Identification of ecdysone 22-long-chain fatty acyl esters in newly laid eggs of the cattle tick *Boophilus microplus*

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1. The five major apolar ecdysone esters present in newly laid eggs of the cattle tick *Boophilus microplus* have been purified by h.p.l.c. 2. The quantities of the apolar esters present in the eggs were increased by administration of ecdysone to the mature females. 3. G.c.–m.s. analysis, as their methyl esters, of the fatty acids released from the apolar ecdysone derivatives by alkali, coupled with positive-ion fast-atom-bombardment m.s. of the intact ecdysone esters, showed that the compounds consisted of a series of fatty acyl esters of ecdysone. 4. The position of esterification of the ecdysone was established by p.m.r. spectroscopy. 5. The combined data show that the novel apolar derivatives of ecdysone consist of the 22-palmitate, -palmitoleate, -stearate, -oleate, and -linoleate esters respectively. Confirmation was obtained by comparison with synthetic ecdysone 22-palmitate. 6. The significance of the ecdysone fatty acyl esters as a possible source of free hormone during embryogenesis is discussed.

INTRODUCTION

Moulting in insects and other arthropods is controlled by the steroidal moulting hormones, the ecdysteroids. It has been demonstrated in many insect species that ecdysteroids occur not only in immature stages, but also in adult females (for a review, see Hoffmann *et al.*, 1980). That the ovaries synthesize ecdysteroids has been demonstrated in several cases and, at least in the migratory locust (*Locusta migratoria*), the follicle cells are the site of hormone synthesis within the ovary (Goltzené *et al.*, 1978). In many, but not all, insect species (e.g. locusts), the bulk of the ovarian ecdysteroids is passed into the eggs (Hoffmann *et al.*, 1980). The ovarian/eggecdysteroids, in many species, occur predominantly as polar conjugates, which have been identified as a series of ecdysteroid 22-phosphate derivatives in locusts (Isaac *et al.*, 1983; for a review, see Rees & Isaac, 1984). The ecdysteroid 22-phosphates in eggs may represent inactive storage forms of maternal hormone being enzymically hydrolysed during embryogenesis, yielding free ecdysteroid, which at least may contribute to peaks in the titre of the hormones. In fact, embryonic tissues of the locust *Schistocerca gregaria* can enzymically hydrolyse ecdysteroid 22-phosphates, releasing free hormones (Isaac *et al.*, 1983; Rees & Isaac, 1984). Other sources of free ecdysteroids in the embryos, such as transformation of precursors also present in the eggs (Meister *et al.*, 1985) or synthesis *de novo*, are not precluded. In a number of insect species there is an apparent correlation between peaks of free ecdysteroids and the formation of embryonic membrane/cuticle (see Rees & Isaac, 1984), implying a likely function for the hormones in controlling cycles of cuticulogenesis in embryos. In *Manduca sexta* (tobacco hornworm) eggs, 26-hydroxyecdysone 26-phosphate has been identified as the major ecdysteroid conjugate (Thompson *et al.*, 1985). However, in some insect species, such as the mosquito

Aedes aegypti, the ovarian ecdysone appears to be largely released into the haemolymph and, after conversion into 20-hydroxyecdysone in the fat-body, may be involved in regulating vitellogenin synthesis (Hanaoka & Hagedorn, 1980).

There is much evidence for hormonal control of development and for physiological effects of ecdysteroids in ticks (for a review, see Solomon *et al.*, 1982). The occurrence of ecdysteroids in nymphs of the hard tick *Amblyomma hebraeum* (Delbecque *et al.*, 1978) and in the soft tick *Ornithodoros moubata* (Germond *et al.*, 1982) is established. That ecdysteroids are involved in the control of moulting in ticks is indicated by the correlation of ecdysteroid titres with changes in integument structure during development in the latter species. The occurrence of ecdysteroids in adult females of the cattle tick *Boophilus microplus* has also been demonstrated, the free immunoreactive ecdysteroid titre exhibiting a peak just before full engorgement and decreasing subsequently to a very low value (Wigglesworth *et al.*, 1985). At peak titre, ecdysone (I, R = H) was the major ecdysteroid, being accompanied by much lower levels of 20-hydroxyecdysone (I, R = OH). Although the exact

(I)

Abbreviations used: f.a.b., fast atom bombardment; TEG, tetraethylene glycol.

