Biochem. J. (1988) 252, 95–103 (Printed in Great Britain)

Formation of apolar ecdysteroid conjugates by ovaries of the house cricket *Acheta domesticus in vitro*

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The newly laid eggs of the house cricket *Acheta domesticus* contain apolar ecdysteroid conjugates, which we have hypothesized to be ecdysone long-chain fatty acyl esters [Whiting & Dinan (1988) J. Insect Physiol., in the press]. The ovaries of mature adult female *A. domesticus in vitro* convert [^{3}H]ecdysone into apolar conjugates identical with those found in newly laid eggs. Comparison of the radioactive metabolites produced on incubation of [^{3}H]ecdysone with various organs of adult female *A. domesticus in vitro* indicate that the fat-body is the major producer of polar ecdysteroid metabolites at this stage of development, whereas the ovaries are the major site of production of apolar metabolites. Apolar metabolites are also produced to a lesser extent by the crop, gut sections and the fat-body. Hydrolysis of radioactive metabolites produced by the ovaries with *Helix* enzymes releases only [^{3}H]ecdysone, and thus ecdysone is not metabolized before conjugation by the ovaries. Formation of chemical derivatives (acetonide and acetates) of these ^{3}H-labelled apolar conjugates strongly indicates that the position of conjugation is through the hydroxy group at C-22 of ecdysone. Extensive chromatographic analysis of the ^{3}H-labelled apolar metabolites produced by the ovaries by t.l.c. and h.p.l.c. and comparison with authenticated reference compounds have conclusively demonstrated that the conjugates consist of ecdysone esterified at C-22 to a mixture of common long-chain fatty acids. The major fatty acyl esters have been identified and their percentage contribution to the mixture determined: laurate (0.5%), myristate (2.8%), palmitate (25.8%), stearate (8.4%), arachidate (1.0%), oleate (15.7%), linoleate (38.8%) and linolenate (2.1%). In addition there are three minor unidentified peaks, one of which has been tentatively identified as ecdysone 22-palmitoleate (2.6%). Comparison of this percentage composition with the previously published fatty acid composition of *A. domesticus* haemolymph [Wang & Patton (1969) J. Insect Physiol. 15, 851–860] reveals remarkable similarities, indicating that the acyl transferase(s) forming the conjugates have a broad specificity with regard to the fatty acyl substrate.

INTRODUCTION

The only class of insect steroids with a proven hormonal function is the ecdysteroids, which have roles in moulting, metamorphosis and reproduction and have been implicated in the regulation of many other physiological processes. In addition to insects, the ecdysteroids have been detected and identified in other arthropods and several other invertebrate phyla (Karlson, 1983). The ecdysteroids can undergo many forms of metabolic conversion (see Lafont & Koolman, 1984, for a review), including hydroxylation, oxidation, epimerization and conjugation. The first ecdysteroid conjugates to be isolated and thoroughly identified were the polar 22-phosphates of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone from newly laid eggs of *Schistocerca gregaria* (Isaac *et al.*, 1983). It has also become apparent that apolar conjugates may also be formed, either as acetate esters (Isaac *et al.*, 1981; Gibson *et al.*, 1984; Modde *et al.*, 1984; Bueckman *et al.*, 1986) or common long-chain fatty acyl esters. The later group was first detected in two species of tick (Connat *et al.*, 1984; Wigglesworth *et al.*, 1985) and later characterized (Diehl *et al.*, 1985; Crosby *et al.*, 1986). In the soft tick *Ornithodorus moubata*, the fatty acyl esters were found to be at the 22-position of 20-hydroxyecdysone and the structures were determined by chemical ionization/desorption m.s. and analysis of the fatty acids released from the purified conjugates after transesterification (Diehl *et al.*, 1985). In the cattle tick *Boophilus microplus*, the apolar ecdysteroid conjugates produced by ovaries and recovered in newly laid eggs have been identified as the 22-palmitate, 22-palmitoleate, 22-stearate, 22-oleate and 22-linoleate esters of ecdysone by a combination of fast atom bombardment (f.a.b.)-m.s., p.m.r. spectroscopy and g.c.-m.s. of the methyl esters of the component fatty acids (Crosby *et al.*, 1986).

Although there is circumstantial chromatographic evidence that similar apolar ecdysteroid conjugates are widespread in insect species (Hoffmann *et al.*, 1985; Connat & Diehl, 1986; Duebendorfer & Maróy, 1986; Slinger *et al.*, 1986; Whiting & Dinan, 1988), more substantial evidence as to the identity and the position of the conjugate moiety has only been obtained for crickets. Hoffmann *et al.* (1985) have shown that adult female Mediterranean field crickets convert injected [^{3}H]-ecdysone into several metabolites, of which the major metabolite accumulating in the ovaries is a mixture of ecdysone C-22 fatty acyl esters. The fatty acids were

Abbreviations used: f.a.b., fast atom bombardment; h.p.t.l.c., high-performance thin-layer chromatography; i.d., internal diameter.
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identified by g.c. after enzymic hydrolysis of the conjugates and methylation, with palmitate, stearate and oleate esters predominating.

We have shown that newly laid eggs of the house cricket *Acheta domesticus* contain apolar ecdysteroid conjugates (Whiting & Dinan, 1988). Our working hypothesis has been that these are also fatty acyl esters. In this paper we demonstrate that (1) the conjugates can be produced in a radiolabelled form after incubation of cricket ovaries *in vitro* with [^{3}H]ecdysone, (2) the [^{3}H]-ecdysone does not undergo modification before conjugation and (3) the position of conjugation is C-22. We also identify the probable composition of the fatty acids involved in esterification. Point (3) has been achieved by developing chromatographic procedures to separate the majority of C-22 fatty acyl esters of ecdysone and comparison of the radiolabelled metabolites with reference compounds. This approach permits extensive characterization of ecdysteroid fatty acyl conjugates in biological extracts without resorting to extensive use of expensive physico-chemical techniques (such as g.c.-m.s. and n.m.r.), which are beyond the scope of many laboratories.

