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IDENTIFICATION AND METABOLIC FATE OF OVARIAN 22-ADENOSINE MONOPHOSPHORIC ESTER OF 2-DEOXYECDYSONE IN OVARIES AND EGGS OF AN INSECT, *LOCUSTA MIGRATORIA*

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Abstract—Newly-laid eggs of *Locusta migratoria* contain as the major ecdysteroid conjugate donated by the female to its offspring the 22-adenosinemonophosphoric ester of 2-deoxyecdysone (**3**); during embryonic development this conjugate is hydrolysed to free 2-deoxyecdysone (**1**), which is subsequently metabolized to 3-dehydro 2-deoxyecdysone (**7**) and 2-deoxy-3-epi-ecdysone(**5**): the latter substance is accumulated at late stages of development as a 3-phosphoric ester (**6**). 22-Phospho-2-deoxyecdysone (**4**) also appears as embryonic development proceeds, either from partial hydrolysis of the maternal conjugate or from phosphorylation of free 2-deoxyecdysone(**1**).

Ecdysteroids are polyhydroxylated derivatives of 5β-cholest-7-en-6-one, which control moult (ecdysis) and metamorphosis in insects. Up to a few years ago, studies on ecdysone and/or its major hydroxylated metabolite 20-hydroxyecdysone were limited to larval and pupal development. It came as a major surprise when several laboratories demonstrated recently that ovaries of adult females synthesize ecdysteroids.[1-3] In several species, it was clearly established that the bulk of these ovarian ecdysteroids was not secreted into the blood of the female, but was accumulated in the oöcytes and was present in the newly-laid eggs;[2,4-10] it was also shown in several insects that the majority of ovarian ecdysteroids was present in the eggs in a conjugated form.[3-5,9-12]

Independently from these studies, eggs had been demonstrated to exhibit several peaks of concentration of free ecdysone and/or 20-hydroxyecdysone during the span of embryonic development.[8,12,13] As several of these peaks were clearly monitored before the differentiation of endocrine glands able to initiate the *de novo* biosynthesis of ecdysone, it was proposed that the hydrolysis of the ovarian ("maternal") conjugates could account for the early peaks of free ecdysone concentration in the eggs.[3,10,11] The experimental demonstration of a hormonal relationship between the female and its offspring still lacked identification of the ovarian ecdysteroid conjugates and of the metabolites formed in the embryo.

We have briefly reported the identification of two of the three major 2-deoxyecdysone conjugates.[14] We now give the details of identification of the structures of the three conjugates and of two ecdysteroid metabolites present under unconjugated form during embryonic development.

RESULTS

In their study on ecdysteroid conjugates in eggs of *Locusta migratoria*, Lagueux *et al.*[11] noted that silicagel thin-layer chromatographic separation of extract from a pool of eggs of various developmental stages allowed the separation of three groups of conjugated ecdysteroids, which they termed "C1", "C2" and "C3" by increasing polarity. The predominant genins were 2-deoxyecdysone for "C1", ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone for "C2" and an unidentified compound (NI_2) for "C3". An observation which served as a guideline in the present study can be summarized as follows: "C1" being present in ovaries and newly-laid eggs, is the predominant maternal ecdysteroid conjugate; "C2" appears as embryonic development proceeds; "C3" is found in eggs after blastokinesis of the embryo and is the predominant ecdysteroid conjugate in ageing eggs.

We describe now the chemical structure of the predominant maternal conjugate referred to by Lagueux *et al.* as "C1"-2-deoxyecdysone, the conjugate appearing later in embryonic development and referred to as "C2"-2-deoxyecdysone, and the predominant conjugate of ageing eggs called "C3"-NI_2.[11] A major aim of the present paper will be to established whether and in which way these compounds are metabolically related to one another.

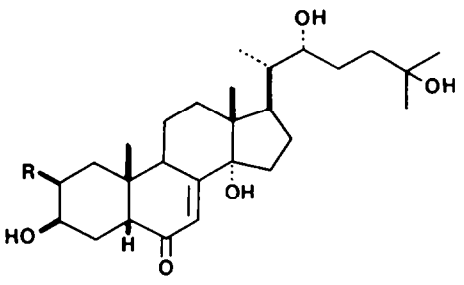
Identification of the predominant maternal conjugate

We have taken advantage of the observation by Lagueux *et al.*[11] that the predominant maternal ecdysteroid conjugate is bound to the major yolk protein vitellin, and have firstly extracted this protein according to well established techniques, given in experimental data. From a total of 1.5 kg of wet semi-purified vitellin, we have extracted and isolated 25 mg of a predominant ecdysteroid conjugate and lower amounts of another ecdysteroid conjugate. In this paper, we will focus our attention exclusively on the predominant conjugate; the identification of the minor compound will be reported later. Following Lagueux *et al.*[11] we assumed the genin of the major ecdysteroid conjugate to correspond to

2-deoxyecdysone. We have first demonstrated the correctness of this assumption.

Identification of the genin of the predominant maternal conjugate.

