Environmental Degradation of the Insect Growth Regulator Methoprene. VII. Bovine Metabolism to Cholesterol and Related Natural Products

Gary B. Quistad,* Luana E. Staiger, B. John Bergot, and David A. Schooley

Samples of fat, muscle, liver, lung, blood, and bile from a steer which received a single dose of [5-^{14}C]methoprene were analyzed for radioactive residues. No primary methoprene metabolites could be characterized, but the majority (16–88%, depending on tissue) of the total tissue radioactivity was positively identified as [^{14}C]cholesterol. A total of 72% of the bile radioactivity was contributed by cholesterol, cholic acid, and deoxycholic acid. Radioactivity from catabolized methoprene was also associated with protein and cholesteryl esters of fatty acids.

Methoprene (**1**, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, ZR-515, trademark Altosid) is the most commercially advanced example of a class of insect growth regulators (IGR's) with juvenile hormone activity (Henrick et al., 1973; Staal, 1975). Methoprene is effective in preventing adult emergence of mosquitoes (Schaefer and Wilder, 1973) and various fecal-breeding flies (Harris et al., 1973). This paper reports the metabolism of methoprene when ingested by a steer, and investigates the identity of residual tissue radioactivity. A radioactivity balance study and identification of urinary–fecal metabolites will be reported for methoprene metabolism in the same steer (Chamberlain et al., 1975). Previous reports on the environmental degradation of methoprene have appeared from this laboratory (for part VI, see Quistad et al., 1974b).

MATERIALS AND METHODS

Treatment of Steer. The oral dosage of this Hereford steer with [5-^{14}C]methoprene (3.55 mCi, 0.58 mCi/mmol) has been described (Chamberlain et al., 1975). The animal was sacrificed 2 weeks after treatment and selected tissues (fat, muscle, liver, lung, blood, and bile) were frozen for shipment (packed in Dry Ice) by air freight to Zoecon Corporation for analysis.

Radioassay and Chromatography. Radioactivity was measured by liquid scintillation counting and total combustion as previously described (Quistad et al., 1974a). Thin-layer chromatography (TLC) plates were precoated (silica gel GF and alumina GF, Analtech). The conditions for gas–liquid chromatography (GLC) and mass spectrometry (MS) have been described (Quistad et al., 1974a).

High-resolution liquid chromatographic (HRLC) separations were performed on an instrument built from selected commercial components: Haskel Engineering pump (part No. 26920-4); pressures up to 3000 psi; Valco loop injector (part No. CV-6-HPax) or a Waters Associates septum injector; Chromatronix Model 230 ultraviolet absorbance detector; stainless steel columns; solvents routinely redistilled in glass.

General Extraction Procedure for Solid Tissues. A 100-g portion of each solid tissue was minced and homogenized in a Waring Blendor with $CHCl_3$ (3 × 250 ml). The combined $CHCl_3$ extracts were washed with water (100 ml). Aqueous washings contained negligible radioactivity and were discarded. $CHCl_3$ was evaporated and residual lipids (2–30 g) were dissolved in CH_2Cl_2 (200 ml) to which Celite (10 g) was added. Agitation at −78° with a stream of nitrogen precipitated lipids adsorbed to Celite for filtration at −18°. This low-temperature precipitation, though ineffective for fat samples, effectively eliminated lipids (all but about 100 mg) from muscle, lung, and liver extracts with little (4%) loss of radioactivity and rendered the residue suitable for TLC analysis. Substitution of CH_3OH for CH_2Cl_2 removed enough lipid from the concentrated fat extract to permit TLC analysis, but with a concomitantly large (61%) incorporation of radioactivity into the precipitate. The concentrates were chromatographed on silica gel (500 μ) and alumina (250 μ) with development in hexane–ethyl acetate (100:15) (SS-1) followed by benzene–ethyl acetate–acetic acid (100:30:3) (SS-2). Radioactive bands were detected with a scanner and quantitated by counting scrapings of each band.

After $CHCl_3$ homogenization the residual tissue was exhaustively extracted in a Soxhlet apparatus for 16 hr with additional $CHCl_3$ (500 ml) and then with methanol (500 ml), and the proceeds analyzed as described above. The average loss for CH_2Cl_2 precipitation of the MeOH Soxhlet extract fractions was 49% and unextractable radioactivity was determined by combustion of the residual tissue solids.

Purification of Liver Cholesterol. The radioprofiles on TLC of $CHCl_3$ extracts of fat, muscle, liver, and lung were quite similar. The main radioactive peak on silica gel (developed with SS-1 and then SS-2) appeared to have the same R_f (0.47) as hydroxy ester **2** and methoxy acid **4**, while a second band seen in varying amounts (11–33% of radioactivity in extract) had R_f the same as the large biomass of neutral lipids on the plate and higher than methoprene or any known metabolite.

