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Isolation and identification of ecdysteroid phosphates and acetylecdysteroid phosphates from developing eggs of the locust, *Schistocerca gregaria*

R. Elwyn ISAAC* and Huw H. REES
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

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Maturing eggs of the desert locust, *Schistocerca gregaria*, contain a variety of ecdysteroid (insect moulting hormone) conjugates and metabolites, four of which have been previously isolated from polar extracts and identified as ecdysonoic acid, 20-hydroxyecdysonoic acid, 3-acetylecdysone 2-phosphate and ecdysone 2-phosphate. In the present study we have isolated eight additional ecdysteroids from similar late-stage eggs by high-performance liquid chromatography. The 22-phosphate esters of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone, all of which were first identified as ecdysteroid components of newly-laid eggs of *S. gregaria*, were identified by co-chromatography with authentic compounds and by physicochemical techniques. The remaining compounds were identified as 3-acetyl-20-hydroxyecdysone 2-phosphate, 3-epi-2-deoxyecdysone 3-phosphate, 3-acetylecdysone 22-phosphate and 2-acetylecdysone 22-phosphate by fast atom bombardment mass spectrometry, p.m.r. spectroscopy and analysis of the steroid moieties after enzymic hydrolysis. The latter two compounds, after isolation, are susceptible to non-enzymic acetyl migration and deacetylation to give mixtures of ecdysone 22-phosphate and its 2- and 3-acetate derivatives. The possible role and significance of these ecdysteroid conjugates with respect to the control of hormone titres in insect eggs is discussed.

Polar ecdysteroid (insect moulting hormone) conjugates are not only prominent inactivation products of the circulating moulting hormone in immature stages of insects, but they are also present in ovaries of adult females and in the newly laid eggs of some insect species (for reviews see Koolman, 1982; Hoffmann *et al.*, 1980).

In the desert locust, *Schistocerca gregaria*, the predominant polar ecdysteroid conjugates of newly-laid eggs have been identified as the 22-phosphate esters of ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone and 2-deoxy-20-hydroxyecdysone (Isaac *et al.*, 1982*a*, 1983*a*). These conjugates can serve as storage forms of ecdysteroids and may release active free hormone at specific stages in embryogenesis by the action of an embryonic phosphatase (Rees *et al.*, 1981; Isaac *et al.*, 1983*b*).

Abbreviations used: h.p.l.c., high-performance liquid chromatography; FAB, fast atom bombardment.

* Present address: Department of Pure and Applied Zoology, Agricultural Sciences Building, University of Leeds, Leeds LS2 9JT, U.K.

Other sources of ecdysteroids may also be available to the developing embryo, for example, synthesis from sterol precursors. Studies on the metabolism of ecdysteroid 22-phosphates and ecdysone in embryos of *S. gregaria* have shown that a number of ecdysteroids may arise from the metabolism of ovarian ecdysteroid 22-phosphates via the free hormone (Rees *et al.*, 1981; Isaac & Rees, 1984). As a consequence, late-stage eggs contain, in addition to the unmetabolized maternal ecdysteroid phosphates, a diversity of ecdysteroid metabolites. Some of these metabolites have been identified as ecdysonoic acid, 20-hydroxyecdysonoic acid, 3-acetylecdysone 2-phosphate and ecdysone 2-phosphate (Isaac *et al.*, 1983*c*, 1984). The latter compound can be a non-enzymic deacetylation product of 3-acetylecdysone 2-phosphate (Isaac *et al.*, 1984). A polar conjugate of 3-epi-2-deoxyecdysone has also been detected as a minor ecdysteroid in developing eggs of *S. gregaria* (Isaac *et al.*, 1981*a*).