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significance of the peak in free-ecdysteroid titre in the females is unknown, it may be related to vitellogenesis. In addition, ecdysteroids may induce degeneration of tick salivary glands after engorgement (Harris & Kaufman, 1985). [^3H]Ecdysone injected into adult females of *B. microplus* to examine the fate of the free ecdysteroids was metabolized primarily into a group of novel 'apolar' metabolites, which were transferred to the newly laid eggs, where they were by far the preponderant forms of the ecdysteroids present (Wigglesworth *et al.*, 1985). Evidence was obtained indicating that these 'apolar' compounds are long-chain fatty acyl derivatives of ecdysone, with the 2- and 3-hydroxy groups being unsubstituted (Wigglesworth *et al.*, 1985; Rees *et al.*, 1984). We now report unequivocal identification of the major 'apolar' ecdysone derivatives in newly-laid eggs of *B. microplus* as ecdysone esterified at C-22 with (respectively) palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid.

MATERIALS AND METHODS

Generally, the materials and methods were as described previously (Wigglesworth *et al.*, 1985).

Chemicals

Palmitoyl chloride was from BDH Chemicals and ecdysone was obtained from Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand.

T.l.c.

T.l.c. separation of ecdysteroid derivatives was carried out on silica gel (0.25 mm-thick Kieselgel GF₂₅₄; E. Merck A.G., Darmstadt, Germany) developed as specified below. Compounds were detected under u.v. light (253 nm) and eluted from the absorbent with ethyl acetate/methanol (1:1, v/v).

H.p.l.c.

Several h.p.l.c. systems were used.

System 1. A Partisil 5 silica column (25 cm long $\times$ 4.6 mm internal diameter; Whatman, Maidstone, Kent, U.K.) eluted isocratically at a flow rate of 1 ml/min with dichloroethane/propan-2-ol/water (125:24:1, by vol.).

System 2. An Ultrasphere ODS reversed-phase column (25 cm long $\times$ 4.6 mm internal diameter; Anachem, Luton, Beds., U.K.) eluted at a flow rate of 1 ml/min with a linear gradient (30 min) of methanol in water changing from 9:1 (v/v) to 1:0 (v/v), followed by isocratic elution for 15 min under final conditions (methanol).

System 3. A Partisil ODS-3 reversed-phase column (25 cm long $\times$ 4.6 mm internal diameter; Whatman), eluted at a flow rate of 2 ml/min with a linear gradient (30 min) of methanol in water changing from 2:3 (v/v) to 1:0 (v/v), followed by isocratic elution for 15 min under final conditions (methanol).

F.a.b.-m.s.

Positive-ion f.a.b. mass spectra were recorded on a VG Micromass 7070H mass spectrometer linked to a Finnigan Incos 2300 data system. The spectrometer

incorporated an adapted saddle-field source atom gun (Ion Tech Ltd., Teddington, Middx., U.K.) operated at 8–9 kV and a tube current of 1–2 mA, using a primary atom beam of xenon (99.98%, BDH). The mass spectrometer was operated at low resolution (about 1000), at an accelerating voltage of 2 kV (scanning m/z 90–1400). Samples were dissolved in methanol for transfer to the matrix (TEG) on the f.a.b. probe tip, excess methanol being removed using a gentle stream of warm air before f.a.b.

G.c.-m.s.

Fatty acid methyl esters were analysed by g.c.-m.s. using a Pye-Unicam 204 gas chromatograph linked to the aforementioned mass spectrometer. Samples (1 μl of a solution in hexane) were injected using an SGE OCI-3 on-column injector on to an SGE BP-20 flexible fused-silica capillary column (12 m long $\times$ 0.33 mm internal diameter; 0.5 μm film thickness). The gas-chromatograph oven temperature was raised ballistically to 170 $^{\circ}\text{C}$ and held for 6 min before programming to 225 $^{\circ}\text{C}$ at 6 $^{\circ}\text{C}/\text{min}$. Other conditions were inlet pressure of helium carrier gas, 0.5 $\text{kg}\cdot\text{cm}^{-2}$; g.c.-m.s. interface and m.s. source temperatures, 250 $^{\circ}\text{C}$; emission current, 200 μA ; ionisation potential, 70 eV; scan range m/z 40–500; accelerating voltage, 4 kV.

