Environmental Degradation of the Insect Growth Regulator Methoprene. VIII. Bovine Metabolism to Natural Products in Milk and Blood

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[5-^{14}C]Methoprene was extensively metabolized by a lactating dairy cow to acetate which was isolated from blood as randomly labeled acetic acid. Radioactive acetate incorporated into milk fat which was degraded to radiolabeled saturated, monoenoic, and dienoic fatty acids. Also isolated from milk were radioactive lactose, lactalbumin, and casein. The presence of [^{14}C]cholesterol (free and esterified) was confirmed in blood, in agreement with a previous study in a steer.

Methoprene (1, isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate; trademark Altosid) is an effective dipteran larvicide (Harris et al., 1973; Schaefer and Wilder, 1973) and is a potent representative of a class of insect growth regulators (IGR's) with juvenile hormone activity (Henrick et al., 1973; Staal, 1975). We now report the chemical identity of the degradation products resulting from the metabolism of 1 by a lactating dairy cow. A radioactivity balance study will be reported for the same cow (Chamberlain et al., 1975). This work is part of a comprehensive investigation of the environmental degradation of methoprene (for part VII, see Quistad et al., 1975).

METHODS AND MATERIALS

A lactating dairy cow was treated orally with (2*E*,4*E*)-[5-^{14}C]methoprene [99.0% 2*E*,4*E* isomer, 1.0% 2*Z*,4*E* isomer, 1.11 mCi/mmol, 0.74 mCi] by W. F. Chamberlain (ARS-USDA, Kerrville, Tex.). The cow was maintained in a metabolism stall and sacrificed 1 week after treatment. Samples of blood and milk were frozen and shipped (packed in Dry Ice) by air freight to Zoecon for analysis.

Radioassay and Chromatography. Radioactivity was measured by liquid scintillation counting (LSC) and total combustion as described previously (Quistad et al., 1974a). Thin-layer chromatography (TLC) plates were precoated (silica gel GF_{254}, Analtech). The conditions for gas–liquid chromatography (GLC) and gel permeation chromatography (GPC) have been described (Quistad et al., 1974a).

Milk Extraction. A sample of milk (100 ml, 44-hr post-treatment) containing maximum radioactivity (1.14×10^6 dpm) was fractionated for residue analysis. Extraction with chloroform (3×300 ml) gave an emulsion which separated into three phases after centrifugation (10 min, 800*g*): aqueous phase (61.6% total radioactivity in milk), $CHCl_3$ extract (16.2%), and cream emulsion (22.2%). The aqueous phase and emulsion were combined and exhaustively extracted with ether for 18 hr in a liquid–liquid continuous extraction apparatus. An additional 3% of the total radiolabel was ether extractable from the cream emulsion and was combined with the $CHCl_3$ extract to give an organic extract containing 19.2% of the ^{14}C label in milk.

Isolation of Fatty Acids. The organic extract was evaporated to dryness and then agitated in acetonitrile (150 ml) to extract methoprene and potential metabolites from precipitated fat (cf. Miller et al., 1975). The acetonitrile phase was diluted with water (600 ml) and extracted with ethyl acetate (200 ml) to give a "metabolite" extract (fat, parent 1, and metabolites). The "metabolite" extract still contained a large biomass of fat which caused aberrant migration during TLC analysis of residues. The interfering fat was removed by GPC; subsequent TLC analysis (silica gel; hexane–ethyl acetate, 100:15) of a GPC fraction (approximating triglycerides in molecular weight) revealed that 86% of the radioactivity in the metabolite extract was lipid. In a separate GPC fraction approximating 1 and metabolites in molecular weight, methoprene was detectable (0.015 ppm, 1.0% total radiolabel in milk), based on TLC cochromatography with an authentic sample, but primary methoprene metabolites were not detectable (<0.01 ppm).