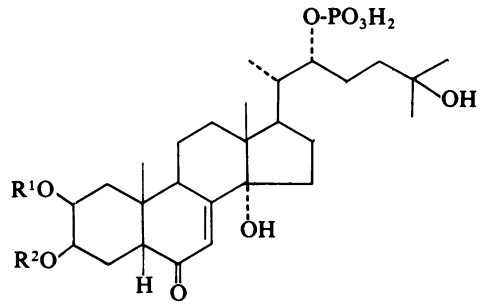
In the present study we have isolated four ecdysteroid metabolites from developing eggs of *S. gregaria* and identified them as 3-acetyl-20-

hydroxyecdysone 2-phosphate (**2**), 3- and 2-acetylecdysone 22-phosphate (**1**, **3**) and 3-epi-2-deoxyecdysone 3-phosphate (**4**). Late-stage eggs also contain the 22-phosphate esters of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone. All of the ecdysteroid conjugates were fully characterized by physicochemical techniques.

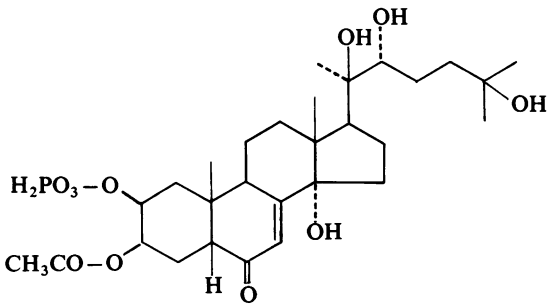
Materials and methods

Chemicals

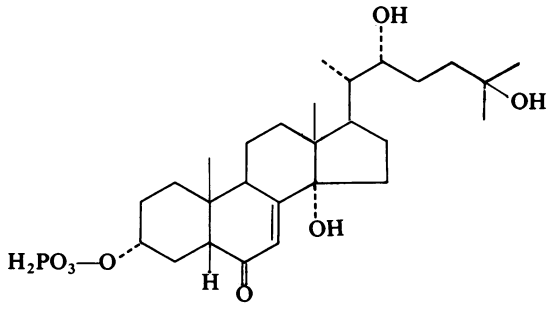
Ecdysone was obtained from Simes, Milan, Italy, and both 20-hydroxyecdysone and 3-epi-2-deoxyecdysone were kindly given by Dr. G. B. Russell, D.S.I.R., New Zealand. Ecdysteroid acetates were synthesized, and purified by silica t.l.c., as described by Galbraith & Horn (1969). *Helix pomatia* arylsulphatase preparation was from Sigma, Poole, Dorset, U.K.



(**1**) $R^1 = H$, $R^2 = COCH_3$
(**3**) $R^1 = COCH_3$, $R^2 = H$



(**2**)



(**4**)

Insects

The rearing of *S. gregaria* and the collection of eggs were carried out as described previously (Dinan & Rees, 1981). Eggs were incubated at 30°C for 15–16 days before being stored at −20°C for extraction.

Extraction and isolation of the polar ecdysteroid fraction from eggs

Ecdysteroids were extracted by macerating eggs four times in methanol/water (7:3, v/v; 250ml/100g of eggs). The extract was partitioned between methanol/water (7:3, v/v) and hexane and the polar ecdysteroids were isolated by chromatography on a silicic acid column as described by Dinan & Rees (1981).

High-performance liquid chromatography

A Waters gradient system (Waters Associates, Northwich, Cheshire, U.K.) incorporating a u.v. detector set at 254nm was used. Polar ecdysteroids were separated on the following systems.

System 1: ion-suppression reversed-phase chromatography on a Partisil ODS-3 column (Whatman, Maidstone, Kent, U.K.; Magnum 9, 50cm × 9.4mm internal diameter) eluted at a flow rate of 3ml/min with (*a*) a linear gradient (25min) of methanol in 20mM-sodium citrate buffer, pH6.5, changing from 3:7 (v/v) to 4:1 (v/v), or (*b*) methanol in 20mM-sodium citrate, pH6.5 (21:29, v/v).

System 2: ion-suppression reversed-phase chromatography on a Resolve column (Waters Associates; 15cm × 4.6mm internal diameter) eluted at a flow rate of 1ml/min with (*a*) methanol in 20mM-sodium citrate buffer, pH6.5 (3:7, v/v) or (*b*) a linear gradient (30min) of methanol in 20mM-sodium citrate buffer, pH6.5, changing from 1:4 (v/v) to 7:3 (v/v).