P.m.r. spectroscopy

Fourier-transform p.m.r. spectra were recorded on a Bruker 400 MHz instrument [S.E.R.C. (Science and Engineering Research Council) High Field NMR Service, Department of Chemistry, University of Sheffield, Sheffield, U.K.]. Samples were dissolved in [^2H]chloroform and spectra were calibrated with reference to tetramethylsilane.

Synthesis of ecdysone 22-palmitate

Ecdysone 2,3-acetonide was prepared by the method of Galbraith & Horn (1969). The crude reaction product was purified by t.l.c. with chloroform/methanol (9:1, v/v) for development and the band (R_F 0.4) co-chromatographing with an authentic sample was eluted.

Introduction of the 22-palmitate group was achieved by a modification of the method of Swell & Treadwell (1955). Palmitoyl chloride (50 μl) was added to ecdysone 2,3-acetonide (7 mg) and to this mixture, dry, redistilled pyridine (750 μl) was added. The mixture was then heated under nitrogen at 65 $^{\circ}\text{C}$ for 17 h. The reaction was terminated by cooling to room temperature, followed by addition of water. The aqueous solution was then extracted four times with ethyl acetate and the combined organic extracts washed twice with NaHCO_3 solution followed by three times with water to remove excess palmitic acid.

The crude ecdysone 2,3-acetonide 22-palmitate was evaporated to dryness under N_2 and the acetonide group removed by treatment with 1 ml of a solution of 0.1 M-HCl in tetrahydrofuran/water (9:1, v/v) at room temperature for 4 h. The reaction mixture was then diluted with water (5 ml), extracted with ethyl acetate (4 $\times$ 5 ml), the combined organic extract washed with water (4 $\times$ 20 ml) and evaporated to dryness under vacuum. The crude product was purified by t.l.c. with chloroform/methanol (4:1, v/v) for development and the ecdysone 22-palmitate (R_F 0.6), which co-chromatographed with an authentic ecdysone 2-(or 3)-acetate

marker, was eluted. Further purification of the ecdysone 22-palmitate was effected by h.p.l.c. on silica (system 1: retention volume 24.5 ml) followed by reversed-phase fractionation (system 2; retention volume 28 ml). The resulting material was characterized by f.a.b.-m.s. (Table 1 below) and p.m.r. spectroscopy (Table 3 below).

Production and isolation of apolar ecdysone esters

To increase the yield of apolar ecdysone esters obtained from ticks, unlabelled ecdysone was administered to adult females and the newly laid eggs extracted. For this, 400 day-22–23 adult female ticks were treated, by injection through the dorsal wall, with 2 μ l (40 μ g of ecdysone) each of a solution containing 16 mg of ecdysone dissolved in ethanol (544 μ l) and made up to 800 μ l with 0.9% (w/v) NaCl solution. The eggs were collected within 24 h of oviposition and extracted and fractionated as described previously (Wigglesworth *et al.*, 1985). Essentially the initial extract was partitioned between methanol/water (7:3, v/v) and hexane and the polar fraction chromatographed on a silicic acid column eluted in a stepwise manner. The fraction eluted with 30% (v/v) methanol in chloroform contained the apolar ecdysone esters as well as any free ecdysteroids. The ecdysone esters were initially purified as a group by successive h.p.l.c. on reversed-phase (system 3; elution volume 74.0 ml) and silica (system 1; elution volume 25.0 ml) columns. The apolar ecdysteroid esters were further fractionated on reversed-phase system 2, yielding at least four distinct u.v.-absorbing peaks, which were quantified by u.v. absorption.

Saponification of the apolar ecdysone esters and methylation of the fatty acids

A modification (Wang & Peter, 1983) of the method of Metcalfe & Schmitz (1961) was used. A 0.5 M solution (0.5 ml) of KOH (purified by fusion) in dry methanol was added to each of the ecdysone ester samples, the mixtures purged with N₂ and then heated at 100 °C for 15 min. BF₃ in methanol (14%, w/v; 0.5 ml) was added and the mixture heated at 100 °C for an additional 10 min. The reaction mixture was cooled, extracted twice with 1.5 ml of dry, redistilled light petroleum (b.p. 40–60 °C) and evaporated to dryness at 37 °C under N₂. The residue was dissolved in hexane (10 μ l) before g.c.-m.s. analysis.