MATERIALS AND METHODS

Ecdysteroids

Ecdysone ($2\beta,3\beta,14\alpha,22R$,25-pentahydroxycholest-7-en-6-one) was purchased from Simes (Milan, Italy). [23,24-$^3H_2$]Ecdysone (60 Ci/mmol) was purchased from NEN (Du Pont U.K.). Reference ecdysone 22-acyl esters and their 2,3-acetonide derivatives were chemically synthesized from ecdysone (L. Dinan, unpublished work). Their identity was confirmed by ^{13}C-, ^{1}H-n.m.r. and/or high-resolution f.a.b.-m.s. Mass spectroscopic analyses were carried out by Professor H. H. Rees (Liverpool University) and Dr. J. A. Ballantine (S.E.R.C. Mass Spectrometry Centre, Swansea, Wales, U.K.). N.m.r. spectroscopy was performed by Dr. O. Howarth (S.E.R.C. High Field NMR Service, Warwick, U.K.).

Other chemicals

Helix pomatia arylsulphatase preparation type H-1, streptomycin and penicillin G were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Merck silicagel 60 F_{254} high-performance (h.p.) t.l.c. plates, RP-8 F_{254} reverse-phase h.p.t.l.c. plates and h.p.l.c.-grade solvents were supplied by BDH (Poole, Dorset, U.K.). Water for h.p.l.c. was de-ionized and double glass distilled. H.p.l.c. columns were purchased from Jones Chromatography (Hengoed, Mid Glamorgan, Wales, U.K.) and were protected by Uptight guard columns (Upchurch Scientific) containing identical packing materials with those in the main column. All chemicals were of analytical grade.

Insects

A. domesticus were purchased from Xenopus Ltd. (Redhill, Surrey, U.K.) and reared as described previously (Whiting & Dinan, 1988).

Ovarian incubations

Mature adult female insects were surface sterilized by brief immersion in ethanol/water, 7:3 (v/v), and the ovaries were removed under semi-sterile conditions in a laminar flow cabinet. The ovaries were transferred to stoppered sterile vials containing the required volume of sterile buffered insect Ringer solution (7.5 g of NaCl, 0.35 g of KCl and 0.31 g of $CaCl_2,6H_2O$/l of 10 mM-Hepes buffer, pH 7.0) or sterile Landureau's medium (modified for *Locusta*; Landureau & Grellet, 1972; Hetru, 1981), each containing 500 units of penicillin G and 500 units of streptomycin sulphate/ml. Ovarian incubations were conducted at 25 °C with continuous shaking.

Tissue incubations

Ten mature adult female insects were dissected to give the following organs: crop, fore-gut, caecae, mid-gut, Malpighian tubules, hind-gut, ovaries, fat-body and carcass. The gut sections were cleared of their contents, but the caecae were not. The respective organs from the ten insects were pooled and transferred into 1 ml of sterile Landureau's medium (plus antibiotics) containing 2 μCi of [^{3}H]ecdysone (33 nM), incubated at 25 °C for 24 h and then extracted. Each extract was analysed by silica h.p.t.l.c. and separated on reverse-phase Sep-Pak cartridges and reverse-phase t.l.c.

Extraction procedure

Incubations were stopped by the addition of ethanol (5 ml/ml of medium) and homogenized with an Ultra-turrax homogenizer (Janke & Kunkel G.m.b.H., Staufen, Germany). The homogenate was centrifuged (2500 ***g*** for 5 min). The supernatant was removed and the pellet was re-extracted twice with further portions of ethanol. The combined supernatants were rotary evaporated to dryness and the residue redissolved in 5 ml of CH_3OH.

In experiments to determine the distribution of metabolites between the ovaries and the medium, the ovaries were removed from the medium at the end of the incubation period and rinsed with medium (2 × 2 ml) before being homogenized in ethanol. The rinses were combined with the incubation medium for extraction.

T.l.c.

Samples were rotary concentrated in the presence of 10 μg of unlabelled carrier ecdysone, redissolved in 20 μl of CH_3OH and separated either on silica h.p.t.l.c. (10 × 10 cm) plates, with either $CHCl_3/CH_3OH$, 4:1 (v/v) or $CHCl_3/CH_3OH$, 9:1 (v/v) for development, or on 10 × 10 cm RP-8 reverse-phase h.p.t.l.c. plates with CH_3OH/water, 9:1 (v/v) for development. Positions of marker compounds were determined by visualization of the developed chromatogram under u.v. light and the distribution of radioactivity was determined by radio-scanning the t.l.c. plate (Berthold Linear Analyser LB2832 fitted with a high-sensitivity detector).

Sep-Pak separation of metabolites

Portions (0.5 ml) of the CH_3OH extracts were diluted to 5 ml with water and applied to activated reverse-phase Sep-Pak C_{18}-cartridges (Waters Chromatography Division of Millipore) which were then sequentially eluted with 5 ml each of CH_3OH/water, 1:9 (v/v), CH_3OH/water 1:3 (v/v), CH_3OH/water, 6:4 (v/v), CH_3OH, acetonitrile and $CHCl_3$. Portions of the fractions were radioassayed and those fractions containing significant amounts of radioactivity were analysed by h.p.t.l.c.

H.p.l.c.

Samples were separated using a dual-pump Gilson 401

h.p.l.c. system operating at ambient temperature with a variable wavelength detector (set at 242 nm). Columns and solvent systems used were as follows.

System 1. Spherisorb SW-5 column (5 μm, 25 cm long × 4.6 mm i.d.) eluted isocratically with dichloromethane/propan-2-ol, 125:25 (v/v) at 1 ml/min.

System 2. Spherisorb octadecylsilane-2 column (5 μm, 25 cm long × 4.6 mm i.d.) eluted with a gradient of CH_3OH in acetonitrile at a constant flow rate of 1 ml/min. The column was equilibrated with acetonitrile. On injection of the sample, the gradient increased linearly from 0% CH_3OH to 100% CH_3OH over 30 min, and was then maintained at 100% CH_3OH.

System 3. Spherisorb C_6-column (5 μm, 15 cm long × 4.6 mm i.d.) eluted with a gradient of CH_3OH/water at 1 ml/min. The column was equilibrated with CH_3OH/water, 4:1 (v/v). The solvent composition was changed 5 min after injection of the sample by means of a linear gradient to CH_3OH/water, 9:1 (v/v) over 30 min, and then maintained at that composition.

System 4. Spherisorb nitrile column (5 μm, 25 cm long × 4.6 mm i.d.) eluted with a gradient of acetonitrile/water. The column was equilibrated with acetonitrile/water, 45:55 (v/v). On injection of the sample, the composition of the eluent was initially maintained for 10 min, then changed linearly over 20 min to acetonitrile/water, 6:4 (v/v), at which composition it was then maintained.