System 3: ion-suppression reversed-phase chromatography on a Partisil ODS-3 column (25cm × 4.6mm internal diameter) eluted at a flow rate of 2ml/min with (*a*) a linear gradient (25min) of methanol in 20mM-sodium citrate buffer, pH6.5, changing from 2:3 (v/v) to 4:1 (v/v) or (*b*) methanol in 20mM-sodium citrate buffer, pH6.5 (9:11, v/v).

Samples collected from h.p.l.c. were concentrated under reduced pressure and were desalted as described previously (Isaac *et al.*, 1982*a*) whenever buffer was employed in the mobile phase. Com-

pounds were finally chromatographed by reversed-phase h.p.l.c. using a Partisil ODS-3 column (25cm × 4.6mm internal diameter) and a linear gradient (20min) of methanol in water, changing from 1:10 (v/v) to 1:1 (v/v), at a flow rate of 2ml/min. Non-conjugated ecdysteroids and ecdysteroid acetates were identified by chromatography on a reversed-phase column (Isaac *et al.*, 1982*b*) and on an aminopropyl column (APS-Hypersil; Shandon Southern Products, Runcorn, Cheshire, U.K.; Dinan *et al.*, 1981).

Enzymic hydrolysis of ecdysteroid conjugates

Samples were incubated with *H. pomatia* enzymes and the released ecdysteroids isolated as described previously (Isaac *et al.*, 1983*a*).

Mass spectrometry and n.m.r. spectroscopy

Electron-impact and negative-ion FAB mass spectra were recorded on a VG Micromass 7070F spectrometer and Fourier transform n.m.r. spectra were recorded on a Bruker 400 MHz instrument (S.E.R.C. High Field NMR Service, Chemistry Department, University of Sheffield) with (^{2}H)methanol as the solvent and the same conditions as described previously (Isaac *et al.*, 1983*a*).

Results

Initial fractionation of polar ecdysteroids by h.p.l.c.

The crude polar fraction obtained from 150g of day 15–16 eggs by silicic acid column chromatography was subjected to semi-preparative reversed-phase h.p.l.c. (system 1*a*) which resolved the mixture into a number of u.v.-absorbing peaks (Fig. 1*a*). Three of the compounds were identified as 20-hydroxyecdysone 22-phosphate, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate by co-chromatography with authentic samples (Fig. 1*a*) isolated from newly-laid eggs, FAB mass spectrometry, p.m.r. spectroscopy and, for the last two compounds, ^{13}C n.m.r. spectroscopy (results not given; see Isaac *et al.*, 1983*a*). The complex elution profile indicated the presence of a number of unidentified compounds, in addition to ecdysteroid acids and 3-acetylecdysone 2-phosphate which have been previously isolated from late-stage eggs of *S. gregaria* (Isaac *et al.*, 1983*c*, 1984). Three broad fractions (I–III) were collected for further purification and analysis.

Isolation of ecdysteroid conjugates (**1–4**)

Fraction I was separated further into three u.v.-absorbing fractions (a, b and c) by reversed-phase h.p.l.c. using isocratic elution (system 1*b*; Fig. 1*b*). Major components of a, b and c were identified as ecdysonoic acid, ecdysone 2-phosphate and 2-deoxy-20-hydroxyecdysone 22-phosphate, respec-