RESULTS

Production and isolation of apolar ecdysone esters

In an initial experiment, the capacity of adult female *B. microplus* to convert greater-than-physiological amounts of ecdysone into apolar esters was examined. For this, 50 day-22–23 adult female ticks were injected with 5 μ Ci of [³H]ecdysone (sp. radioactivity 80 Ci/mmol) plus 800 μ g of unlabelled ecdysone (16 μ g/tick) and the newly laid eggs collected. After extraction and h.p.l.c. fractionation, the ³H detected in the three apolar ester peaks indicated that 4% of the administered ecdysone had been recovered in the apolar esters (μ g of steroid moiety; peak 1, 13; peak 2, 16; peak 3, 3.4). This conversion was only marginally less than that found when [³H]ecdysone undiluted with unlabelled material was used as substrate. Furthermore, the greater amount of ecdysone did not produce apparent

Fig. 1. Reversed-phase h.p.l.c. fractionation of the apolar ecdysone esters

Apolar ecdysone esters isolated as a group by h.p.l.c. successively on reversed-phase (system 3) and silica (system 1) columns were resolved into four major peaks (1A, 1B, 2 and 3) on reversed-phase system 2 (see the Materials and methods section). Chromatography was monitored by measuring the u.v. absorbance at 254 nm.

adverse effects on the ticks in terms of time of commencement of oviposition as well as viability and quantity of eggs produced.

Having established the feasibility of producing workable quantities of apolar ecdysone esters for identification by administration of unlabelled ecdysone to ticks, a larger-scale experiment was conducted. In this, 400 ticks were injected with 40 μ g of ecdysone/tick and the newly laid eggs extracted and fractionated by successive h.p.l.c. on reversed-phase (system 3), silica (system 1) and reversed-phase (system 2), using [³H]ecdysone esters produced previously as marker compounds. The u.v. chromatogram obtained on the final reversed-phase fractionation is shown in Fig. 1. It is apparent that the greater resolution of this column compared with that used previously (Wigglesworth *et al.*, 1985) has resulted in fractionation into two of the first peak of apolar compounds previously observed. The amount of ecdysone in each peak was: peak 1A, 53 μ g; peak 1B, 30 μ g; peak 2, 114 μ g, peak 3, 50 μ g.

F.a.b.-m.s.

Since preliminary work on f.a.b.-m.s. of synthetic ecdysone 22-palmitate employing a range of commonly used matrices indicated that the best results were obtained with TEG and operation in the positive-ion mode, these conditions were used in the present work. Each of the apolar ecdysone ester peaks purified by h.p.l.c. from newly laid eggs of *B. microplus*, as well as the synthetic ecdysone 22-palmitate, were subjected to positive-ion f.a.b.-m.s. analysis. Partial spectra are shown in Fig. 2, and interpretations are tabulated (Table 1). In each case, significant ions are observed for [M+H]⁺, the assignment being corroborated by ions

Fig. 2. Partial positive-ion f.a.b. mass spectra (background subtracted) of apolar ecdysone esters from newly laid eggs of *B. microplus* (h.p.l.c. peaks 1A, 1B, 2 and 3) and of synthetic ecdysone 22-palmitate

The spectra show distinct ions for $[M+H]^+$ and fragmentation of fatty acid moieties $[M+H-FA]^+$ (where FA is fatty acid). Matrix adducts ($[M+H+TEG]^+$) facilitate confirmation of M_r values. See Table 1 for further spectral interpretations.

Table 1. F.a.b.-m.s. of synthetic ecdysone 22-palmitate and apolar ecdysone esters isolated from *B. microplus* eggs by h.p.l.c.Abbreviation used: FA, fatty acid. The M_r of TEG is 194.