Lipids in the organic extract represented 15.6% of the total radiolabel in milk and a sample (ca. 500 mg) was saponified by dissolving in ether (6 ml) and stirring for 18 hr with methanolic KOH (12 ml, 0.5 *M*). The reaction mixture was acidified and extracted with ether. The ether extract (fatty acids) was methylated with diazomethane. Fatty acid methyl esters were resolved into three classes by TLC on $AgNO_3$-impregnated plates developed with hexane–ether (95:5). These plates were prepared by dipping precoated silica gel GF plates (Analtech) for 5 min in an ethanolic solution of 5% $AgNO_3$ + 5% CH_3CN, followed by air drying prior to use. Useful separations were not achieved with Analtech precoated 5% $AgNO_3$ plates. The three classes of fatty acid methyl esters isolated were: saturated (R_f 0.45), monoenoic (R_f 0.33), and dienoic (R_f 0.09) which contributed 1.1, 5.0, and 3.8% of the total radiolabel in milk. Each class of methyl esters was analyzed for chemical composition by GLC. The potential primary metabolite dienoic acids (methoxy acid 4 and hydroxy acid 3) were *not* part of the dienoic ester fraction from saponification of milk lipids (TLC on normal silica; hexane–ethyl acetate, 4:1).

Fatty acids were also obtained from the cream emulsion. An aliquot of the cream emulsion (10 ml/246 ml) was evaporated to dryness. The residue was extracted with ether (3×25 ml). Saponification of the ether extract of lipids followed by acidification and methylation gave the three classes of fatty acid esters: saturated, monoenoic, and dienoic, representing 1.0, 4.5, and 4.2% of the total radioactivity in milk.

Lactalbumin, Lactose, and Casein. These natural products were isolated by a standard method similar to that described by Kleiber et al. (1952a). Since the milk was already slightly acidic (pH ~4), the casein had already precipitated and was part of the cream emulsion. An aliquot of the cream emulsion (10 ml/246 ml) was evaporated to dryness. The residue was washed with ether to remove lipids. The white casein solid was dissolved in 6% ammonium hydroxide and reprecipitated by adding 10% acetic acid to give casein (66 mg) which represented 2.5% of the total radioactivity in milk.

Lactalbumin and lactose (3.8 and 2.0% total ^{14}C in milk, respectively) were isolated exactly as previously described (Kleiber et al., 1952a). Lactose was recrystallized from ethanol–water. Examination of lactose by TLC on cellulose (MN 300F cellulose, normal, Analtech; butanol–methanol–water, 20:12:4) showed no contamination by monosaccharides after detection by spraying (Quistad et al., 1974a). The aqueous supernatant from lactose isolation was treated with phenylhydrazine hydrochloride to precipitate addi-

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Table I. Analyzed Primary Metabolites of Methoprene[a]

Isopropyl 11-hydroxy-3,7,11-trimethyl-2,4-dodecadienoate (hydroxy ester, 2)
11-Hydroxy-3,7,11-trimethyl-2,4-dodecadienoic acid (hydroxy acid, 3)
11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid (methoxy acid, 4)
7-Methoxycitronellic acid (5)
7-Hydroxycitronellic acid (6)
7-Methoxycitronellal (7)

[a] For chemical structures see Quistad et al. (1975).

tional lactose (8.1% total ^{14}C in milk) as its osazone [mp 202° dec] (Shriner et al., 1964).

Acetic Acid from Blood. Volatile acids were isolated from a sample of cow blood (48 hr) by the method of McAnally (1945). Unlabeled acetic acid (53 mg) was added to the blood (15 ml, 18,400 dpm) to facilitate isolation. Steam distillation gave a total volatile acid content of 2030 dpm. Acetic acid was separated from other volatile fatty acids by column chromatography (Moyle et al., 1948). The efficiency of this method was calibrated by elution of an authentic sample of [2-^{14}C]acetic acid from a separate column (to avoid possible contamination). The buffered silica gel column was eluted with 10% butanol in chloroform to remove acids of greater molecular weight than C_2 (acetic). Subsequent elution with methanol gave acetic acid (1540 dpm, 8% total radioactivity in blood).

