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PROBABLE OCCURRENCE OF ECDYSTEROID FATTY ACID ESTERS IN DIFFERENT CLASSES OF ARTHROPODS

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Abstract—We investigated the fate of injected [^{3}H]ecdysone or [^{3}H]20-hydroxyecdysone in various species of ticks, spiders, scorpions, myriapods, crustaceans and insects. Most of these arthropods were able to convert the ecdysteroids to esterase-labile metabolites with a very apolar behaviour in reverse-phase HPLC. Some of them have retention times similar to the apolar conjugates AP2 of the tick, *Ornithodoros moubata*, which have been identified recently as ecdysteroids esterified as C_{22} with palmitic, stearic, oleic or linoleic acid [Diehl *et al.* (1985a) *Int. J. invert. Reprod. Devl.* **8**, 1–13]. Others are less apolar and could correspond to the AP1 from *O. moubata*. The possible function of these metabolites remains to be established. They could represent inactivation products and/or a hormone storage-form for embryos.

Key Word Index: Arthropods, ecdysteroid metabolism, apolar ecdysteroid conjugates

INTRODUCTION

"Apolar" ecdysteroid conjugates, corresponding to ecdysone-3-acetate, were first discovered in *Schistocerca gregaria* embryos (Isaac *et al.*, 1981) and later were found in *Locusta migratoria* (Modde *et al.*, 1984). Recently, however, other less polar conjugates have been observed in the soft tick, *Ornithodoros moubata*, after injection of ecdysone, 20-hydroxyecdysone, or supposed 20,26-dihydroxyecdysone in the last (fifth) nymphal instar. These products were always prominent, constituting 30–75% of the injected label after 24 hr of metabolism (Bouvier *et al.*, 1982). This metabolic pathway was very efficient during periods of low levels of endogenous ecdysteroids and was also observed in females during vitellogenesis (Connat *et al.*, 1984). In both stages such low-polarity metabolites were produced more rapidly after ingestion of ecdysone and 20-hydroxyecdysone. Recent studies with ingested 20-hydroxyecdysone revealed that the least polar of these compounds (AP2) are conjugates of 20-hydroxyecdysone esterified at C_{22} with the common long-chain fatty acids $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ or $C_{18:2}$ (Diehl *et al.*, 1985a). This represented a new class of ecdysteroid conjugates.

Conjugates AP2 were not however the final products of the low-polarity pathway. Concomitant with their gradual disappearance, slightly more polar conjugates (AP1) were formed. Hydrolysis of these AP1 with hog liver esterase also liberated the original free hormones, indicating that these products had remained esters (Connat *et al.*, 1984, 1985); but their exact chemical nature has not been determined.

In *O. moubata*, the role of these new ecdysteroid fatty acid esters is not yet clear. They may be the final products of an inactivation pathway in nymphs (Bouvier *et al.*, 1982) or a hormone storage form for embryonic development as suggested by Connat *et al.* (1984).

Apolar ecdysteroid fatty acid esters have also been demonstrated in the females and the eggs of the hard tick, *Boophilus microplus*, where they act presumably as a hormone source for the embryo (Wigglesworth *et al.*, 1985).

However, such apolar conjugates are not restricted apparently to the Acarina, since comparable hydrolyzable ecdysteroid esters were also found in different tissues of *Drosophila melanogaster* incubated *in vitro*. These products accounted for more than 50% of the total label and were exclusively associated with the tissues and not with the medium (Dübendorfer and Maroy, 1983; Dübendorfer, pers. commun.).

We have thus attempted to survey the occurrence of such apolar metabolites in various species from different arthropod classes. With this preliminary comparative study we hope to demonstrate their wide distribution and to suggest further studies which might elucidate the role of these new apolar conjugates.

MATERIALS AND METHODS

Animals

Ticks, scorpions and several insect species come from different laboratory colonies; others were collected in the field. Depending on the experiment, the number of animals used varied from one to ten, but most often was two.