Treatment of a portion of the $CHCl_3$ extract of liver with ethereal diazomethane and subsequent TLC analysis revealed no change in chromatographic properties (hence, dienoic acid **4** absent). The entire sample of $CHCl_3$ extract was subjected to preparative TLC separation (four 20 × 20 × 0.1 cm silica gel plates) in SS-2. After scraping the radioactive band and eluting with redistilled THF, the sample (4.05×10^5 dpm, 150–300 mg) was further purified by preparative HRLC using a 0.5 m × 22 mm i.d. slurry-packed column of LiChrosorb SI 60, 20 μ, eluted with 25% ether in pentane at a flow rate of 16 ml/min. After elution for 91 min post-injection, the system pressure was increased from 400 to 1000 psi. The radioactive cholesterol (3.43×10^5 dpm) eluted in the next fraction (20 min). Little uv activity was evident in this fraction (much less than expected for 98 μg of **2** equivalent to 4.05×10^5 dpm). Evaporation of solvent gave a white, crystalline solid (2920 dpm/mg, 143 mg, mp 143–146°) [cholesterol mp 146–149° (Eastham and Teranishi, 1963)]. GLC analysis (3% OV-101 on Chromosorb W-AW-DMCS, 230°) of this material showed it to contain 99.7% of a single component with identical retention time to a cholesterol standard; subsequent GLC–MS analysis revealed that its mass spectrum was identical with that of cholesterol.

The potential primary metabolite **2** and cholesterol were unresolvable on silica gel (R_f 0.47, SS-1 followed by SS-2) or by HRLC on a silica (LiChrosorb, Merck) preparative

Zoecon Corporation Research Laboratory, Palo Alto, California 94304.

column. However, a consistently good separation (R_f 0.65 vs. 0.59) was found on alumina (SS-2). The same TLC behavior was observed for methoprene metabolites in lung, muscle, and fat tissues although the large biomass of fat caused slightly anomalous TLC migrations.

Preparation of Cholesterol Derivatives. The steer liver cholesterol (1.3 mg) and digitonin (12.4 mg) in ethanol (95%, 1 ml) and acetone (5 ml) were heated for 10 min at 55–60° and the solvent then evaporated. No radioactivity was extractable from the precipitated *digitonide* with acetone and no free cholesterol remained. The digitonide was cleaved by heating the precipitate in glacial acetic acid (1.0 ml) for 5 min at 55–60°. The acetic acid was neutralized with saturated potassium bicarbonate (40 ml) and the aqueous phase extracted with $CHCl_3$ (2 × 50 ml). Evaporation of solvent followed by TLC gave free cholesterol containing 76% of the plated radioactivity.

The liver cholesterol (1.1 mg) was acetylated in acetic anhydride (20 μl), pyridine (20 μl), and $CHCl_3$ (300 μl) at 100° for 0.5 hr. The reaction mixture in $CHCl_3$ (20 ml) was washed with water (5 ml) and the aqueous phase was extracted with $CHCl_3$ (10 ml). The combined $CHCl_3$ extracts were evaporated for TLC. *Cholesteryl acetate* (R_f 0.77, alumina, developed in SS-2) represented 83% of the radioactivity on the plate. An additional 11% corresponded to unreacted cholesterol.

The same procedure using liver cholesterol (1.2 mg), benzoyl chloride (20 μl), and pyridine (20 μl) afforded *cholesteryl benzoate* (R_f 0.81, alumina, developed in SS-2) in 90% yield as determined by scanning radioactivity on a TLC plate. No other distinct product bands were observed.

Attempted Solubilization of Unextractable Radioactivity from Exhaustively Extracted Steer Liver Tissue. All tissues examined were homogenized and exhaustively extracted with solvent. Nevertheless, total combustion of the remaining predominantly proteinaceous material demonstrated the presence of residual radiolabel. Of those tissues so analyzed, liver showed the highest percentage (52%) of unextractable radioactivity. Samples of this residue were therefore digested with Pronase (from *Streptomyces griseus,* Calbiochem), in an effort to solubilize the label for chemical identification.

In order to facilitate Soxhlet extraction, the homogenized liver sample had been mixed with Celite (20 g of Celite to 100 g of dry tissue homogenate). A 1.1-g sample of this heterogeneous material was suspended in 5.0 ml of 0.75 *M* phosphate buffer (pH 7.5), treated with 50 mg of fresh Pronase, and shaken at 32° for 18 hr, followed by 6 hr at 38°. A control sample (1.0 g) was run with identical conditions, lacking only the enzyme. After addition of 5.0 ml of methanol to each sample, solid residues were removed by filtration, the filtrates concentrated to near dryness under reduced pressure, and the resultant syrups redissolved in 2.0 ml of water. The aqueous solution was extracted with $CHCl_3$ and this extract and both aqueous solutions were then radioassayed in Insta-Gel (Packard). The solid residues were radioassayed by total combustion of duplicate 100-mg samples followed by liquid scintillation counting.

Pronase digestion of the tissue solubilized 83% of the previously unextractable radioactivity. Aqueous and $CHCl_3$ extracts were examined on TLC (silica gel, developed with hexane–ethyl acetate, 2:1; the "aqueous extract" was developed again with $CHCl_3$–acetone–acetic acid, 100:20:2). Despite heavy loading, the specific activity of the samples was so low that radioscans of the TLC plates were extremely noisy. However, the scans suggested that most of the activity was associated with amino acids at the origin.

Analysis of Radioactivity in Blood. Samples of frozen whole steer blood (2-weeks post-treatment) were thawed (cells fully lysed by freeze–thaw treatment) and examined for (a) total activity, (b) inorganic radioactivity as $H^{14}CO_3^-$, (c) organic extractable activity, (d) water soluble activity, and (e) unextractable label associated with blood proteins.