Sample	Principal ions . . .	<i>m/z</i> (intensities relative to base peak in parentheses)							Ester composition (ecdysteroid + FA)
		[<i>M</i> + <i>H</i>] ⁺	[<i>M</i> + <i>H</i> + TEG] ⁺	[<i>M</i> + <i>H</i> − <i>H</i> ₂ O] ⁺	[<i>M</i> + <i>H</i> − 2 <i>H</i> ₂ O] ⁺	[<i>M</i> + <i>H</i> − FA] − <i>H</i> ₂ O] ⁺	[<i>M</i> + <i>H</i> − FA − 2 <i>H</i> ₂ O] ⁺	[<i>M</i> + <i>H</i> − FA − 3 <i>H</i> ₂ O]	
Synthetic ecdysone 22- palmitate H.p.l.c. peaks		703(6)	897(2)	685(56)	667(3)	447(8)	429(100)	411(68)	Ecdysone C _{16:0}
	1A	701(8)	895(3)	683(38)	665(4)	447(12)	429(100)	411(66)	Ecdysone C _{16:1}
	1B	727(8)	921(2)	709(31)	691(2)	447(9)	429(100)	411(80)	Ecdysone C _{18:2}
	2	703(2)	897(2)	685(12)	667(1)	447(11)	429(100)	411(40)	Ecdysone C _{16:0}
	2	729(8)	923(4)	711(37)	693(4)	447(11)	429(100)	411(40)	Ecdysone C _{18:1}
	3	731(7)	925(1)	713(40)	695(5)	447(11)	429(100)	411(45)	Ecdysone C _{18:0}

Table 2. G.c.-m.s. of fatty acid methyl esters obtained from apolar ecdysone esters by saponification and methylation

For details see the Materials and methods section.

H.p.l.c. peak	Retention time relative to methyl palmitate	M^+	m/z of other principal ions (intensities relative to base peak in parentheses)	Identity of compound
1A	1.035	268(1)	237(6), 236(9), 194(7), 152(7), 138(5), 137(6), 123(11), 111(11), 110(13), 101(5), 98(23), 97(26), 96(29), 87(35), 84(35), 81(28), 74(59), 69(50), 55(100), 43(68)	Methyl palmitoleate
1B	1.481	294(4)	263(2), 262(2), 220(1), 178(2), 164(3), 150(5), 137(4), 136(5), 135(5), 123(8), 110(11), 109(18), 96(22), 95(43), 81(72), 79(31), 67(100), 55(74), 43(31)	Methyl linoleate
2	1.000	270(3)	239(2), 227(4), 199(1), 185(2), 171(2), 157(1), 143(8), 129(4), 115(2), 101(4), 97(4), 87(65), 83(7), 74(100), 69(13), 55(30), 43(49)	Methyl palmitate(1)*
	1.424	296(1)	265(6), 264(8), 222(5), 180(4), 166(2), 152(3), 151(3), 137(5), 123(9), 111(11), 110(11), 101(5), 97(26), 96(25), 87(33), 83(36), 24(51), 69(53), 55(100), 43(67)	Methyl oleate(2)*
3	1.417	298(3)	267(2), 255(4), 199(3), 185(1), 143(9), 129(4), 115(2), 111(2), 101(4), 97(6), 87(68), 83(8), 74(100), 69(16), 55(34), 43(52)	Methyl stearate

* Two major fatty acid methyl esters observed in a ratio of approx. 1:2.

Table 3. ^1H chemical shifts (δ) of characteristic protons of the ecdysone moiety of the apolar ecdysone esters isolated from *B. microplus* eggs and of authentic ecdysone esters
P.m.r. spectra were recorded with $[\text{2H}]\text{chloroform}$ as the solvent and tetramethylsilane as reference. Chemical shifts are given downfield from tetramethylsilane.

Compound	Signal ...	δ (p.p.m.)						
		^3H			^1H			
		C-18	C-19	C-21	C-26/27	C-2	C-3	C-7
Apolar ecdysone esters:								
Peak 1A		0.678 (s)	0.993 (s)	0.950 (d, J 7Hz)	1.233* (s)	3.92† (m, $W_{1/2}$ 22Hz)	4.05 (m, $W_{1/2}$ 13Hz)	5.855 (d, J 2Hz)
Peak 2		0.675 (s)	0.993 (s)	0.949 (d, J 7Hz)	1.232, 1.246 (s)	3.92 (m, $W_{1/2}$ 22Hz)	4.06 (m, $W_{1/2}$ 10Hz)	5.857 (d, J 2Hz)
Peak 3		0.677 (s)	0.993 (s)	0.949 (d, J 7Hz)	1.232* (s)	3.92 (m, $W_{1/2}$ 22Hz)	4.06 (m, $W_{1/2}$ 10Hz)	5.855 (d, J 2Hz)
Synthetic ecdysone 22-palmitate		0.677 (s)	0.993 (s)	0.950 (d, J 7Hz)	1.232* (s)	3.92 (m, $W_{1/2}$ 22Hz)	4.06 (m, $W_{1/2}$ 9Hz)	5.855 (d, J 2Hz)
Ecdysone 22-acetate		0.677 (s)	0.993 (s)	0.954 (d, J 7Hz)	1.233, 1.248 (s)	3.92 (m, $W_{1/2}$ 22Hz)	4.06 (m, $W_{1/2}$ 8Hz)	5.857 (d, J 2Hz)
Ecdysone 2-acetate		0.691 (s)	0.996 (s)	0.947 (d, J 7Hz)	1.256, 1.264 (s)	5.03 (m, $W_{1/2}$ 21Hz)	4.12 (m, $W_{1/2}$ 8Hz)	5.857 (d, J 2Hz)