Chemicals

[23,24-$^3H_2$(N)]ecdysone (sp.act. about 50 Ci/mmol) was purchased from New England Nuclear [23,24-$^3H_2$(N)]-20-hydroxyecdysone was synthesized *in vitro* by incubation of labelled ecdysone with *Locusta* Malpighian tubules followed by purification by high performance liquid chromatography (HPLC). Labelled hormones ($1-2 \times 10^5$ cpm) were dissolved in 1–2 μl TC 199 and then injected into the hemocoel of each animal.

Extractions

The animals or the organs were homogenized in methanol and sonicated for 1 min. After centrifugation the supernatants were dried, redissolved in methanol and kept at −18 C.

High-performance liquid chromatography (HPLC)

HPLC analysis were performed on a Perkin–Elmer series 3 chromatograph equipped with a variable wavelength spectrophotometer operated at 242 nm and a RP-18 column (Merck, Lichrosorb, 7 μm; column dimensions: 4 × 250 mm). Solvent system: linear gradient of methanol–tris/perchloric acid buffer (20 mM, pH 7.5) at 0.8 ml/min from 30 to 45% in 10 min, 45% for 15 min, 45–100% for 20 min followed by 100% methanol for 20 min. Fractions were collected every 30 sec and their radioactivity (cpm) was monitored by liquid scintillation counting (LSC) in a Kontron MR 300 counter. Samples were dissolved in Riatron scintillation cocktail (Kontron; 1:1.75 v/v). Isocratic HPLC on silica columns (Merck, Lichrosorb, 7 μm; column dimensions: 4 × 250 mm) was performed with chloroform–isopropanol–water (100:25:1.25) at 0.8 ml/min. For more technical details see Connat *et al.* (1984) or Diehl *et al.* (1985a).

Hydrolysis by esterase

Hydrolysis of apolar conjugates was accomplished with 50 μl (corresponding to 50 IU) hog liver esterase (EC 3.1.1.1; Boehringer) in 950 μl of borate buffer (100 mM, pH 8). After an overnight incubation at 37°C, ecdysteroids were extracted with methanol.

RESULTS AND DISCUSSION

Occurrence of apolar conjugates in ticks

Ingested [^{3}H]20-hydroxyecdysone in the soft tick *Ornithodoros parkeri*, as in the closely related *O. moubata* (cf. Connat *et al.*, 1985; Diehl *et al.*, 1985a), was converted to apolar products (Fig. 1b). Compounds with retention times similar to the *O. moubata* AP2 (Fig. 1a) appeared first and were thereafter gradually converted to metabolites comparable to AP1. However, in contrast to *O. moubata* where AP1 accumulated in the midgut cells and content, in *O. parkeri* 42% of the ingested hormone was excreted as AP1 in the faeces within 1 week following the blood-meal. Presumably this difference simply stems from the fact that, in contrast to *O. moubata* which has a blocked midgut, *O. parkeri* has a functional midgut which communicates with the hindgut allowing for excretion. In view of the similar retention times of apolar products from *O. parkeri* and *O. moubata* and of their lability to hydrolysis by esterase, we suggest that these conjugates may be of the same nature.