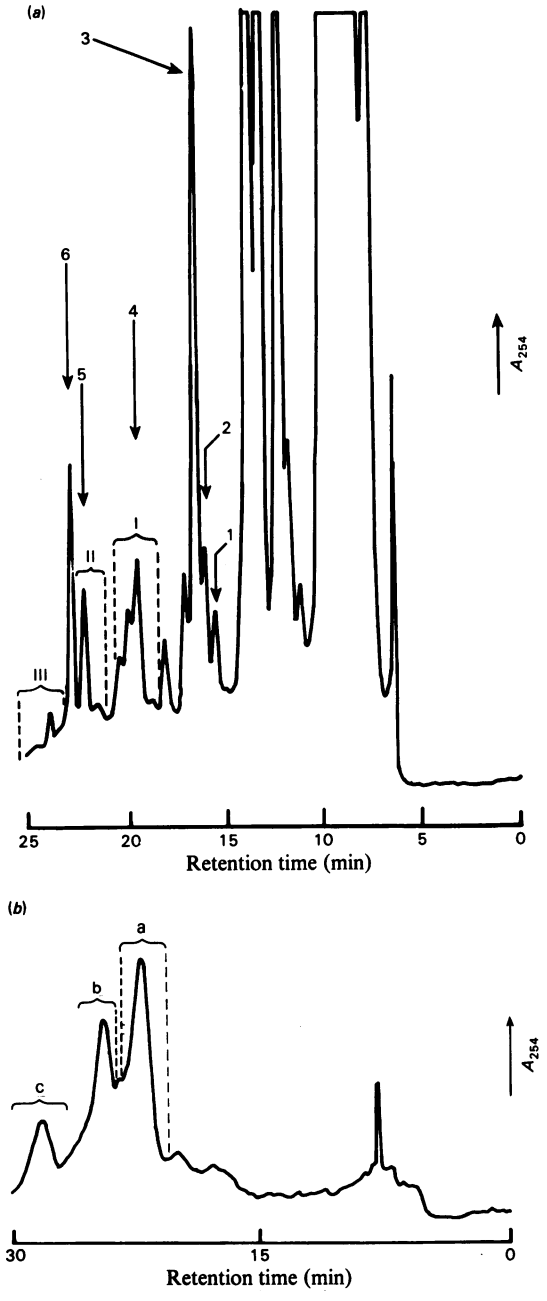


Fig. 1. *Semi-preparative reversed-phase h.p.l.c. of ecdysteroid conjugates*

(*a*) Fractions I–III were collected from the chromatography of the crude ecdysteroid conjugate fraction on h.p.l.c. system 1*a*. Arrows indicate positions of elution of authentic (1) 20-hydroxyecdysone 22-phosphate, (2) 20-hydroxyecdysonoic acid, (3) ecdysone 22-phosphate, (4) ecdysonoic acid, (5) 3-acetylecdysone 2-phosphate and (6) 2-deoxyecdysone 22-phosphate. (*b*) Fraction I was resolved into components a, b and c by isocratic elution (system 1*b*). Chromatography was monitored by measuring the u.v. absorbance at 254nm.

tively by co-chromatography with authentic compounds on ion-suppression reversed-phase h.p.l.c., analysis of the ecdysteroids released after incubation with *H. pomatia* enzymes and FAB mass spectrometry (Isaac *et al.*, 1983*a*,*c*, 1984). Fraction Ia also contained compounds (**1**) (55 μg) and (**2**) (35 μg) which were resolved by chromatography on an analytical column [system 2*a*; retention volumes: authentic ecdysonoic acid, 12ml; (**1**), 17ml; (**2**), 22ml]. Compound (**3**) was isolated from fraction II and separated from 3-acetylecdysone 2-phosphate, the major ecdysteroid present, by chromatography on h.p.l.c. system 3*b* [retention volumes: 3-acetylecdysone 2-phosphate, 20ml and (**3**), 18ml). Removal of contaminating 3-acetylecdysone 2-phosphate was completed by the re-chromatography of (**3**) (30 μg) on the same system. Fraction III was re-chromatographed on h.p.l.c. system 3*a* to yield compound (**4**) (125 μg; retention volume 26ml). All four compounds (**1**–**4**) showed an absorbance maximum at 242nm (methanol) and could be hydrolysed by incubation with *H. pomatia* enzymes, indicating that they were ecdysteroid conjugates.