Enzymatic hydrolysis of a fraction of the maternal conjugate yielded a free ecdysteroid which co-migrated on TLC and co-eluted in reversed phase C18 HPLC with reference 2-deoxyecdysone (1). Our final identification of 2-deoxyecdysone (1) as the source of the predominant maternal conjugate was based on the following criteria: (a) GC-MS after trimethylsilylation; the main peaks were at *m/e* 736(1.5%), 721(3%), 708(22%), 646(3%), 631(3%), 618(6%), 556(10%), 541(7%), 356(28%), 261(15%), 171(95%), 131(66%), 73(100%); this fragmentation is identical to that reported in the same conditions for reference 2-deoxyecdysone (1) by Hetru *et al.*[15] (b) ^{1}H NMR spectra; the chemical shifts (δ) of methyl proton resonances (Table 1) correspond to those reported for 2-deoxyecdysone (1);[16] (c) ^{13}C NMR spectra Table 2 gives the signals of the 27 carbon atoms of authentic ecdysone (2) and of the deoxyecdysone from our extract



R = H : 2-Deoxyecdysone (1)

R = OH: Ecdysone (2)

obtained in the same conditions. In these spectra, the signals of all carbon atoms are identical for ecdysone (2) and deoxyecdysone, except for the carbons of the A ring. The differences observed concern the signals of carbons C-1, C-2, C-3, C-4 and C-10. The identity of the signals of carbon C-5 in authentic ecdysone (2) and the endogenous deoxyecdysone eliminates the possibility that the latter compound either lacked a C-3 hydroxyl group or was a 3α-epimer, as both of these modifications would annihilate the synaxial interaction between 3β-OH and 5β-H (γ effect). In addition, the presence, in the spectrum of the endogenous deoxyecdysone, of a quartet signal at δ = 24.30 ppm, which corresponds to C-19 in the case of ecdysone (2), ensures that the A/B ring junction is *cis* in this deoxyecdysone. From these data we may conclude that the source of the maternal conjugate corresponds to 2-deoxyecdysone (1).

Identification of the non-hydrolysed predominant maternal conjugate.

For the purified predominant maternal conjugate, the following spectra data were recorded: IR^{KBr} cm^{-1}: 3400 (OH), 1640 (CO) and a large absorption band at 1050–1100 (P-O-C); UV^{MeOH} 250 nm. Microanalysis showed the presence of phosphorus (estimated ratio: one phosphorus atom per molecule of conjugate). ^{31}P NMR spectra presented a signal at δ = 1 ppm relative to phosphoric acid, which demonstrates the presence of a phosphate group. The ^{13}C NMR spectrum of the maternal conjugate (Table 2) shows all the signals related to the carbon atoms of 2-deoxyecdysone (1) together with nine additional signals (Table 3); conjugation appears to have modified three of the signals of the carbons of 2-deoxyecdysone (1) (Fig. 1). In the conjugate, the signal at δ = 78.5 ppm, which is present as a doublet (J = 4 Hz) in proton-noise decoupled spectra, corresponds to a carbon carrying a phosphate substituent; this signal can be attributed to C-22 which has undergone a downfield shift of 4.6 ppm as a result of the electronegative phosphate group. The conclusion that the attachment of the phosphate moiety has occurred at C-22 is in agreement with the observation that the signals of C-20 and C-23 are shifted upfield by 2 ppm in the conjugated steroid, as compared to free 2-deoxyecdysone (1).[17,19] We may therefore conclude that the maternal conjugate contains 22-phospho-2-deoxyecdysone.

Direct introduction mass spectrometry on gold support of the non-derivatized conjugate[18] shows the presence of fragments characteristic of 2-deoxyecdysone (1) (Fig. 1a), plus fragments at m/e 136, 164, 178, 237, 251, 268, 368 (Fig. 1b). The molecular ion of the conjugated ecdysteroid is not observed, due to the chemical instability of this compound containing a C–O–P bond. The fragment at *m/e* 136 which was present in our spectra, is a characteristic fragment for adenine in adenosine monophosphate. This has led us to compare the mass spectra obtained in the same experimental conditions for reference adenosine monophosphate (AMP; Fig. 1b) and our conjugate (Fig. 1c). The fragments observed in the spectra of AMP are present in the spectra of the maternal conjugate. Partial hydrolysis of the maternal conjugate by HCl at pH 2 liberated a putative nucleoside which was purified by reversed phase C8 liquid chromatography. This compound, which absorbs UV with a λ max of 260 nm, as does reference adenosine, was subsequently analysed by direct introduction mass spectrometry on gold support both with electron impact ionization and chemical (NH_3) ionization. In these various conditions, reference adenosine and the putative nucleoside from the maternal conjugate behaved similarly. High resolution mass spectrometry of the principal fragment of the putative nucleoside liberated by

Table 1. ^{1}H NMR data of compounds **1**, **2** and **5**. Chemical shifts (δ) of methyl proton resonances in d$_5$-pyridine (250 MHz; internal standard: TMS)