Total radioactivity in blood was 5760 dpm/ml by total combustion of whole blood adsorbed on cellulose powder. A 20.0-ml sample (112,000 dpm) of whole blood was decanted into a 250-ml gas-washing bottle containing 50 ml of 3 *M* aqueous HCl. The outlet was connected in series to two gas-washing bottles containing 100 ml each of 5% aqueous KOH solution to trap $^{14}CO_2$. The entire gas train was then purged with a slow stream of nitrogen, which bubbled through the acidified blood for about 2.5 hr. Aliquots (2 ml) of each trap were then added to Insta-Gel for radioassay revealing only 8 dpm/ml of volatile radioactivity (as $^{14}CO_2$).

To facilitate extraction with organic solvents, 5.0 ml of whole blood (28,800 dpm), diluted with 15 ml of water, was saturated with $(NH_4)_2SO_4$ (about 15–20 g), and the combined aqueous phase and red precipitate extracted once with 40 ml of acetonitrile–1-butanol (1:1), followed by extractions with 1-butanol (3 × 20 ml). The organic extracts were combined, radioassayed, concentrated under reduced pressure, and set aside for further analysis. The aqueous residue, containing the "curdled" blood proteins, was filtered, the solids washed with water, and the colorless filtrate radioassayed in Insta-Gel. The brick-red precipitate was freeze dried, pulverized, and then mixed with powdered cellulose prior to total combustion.

The organic extract of blood was preparatively chromatographed on silica gel to yield a crude cholesterol zone (5.8 mg) and radioactive material less polar than methoprene (1.4 mg). To obtain more material an additional 10 ml of blood was extracted and chromatographed, providing 7.1 and 3.5 mg, respectively. An aliquot of the material from the cholesterol zone was then benzoylated in pyridine (100 μl) and benzoyl chloride (80 μl) at 23° for 18 hr, yielding a single radiolabeled substance which co-migrated on silica gel TLC with authentic cholesteryl benzoate. To further characterize the [^{14}C]cholesteryl benzoate, an aliquot was subjected to HRLC (0.5 m × 2.4 mm i.d., LiChrosorb SI 60, 10 μ, eluted with 0.5% ether in pentane) and coincidence of radiolabel with uv absorbance of an authentic standard was observed.

The less-polar radioactive material (4.9 mg) was saponified by refluxing with CH_3OH (800 μl) and 50% aqueous NaOH (100 μl) for 3.5 hr. Following concentration to a volume of 200–300 μl, the basic solution was diluted to 1.0 ml with water and extracted with hexane (4 × 0.5 ml). The concentrated hexane extracts (2.3 mg of solid; 5900 dpm) were chromatographed on silica gel as before, and a single radioactive band with identical mobility to free cholesterol was observed, with no radioactivity observed in the less polar zone. Benzoylation of this neutral material produced by saponification gave a derivative with identical chromatographic mobility (TLC and HRLC) to authentic cholesteryl benzoate.

The aqueous alkaline extract from the saponification was acidified with $KHSO_4$, saturated with NaCl, and extracted with hexane (3 × 1 ml) to give only 440 dpm. After evaporation of the hexane, the residue was treated with ethereal diazomethane. The methylated extract was preparatively chromatographed by silica gel TLC, and zones corresponding to cholesterol (R_f 0.4–0.7) were eluted and radioassayed. Approximately 275 dpm was recovered from the cholesterol zone, and about 60 dpm (<2% of the starting radioactivity) was found in the less polar fatty ester zone which was analyzed by GLC–MS.

Exhaustive Analysis of Steer Bile. Steer bile (10 ml, 2.01×10^5 dpm/ml) was extracted with $CHCl_3$ (3 × 20 ml). The $CHCl_3$ extract (2.6% total bile radioactivity) was fractionated into equal amounts of cholesterol and a nonpolar unknown. Cholesterol was identified by TLC on silica gel (SS-2) and TLC of cholesteryl benzoate (benzene–ethyl acetate, 50:15) which was quantitatively formed from choles-

terol.

The aqueous bile after preliminary $CHCl_3$ extraction was acidified (2 *N* HCl) to pH 2 and extracted with $CHCl_3$ (3 × 20 ml). TLC (toluene–acetic acid–H_2O, 1:1:1) of the free bile acids gave cholic acid (**9**, 20.9% total bile radioactivity) and deoxycholic acid (**10**, 11.1%). The crude mixture of bile acids (0.112 g) was methylated with diazomethane and resolved by TLC (benzene–acetone, 70:30, two 20 × 20 cm plates, 500 μ) to give methyl cholate (0.059 g) and methyl deoxycholate (0.030 g) in quantitative yield. The assigned structures were verified by GLC of the silylated ethers: bis-(trimethylsilyl)trifluoroacetamide–DMF, room temperature; 3% OV-101; 1 m × 2 mm i.d. column. Methyl cholate crystallized from hexane after cold storage [mp 151–155°; standard mp 155° (Pollack and Stevens, 1965)]. Methyl cholate was also characterized by GLC–MS. The mass spectrum of the trimethylsilylated methyl cholate was identical with a standard spectrum.