Acetic acid was precipitated as its silver salt (Cornforth et al., 1959) to give silver acetate (92 mg, 1040 dpm). This represented a 63% recovery of the cold carrier acetic acid after its addition to whole blood. The specific activity of the silver acetate was determined by LSC of the solid suspended in a gel [Insta-Gel (Packard) + 4 ml of water]. If the silver acetate (first dissolved in water) were not cooled (ice) before addition of Insta-Gel, it turned purple rather quickly. The silver acetate (1920 dpm/mmol) was decarboxylated by modifying the procedure of Cornforth et al. (1959). Carbon dioxide from triplicate Hunsdiecker decarboxylations of silver acetate (ca. 12 mg from blood and an additional 20 mg cold carrier) was trapped in KOH (5%, 10 ml). Barium chloride (0.4 *M*, 5 ml) was added to the KOH to precipitate barium carbonate (11–16 mg, 27–40%). Barium carbonate was washed with water (2×), ethanol (2×), and ether (1×) before quantitation by LSC in a gel as above. The yield of barium carbonate (828 dpm/mmol) was considerably reduced when BaOH (Cornforth et al., 1959) was used as trapping medium for CO_2 because barium carbonate was lost as an aerosol.

Cholesterol and Cholesteryl Esters from Blood. A sample of 1-week cow blood (100 ml) was extracted with acetonitrile–butanol as previously described (Quistad et al., 1975). Radioactivity in precipitated proteins was quantitated by total combustion, and soluble radioactivity by LSC. The blood organic extract was examined by TLC (hexane–ethyl acetate, 80:20). Cholesterol was the main radiolabeled component (68% blood organic extract) and it was quantitatively acylated to cholesteryl benzoate (see Quistad et al., 1975). A lipid fraction less polar than cholesterol (18% extractable radiolabel) was saponified to cholesterol and fatty acids (12 and 1% of organic extractable radiolabel) as determined by TLC (hexane–ether, 85:15) after methylation of the product mixture.