Identification of 3-acetyl-20-hydroxyecdysone 2-phosphate (**2**)

Hydrolysis of (**2**) with *H. pomatia* enzymes gave compounds which co-chromatographed with 20-hydroxyecdysone, 20-hydroxyecdysone 3-acetate and 20-hydroxyecdysone 2-acetate (relative amounts, 3:17:0.2) on reversed-phase h.p.l.c., suggesting that (**2**) was a polar conjugate of 20-hydroxyecdysone 3-acetate. Negative-ion FAB mass spectrometry gave ions at *m/z* 623 $(M-H \text{ of Na salt})^-$, 601 $(M-H)^-$, 97 $(H_2PO_4)^-$ and 79 $(HPO_3)^-$, and was consistent with (**2**) being a phosphate ester of 20-hydroxyecdysone 3-acetate. The p.m.r. spectrum of (**2**) gave signals at δ 0.885 (3H, s, 18-H_3), 0.995 (3H, s, 19-H_3), 1.19 (3H, s, 21-H_3), 1.19/1.20 (6H, 2s, 26/27-H_3) and 2.09 (3H, s, CH_3CO) p.p.m. These methyl signals only differ from those of the 20-hydroxyecdysone spectrum in the downfield shift of the C-19 methyl (+0.035p.p.m.), indicating that both the phosphate and acetate groups are substituents on the A ring of the steroid nucleus (c.f. the p.m.r. spectrum of 3-acetylecdysone 2-phosphate; Isaac *et al.*, 1984). Thus, the evidence presented is consistent with (**2**) being 3-acetyl-20-hydroxyecdysone 2-phosphate.

Identification of 3-acetylecdysone 22-phosphate (**1**) *and 2-acetylecdysone 22-phosphate* (**3**)

Reversed-phase h.p.l.c. of purified (**1**) and (**3**) revealed that both compounds were unstable on storage and each gave rise to two additional u.v. peaks. Comparative h.p.l.c. analysis of the mixtures obtained showed that (**1**) and (**3**) were in equilibrium with each other and that they formed a compound which co-chromatographed with ecdysone 22-phosphate (Figs. 2*a* and 2*b*). Enzymic hydrolysis of the samples released ecdysone 3-acetate, ecdysone 2-acetate and ecdysone which were identified by chromatography on a reversed-phase column and an APS-Hypersil column. Negative-ion FAB mass spectra of samples of (**1**) and (**3**) were similar with peaks at *m/z* 607 $(M-H \text{ of Na salt})^-$, 585 $(M-H)^-$, 97 $(H_2PO_4)^-$ and 79 $(PO_3)^-$, which were indicative of phosphate esters of

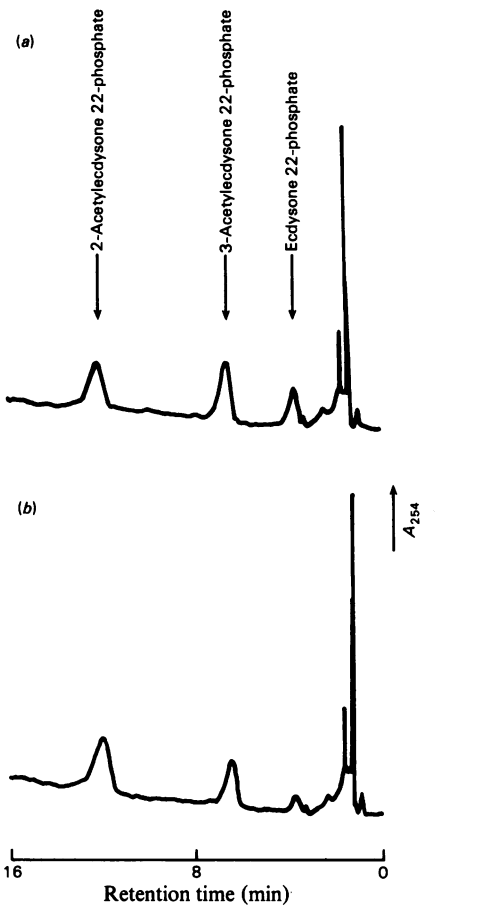


Fig. 2. *Reversed-phase h.p.l.c. separation of ecdysone 22-phosphate, 3-acetylecdysone 22-phosphate and 2-acetylecdysone 22-phosphate*