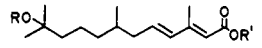
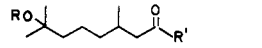
Radioactive cholic and deoxycholic acids were further verified by HRLC of the benzoates of their methyl esters. Methyl cholate (29 mg, 0.07 mmol) reacted with benzoyl chloride (148 mg, 1.05 mmol) for 18 hr in pyridine (300 μl) to give methyl 3,7,12-tris(benzoyl)cholate in 77% yield (2200 dpm/μmol). The remaining 23% was partially benzoylated methyl cholate which could be converted into tribenzoate when treated with additional benzoyl chloride (incomplete benzoylation was also found for authentic methyl cholate where a 67% yield of tribenzoate was obtained). Structures of the benzoates were confirmed by comparison of NMR spectra with di- and tribenzoates prepared from authentic methyl cholate and methyl deoxycholate. The methyl 3,7,12-tris(benzoyl)cholate from bile was radioactive as determined by HRLC: LiChrosorb SI 60, 20 μ, 7.8 mm i.d. × 0.5 m column, 30% ether in pentane, 6.5 ml/min, 500 psi, LDC uv detector. Quantitative recovery of radioactivity from the column was obtained and 89% of the injected radioactivity coincided with the tribenzoate of methyl cholate.

Methyl deoxycholate (15 mg) was completely benzoylated under the same conditions to methyl 3,12-bis(benzoyl)deoxycholate (2530 dpm/μmol). The HRLC characterization was as described but with 15% ether in pentane. 96% of the injected radioactivity was coincident with standard methyl 3,12-bis(benzoyl)deoxycholate. A trace of methyl 3,7-bis(benzoyl)chenodeoxycholate (<2% of total deoxycholate) was also observed.

The aqueous bile (10 ml) after $CHCl_3$ extraction at pH 2 was added to HCl (0.1 *M*, 10 ml) and stirred 18 hr. Extraction with $CHCl_3$ gave additional cholic acid (17.9% total bile radiolabel) and deoxycholic acid (21.1%). Neutralization with base followed by conjugate cleavage in NaOH (0.03 *M*) and $CHCl_3$ extraction failed to produce additional organic extractables. The aqueous phase retained 25.6% of the total radioactivity in bile. Neither methoprene nor primary metabolites were detectable (<0.01 ppm).

Limits of Detection for Methoprene and Its Known Primary Metabolites. The limit of detection of methoprene and known primary metabolites was determined by TLC analysis of extracts. Bands of silica gel corresponding to standard parent compound and metabolite side markers were scraped into scintillation vials and counted. The specific activity of the methoprene was sufficiently high (0.58 mCi/mmol, 4.1×10^6 dpm/mg) to easily establish a detection limit of 0.01 ppm for most tissues (i.e., 0.01 ppm equiv = 4.1×10^3 dpm/100 g of tissue). The analysis for methoprene and its metabolites **3**, **5**, **6**, and **7** was straightforward, and less than 0.01 ppm of any of these substances was present in any tissue. The analysis for **2** and **4** was complicated by their similar chromatographic properties to [^{14}C]cholesterol on silica gel. However, routine treatment of extracts with diazomethane allowed analysis for **4**, the methyl ester of this acid having identical R_f to the isopropyl ester **1** by TLC. Thus, by methylation of extracts and TLC analysis, it was established that <0.01 ppm of **4** was present in any tissue. The limit of detection for **2** was substantially higher than for **1** and other metabolites since the alumina TLC analysis gave close R_f values (R_f 0.65 vs. 0.59) so that some (<5%) of the cholesterol radioactivity could "spill over" into the **2** zone. Nevertheless, a detection limit of 0.01 ppm was established for **2** in all tissues except fat, where the large biomass rendered the cholesterol band broader and gave a detection limit of only <0.1 ppm.

Table I. Analyzed Primary Metabolites of Methoprene

Compd No.	Structure
	
1	R = CH_3; R′ = $CH(CH_3)_2$
2	R = H; R′ = $CH(CH_3)_2$
3	R = H; R′ = H
4	R = CH_3; R′ = H
	
5	R = CH_3; R′ = OH
6	R = H; R′ = OH
7	R = CH_3; R′ = H

RESULTS AND DISCUSSION

Identification of Radioactive Residues in Tissues. Since significant amounts of ^{14}C-labeled residues (4–40 × 10^3 dpm/g of tissue) were found in steer tissues (Chamberlain et al., 1975), the identity of the radiolabeled constituents extractable from these tissues was carefully investigated. Samples of lung, liver, muscle (hind quarter), and fat (subcutaneous) were exhaustively extracted to give chloroform fractions (nonpolar metabolites) and a methanol fraction (polar metabolites). The chloroform fractions representing unconjugated metabolites were extensively investigated for all known methoprene metabolites (i.e. **2–7**, Table I). The absence of **1** and **3–7** was readily shown by TLC behavior since these metabolites (or their methyl esters) migrated much differently from cholesterol and the nonpolar lipid-like radioactive band. However, the major nonpolar "metabolite" (55–95% of radioactivity in chloroform extract) migrated on silica gel in the region of **2**; hence, initially we thought **2** was the major metabolite. Attempts to separate the ^{14}C-labeled product from residual cholesterol by low-temperature precipitation of lipids were completely ineffective. Preparative high-resolution liquid chromatography showed elution of ^{14}C label and almost 150 mg of white solid in the same fraction as a separate injection of standard **2**, but during the HRLC purification it was noted that uv detector response was much lower than that calculated (assuming all radioactivity to be **2**). The first indication that the metabolite was not **2** came from TLC on alumina where a consistent separation (R_f 0.59 vs. 0.65) between cholesterol and **2** appeared, with the radioactivity clearly not associated with **2**. Dilution of the metabolite with an equal mass of unlabeled **2** and separation on alumina TLC still showed the radioactivity to chromatograph with cholesterol.

