glyceride [^{14}C]fatty acids and 8% of the total ^{14}C residue was [^{14}C]cholesterol. Thus, almost 80% of the total ^{14}C in egg yolk was contributed by radiolabeled natural products.

Liver. Since chicken liver contained about 1% of the applied ^{14}C from 1 after 2 days and the liver is a notable site for metabolic transformations, we examined the ^{14}C residue in this organ (Table V). At a high dose of 64 mg/kg the major product was the cholesteryl ester of MTDA (10, 15% total liver ^{14}C). This unusual conjugate (at least for pesticide metabolites) was of minor importance at a lower dose (0.6 mg/kg) and radiolabeled natural products became quantitatively important depots of radioactivity for nonpolar products.

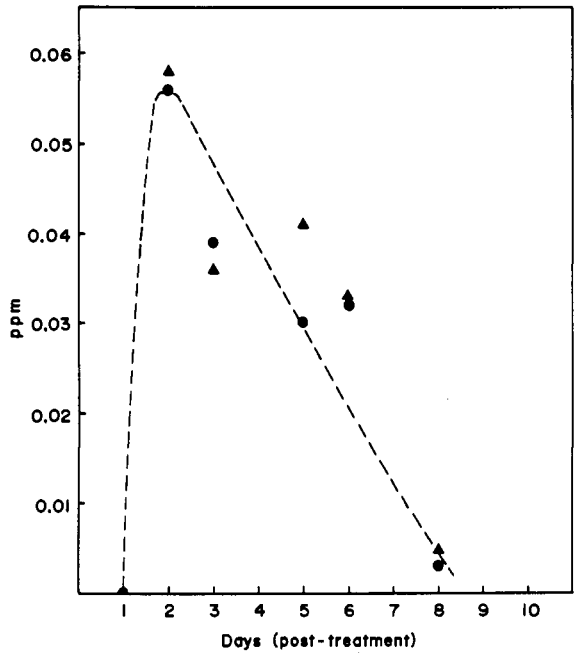

Figure 1. Residues of methoprene (circles) and MTDA (triangles) in chicken eggs after single oral dosage with methoprene at 0.6 mg/kg.

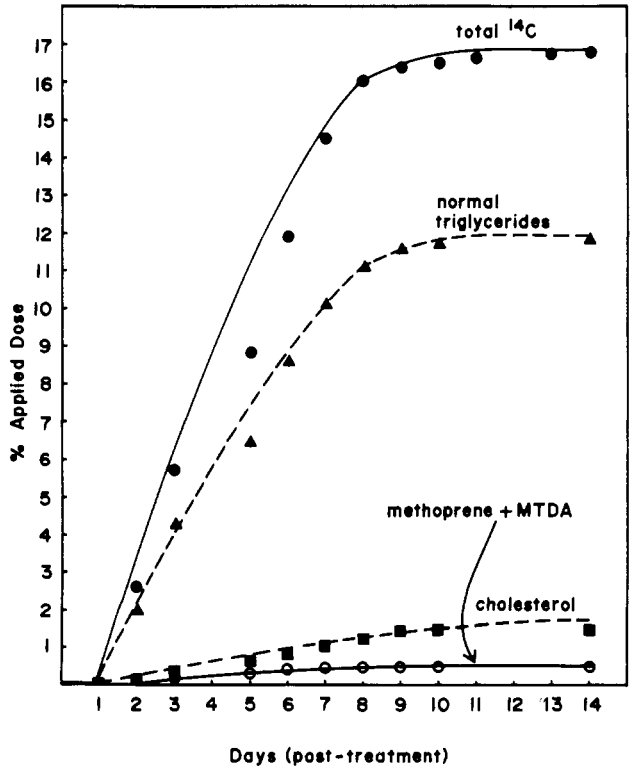

Figure 2. Cumulative radioactive residues in chicken egg yolk after dosage with methoprene at 0.6 mg/kg.

Muscle and Fat. Although muscle contained only trace amounts of 1 and primary metabolites (<0.01 ppm, Table VI), fat contained residues of 1 (2.1 ppm) and MTDA as

glyceride (0.9 ppm), but at considerably lower levels than in yolk even at the exaggerated dose of 59 mg/kg. Again radiolabeled natural triglycerides and cholesterol contributed major portions of the total ^{14}C residue in fat.

A meat-residue study was conducted to determine if detectable 1 or metabolites would be found in a chicken dressed for consumption. Two days after a single oral dose of 1 at 0.6 mg/kg the edible carcass contained 4.2% of the applied dose. However, methoprene and MTDA (as glyceride) were not detectable (<0.01 ppm) in the edible chicken carcass.

Significance of Results. Two new metabolites from methoprene were identified as saturated methoxy (MTDA) and hydroxy (HTDA) acids. Our evidence indicates that these metabolites were absent in previous work on bovine metabolism (Quistad et al., 1975a,b). These reduced metabolites were minor constituents as free acids, but at an elevated dose rate MTDA was a relatively important portion of the total ^{14}C residue in egg yolk (as conjugated glycerides) and in liver (as a cholesteryl ester). These reduced metabolites are possibly intermediates in the conversion of methoprene (1) to acetate (Quistad et al., 1974b, 1975a). An excellent analogy is provided by the natural diterpenoid phytanic acid (13), which bears a striking structural resemblance to the reduced metabolites MTDA and HTDA. Phytanic acid is the major mammalian metabolite of phytol and is apparently formed by reduction of the intermediate phytenic acid. Saturation of the 2-ene double bond generates a substrate more suitable for α-oxidation (Lough, 1975), a step necessary in order to render the β-methyl-branched chain susceptible to β-oxidation. That MTDA and HTDA should be incorporated into cholesterol esters and glycerides is not surprising, since phytanic acid is found in all lipid classes of fish and animals, comprising up to 10% of total fatty acids in ruminant plasma lipids (Lough, 1975). To our knowledge glyceride conjugates of pesticide metabolites are previously unknown, or at least very uncommon.

Total residues of methoprene and MTDA (as glyceride) in all eggs for 2 weeks after treatment were still less than 0.3% of the applied dose. Almost 80% of the total ^{14}C in egg yolks was identified as radiolabeled natural biochemicals which again is indicative of the extensive degradation of methoprene to anabolic precursors of natural products as in previous work in ruminants (Quistad et al., 1975a,b) and fish (Quistad et al., 1976).

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