RESULTS AND DISCUSSION

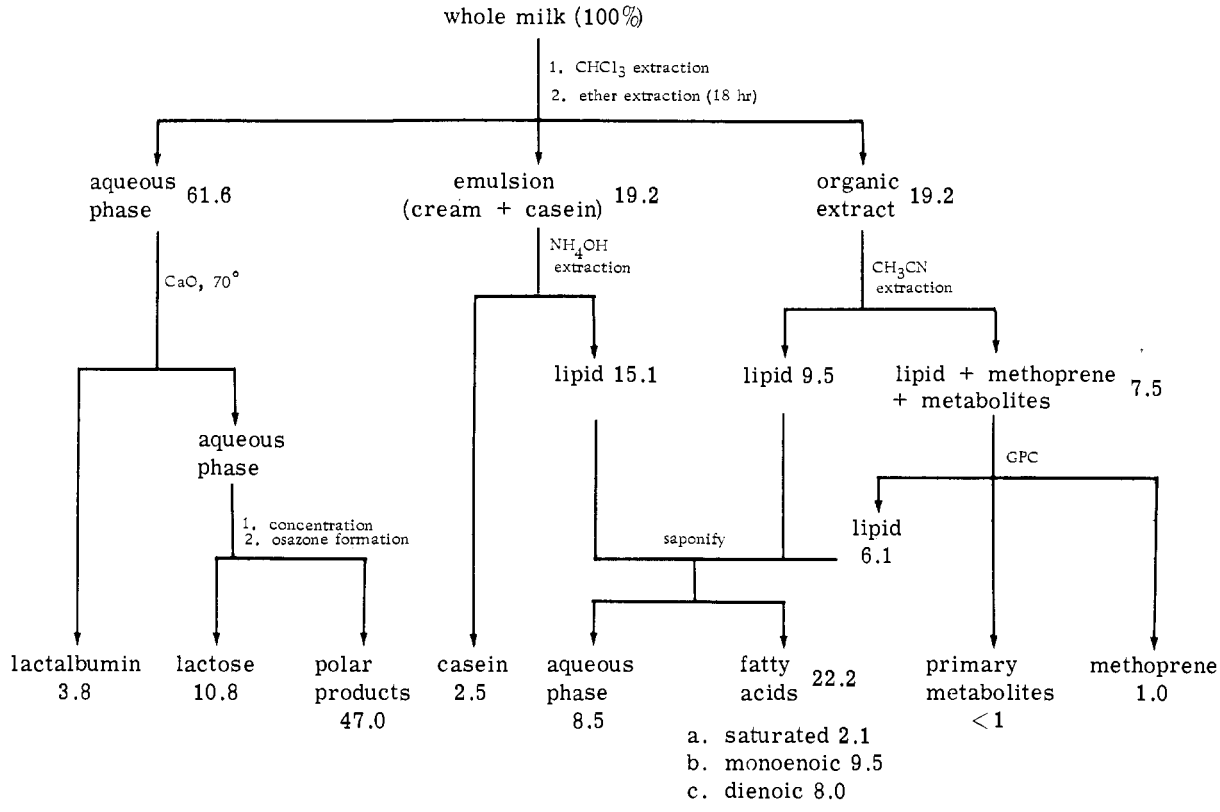
When a lactating Jersey cow was given a large single dose of [5-^{14}C]methoprene (**1**), 8% of the radioactivity appeared in milk (Chamberlain et al., 1975). The chemical nature of the constituent radioactivity in milk was examined in order to analyze for possible presence of **1** and/or its primary metabolites. Thorough extraction of a 44-hr sample (maximal radioactivity) of whole milk, and subsequent analysis of the extract, revealed a trace of methoprene (0.015 ppm) but primary metabolites were not detectable (<0.01 ppm for **2–7**; Table I). Thus, 1% of the radioactivity in the 44-hr milk was contributed by **1**, which by extrapolation means that only about 0.08% of the applied dose was excreted as **1** or its primary metabolites in milk. This rather small amount of primary residues prompted a more extensive analysis of the chemical identity of the radioactivity in the various fractions of milk (i.e. organic extractable, 19%; emulsion, 19%; and aqueous soluble, 62%).

Incorporation of Radioactivity into Fatty Acids. The isolation of radioactive milk lipids (Scheme I), which are 93–98% triglycerides (Morrison, 1970), strongly implicated degradation of **1** into common precursors of intermediary metabolism (e.g. acetate) that would incorporate radiolabel into fatty acids. Saponification of the milk lipids (31% of total radioactivity in milk) released fatty acids which represented 22% of the total radiolabel in milk. The fatty acids (as methyl esters) were resolved by TLC on silica gel impregnated with $AgNO_3$ (Litchfield, 1972) into three classes of fatty acids based on degree of unsaturation: saturated, monoenoic, and dienoic. The saturated fatty acid class contained only 2% of the radiolabel in milk and GLC analysis gave the following chemical composition: myristic (1%), palmitic (35%), and stearic (56%). The monoenoic fatty acid fraction (10% total radiolabel in milk) was predominantly oleic acid (61%) by GLC, and the major component of the dienoic fatty acid class (8% total radiolabel in milk) was linoleic acid (73%). Of course, since individual fatty acids within each class were not isolated, the chemical composition by GLC may not be indicative of the relative specific activities of the component fatty acids.