Because of the rather unexpected nature of this finding and the relatively large amounts of [^{14}C]cholesterol present, we have sought to prove definitively the radiochemical identity of this species. GLC analysis of the crystalline white solid demonstrated that 99.7% of the material isolated co-eluted with cholesterol, whose chemical structure was confirmed not only on the basis of retention time but also by coupled GLC–mass spectral analysis. When the metabolite was treated with digitonin, the precipitated digitonide contained all of the radioactivity [digitonin precipitation is

Table II. Analysis of Radioactive Residues in Liver and Fat

	$CHCl_3$ extract	Aq wash	$CHCl_3$ Soxhlet	MeOH Soxhlet	Solid residue	Total
			Liver			
Radioactivity, dpm $\times 10^{-5}$	4.57	0.14	2.08	4.99	12.8	24.6
(% of total)	(18.6)	(0.6)	(8.5)	(20.3)	(52.0)	(100)
% cholesterol	59	<1	40	11		16
% less polar than **1**	15	<1	20	6		6
			Subcutaneous Fat			
Radioactivity, dpm $\times 10^{-5}$	5.68		0.11	0.26	0.43	6.48
(% of total)	(87.6)		(1.8)	(4.0)	(6.6)	(100)
% cholesterol	94	<1		91		88
% less polar than **1**		<1		5		<1

Table III. Analysis of Radioactive Residues in Muscle and Lung

	$CHCl_3$ extract	Aq wash	$CHCl_3$ Soxhlet	MeOH Soxhlet	Solid residue	Total
			Muscle (Hindquarter)			
Radioactivity, dpm $\times 10^{-5}$	1.48	0.21	0.17	0.35	1.26	3.47
(% of total)	(42.7)	(6.0)	(4.9)	(10.1)	(36.3)	(100)
% cholesterol	57	<1	23	24		28
% less polar than 1	17	<1	22	7		8
			Lung			
Radioactivity, dpm $\times 10^{-5}$	12.0		16.9	8.5	3.2	40.6
(% of total)	(29.6)		(41.6)	(20.9)	(8.0)	(100)
% cholesterol	89		41	7		45
% less polar than **1**	2		5	4		3

Table IV. Distribution of Radioactivity in Pronase-Treated Liver Tissue and Control

Sample	Wt before treatment, g	Wt after treatment, g	Radioact. after treatment, dpm: In tissue residue	Water soluble	$CHCl_3$ soluble
Treated with Pronase	1.1 (0.92)[a]	0.534 (0.35)[a]	3,600	15,000	1,600
Control	1.0	1.3	40,000	4,300	Not done

[a] Corrected mass of tissue only, determined by subtracting 0.18 g Celite (calculated as present in starting tissue sample) from observed mass.

Table V. Radiolabel Distribution in Whole Steer Blood

Fraction	dpm of ^{14}C/ml (% of whole blood)	ppm, methoprene equiv
Whole blood	5,760(100)	1.4
Organic soluble	2,316 (40.2)	0.56
Aqueous soluble	<10 (<0.2)	<0.002
Volatile (as $^{14}CO_2$)	8 (0.14)	0.002
Associated with blood proteins	3,205 (55.6)	0.78
	Total (96.1)	

specific for 3β-hydroxysterols (Fieser and Fieser, 1959)]. Cleavage of the filtered and washed digitonide in warm acetic acid released the radioactivity as cholesterol (76%). In addition to the digitonide, the "metabolite" was converted to the acetate (83% yield) and benzoate (90% yield) esters, demonstrating that the radiolabel had the same chromatographic mobility as the cholesterol derivatives (**2** does not form esters under the reaction conditions).

The relatively large amounts of [^{14}C]cholesterol in tissues (Tables II and III), combined with the very similar R_f values of cholesterol and certain metabolites (e.g. **2, 4,** and **5**) on the particular silica gel system used (see Materials and Methods section), rendered setting detectability limits somewhat difficult. Nevertheless, the specific activity of the parent methoprene (4.1×10^3 dpm/μg) was quite adequate to detect 1 μg of 1 or metabolites **2–7** per 100 g of tissue (0.01 ppm) after chemical derivatization of metabolites as their methyl esters. Detection limits of <0.01 ppm could be set for metabolites **3–7** in fat, muscle, lung, and liver and for **2** in all tissues except fat where 0.1 ppm was the minimal detectable limit.