In order to determine the separation of ^{3}H-labelled apolar metabolites, fractions of 0.5 or 0.25 min duration were collected (Gilson model 203 fraction collector; Anachem, Luton, U.K.) into Eppendorf-type vials. The mobile phase was evaporated (Gyrovap rotary concentrator; V. A. Howe and Co., London, U.K.) and then 0.5 ml of scintillation fluid (Scintillator 299; United Technologies Packard) was added before radioassay.

Enzymic hydrolyses

Enzymic hydrolysis of ovarian apolar ecdysteroid conjugates (Whiting & Dinan, 1988) was achieved by dissolving fractions containing apolar ecdysteroid conjugates in ethanol (5 μl) and adding 95 μl of *Helix* arylsulphatase preparation (10 mg/ml in 0.1 M-sodium acetate buffer, pH 5.4). Hydrolysis mixtures were incubated at 37 °C for 5 days and terminated by the addition of 1 ml of cold ethanol to precipitate protein. Supernatant, after centrifugation, was diluted with water (9 ml) and separated by RP-Sep-Pak before analysis by t.l.c. or h.p.l.c.

Acetonide formation

Acetonide formation was carried out according to the procedure of Galbraith & Horn (1969). Carrier unlabelled ecdysone (10 μg) was mixed with the ^{3}H-labelled apolar metabolites (0.04 μCi) and dissolved in acetone (100 μl; dried over molecular sieve 4A). Phosphomolybdic acid dodecahydrate (three small crystals) was added and the reaction left for 20 min at 22 °C. Butanol (1 ml) and saturated $NaHCO_3$ solution (0.4 ml) were added and mixed. The butanol phase was washed three times with water (0.4 ml), before being rotary concentrated to dryness and redissolved in CH_3OH (80 μl). A portion was analysed by silica h.p.t.l.c. [with $CHCl_3$/ethanol, 9:1 (v/v) for development] with authentic markers of ecdysone and ecdysone 2,3-acetonide.

Acetate formation

Acetate derivatives of the apolar metabolites were prepared according to Galbraith & Horn (1969). The ^{3}H-labelled apolar metabolites (0.75 μCi) and 200 μg of unlabelled ecdysone were dissolved in 200 μl of pyridine (dried over NaOH pellets) and acetic anhydride (100 μl) was added. Portions (20 μl) were removed at known time intervals (0 min, 10 min, 20 min, 40 min, 1 h, 4 h and 24 h) and 500 μl of water was added to each. After rotary concentration, the residue was redissolved in CH_3OH (20 μl), separated by silica h.p.t.l.c. [with $CHCl_3$/CH_3OH, 9:1 (v/v) for development] and radioscanned.

Radioassay

Portions of samples were radioassayed in 2 ml of Scintillator 299 (United Technologies Packard) in 6 ml polyethylene Mini-Vials (Zinsser Analytic GmbH, Frankfurt, Germany) inserted into 20 ml glass scintillation vials. Values are expressed as d.p.m., corrected for counting efficiency, chemical quenching and background.

RESULTS

Metabolism of [^{3}H]ecdysone by *Acheta* ovaries *in vitro*

In an initial experiment, ten pairs of ovaries were incubated in 2 ml of sterile medium containing 5 μCi of [^{3}H]ecdysone (42 nM) for 15 h at 25 °C. Analysis of the alcoholic extract by silica h.p.t.l.c. [with $CHCl_3$/CH_3OH, 4:1 (v/v) for development] and radioscanning revealed significant (60%) metabolism of the ecdysone to give a peak at a position (R_F 0.68) indicative of a lower polarity than ecdysone (R_F 0.51). This extensive metabolism was achieved whether the ovaries were incubated in Ringer solution or in Landureau's medium. Controls from which the ovaries were omitted showed no conversion of [^{3}H]ecdysone. A time-course study of the conversion of [^{3}H]ecdysone (1 μCi, 8.3 nM) into apolar metabolites by ten ovary pairs in buffered insect Ringer (2 ml) at 25 °C showed that 50% and 80% conversions are achieved after 12 h and 29 h respectively (results not shown).

Mobility of the apolar metabolites was compared with those of ecdysone acetates on silica h.p.t.l.c. [with $CHCl_3$/CH_3OH, 9:1 (v/v) for development]. The apolar metabolites migrate to a position (R_F 0.28) which means they are slightly less polar than ecdysone 22-acetate (R_F 0.20), but not as apolar as ecdysone 2-acetate (R_F 0.35).

Separation of the extract on a Sep-Pak cartridge revealed that significant amounts of radioactivity were found only in the 60% (v/v) CH_3OH and CH_3OH fractions. All the radioactivity eluting in the 60% CH_3OH fraction co-chromatographed with ecdysone and all that in the CH_3OH fraction corresponded to the apolar metabolites.

Production of apolar metabolites of [^{3}H]ecdysone by other organs of *A. domesticus in vitro*

Metabolism of [^{3}H]ecdysone by the various organ preparations *in vitro* as revealed by separation of the crude extracts on silica t.l.c. [with $CHCl_3$/CH_3OH, 9:1 (v/v) for development] is shown in Fig. 1 and distribution of radioactivity after separation of the various organ