We have also investigated the metabolism of injec-

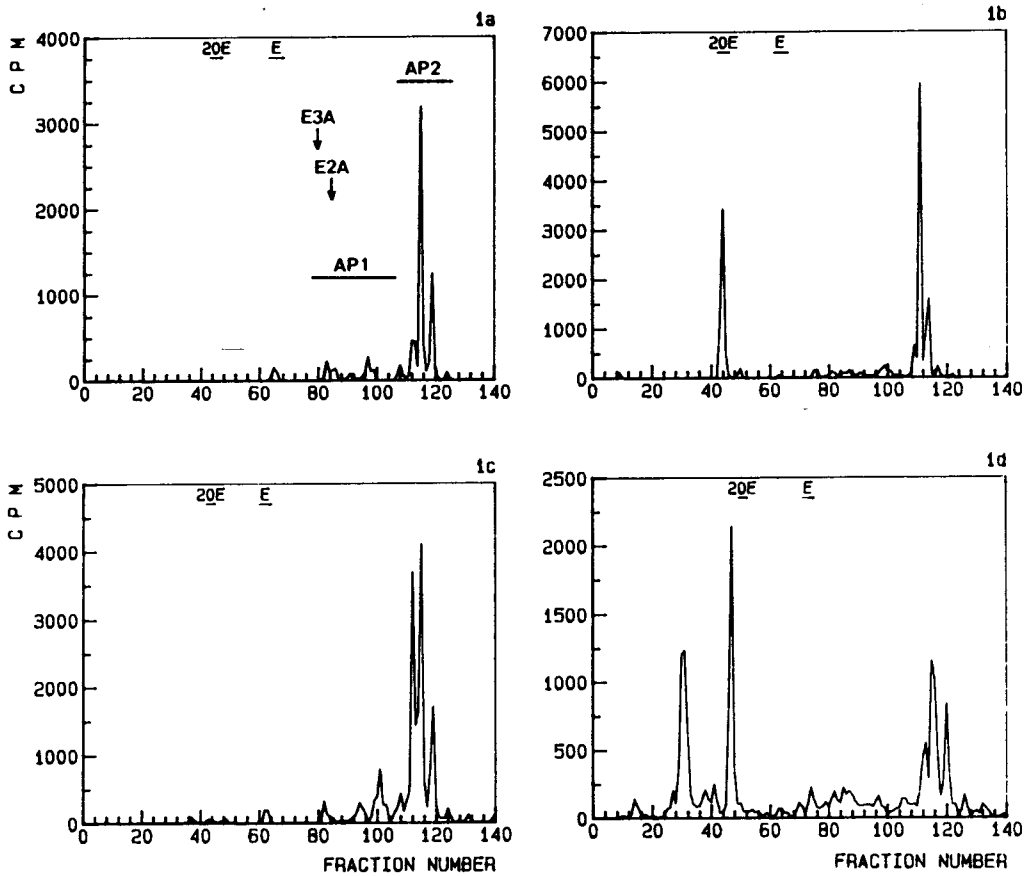

Fig. 1. Reverse phase HPLC radiochromatograms of ecdysteroid metabolites from different ticks. Retention times of internal authentic ecdysone (E) and 20-hydroxyecdysone (20E) are indicated. (a) Metabolism of ingested [^{3}H]ecdysone in adult females of *Ornithodoros moubata*, 24 hr after the blood meal. The less polar conjugates AP2 are formed rapidly and thereafter are gradually transformed into more polar conjugates AP1. Retention times of authentic ecdysone-3-acetate (E3A) and ecdysone-2-acetate (E2A) are marked. (b) Metabolism of ingested [^{3}H]20-hydroxyecdysone supplemented with cold 20-hydroxyecdysone (5 μg/ml blood) in adult females of *Ornithodoros parkeri*, 24 hr after the blood meal. (c) Metabolism of [^{3}H]ecdysone injected into females of *Boophilus microplus* (Biarra strain) three days post-detachment, 24 hr after injection. (d) Metabolism of [^{3}H]ecdysone injected into female *Amblyomma hebraeum* at the beginning of oviposition, 24 hr after injection.

ted [^{3}H]ecdysone in two species of the family Ixodidae (hard ticks). Females of both species, *Amblyomma hebraeum* and *Boophilus microplus* (Biarra strain), were able within 24 hr to produce apolar metabolites having the same retention time as *O moubata* AP2 (Fig. 1c, d). However, in *A. hebraeum* these compounds remained in the female and were gradually converted to products of the same retention times as *O. moubata* AP1. Free hormones (ecdysone and, principally, 20-hydroxyecdysone) accumulated in the eggs. In contrast to *A. hebraeum*, in *B. microplus* the products corresponding to AP2 accumulated in the eggs thus confirming the results by Wigglesworth *et al.* (1985). In both cases hydrolysis of the apolar metabolites with esterase liberated the free injected hormone. The nymphal stage of *A. hebraeum*, injected with [^{3}H]ecdysone or [^{3}H]20-hydroxyecdysone, also utilized this apolar pathway, and, as in *O. moubata* nymphs (Bouvier *et al.*, 1982), this mechanism seems to be more efficient at the time when endogenous ecdysteroid levels are low.