In liver and muscle relatively large amounts (40–50%) of the radioactivity were unextractable with organic solvents. We therefore treated the exhaustively extracted tissue residue with Pronase (a nonspecific, proteolytic enzyme) in an attempt to solubilize residual radiolabel (Table IV). The Pronase treatment was clearly very effective at solubilizing both 83% of the bound radiolabel and at least 62% of the tissue mass. Furthermore, on the basis of solubility and limited chromatographic data, 90% of the solubilized radiolabel seems to be associated with water-soluble natural

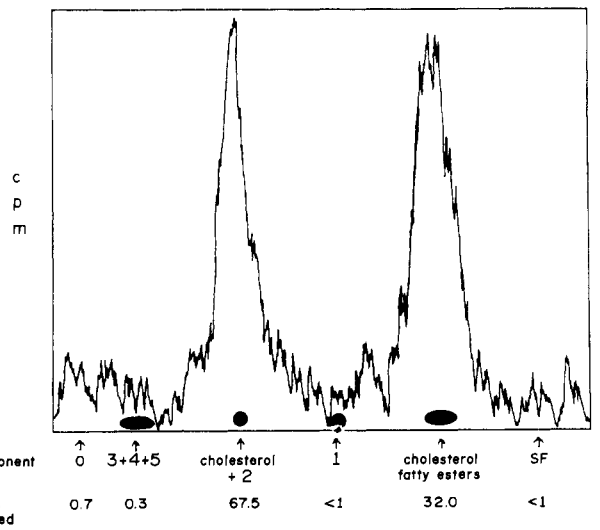


Figure 1. Radioscan of organic extract of steer blood. TLC on silica gel developed with hexane–ethyl acetate (80:20).

amino acids rather than known organic extractable methoprene metabolites. Analysis of individual amino acids in the hydrolysate with an automated column amino acid analyzer was considered. However, a commercial laboratory specializing in such analyses indicated that the maximum sample that could presently be analyzed would be ca. 0.6 mg of protein hydrolysate, corresponding for our sample to only 18 dpm of radioactivity (we observed ~30,000 dpm/g of extracted tissue). This mass is about two orders of magnitude lower than feasible for such an experiment. Considerable radioactivity was also associated with precipitated, organic-extracted blood proteins, but again dilution was so great that no identification or solubilization was attempted.

Identification of Radioactive Residues in Blood. Since the Kerrville group (Chamberlain et al., 1975) found appreciable $^{14}CO_2$ evolution from the steer, we analyzed blood for [^{14}C]bicarbonate by acidification and KOH trapping of liberated $^{14}CO_2$. However, less than 0.2% of the radiolabel present in blood was $^{14}CO_2$. The data of Chamberlain et al. (1975) showed that the rate of evolution of expired $^{14}CO_2$ peaked 30 hr after treatment, so such a small amount of blood [^{14}C]bicarbonate 2 weeks after treatment does not seem inconsistent. To facilitate extractions, blood proteins (56% of total blood radioactivity) were precipitated with $(NH_4)_2SO_4$ and the combined precipitate and supernatant extracted thoroughly with acetonitrile–butanol (Table V). In contrast to solid tissues, the organic extract (40% of blood radioactivity) had relatively little biomass and the two radioactive zones were quite easily purified by TLC (Figure 1). The particular silica gel TLC system used gave excellent resolution of the acidic metabolites **3, 4, 5,** and **6** from the [^{14}C]cholesterol band (Figure 1). The combined mass equivalent to these metabolites was below 0.003 ppm, and the detection limit of methoprene was even lower. Again it was found that **2** and [^{14}C]cholesterol cochromatographed on the silica gel plate, but benzoylation of the eluted cholesterol zone followed by rechromatography of the cholesteryl benzoate showed no radioactivity remaining in the **2**–cholesterol zone. The limit of detection of **2** was therefore about 0.01 ppm. The identity of the [^{14}C]cholesteryl benzoate from blood was shown on the basis of identical chromatographic mobility (HRLC, TLC) to standards. The band of radioactivity less polar than **1** was not massively diluted with lipids as encountered in extracts of solid tissues. After additional TLC purification, this "lipid" band was saponified. All (>98%) of the radioactivity was liberated as free cholesterol, again identified via the benzoate ester. The acidic substances liberated on saponification were shown to contain negligible radioactivity (<2%) and to consist of a complex mixture of linoleic, myristic, palmitic, and other fatty acids (based on GLC–MS of methyl esters). Although fatty acids conjugated with cholesterol in steer blood contained little radioactivity, in a separate study radioactive fatty acids have been isolated from bovine milk fat (Quistad et al., 1975).

Table VI. Identity of Steer Bile Constituents

Bile product	% total bile radioact.
Cholesterol	1.3
Cholic acid (**9**)	38.8
Deoxycholic acid (**10**)	32.2
Chenodeoxycholic acid (**11**)	<0.6
Total identified residues in bile	72.3

Similarly, extracts of solid tissues had shown a lesser metabolite fraction (11–22% of the radioactivity in the chloroform extract) associated with a broad region *less polar* than methoprene (Tables II and III). The radioactivity of this fraction co-chromatographed with a very large biomass of unresolved lipids (e.g., triglycerides, cholesterol esters, etc.). This very nonpolar fraction was apparently of particular importance for fat tissue where much of the radioactivity (61% of that present in the chloroform extract) was incorporated into the solid after low-temperature precipitation of lipids. Due to the very high dilution of activity in solid tissues, investigation of metabolite identity was not pursued, but by analogy with blood it seems likely this band may well be fatty acid esters of [^{14}C]cholesterol or fat (cf. Quistad et al., 1975).

Identification of Radioactive Residues in Bile. The comparatively large amount of radioactivity (48 μg/g equiv of **1**) associated with the bile prompted an examination of the chemical identity of the residues. Only 3% of the radioactivity was extractable with an organic solvent; again [^{14}C]cholesterol was identified (1.3% of total bile radioactivity). Neither **1** nor any known primary metabolite (**2–7**) could be identified (<0.01 ppm).