Chromatography of (*a*), compound (**1**) and (*b*), compound (**3**) on a Partisil ODS-3 column (25cm × 4.6mm internal diameter) eluted at a flow rate of 2ml/min with a linear gradient of methanol in sodium citrate buffer, pH6.5, changing from 2:3 (v/v) to 1:1 (v/v) over 25min. Chromatography was monitored by measuring the u.v. absorbance at 254nm.

ecdysone monoacetates (Isaac *et al.*, 1984). In the FAB mass spectrum of one sample an additional ion was observed at *m/z* 543 which indicated that this sample had undergone partial breakdown to give ecdysone phosphate. The p.m.r. spectrum of (**1**) gave signals at δ 0.74 (3H, s, 18-H_3), 0.99 (3H, s, 19-H_3), 0.975, 0.99 (3H, d, 21-H_3), 1.17/1.185 (6H, 2s, 26/27-H_3) and 2.06/2.18 (3H, s, CH_3CO). The chemical shift of the 21-methyl was characteristic of a 22-phosphate ester linkage (Isaac *et al.*, 1983*a*) and the occurrence of two signals in the 2.00 p.p.m. region of the spectrum suggested the presence of a mixture of axial and equatorial acetate substituents (Horn, 1971). This spectral data, together with the earlier analytical results, suggested that (**1**) was a 22-phosphate of an ecdysone monoacetate with the acetate group migrating between the C-2 and C-3 hydroxyls (Isaac *et al.*, 1981*b*). The h.p.l.c. analysis of purified (**1**) and (**3**) (Figs. 2*a* and 2*b*) can be explained by acyl migration to give a mixture of 2- and 3-acetyl derivatives and partial deacylation to give ecdysone 22-phosphate. The corresponding non-phosphorylated ecdysteroids are easily resolved on reversed-phase h.p.l.c., with ecdysone being eluted before ecdysone 3-acetate, which in turn is eluted before ecdysone 2-acetate (Isaac *et al.*, 1982*b*). Therefore, it is likely that under ion-suppression reversed-phase chromatographic conditions, the same elution sequence would be maintained for the phosphate derivatives of ecdysone 3- and 2-acetates. Compound (**1**) at the time of isolation probably represented the 22-phosphate of ecdysone 3-acetate, with (**3**) being the corresponding 2-acetyl derivative.

Identification of 3-epi-2-deoxyecdysone 3-phosphate (**4**)

Enzymic hydrolysis of (**4**) released 3-epi-2-deoxyecdysone, which was identified by co-chromatography with authentic material by h.p.l.c. on both reversed-phase and APS-Hypersil columns. The negative ion FAB mass spectrum [*m/z* 549 $(M-H \text{ of Na salt})^-$, 527 $(M-H)^-$, 97 $(H_2PO_4)^-$ and 79 $(PO_3)^-$] was consistent with (**4**) being a phosphate ester of 3-epi-2-deoxyecdysone. The p.m.r. spectrum gave signals at δ 0.73 (3H, s, 18-H_3), 0.91 (3H, s, 19-H_3), 0.94/0.96 (3H, d, 21-H_3), 1.19/1.20 (6H, 2s, 26/27-H_3), 3.60 (1H, m, C-22-*H*, $W_{\frac{1}{2}}$ 19 Hz) and 4.08 (1H, m, C-3-*H*, $W_{\frac{1}{2}}$ 20 Hz) p.p.m. An upfield shift of the C-19 methyl signal relative to that of ecdysone and 2-deoxyecdysone (−0.06 p.p.m. and −0.05 p.p.m., respectively; Isaac *et al.*, 1983*a*) was also observed in the p.m.r. spectrum of non-conjugated 3-epi-2-deoxyecdysone (Isaac *et al.*, 1981*a*). The broad signal at 4.08 p.p.m. was assigned to the axial C-3 proton on the basis of its peak width at half height (Horn, 1971; Isaac *et al.*, 1981*a*). The downfield shift of this signal (+0.10 and +0.12 p.p.m. relative to 2-deoxyecdysone 22-phosphate and ecdysone, respectively), and the lack of any significant shifts of the methyl (other than the C-19) and the C-22 proton signals, relative to those of ecdysone and 2-deoxyecdysone, showed that the phosphate ester is positioned at C-3. Compound (**4**) is, therefore, identified as 3-epi-2-deoxyecdysone 3-phosphate.