Thus occurrence of apolar conjugates in ticks appears to be frequent and is perhaps general, but the role of these compounds is not yet clear and could vary among the different species and/or life stages. They could represent an inactivation mechanism for endogenous and exogenous ingested ecdysteroids (see Connat *et al.*, 1985; Diehl *et al.*, 1985a), but they could also be a storage form of maternal ecdysteroids in embryos of certain tick species.

Apolar conjugates in scorpions and spiders

Three adults each of two insectivorous scorpions *Androctonus australis* and *Buthus occitanus* were injected with [^{3}H]ecdysone or [^{3}H]20-hydroxyecdysone and were left for 88 hr at 20°C. Both species converted the hormones into polar and apolar metabolites. In addition, in the case of injection of [^{3}H]ecdysone, a small part of the label comigrated with unlabelled 20-hydroxyecdysone. The apolar pathway was more efficient in *B. occitanus*; 35–40% of the recovered label had retention times similar to AP1 of ticks, and 30–35% similar to AP2. Thus these apolar metabolites represented about 70% of the total cpm in *B. occitanus*, whereas they represented only 40–50% of the total labelling in *A. australis*. Hydrolysis by esterase of these products liberated polar compounds in addition to the original free hormone (Fig. 2a, b).

The metabolism of injected [^{3}H]ecdysone in five different species of spiders, *Araneus sclopetarius*, *Pardosa* sp., *Pholcus phalangioides*, *Tegenaria atrica*, and *Zygiella-x-notata* was also investigated. After 48 hr the different species showed a high conversion of the injected hormone to apolar esterase-labile compounds with retention times similar to AP1 and AP2 of *O. moubata* (Fig. 2c, d). Kinetic studies in *T. atrica* demonstrated that, as in ticks, metabolites corresponding to AP2 decreased with time and less apolar hydrolyzable conjugates with retention times of AP1 accumulated.

Our results from several species of scorpions and spiders together with those from ticks seem to indicate that chelicerates have a high capacity for converting injected ecdysone or 20-hydroxyecdysone by the apolar pathway, producing esterase-labile conjugates. This pathway may inactivate endogenous ecdysteroids, but it may also be a detoxification mechanism against the ecdysteroids present in the prey of these insectivorous species, which, if absorbed by these predators, would interfere with their physiological processes.

Apolar conjugates in myriapods

[^{3}H]ecdysone injected into two adults of *Lithobius* sp. (Chilopoda) was metabolized to partially hydrolyzable apolar compounds with retention times comparable to AP1. They accounted for 25% of the total labelling 16 hr after injection.

Apolar metabolites in crustaceans

Two isopods belonging to the family Porcellionidae were injected with [^{3}H]ecdysone. In the methanolic extract obtained from the two animals 18 hr after the injection, 34% of the total labelling corresponded to apolar compounds and 14% to products with retention times similar to AP2 yielding free ecdysone after hydrolysis. The apolar compounds with retention times of AP1 were esterase-resistant.

Three *Carcinus maenas* larvae injected with [^{3}H]ecdysone contained only small amounts of apolar material after 3 days of metabolism. 2.9% of the total labelling migrated like AP1 and 5.3% like AP2. On the other hand, about 13.3% of the total label had been released into the seawater. About 23.2% of this material corresponded to esterase-resistant apolar material with retention time of AP1.

Adult *Orchestia cavimana* injected with [^{3}H]ecdysone before (stage D_1''), during (stage D_1''') and after (stage D_2) the endogenous ecdysteroid peak, produced large amounts of apolar metabolites comigrating with the AP of ticks (about 95% of total labelling recovered in animals 48 hr after injection). No conversion into 20-hydroxyecdysone was observed. Esterase hydrolysis liberated free ecdysone and also minute quantities of polar compounds (Figs 2e, f).