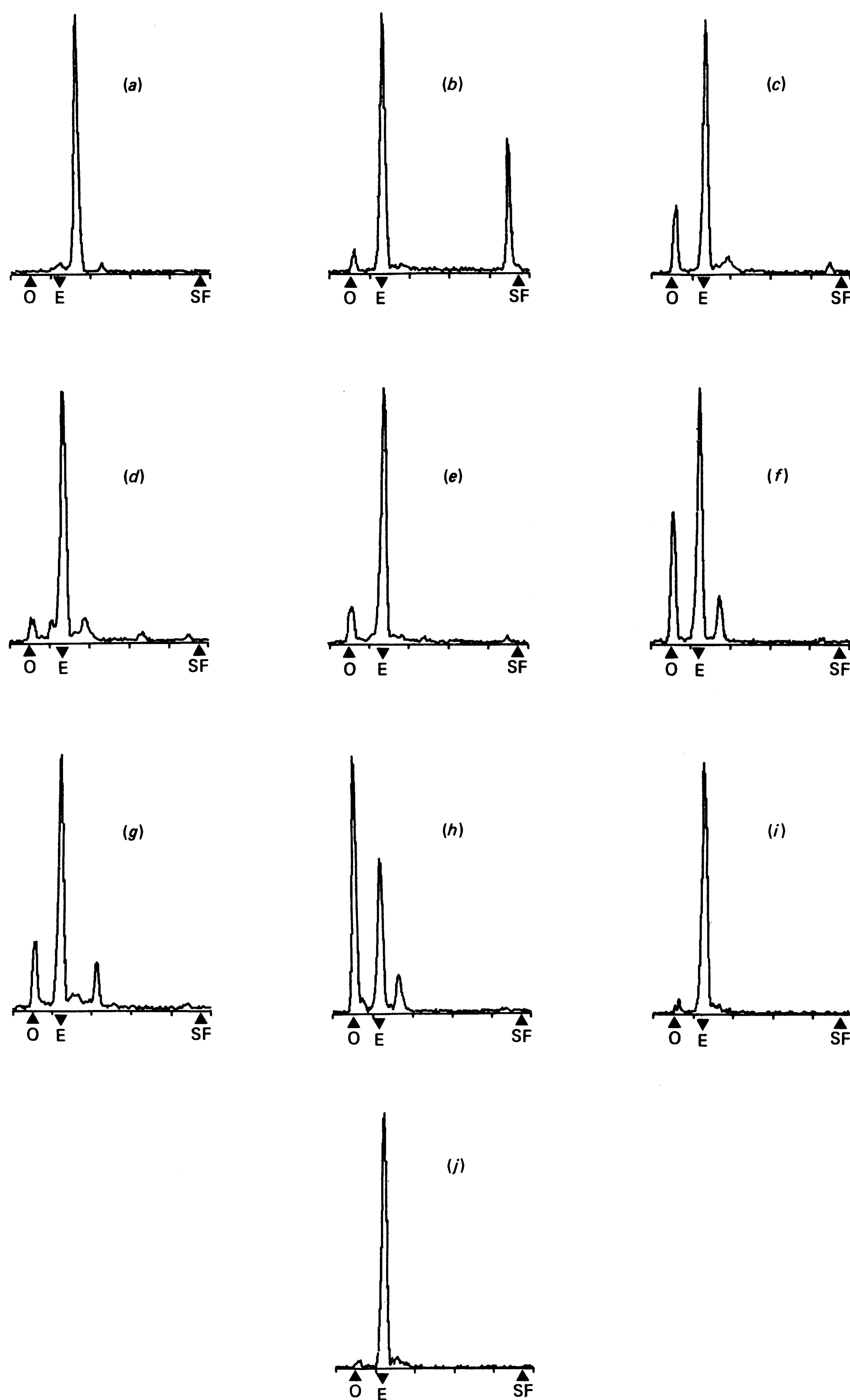

Fig. 1. Metabolism of [^{3}H]ecdysone by organs of *A. domesticus in vitro*

Organs were incubated at 25 °C with [^{3}H]ecdysone (2 μCi) in 1 ml of Landureau's medium for 24 h and then extracted as described under Materials and methods. The extracts were separated by h.p.t.l.c. on silica with $CHCl_3/CH_3OH$, 9:1 (v/v), for development and radioscanned. (*a*) Ovaries, (*b*) crop, (*c*) fore-gut, (*d*) caecae, (*e*) mid-gut, (*f*) Malpighian tubules, (*g*) hind-gut, (*h*) fat-body, (*i*) carcass, (*j*) no tissue (control). The positions of the origin, solvent front and ecdysone reference, are denoted by O, SF and E, respectively.

Table 1. Metabolism of [^{3}H]ecdysone by organs of *A. domesticus in vitro* as determined by separation of extracts on Sep-Pak C_{18}-cartridges and radioassay

Organ	Eluent composition ...	Radioactivity (% recovered)				
		10% CH_3OH	25% CH_3OH	60% CH_3OH	100% CH_3OH	100% Acetonitrile
Crop		10	2	81	6	0
Fore-gut		2	5	87	6	0
Caecae		0	2	89	9	0
Mid-gut		0	3	94	3	0
Malpighian tubules		5	12	75	8	0
Hind-gut		0	4	94	2	0
Ovaries		0	7	8	86	0
Fat-body		4	30	59	8	0
Carcass		1	3	92	4	0

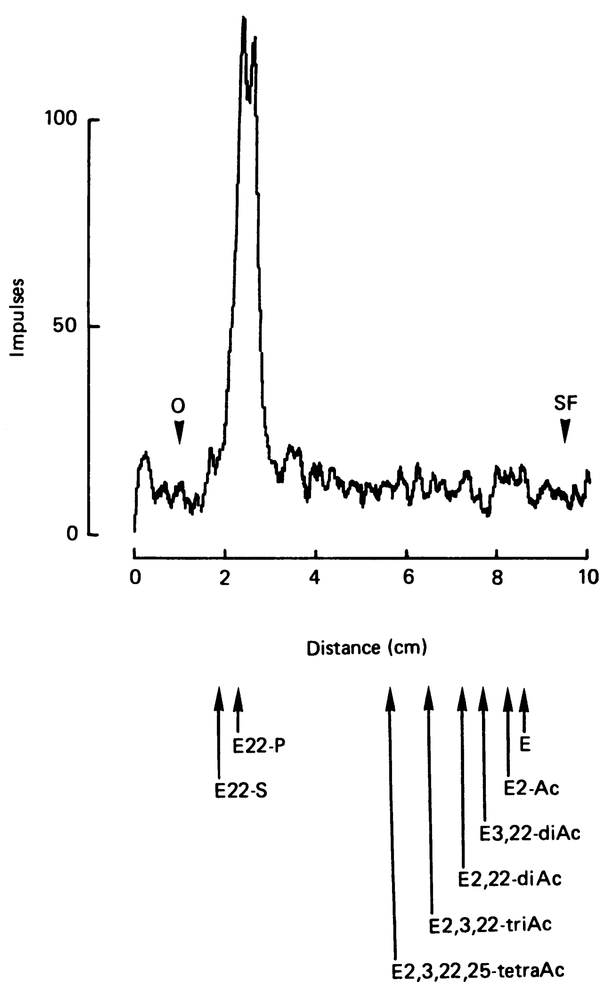

Fig. 2. Reverse-phase RP-8 h.p.t.l.c. separation of the apolar metabolites of [^{3}H]ecdysone produced by *A. domesticus* ovaries *in vitro*