Glyceride fatty acids in bovine milk fat originate from blood acetate (C_4 to C_{14}) and blood glycerides (C_{16} to C_{18}) (Gerson et al., 1968). The isolation of [^{14}C]acetate from bovine blood in this study and demonstration that [^{14}C]acetate was the precursor of steroids in previous work on bovine metabolism of **1** (Quistad et al., 1975) both provide an adequate explanation for the inclusion of radiolabel into milk fatty acids. Lipogenesis of milk glyceride fatty acids has received considerable attention because of the obvious importance of milk fat content to consumers. Gerson et al. (1968) found that exogenous [^{14}C]acetate administered intravenously was incorporated mostly into saturated fatty acids when glycerides were isolated after short intervals (30 hr). We found that 90% of the radioactivity in milk glyceride fatty acids was about equally divided between monoenoic and dienoic fatty acids with only 10% in saturated fatty acids. The preponderance of radiolabel in unsaturated fatty acids from milk in this work may be a reflection of biochemical differences between exogenous [^{14}C]acetate (Gerson et al., 1968) and endogenous acetate generated by catabolism of **1** (this work). Thus, the preferential biosynthesis of radioactive unsaturated fatty acids observed here may be a factor of compartmentation of biosynthesis, although undoubtedly the time of sampling milk (44 hr) and the metabolic state of the cow (considerable reduction in animal weight; Chamberlain et al., 1975) influenced the disposition of biosynthesis. It is noteworthy that two potential primary metabolites of **1** are also dienoic acids (i.e., **3** and **4**), but although **3** and **4** are structurally similar to branched fatty acids, they were chromatographically dissimilar to the dienoic fatty acids released by milk lipid saponification. Thus, **3** and **4** were not part of the isolated dienoic fraction or conjugated as glycerides in milk.

Incorporation of Radioactivity into Milk Protein and Lactose. Since a previous report (Kleiber et al., 1952b) showed that acetate was a precursor of milk constituents in

Scheme I. Analysis of Milk[a]



[a] All numbers expressed as percent total radioactivity in milk.

Table II. Comparison of Calculated and Experimentally Determined Specific Activities of Silver Acetate and Its Degradation Product ($BaCO_3$)

	Specific act., dpm/mmol			
	Predicted			
Product	Random [^{14}C]-acetate	[1-^{14}C]-Acetate	[2-^{14}C]-Acetate	Found[a]
CH_3CO_2Ag				1920
$BaCO_3$	960	1920	0	828[b]

[a] Specific activities were determined by LSC to 1-2% standard error. [b] 1 dpm above background was 49 dpm/mmol.

the dairy cow, and we strongly suspected catabolism of 1 to acetate (cf. Quistad et al., 1974b), milk protein and lactose were isolated for quantitation of radioactivity. Significant amounts of radioactivity were associated with lactalbumin, casein, and lactose, which represented 4, 3, and 11% of the total radiolabel in milk, and these quantities compare favorably to results reported for [^{14}C]acetate incorporation into these same milk constituents (Kleiber et al., 1952b) although we recovered more lactose (an additional 8% via osazone precipitation).

The isolation of [^{14}C]lactose should not be viewed as gluconeogenesis from [^{14}C]acetate since metabolism of acetate cannot lead to net synthesis of glucose (and hence lactose) (Ballard et al., 1969). However, inclusion of radiolabel into sugars from acetate via oxaloacetate is the result of obligatory crossover of common metabolic pathways (Krebs cycle and gluconeogenesis), even though there is no overall synthesis of sugars from acetate in mammals (Dagley and Nicholson, 1970).

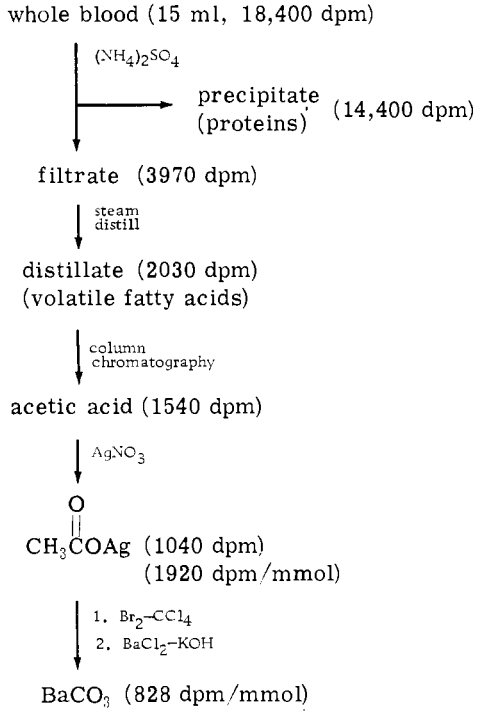
Polar unknowns contributed 47% of the total radioactivity in milk (Scheme I). This large quantity of partially characterized radiolabel was apparently a complex mixture of natural biochemicals and was refractory to further analysis. Conjugates of primary metabolites from 1 constituted less than 5% of the unknown radioactivity as determined by enzymatic cleavage with β-glucuronidase and sulfatase (Quistad et al., 1974a); thus, aglycones from 1 (i.e. 2–7) were minor components (<0.01 ppm). Additional radiolabeled protein could not be precipitated from the aqueous solution of polar unknowns and only 3% of this unidentified radioactivity could be trapped by steam distillation.