Discussion

The occurrence of 3-acetyl-20-hydroxyecdysone 2-phosphate in developing eggs of *S. gregaria* was not unexpected, since the corresponding ecdysone derivative has been identified previously as a major metabolite of ecdysone in this system (Isaac *et al.*, 1984; Isaac & Rees, 1984). Polar conjugates of the acetates of ecdysone and 20-hydroxyecdysone have also been identified as metabolites of ecdysone in fifth instar larvae of both *S. gregaria* (Gibson, 1982; Gibson *et al.*, 1984) and *Locusta migratoria* (Gibson, 1982; Gibson *et al.*, 1984; Lafont *et al.*, 1983). 20-Hydroxyecdysone is generally believed to be the active hormone at least in immature stages of insect post-embryonic development (Gilbert & King, 1973). If this also holds for embryos, the formation of 3-acetyl-20-hydroxyecdysone 2-phosphate in addition to that of 20-hydroxyecdysonoic acid in the closed system of the developing insect egg can be considered to be an important inactivation pathway of the hormone. We have already shown in the case of 3-acetylecdysone 2-phosphate that substitution of the A ring with acetate and phosphate groups is likely to result in an end product of ecdysteroid metabolism (Isaac *et al.*, 1984). It is not known whether the acetates of ecdysteroid 22-phosphates can result from direct acetylation of maternal conjugate or the combined phosphorylation and acetylation of non-conjugated hormone. The demonstration that gut and Malpighian tubules of *L. migratoria* can metabolize 20-hydroxyecdysone, *in vitro*, to 3- (or 2-) acetyl-20-hydroxyecdysone 22-phosphate indicates that such reactions could be involved in regulating ecdysteroid titres (Tsoupras *et al.*, 1983*a*). In addition, the latter compound has also been isolated from developing eggs of *L. migratoria*, albeit in very low amounts (Tsoupras *et al.*, 1982). However, the lability of acetylecdysteroid phosphate compounds during isolation and storage might result in an underestimate of their significance, and for this reason, the amounts of the 22-phosphate esters of ecdysone acetates isolated in the present study were limited by the time taken to carry out the numerous purification steps. A reliable estimation of these compounds in insect extracts will require a mild and expeditious analytical procedure. Conversely, caution must be exercised whenever

ecdysteroid 22-phosphates or ecdysteroid 2-phosphates are identified as metabolites of ecdysone or 20-hydroxyecdysone, as these may arise from the non-enzymic deacetylation of the corresponding acetate derivatives (Isaac *et al.*, 1984). The 3-phosphate ester of 3-epi-2-deoxyecdysone has been recently identified as a major ecdysteroid in the late eggs of *L. migratoria* (Tsoupras *et al.*, 1983*b*). In contrast, although 3-epi-2-deoxyecdysone 3-phosphate is present in developing eggs of *S. gregaria*, it does not accumulate to any great extent during embryogenesis in this species (R. E. Isaac & H. H. Rees, unpublished work). This may reflect the lower amount of 2-deoxyecdysone conjugate present in newly laid eggs of *S. gregaria*, compared with eggs of *L. migratoria* where the conjugate is the predominant ecdysteroid in the early stages of embryogenesis (Sall *et al.*, 1983).

The identification of phosphate conjugates, double phosphate/acetate conjugates of ecdysteroids and ecdysteroid acids, has provided an insight into the array of reactions involved in inactivating insect moulting hormones. Studies on the reactions involved in the synthesis of these compounds will help to indicate the control of ecdysteroid titres in insects.

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