The ovarian extract was partially purified by chromatography on a Sep-pak cartridge and then separated by RP-8 h.p.t.l.c. with CH_3OH/water, 9:1 (v/v) for development before radioscanning. Mobility of reference compounds is indicated (E = ecdysone, P = palmitate, S = stearate, Ac = acetate), as are the positions of the origin (O) and solvent front (SF).

extracts by reverse-phase Sep-Pak is given in Table 1. In conjunction, these results indicate that the fat-body is the major producer of polar metabolites in the adult female house cricket, whereas the ovary is the major producer of apolar metabolites. As the large majority of the radioactivity elutes in the 60 and 100% CH_3OH fractions from the Sep-Pak for all the extracts (except the fat-body), these fractions were analysed for apolar metabolites by C_8 reverse-phase h.p.t.l.c., developed with CH_3OH/water, 9:1 (v/v), a system which separates ecdysteroid acetates and long-chain fatty acyl esters well (Dinan, 1987; see Fig. 2). In this system the ovarian apolar metabolites (73% of the recovered radioactivity) show a characteristic double peak at R_F values (0.13 and 0.15) similar to that (0.13) of the ecdysone 22-palmitate marker (Fig. 2). Thus, other organs also producing the apolar metabolites characteristic of the ovaries, could be identified.

The crop produces a very apolar metabolite (AP1 at R_F 0.05), which forms 28% of the recovered radioactivity. On silica h.p.t.l.c. [$CHCl_3/CH_3OH$, 9:1 (v/v) for development] this peak has an R_F value of 0.94 (cf. R_F for ecdysone 0.18). The identity of this metabolite remains unknown.

The fore-gut produces two apolar metabolites. One (2% of the recovered radioactivity) elutes in the 100% CH_3OH fraction and corresponds to AP1. The other (AP2; 8% of the recovered radioactivity) elutes in the 60% CH_3OH fraction and co-chromatographs with ecdysone 2,22-diacetate on reverse-phase h.p.t.l.c.

The caecae produce two closely migrating apolar metabolites (AP3; together 9% of the recovered radioactivity, eluting in the 100% CH_3OH fraction) with R_F values (0.17 and 0.19) very similar to that (0.17) of ecdysone 22-palmitate.

The mid-gut produces one apolar metabolite (3% of the recovered radioactivity) eluting in the 100% CH_3OH fraction, which co-chromatographs with ecdysone 2,22-diacetate on reverse-phase h.p.t.l.c.

The Malpighian tubules produce one class of apolar metabolite (11% of the recovered radioactivity) which corresponds to AP3. The hind-gut produces one major apolar metabolite (10% of the recovered radioactivity) which elutes in the 60% CH_3OH fraction and co-chromatographs with ecdysone 2,22-diacetate on reverse-phase h.p.t.l.c.

The fat-body produces two types of apolar metabolite, both eluting in the 100% CH_3OH fraction. The first (20% of ^3H in the 100% CH_3OH fraction) corresponds to AP3 and the second (55%) to AP2. The carcasses do not metabolize ecdysone to any significant extent.

Distribution of metabolites between the ovaries and the medium

Twenty pairs of ovaries were incubated in 2 ml of Landureau's medium with 1 μCi (8.4 nM) of [^{3}H]-ecdysone for 15 h. Ovaries and medium were extracted separately. Radioactivity recovered from the ovaries was 29% and that from the medium 71%. T.l.c. separation of the ovarian and medium extracts revealed that the ovaries contain essentially only apolar metabolites (97%), whereas the incubation medium contains apolar metabolites (68%) and unmetabolized ecdysone (32%).

Hydrolysis of ovarian ^{3}H-labelled apolar metabolites

Complete enzymic hydrolysis of the ^{3}H-labelled apolar metabolites with the *Helix* enzyme preparation released [^{3}H]ecdysone as the sole product. Released ecdysone was identified by co-chromatography with authentic ecdysone on silica h.p.t.l.c. (Fig. 3) and reverse-phase h.p.l.c. (results not shown).

Formation of derivatives

Acetonide formation. Ovarian ^{3}H-labelled apolar metabolites form an acetonide derivative as indicated by the shift in the R_F value from 0.38 (underivatized metabolites) to 0.71 (derivatized metabolites) on silica h.p.t.l.c. with $CHCl_3$/ethanol, 9:1 (v/v) for development. Thus, the 2,3-*cis*-diol must be still available for derivatization in the metabolites.

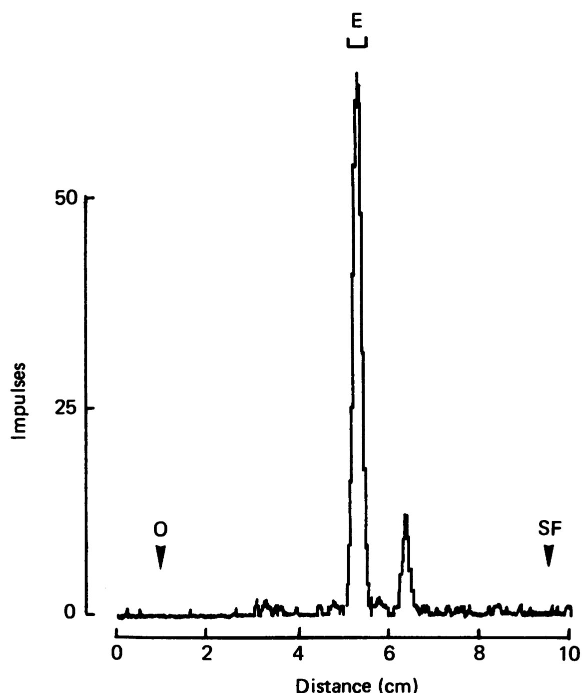

Fig. 3. Identification by silica h.p.t.l.c. with $CHCl_3/CH_3OH$, 4:1 (v/v) for development of the [^{3}H]ecdysteroid released from the ^{3}H-labelled apolar metabolites on hydrolysis with *Helix* enzymes

E denotes the mobility of reference ecdysone, O the position of the origin and SF that of the solvent front.