* Parts of these signals were masked by the large signal of the $(\text{CH}_2)_n$ of the fatty acid moiety.

† Width at half height.

for $[M+H+TEG]^+$ and $[M+H-H_2O]^+$. Furthermore, the ions at m/z 447, 429 and 411 confirm that, in each case, the ecdysteroid moiety is ecdysone. The combined results are consistent with the compounds being monoesters of ecdysone and the respective fatty acids noted in Table 1.

G.c.-m.s. of fatty acid methyl esters

Each of the h.p.l.c. peaks for the apolar ecdysone esters were saponified, and the fatty acids released were methylated before g.c.-m.s. analysis. Mixtures of appropriate authentic fatty acid methyl esters were analysed at the same time. Comparison of both the retention times and mass-spectral data [for interpretation, see McCloskey (1969) and Odham & Stenhagen (1972)] for the compounds derived from *B. microplus* (Table 2) with those of authentic compounds, confirmed the identity of the fatty acid moieties of the ecdysone esters indicated in Table 1. Analysis of h.p.l.c. peak 2 by both f.a.b.-m.s. and by g.c.-m.s. of the derived fatty acid methyl esters indicate that this material contains both oleic acid and palmitic acid in a ratio of approx. 2:1.

Fourier-transform p.m.r. spectroscopy

The p.m.r. spectra of the apolar ecdysone esters (peaks 1A, 2 and 3) were compared with the spectra of the synthetic ecdysone 22-palmitate (see the Materials and methods section). and of the 22- and 2-acetates of ecdysone obtained by using the same solvent (Table 3; cf. assignments in Horn, 1971). The quantity of peak 1B available was insufficient for p.m.r. spectroscopy. The proton signals for the ecdysone moiety of the apolar esters (1A, 2 and 3) are very similar to those of synthetic ecdysone 22-palmitate and of ecdysone 22-acetate. In all these compounds, the only major difference between the signals of the steroid protons and those of ecdysone 2-acetate (with the exception of the C-2 and C-3 proton signals as expected) is the marked downfield shift (+ approx. 1.21 p.p.m.) in the C-22 signals of the former substances. The p.m.r. spectra provide conclusive evidence that the long-chain fatty acyl groups of the apolar ecdysone esters are attached to ecdysone through an ester linkage at C-22. There is no evidence for a downfield shift in the C-2 or C-3 proton signals, compared with those of ecdysone 22-acetate, confirming previous evidence from derivatization studies that the C-2 and C-3 hydroxy groups are free in the esters.

The fatty acid moieties of each of the ecdysone esters examined gave signals as expected in the p.m.r. spectra centred at δ 0.882 (t, J 7 Hz, $-CH_3$), 1.25–1.26 [m, $-(CH_2)_n-$], 1.5–1.58 (m, $-CH_2 \cdot CH_2 \cdot CO_2R$), 2.31 (t, J 8 Hz, $-CH_2 \cdot CO_2R$) p.p.m. In addition, peaks 1A and 2 yielded signals characteristic of olefinic bonds centred at 2.01 (m, $-CH_2 \cdot CH=CH \cdot CH_2-$) and 5.34 (m, $-CH=CH-$).