Radiolabeled Natural Products from Blood. Bovine blood contains relatively large amounts of acetic acid (McClymont, 1949). In order to obtain direct proof that 1 was metabolized to acetate (cf. Quistad et al., 1974b), acetic acid was isolated from the volatile components of a 48-hr blood sample (Scheme II). The majority of the whole blood radioactivity (88%) was associated with precipitated proteins, but [^{14}C]acetate constituted 8% of the total radioactivity in blood. We were naturally curious to ascertain the specificity of labeling in acetate since previous work (Quistad et al., 1974b) showed metabolism of 1 to [2-^{14}C]acetate. Chemical degradation of the blood acetate by classical methods revealed that blood acetate was approximately equally labeled in the carbonyl and methyl groups (Table II). The utilization of specifically labeled [2-^{14}C]acetate from 1 for steroid synthesis (Quistad et al., 1974b) may be a compartmentation effect since there is ample opportunity for randomization of radiolabel in specifically labeled acetate by normal metabolic pathways (e.g., Krebs cycle; cf. White et al., 1964). Also, since large amounts of radioactivity (88% total in blood) were associated with proteins, and amino acid oxidation contributes to significant quantities of plasma acetate (Annison et al., 1967), secondary catabo-

Table III. Radiolabel Distribution in Whole Cow Blood (1 Week Post-treatment)

Fraction	dpm of ^{14}C/100 ml (% of whole blood)
Whole blood	85,600 (100)
Organic extract	6,670 (7.8)
Aqueous soluble	3,220 (3.8)
Associated with blood proteins (precipitated)	75,700 (88.4)

Scheme II. Isolation of Acetic Acid from Blood (48 hr)



lism of proteins may also add to the randomization of isotopic labeling in blood acetate.

A sample of blood (1-week post-treatment) was analyzed in order to verify that **1** was metabolized by similar pathways in a steer and cow (Table III) since in the latter, tissues were analyzed only for total radioactivity content (Chamberlain et al., 1975). While considerably more radioactivity in cow blood was found in proteins than in steer blood (88% vs. 56%; cf. Quistad et al., 1975), the metabolic conditions were not identical (e.g., 1-week vs. 2-week post-treatment samples). The organic extracts of both cow and steer blood were qualitatively and quantitatively similar. As in the steer, the blood contained no methoprene or its known primary metabolites (i.e. **2–7**). Radiolabel in the organic extract of cow blood consisted mostly of cholesterol (68%) and cholesterol esters of fatty acids (18%). Saponification of cholesterol esters gave free cholesterol which contained greater than 90% of the radioactivity in the conjugated steroid. Thus, although fatty acids in milk contained large amounts of radioactivity, blood fatty acids (esterified with cholesterol) contained little radiolabel.

Significance of Results. These results accentuate the importance of rigorously investigating the chemical nature of radioactive residues from metabolic transformations of methoprene. As in previous studies (Quistad et al., 1974a,b, 1975) the degradation of methoprene to natural products is considerably more important quantitatively than formation of isolable primary metabolites. Presumably this extensive biodegradability is indicative of favorable environmental impact.

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