Occurrence of ecdysteroid fatty acid esters in insects

We have investigated the fate of injected [^{3}H]ecdysone or [^{3}H]20-hydroxyecdysone in different insects of several orders. Table 1 summarizes our results concerning the occurrence of metabolites having retention times on RP-18 columns similar to the apolar conjugates in ticks (Fig. 3). Such apolar products occurred in 7 out of 8 orders examined. The presence and quantity of these metabolites varied among the different orders, among the species in the same order, and among the different stadia of the same species. In the hemipteran *Dysdercus cingulatus* and the lepidopteran *Pieris brassicae* relatively low quantities were synthesized, while the adult cockroaches produced large quantities. Within the order Orthoptera, these products did not appear to be present in *Locusta migratoria* (they possess on the other hand the comparable acetates; (Modde *et al.*, 1984), but they are found in *Gryllus bimaculatus* adults (K. Hoffmann, pers. commun.). Little or no apolar metabolites were produced in larvae or pupae of the hemipteran, coleopteran, dipteran and lepidopteran species investigated while adults were able to

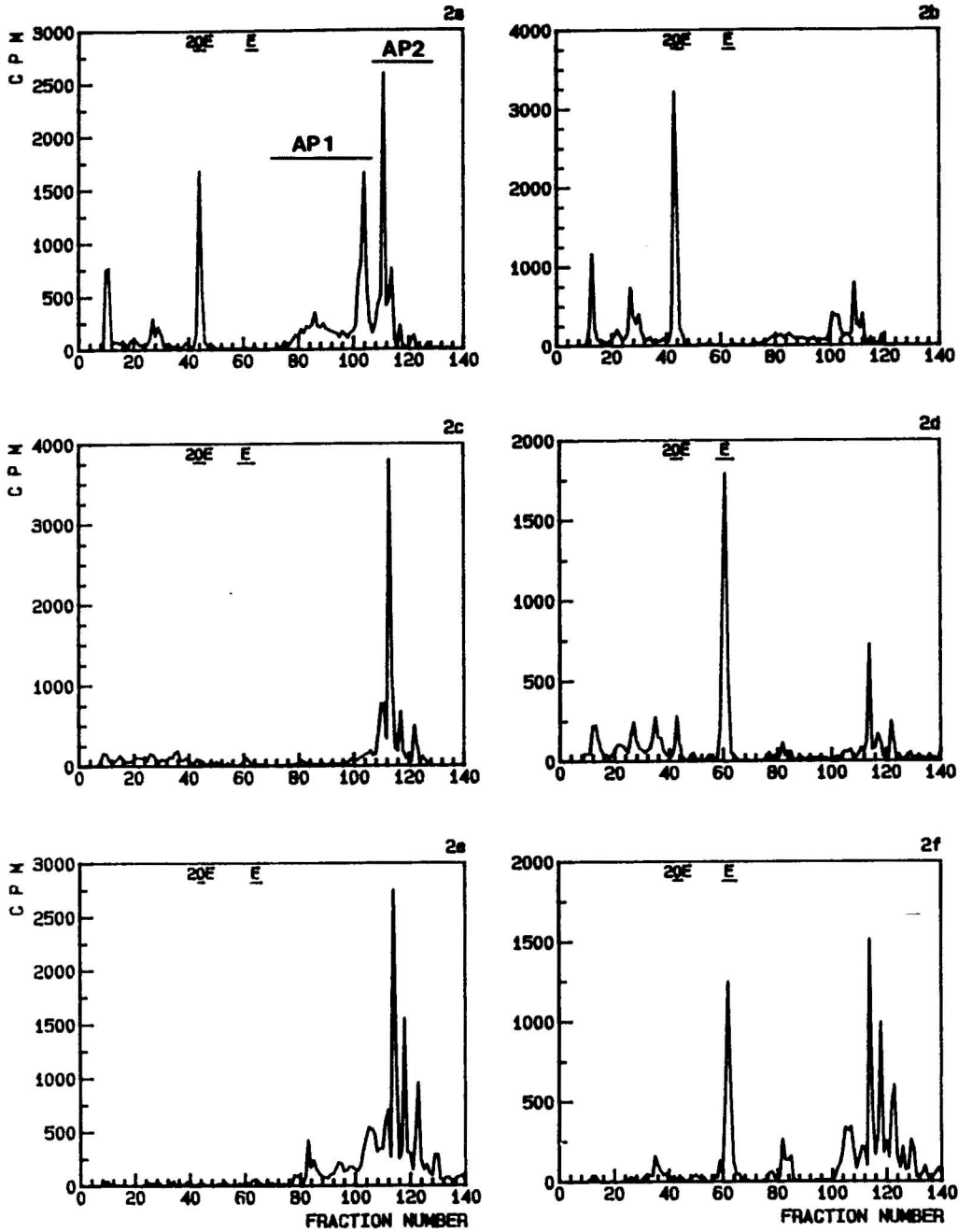

Fig. 2. Reverse phase HPLC radiochromatograms of ecdysteroid metabolites from scorpions, spiders and crustaceans before and after hydrolysis by esterase. AP1 and AP2 correspond to the retention times of apolar compounds from *Ornithodoros moubata*. (a) Adult scorpion *Buthus occitanus* 88 hr after injection of [^{3}H]20-hydroxyecdysone and (b) after hydrolysis. (c) Male of the spider *Araneus sclopetarius* 48 hr after injection of [^{3}H]ecdysone and (d) after hydrolysis. (e) Adult *Orchestia cavimana* (stage D_1°) 48 hr after injection of [^{3}H]ecdysone and (f) after hydrolysis.

synthesize them in larger quantities. Generally both sexes were able to metabolize the injected hormones using the apolar pathway, but surprisingly in the case of *Dysdercus cingulatus*, only the male produced these apolar products.

In all species investigated, the apolar compounds with retention times similar to AP2 of *O. moubata* were always esterase-labile and generally they liberated free ecdysone. The more polar products with similar retention times to AP1 from *O. moubata* were partially or totally hydrolyzable. In some cases, products other than the injected hormone could be liberated after hydrolysis by esterase of the extracts, e.g. in *Tenebrio molitor*, in addition to small quantities of ecdysone and 20-hydroxyecdysone, a very polar compound (P) was liberated (Fig. 3e). Esterification of