DISCUSSION

The combined data demonstrate that the apolar metabolites isolated from newly laid eggs of *B. microplus* consist of ecdysone conjugated at C-22 to palmitoleic ($C_{16:1}$), linoleic ($C_{18:2}$), palmitic ($C_{16:0}$), oleic ($C_{18:1}$) and stearic ($C_{18:0}$) acids respectively. The h.p.l.c. trace suggests that there may also be minor amounts of esters of other fatty acids. Since the ecdysone esters are separated on reversed-phase h.p.l.c. according to chain

lengths and polarity, it is not surprising to encounter overlap between the palmitate and oleate esters. Although the esters were extracted in sufficient amounts for full identification from eggs derived from ecdysone-injected females, the profile of the u.v.-h.p.l.c. chromatogram was similar to that for 3H in the case of esters derived from high-specific-radioactivity [3H]ecdysone, suggesting that the esters identified reflect those occurring under physiological conditions. Furthermore, when the apolar ecdysone ester-containing silicic acid column fraction from a batch of newly laid eggs derived from uninjected females was hydrolysed with a porcine esterase enzyme preparation, there was a nearly 100-fold increase in the amount of ecdysteroid immunoreactivity (Crosby *et al.*, 1986). This demonstrates that the apolar ecdysone esters greatly predominate over free hormones in newly laid eggs under physiological conditions. Previously observed significant overlap in the fatty acid profile of the peaks resolved on h.p.l.c. can be attributed to the difficulties of analysing small amounts of samples. Although several tissues from adult female *B. microplus* can effectively synthesize the apolar esters when incubated with [3H]ecdysone *in vitro*, it is not clear whether the metabolites in the newly laid eggs originate solely in the ovaries or are also transferred from other tissues (Crosby *et al.*, 1986).

The production of the apolar ecdysone 22-fatty acyl esters in female *B. microplus* and their transfer into the eggs is analogous to the formation of polar ecdysteroid 22-phosphates in locusts. Since the 22R hydroxy group of ecdysteroids is essential for hormonal activity (for a review, see Bergamasco & Horn, 1980), it could be that the apolar ecdysone 22-esters serve as inactive storage forms of hormone and be enzymically hydrolysed during embryogenesis, releasing free hormone. This could at least contribute to the distinct peaks in immunoreactive ecdysteroid titre observed in developing eggs (K. P. Wigglesworth, D. Lewis & H. H. Rees, unpublished work), without precluding synthesis *de novo* of hormone. In fact, effective hydrolysis of [3H]ecdysone 22-fatty acyl esters by a homogenate of partly developed *B. microplus* eggs has been demonstrated (Wigglesworth *et al.*, 1985). However, the specificity of this enzymic activity is unclear.

Formation of analogous apolar metabolites of [3H]ecdysteroids has been reported recently in nymphs (Bouvier *et al.*, 1982) and adult females of the soft tick *Ornithodoros moubata* (Connat *et al.*, 1984). In the nymphs, the apolar metabolites of ingested 20-hydroxyecdysone have been identified as the 22-fatty acyl esters (Diehl *et al.*, 1985). In fact, a sample of corresponding [3H]20-hydroxyecdysone 22-fatty acyl esters ($C_{18:2}$, $C_{18:1}$, $C_{16:0}$ and $C_{18:0}$; kindly furnished by Dr. J.-L. Connat & Dr. P. A. Diehl) was resolved on h.p.l.c. system 2 into three peaks [(i) $C_{18:2}$, (ii) $C_{18:1}$ plus $C_{16:0}$ and (iii) $C_{18:0}$; Diehl *et al.*, 1985], co-chromatographing with peaks 1B, 2 and 3 respectively from *B. microplus*. The corresponding 22-fatty acyl esters of ecdysone and of 20-hydroxyecdysone do not separate, presumably because the effect of the 20-hydroxy group is masked by the 22-acyl group. Apolar ecdysteroid derivatives which may well be similar to the ecdysteroid fatty acyl esters characterized from ticks have also been detected recently in insects (Hoffmann *et al.*, 1985; Slinger *et al.*, 1986) and other arthropods (Connat & Diehl, 1986). It is also interesting that several fatty acyl ester (lipoidal) derivatives of

steroid hormones have been characterized from mammalian tissues (Hochberg *et al.*, 1977; Abul-Hajj, 1982) and may serve as biosynthetic intermediates (Mellon-Nusbaum *et al.*, 1980).

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