Acetate derivatives. Hydroxy groups of ecdysone acetylate in the preferential order C-2 > C-22 > C-3 ≫ C-25. Comparison of the time course of the acetylation sequence for the ovarian ^{3}H-labelled apolar metabolites and the R_F values of the acetate derivatives with those for ecdysone shows that the C-2 and C-3 hydroxy groups are readily available for acetylation, but that the C-22 hydroxy group is not. On heating (50 °C) the ecdysone acetylation mixture for 4 h, ecdysone is partially converted to the 2,3,22,25-tetra-acetate derivative. Under identical conditions, the apolar metabolite produces a triacetate derivative. This demonstrates that only the C-22 secondary hydroxy group is unavailable for acetylation in the apolar metabolites and, consequently, the position of conjugation must involve this hydroxy group.

Acetonide/acetate derivatives. The acetonide derivative of ^{3}H-labelled apolar metabolites ([^{3}H]APA; 0.1 μCi) was purified by chromatography on silicic acid and then mixed with unlabelled carrier ecdysone 2,3-acetonide (50 μg). The mixture was divided into aliquots which were concentrated to dryness and then subjected to acetylation at 22 °C for 60 min or 24 h. The reaction mixtures were separated by silica h.p.t.l.c. [with $CHCl_3$/ethanol, 9:1 (v/v) for development]. After 60 min, the unlabelled ecdysone 2,3-acetonide (R_F 0.39) was largely (approx. 70%) converted to 22-acetoxyecdysone 2,3-acetonide (R_F 0.48), whereas the [^{3}H]APA (R_F 0.49) was not converted at all, showing that the hydroxy group at C-22 is not available for acetylation. After 24 h, the ecdysone 2,3-acetonide had been converted to a mixture of 22-acetoxyecdysone 2,3-acetonide (approx. 90%) and 22,25-diacetoxyecdysone 2,3-acetonide (approx. 10%; R_F 0.62), while 10% of the [^{3}H]APA had been converted to a less polar derivative (R_F 0.62), showing that the hydroxy group at C-25 is free for acetylation in [^{3}H]APA.

H.p.l.c. separation of apolar metabolites

Apolar metabolites were separated by silica h.p.l.c. (system 1) (Fig. 4). The metabolites elute as a single peak at a retention time of 11 min (cf. ecdysone, 19.5 min).

On C_{18} reverse-phase h.p.l.c., eluted with a gradient of CH_3OH in acetonitrile (system 2), apolar metabolites separate into at least six peaks (Fig. 5*a*). Separation of synthetic ecdysone 22-acyl esters under identical conditions shows that their h.p.l.c. behaviour is similar to that of the ^{3}H-labelled metabolites, with many of the radioactive peaks co-chromatographing with the reference compounds. However, some of the reference compounds co-elute, making identification of the radioactive metabolites ambiguous. Saturated fatty acyl esters separate clearly from each other, as do unsaturated C_{18} fatty acyl esters of ecdysone. Linoleate and myristate esters are not, however, completely resolved and linolenate and laurate esters do not resolve at all. On a C_6 reverse-phase column, eluted with a gradient of CH_3OH/water (system 3), selectivity for ecdysone 22-fatty acyl esters is somewhat different from that of the C_{18} column (Dinan, 1988). This system retains the resolution of the saturated fatty acyl esters, but the retention times of the

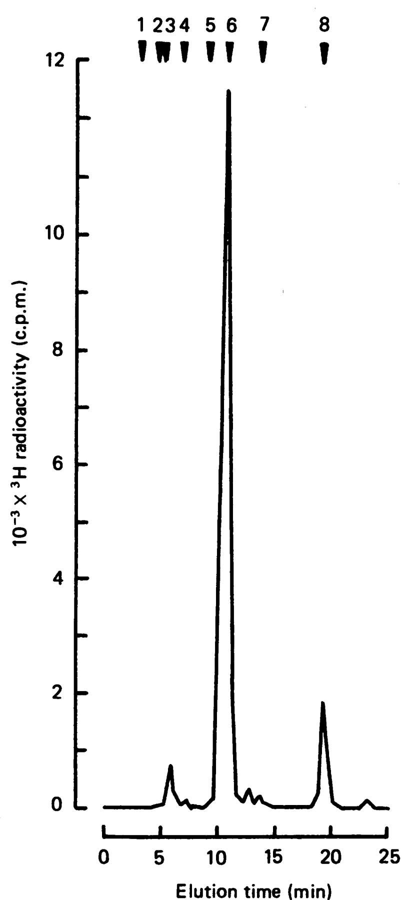

Fig. 4. Separation of ovarian ^{3}H-labelled apolar ecdysone metabolites by normal-phase h.p.l.c. (system 1)

Ovaries were incubated with [^{3}H]ecdysone (5 μCi) for 16 h and extracted as described under Materials and methods. The extract was separated on a Spherisorb SW5 column. Effluent fractions of 0.5 min duration were collected and radioassayed. Elution times of the radioactive metabolites are compared with those of reference ecdysteroids: 1, ecdysone 2,3,22-triacetate; 2, ecdysone 2,3-diacetate; 3, ecdysone 2,22-diacetate; 4, ecdysone 2-acetate; 5, ecdysone 25-acetate; 6, ecdysone 22-long-chain fatty acyl esters; 7, ecdysone 22-acetate; 8, ecdysone.

unsaturated fatty acyl esters are now extended, moving them closer to that of ecdysone 22-stearate. Thus, oleate now elutes after palmitate. Linoleate is completely resolved from myristate and linolenate now elutes with myristate, rather than the laurate ester. This system resolves ^{3}H-labelled apolar metabolites into ten peaks (Fig. 5*b*), eight of which co-chromatograph with available reference compounds. The identity of major components in the mixture is confirmed by separation on a nitrile column (system 4) in the reverse-phase mode eluted with a gradient of acetonitrile/water (Fig. 5*c*).

When ^{3}H-labelled apolar metabolites are subjected to acetonide formation and the reaction products are separated with the C_6 reverse-phase system (system 3; Fig. 5*d*), most of the radioactivity now co-elutes with the appropriate 22-acylecdysone 2,3-acetonide reference compounds, giving further proof of the identity of the apolar metabolites.