Table 1. Occurrence and abundance of apolar metabolites (% total radioactivity in the extract) in various insects after injection of [^{3}H]ecdysone (E)

Order	Genus species	Stadium	Occurrence of apolar metabolites (% total labelling recovered)		Hydrolysis by esterase (esterase-labile: +, esterase-resistant: −)
Odonata	*Aeschna* sp.	Larva	18.3% after 48 hr (Fig. 3a)		Yielding E and 20E
Orthoptera	*Locusta migratoria*	L 5			
		Ad ♂	Not found		
		Ad ♀			
	Gryllus bimaculatus	Ad ♀	Presence of esterase-labile apolar conjugates in the haemolymph, fat body and ovaries (Dr K. H. Hoffmann, Ulm University, pers. comm.)		
Phasmoptera*	*Carausius morosus*	Larvae	Not found		
Dichtyoptera	*Blatella germanica*	Last larva	31% after 48 hr	with a similar radiochromatographic pattern	+, Yielded E and 20E peak X: −
		Young ♀	71% after 48 hr		
		♀ with ootheca	63% after 48 hr		
		Ad ♂	80% after 48 hr (Fig. 3b)		
	Periplaneta americana	Larva	50% after 48 hr (Fig. 3c)		+
		Ad ♀	86% after 48 hr (Fig. 3d)		+, Peaks 1 and 2: −
		Ad ♂	81% after 48 hr		
	Nauphoeta cinerea	Vitellogenic ♀	Presence of products comigrating with AP2 from *O. moubata*. Proportion not known, collaborative work with Dr Lanzrein (Bern).		+, Yielded E
Hemiptera	*Dysdercus cingulatus*	L 5	Not found		
		Ad ♂	12.3% after 48 hr		
		Ad ♀	Not found (negligible amount)		
Coleoptera	*Donacia* sp.	Larva	Not found (negligible amount)		
	Tenebrio molitor	Last larval stage (A period)	10% after 48 hr		Not tested
		Pupa PS 0	7.5% after 48 hr		
		PS 5	43.6% after 48 hr		+
		Ad ♂	59.3% after 48 hr (Fig. 3e)		
		Ad ♀	32% after 48 hr	with a similar radiochromatographic pattern	Yielded E, 20E, and principally the product P
Diptera	*Prionocera turcica*	Larva	3.3% after 48 hr		
	Drosophila melanogaster	L2, L3	Not found after 12 hr incubation.		
		Ad ♀	Presence of esterase-labile products comigrating with AP2 from *O. moubata* on RP 18 or Silica column, proportion not known. collaborative work with Dr Dübendorfer (Zürich).		
	Phormia sp.	Larva	11% after 6 hr		+
		Pupa	11% after 6 hr		+
		Ad	21% after 17 hr		Not tested
	Lucilia serrata	Ad	41.4% after 48 hr (Fig. 3f)		+, except the peak marked with an asterisk in Fig. 3f
Lepidoptera*	*Pieris brassicae*	Pupa	Not found		
		Ad	12.3% after 24 hr		+