After acidification of bile to release conjugated bile acids, extraction with chloroform gave [^{14}C]cholic acid (**9**, 39% total radiolabel in bile), [^{14}C]deoxycholic acid (**10**, 32%), and a trace of [^{14}C]chenodeoxycholic acid (**11**, <0.6%) which were characterized by TLC, TLC of methyl esters, GLC of Me_3Si ethers, and HRLC of benzoates (Figure 2). Thus, 72% of the total bile radioactivity could be attributed to natural steroids (Table VI).

Incorporation of Radiolabel into Natural Products. The extensive biodegradability of methoprene ingested by a steer has been demonstrated by (1) significant evolution of respired $^{14}CO_2$ observed by Chamberlain et al. (1975) and (2) the incorporation of (at least) C-5 of [5-^{14}C]methoprene into cholesterol, bile acids, and apparently proteins. The production of radiolabeled steroids is particularly noteworthy, although it is not the first example of incorporation of radioactivity from an insecticide into cholesterol (cf. [^{14}C-vinyl]dichlorvos in swine; Page et al., 1971). The high level of radioactivity in the steer adrenal glands (Chamberlain et al., 1975) is also suggestive of the presence of natural radiolabeled adrenal cortical steroids which arose from cholesterol catabolism. These results imply degradation of primary metabolites of **1** into common precursors of intermediary metabolism, such as acetate or pyruvate (with radiolabel in these metabolic intermediates arising from C-5 of the dienoate). Catabolism of [^{14}C]acetate would give $^{14}CO_2$ via the Krebs cycle, while [^{14}C]ace-

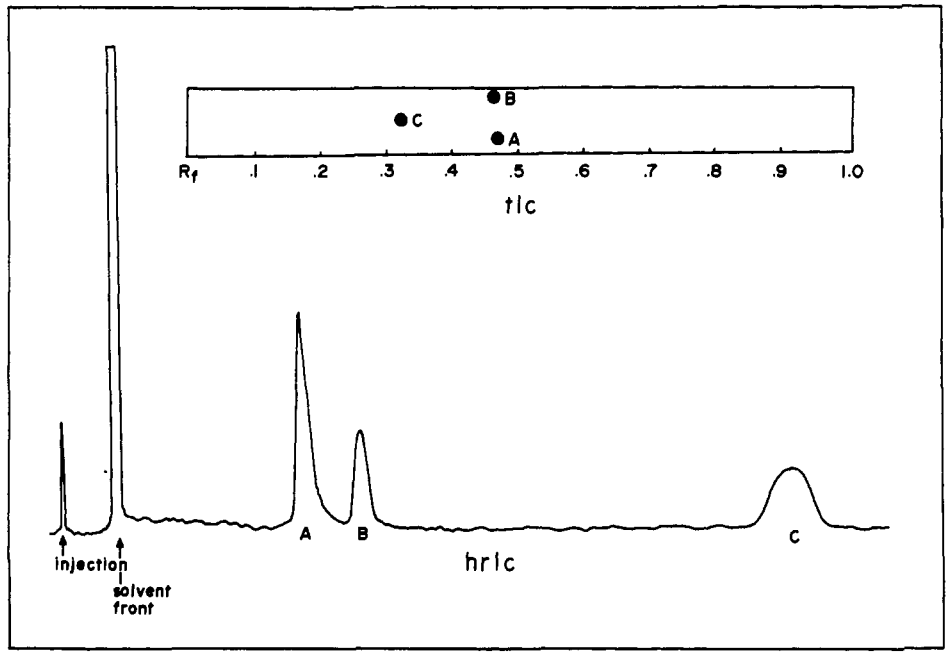


Figure 2. Chromatographic separation of benzoates of standard bile acid methyl esters: methyl deoxycholate dibenzoate (A), methyl chenodeoxycholate dibenzoate (B), and methyl cholate tribenzoate (C). For TLC: silica gel GF, 250 μ (Analtech), developed in hexane–ethyl acetate (2:1). For high-resolution liquid chromatography (HRLC): LiChrosorb SI 60 (E. Merck), 20 μ, 7.8 mm × 0.5 m column, 500 psi, 6 ml/min, developed with 15% ether in pentane.

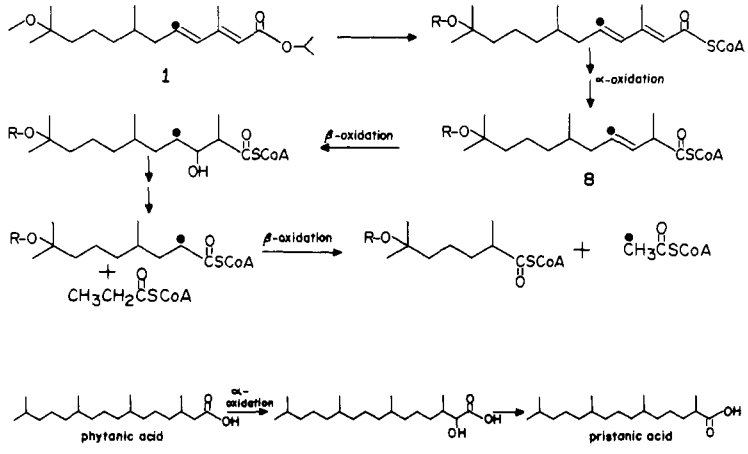


Figure 3. Possible mechanism for formation of [2-^{14}C]acetate from methoprene and the analogous conversion of phytanic acid to pristanic acid (Lough, 1973). Solid circle (●) denotes ^{14}C-labeled position.

tate and [^{14}C]pyruvate could be converted into numerous biochemicals. Incorporation of [^{14}C]acetate into cholesterol in mammalian liver (Bloch et al., 1954) is known to be very efficient, and presumably this organ is the major site of methoprene degradation as well. The observed specific activity of steer liver [^{14}C]cholesterol in this work (1130 dpm/μmol) was only 1100× lower than that of the administered methoprene. In view of the probable size of endogenous pools of acetate, this was a remarkably high incorporation rate.