DISCUSSION

Newly laid eggs of *A. domesticus* have been demonstrated to contain ecdysteroid conjugates (Whiting & Dinan, 1988). In common with other Orthopteran species [*Locusta migratoria* (Lagueux *et al.*, 1984); *Schistocerca gregaria* (Rees & Isaac, 1984)], these conjugates are believed to be produced in ovaries of adult female insects and incorporated into the developing oocytes. Unlike the locust species, the eggs of which contain readily hydrolysable polar ecdysteroid phosphates, cricket eggs were found to contain apolar conjugates of ecdysone, which could only be hydrolysed enzymically by prolonged incubation with *Helix* gut enzymes or pig liver esterases (Whiting & Dinan, 1988).

Since prolonged hydrolysis coupled to radioimmunoassay for the released ecdysteroid is very time-consuming, we sought a way of radiolabelling the apolar conjugates. Initial attempts to achieve this goal by injecting [^{3}H]-ecdysone into mature adult female insects and extracting the eggs they deposited failed, owing to a poor incorporation of radioactivity into the eggs and rapid metabolism and excretion of the [^{3}H]ecdysone. We therefore considered incubation *in vitro* of mature ovaries with [^{3}H]ecdysone. Ovaries convert ecdysone essentially into only one type of apolar metabolite, which has the same R_F on silica h.p.t.l.c. as the endogenous apolar ecdysteroid conjugates extracted from *A. domesticus* eggs.

Distribution of apolar metabolites between the ovaries and the medium has been studied and reveals that essentially only the apolar metabolites accumulate in the ovaries, in accordance with the finding that newly laid eggs of *A. domesticus* contain approx. 90% of endogenous ecdysteroid in the form of apolar conjugates (Whiting & Dinan, 1988). The finding that the medium contains both apolar conjugates and unmetabolized ecdysone is perhaps in accord with the results of Renucci & Strambi (1981), who have shown that the ovaries are the site of biosynthesis of ecdysteroid *in vivo* and that 33% of the ecdysteroid present in adult female *A. domesticus* is to be found in the haemolymph.

The ovaries are not the sole organ capable of metabolizing [^{3}H]ecdysone *in vitro*. Most tissues of adult female *A. domesticus* metabolize [^{3}H]ecdysone *in vitro* (Fig. 1) and produce apolar metabolites to a greater or lesser extent. We have used adsorption and reverse-phase h.p.t.l.c. to begin to determine whether the type of apolar conjugate produced by the ovaries is produced by any other tissue and whether tissues of *A. domesticus* produce multiple classes of apolar ecdysone metabolites. The results to date can be summarized as follows. Three classes of apolar metabolite are distinguishable: (1) an unidentified very apolar metabolite produced primarily by the crop (AP1); (2) a moderately apolar metabolite (AP2), produced by the fore-gut, mid-gut, hind-gut and fat-body, which co-chromatographs with ecdysone 2,22-diacetate on reverse-phase h.p.t.l.c.; (3) the ovarian-type of apolar metabolite (AP3), produced also in lesser amounts by the caecae, the Malpighian tubules and the fat-body and with a low mobility on reverse-phase h.p.t.l.c. (Fig. 2), similar to that of the ecdysone 22-fatty acyl ester reference compounds. Thus, many organs produce apolar metabolites of ecdysone and several organs produce, albeit in smaller amounts, the same class of apolar metabolite as the ovaries. The ovarian

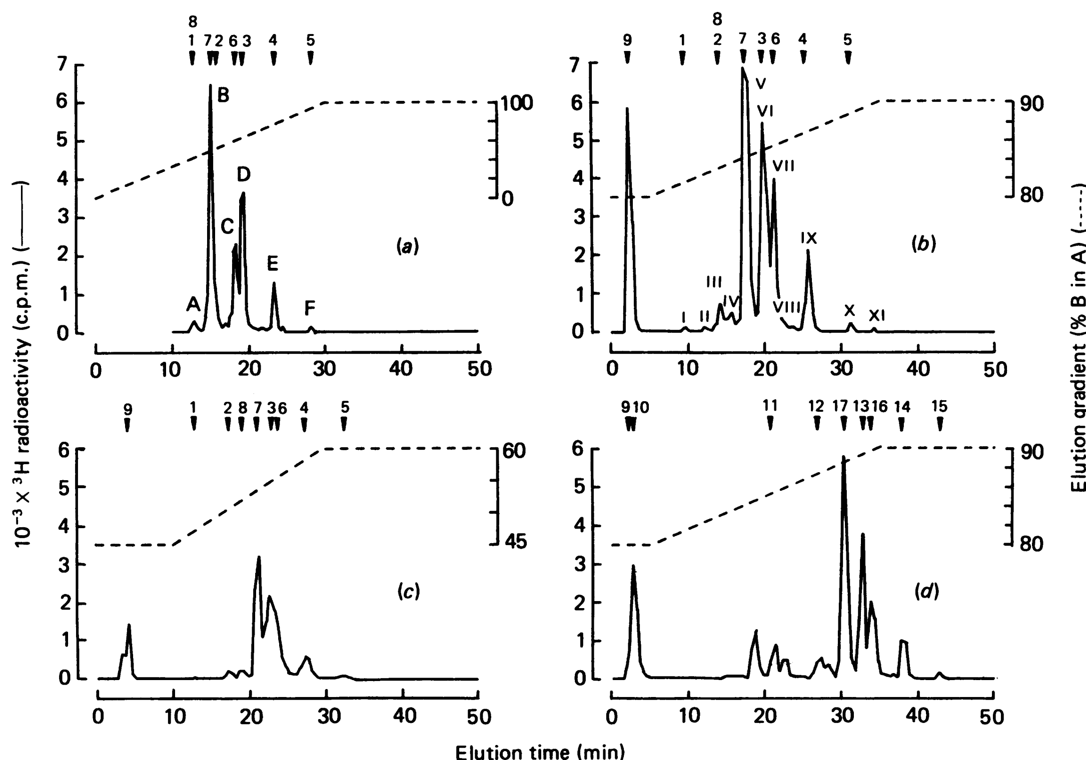

Fig. 5. Separation of ovarian [^{3}H]ecdysone metabolites by reverse-phase h.p.l.c. (systems 2–4)

^{3}H-labelled apolar conjugates were separated directly on (*a*) a C_{18}-column (system 2), (*b*) a C_6-column (system 3) and (*c*) a nitrile column (system 4) or (*d*) a C_6-column (system 3) after acetonide formation. Elution times of radioactive metabolites are compared with those of reference ecdysteroids: 1, 22-lauroyl; 2, 22-myristoyl; 3, 22-palmitoyl; 4, 22-stearoyl; 5, 22-arachidoyl; 6, 22-oleoyl; 7, 22-linoleoyl; 8, 22-linolenoyl esters of ecdysone; 9, ecdysone; 10, ecdysone 2,3-acetonide; 11, 22-lauroyl; 12, 22-myristoyl; 13, 22-palmitoyl; 14, 22-stearoyl; 15, 22-arachidoyl; 16, 22-oleoyl; 17, 22-linoleoyl esters of ecdysone 2,3-acetonide.

metabolite pattern is, however, the simplest and this organ was used for further characterization of the AP3 metabolites.