*Insects injected with [^{3}H]20-hydroxyecdysone (20E). "Not found" means that no labelled compound less polar than ecdysone was present after 48 hr of metabolism and after 24 hr. PS = physiological stage; for peaks ×1,2,P.

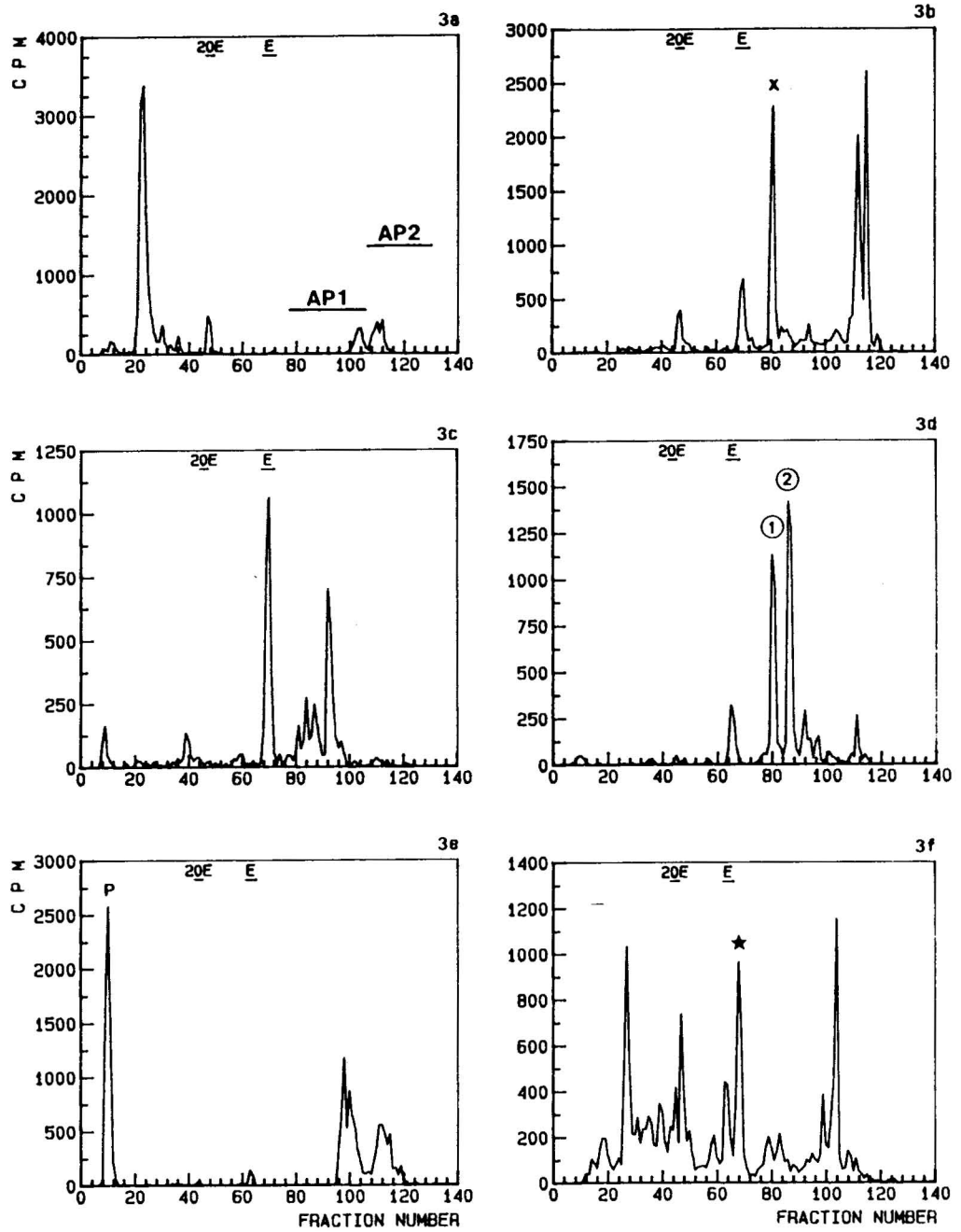

Fig. 3. Reverse phase HPLC radiochromatograms of ecdysteroid metabolites from various species and stadia of insects, 48 hr after injection of [^{3}H]ecdysone. Retention times of ecdysone (E) and 20-hydroxyecdysone (20E) are marked. Metabolites with retention times similar to AP1 or AP2 from *Ornithodoros moubata* were produced. Most of them were hydrolyzable by esterase. (a) *Aeschna* larvae. (b) Male of *Blatella germanica*. X was esterase-resistant. (c) *Periplaneta americana* (larva). (d) *P. americana* (female); peaks 1 and 2 were esterase-resistant. (e) *Tenebrio molitor* (male) before hydrolysis. Hydrolysis of the extract yielded ecdysone, 20-hydroxyecdysone, and, principally, the polar peak P. (f) *Lucilia serrata* (adults); metabolite * was esterase-resistant.

this polar metabolite probably occurred only secondarily. A comparable situation was observed in mature embryos of the tick *B. microplus*; hydrolysis mainly liberated polar compounds (presumably ecdysonoic and 20-hydroxyecdysonoic acid; Diehl *et al.* 1985b).

In the case of *Periplaneta americana* (Fig. 3c,d) low quantities of products with retention times of AP2 were present after 48 hr of metabolism. The larvae produced hydrolyzable metabolites with retention times of AP1 (Fig. 3c). In the female (Fig. 3d) two important peaks of esterase-resistant apolar material with retention times of about 40 and 43 min were found.

We have not yet elucidated the exact structure of

the different apolar conjugates in the insects investigated. However, the esterase-labile ones having retention time on RP-18 columns similar to those of conjugates AP2 identified in *O. moubata* are likely to be of the same nature. This is supported by collaborative work with Dübendorfer and colleagues which demonstrated that the hydrolyzable apolar ecdysteroid esters from *D. melanogaster* females co-chromatographed on RP-18 or silica columns with the AP2 from *O. moubata*.

To conclude our account of these preliminary metabolic experiments with various arthropods, we can say that the occurrence of apolar conjugates with long-chain fatty acids seems to be very common. They may have escaped previous detection methods because they were lost during the purification steps before HPLC; or they may have been confused with other ecdysteroids such as 22,25-dideoxyecdysone on TLC because of the poor resolution of this method (Briers and De Loof, 1983; Briers *et al.*, 1983a,b). The wide distribution of these apolar conjugates indicates that further studies on endogenous ecdysteroids should include hydrolysis by esterase in order to determine whether such conjugates are present. The role of these compounds is not yet clear. They could correspond to inactivated endogenous or exogenous ecdysteroids. However, several results indicate that they may be a storage form in eggs for future use in the development of the embryos. More detailed studies are needed to verify these different hypotheses.

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