From partial chemical degradation of the radioactive steer cholesterol and deoxycholic acid isolated in this study we have shown that methoprene was catabolized by the steer to [2-^{14}C]acetate (Quistad et al., 1974b). The catabolism of 1 to [2-^{14}C]acetate was necessarily a multistep process, but 1 was apparently degraded like a methyl-branched fatty acid. Although β oxidation of fatty acids is blocked by a β-methyl substituent, mammals have circumvented this biochemical problem by possessing the ability to perform α oxidations of methyl-branched fatty acids (for a recent review, see Lough, 1973). This pathway is apparently of particular importance for metabolism of certain normal dietary constituents such as phytanic acid (Lough, 1973). Of particular interest are observations that the α-oxidation system consists of an unusual mitochondrial oxidase that converts phytanic acid to its α-hydroxy derivative as an intermediate (Tsai et al., 1969), that the methyl branches are then removed by β oxidation as propionyl-CoA (Hutton and Steinberg, 1973; Steinberg et al., 1967), and that herbivores (consuming much phytol) possess a greater capacity for α oxidation than carnivores (Stokke, 1967). The mechanism postulated in Figure 3 demonstrates that after α oxidation of a derivative of 1 (resulting from ester cleavage, etc.), successive β oxidations of thio ester 8 will generate [2-^{14}C]acetate. The only unprecedented feature of this scheme is apparent lack of any published data on the effect of a 2-ene bond on α oxidation. Since α and β oxidations occur within the mitochondria [also apparently

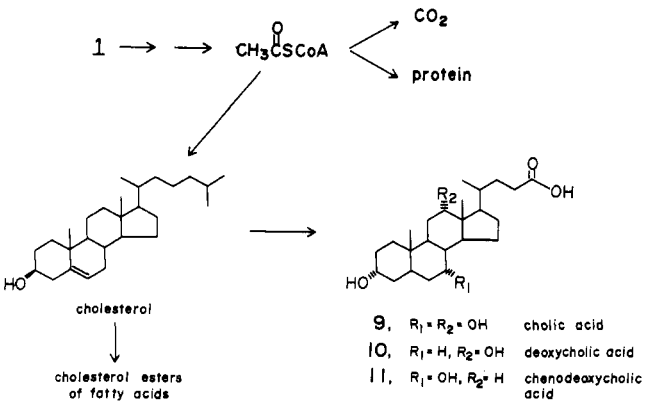


Figure 4. Conversion of methoprene to natural products.

the site of conversion of acetate to the cholesterol precursor 3-hydroxy-3-methylglutaryl-CoA in mammals (Sauer and Erfle, 1966; Williamson et al., 1968)], label from [2-^{14}C]acetate could incorporate into steroids efficiently (Figure 4) before it has a chance to randomize via operation of the Krebs cycle (White et al., 1964). Thus, compartmentation effects could rationalize the apparent disparity between our characterization of [2-^{14}C]acetate from 1 as the precursor of the radiolabeled steroids in this work (Quistad et al., 1974b), and our isolation of randomly labeled [^{14}C]acetate from the blood of a lactating cow after ingestion of [5-^{14}C]-1 (Quistad et al., 1975).

[^{14}C]Cholesterol was an important component of the total radioactivity in each tissue examined (16–88%, Tables II and III). The exact amount of [^{14}C]cholesterol produced from metabolism of 1 by a steer is difficult to estimate, but the value appears to be 2–6%. Since [^{14}C]acetate probably contributed to production of substantial amounts of $^{14}CO_2$ (Chamberlain et al., 1975), presumably via the Krebs cycle, the quantitative importance of the degradation of 1 to acetate is accentuated. Metabolism of 1 to acetate appears to be a rather common mammalian pathway. Chasseaud (1974) has also shown apparent metabolism of 1 to natural steroids in rats by autoradiography where tissue distribution of radioactivity from metabolism of 1 was very similar to that found from an autoradiographic study of [^{14}C]cholesterol administered to rats (Appelgren, 1967).

Significance of Results. The quantitative importance of acetate production from bovine metabolism of 1 implies that methoprene is metabolized as a methyl-branched fatty acid "food" in addition to being detoxified and excreted like most xenobiotics. The degradation of 1 to natural "nonmetabolite residues" (Figure 4) (Rosenblum et al., 1971) is indicative of extensive biodegradability. The production of nonmetabolite residues (i.e. natural products) from 1 introduces certain problems in metabolite analysis. Care must be exercised in the interpretation of data, especially analysis based mainly on TLC behavior. Unless radioactive residues are rigorously characterized as primary metabolites rather than natural products, radiotracer methodology can lead to mistaken conclusions in assessing the metabolic fate of insect control agents such as methoprene.

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