Ovarian radiolabelled ^{3}H-labelled apolar metabolites have been used to deduce their identity and, by analogy, the identity of the conjugates in newly laid eggs. Several lines of evidence (chemical formation of derivatives, enzymic hydrolysis and chromatographic behaviour) indicate that the ^{3}H-labelled metabolites are C-22 fatty acyl esters of ecdysone. This prompted us to synthesize chemically a series of eight ecdysone 22-fatty acyl esters (L. Dinan, unpublished work) and to use these to develop a range of suitable chromatographic separation systems for these compounds (Dinan, 1987, 1988). These chromatographic systems have now been applied to the ^{3}H-labelled apolar metabolites. They elute as one peak co-chromatographing with the reference ecdysone 22-long-chain fatty acyl esters on silica h.p.t.l.c. (Dinan, 1987) and silica h.p.l.c. (Fig. 4). On C_8 reverse-phase h.p.t.l.c. (Dinan, 1987) or on reverse-phase h.p.l.c. columns (Fig. 5), the metabolites separate into several constituent peaks. In all the systems the behaviour of the ^{3}H-labelled apolar metabolites is the same as the reference ecdysone 22-fatty acyl esters. The most useful h.p.l.c. systems for identification of the fatty acyl esters were found to be a gradient of CH_3OH/acetonitrile to elute the fatty acyl esters from an octadecylsilane-2 column (system 2), a C_6-column eluted with a shallow CH_3OH/water gradient (system 3) or a nitrile column eluted with a gradient of acetonitrile/water (system 4). These three reverse-phase systems have different selectivities for the unsaturated ecdysone 22-acyl esters and this permits the certain identification of most of the fatty acids involved in esterification.

On the C_{18}-column, the ^{3}H-labelled metabolites separate into six major peaks (A–F in Fig. 5*a*) which co-chromatograph with the available ecdysone 22-fatty acyl reference compounds; peaks C–F co-chromatograph with the 22-oleoyl, 22-palmitoyl, 22-stearoyl and 22-arachidoyl esters of ecdysone, respectively. Peak A co-elutes with the 22-lauroyl and 22-linolenoyl references, whereas peak B elutes at a retention time similar to those of both the 22-myristoyl and 22-linoleoyl references.

Comparison of the results shown in Fig. 5(*a*) with those shown in Fig. 5(*b*) for the C_6-h.p.l.c. separation of the same extract reveals that peak A in Fig. 5(*a*) corresponds to both ecdysone 22-linolenate and ecdysone 22-laurate. Peak B consists of predominantly ecdysone 22-linoleate, the amount corresponding to ecdysone 22-myristate being relatively small. The composition is confirmed by the separation of the ^{3}H-labelled metabolites on a CN-column, which shows a further difference in selectivity for the ecdysone 22-fatty acyl esters (Fig. 5*c*). The putative composition of the ^{3}H-labelled apolar metabolites is summarized in Table 2. The absolute composition must remain tentative because some possible candidates (e.g. ecdysone 22-palmitoleate) were not available as reference compounds. We have, however,

Table 2. Composition of the ecdysone 22-fatty acyl mixture produced by *A. domesticus* ovaries *in vitro* and comparison with the previously published fatty acid composition of *A. domesticus* haemolymph

The numbering of the peaks refers to the designations shown in Fig. 5(*b*).

Peak	Identity of fatty acid	Amount in conjugates (%)	Amount in haemolymph* (%)
I	Lauric acid ($C_{12:0}$)	0.5	0.11
II	Unknown	0.7	–
IIIa	Myristic acid ($C_{14:0}$)	2.8	1.2
IIIb	Linolenic acid ($C_{18:3}$)	2.1	0.7
IV	Palmitoleic acid ($C_{16:1}$)?	2.6	1.7
V	Linoleic acid ($C_{18:2}$)	38.8	37.0
VI	Palmitic acid ($C_{16:0}$)	25.8	23.5
VII	Oleic acid ($C_{18:1}$)	15.7	28.0
VIII	Unknown	1.0	–
IX	Stearic acid ($C_{18:0}$)	8.4	2.5
X	Arachidic acid ($C_{20:0}$)	1.0	1.9

* From Wang & Patton (1969).

been able to identify most of the endogenous ecdysone 22-fatty acyl esters (including the 22-palmitoleate) present in newly laid eggs of *A. domesticus* by f.a.b.-m.s. after extensive purification by h.p.l.c. The percentage composition of the various endogenous acyl esters is very similar to that found *in vitro* (Whiting & Dinan, unpublished work).

Apparently no data are available for the fatty acid composition of *A. domesticus* ovaries. However, Wang & Patton (1969) have investigated the fatty acid composition of *A. domesticus* haemolymph and comparison of their data with the fatty acid composition of the ecdysone 22-fatty acyl esters produced by *A. domesticus* ovaries (Table 2) reveals remarkable similarities. This suggests that the acyl transferase responsible for esterification of ecdysone is not selective with regard to its fatty acyl substrate.

The financial support of the Science and Engineering Research Council is gratefully acknowledged. F.a.b.-m.s. and n.m.r. spectroscopy for the characterization of the ecdysone acyl esters were kindly carried out for us by Professor H. H. Rees, Department of Biochemistry, Liverpool University, Dr. J. A. Ballantine, S.E.R.C. Mass Spectrometry Centre, University College Swansea, and Dr. O. Howarth, S.E.R.C. High Field NMR Service, Warwick University. L.D. also wishes to thank Dr. R. E. Isaac for helpful discussions.

Received 3 September 1987/30 November 1987; accepted 15 January 1988

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