COMPOUND	C-18 H_3	C-19 H_3	C-21 H_3	C-26 H_3;C-27 H_3
1	0.74(s)	1.07(s)	1.30(d) J=6.7 Hz	1.39(s)
2	0.72(s)	1.07(s)	1.28(d) J=6.4 Hz	1.38(s)
5	0.74(s)	0.99(s)	1.30(d) J=6.6 Hz	1.40(s)

Table 2. ^{13}C NMR data of compounds **1**, **2**, **5** in d_5-pyridine and **3**, **6** in D_2O; δ in ppm from TMS; multiplicity of signals indicated as: s, singlet; d, doublet; t, triplet; q, quartet

Number of carbon atom	1	2	3 [a]	5	6 [a]
1	29.48(t)	37.90(t)	28.90(t)	34.50(t)	34.00(t)
2	29.07(t)	68.03(d)	27.90(t)	32.10(t)	29.70(t)
3	64.06(d)	68.03(d)	65.50(d)	69.30(d)	74.60[b](d)
4	33.07(t)	32.30(t)	32.40(t)	35.90(t)	33.60(t)
5	51.61(d)	51.30(d)	51.70(d)	57.40(d)	56.90(d)
6	203.20(s)	203.20(s)	209.50(s)	202.20(s)	207.80(s)
7	121.30(d)	121.50(d)	121.00(d)	121.50(d)	121.70(d)
8	166.01(s)	165.60(s)	170.00(s)	166.06(s)	169.60(s)
9	34.40(d)	34.50(d)	34.50(d)	34.20(d)	34.60(d)
10	36.96(s)	38.60(s)	37.10(s)	37.00(s)	37.20(s)
11	21.00(t)	21.10(t)	21.30(t)	21.00(t)	21.00(t)
12	31.70(t)	31.40(t)	31.30(t)	31.60(t)	31.70(t)
13	48.00(s)	47.50(s)	48.10(s)	47.80(s)	48.00(s)
14	84.01(s)	83.80(s)	86.30(s)	83.90(s)	85.90(s)
15	31.70(t)	31.80(t)	31.60(t)	31.80(t)	32.20(t)
16	25.50(t)	25.50(t)	25.80(t)	25.80(t)	25.20(t)
17	48.32(d)	48.30(d)	48.50(d)	48.50(d)	48.50(d)
18	15.80(q)	15.80(q)	16.20(q)	16.00(q)	16.40(q)
19	24.30(q)	24.40(q)	24.00(q)	24.10(q)	23.80(q)
20	42.99(d)	42.90(d)	40.20(d)	43.20(d)	42.70(d)
21	13.60(q)	13.60(q)	13.40(q)	13.90(q)	13.50(q)
22	73.90(d)	73.90(d)	78.50[b](d)	74.20(d)	75.50(d)
23	26.70(t)	26.60(t)	24.40(t)	26.80(t)	26.60(t)
24	42.46(t)	42.40(t)	41.70(t)	42.70(t)	41.50(t)
25	69.60(s)	69.70(s)	72.80(s)	69.80(s)	72.70(s)
26	29.98(q)	30.01(q)	28.80(q)	30.00(q)	29.10(q)
27	30.20(q)	30.20(q)	28.60(q)	30.20(q)	28.80(q)

[a] Only the signals attributed to the carbons of the genins are given for this conjugate; for **3**, see also Table 3.
[b] In proton-noise decoupled spectra this signal appears as a doublet.

Table 3. ^{13}C NMR of reference adenosine-monophosphate and the nucleotide of compound **3** in D_2O; δ in ppm from TMS

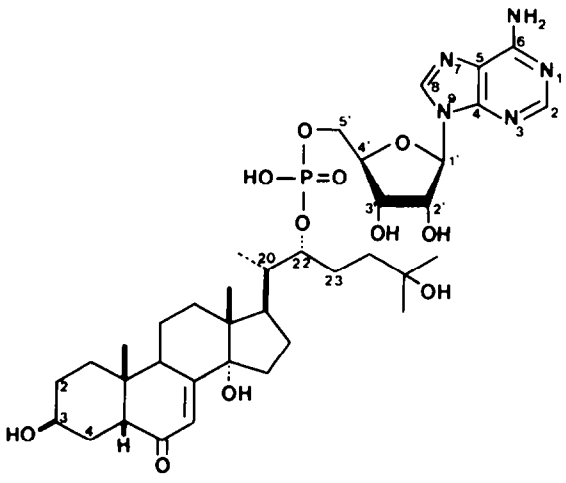
Number of carbon atom	AMP	3
2	152.6	153.5
4	149.0	-
5	119.5	116.5
6	156.3	156.6
8	140.2	141.0
1'	89.6	89.0
2'	75.0	74.8
3'	71.0	71.6
4'	85.2	86.7
5'	68.0*	67.5*

* In proton-noise decoupled spectra this signal appears as a doublet.

hydrolysis of the maternal conjugate showed the exact mass of 135.0543 ± 0.0004, which is compatible with the composition of adenine ($C_5H_5N_5$, calc 135.0545).

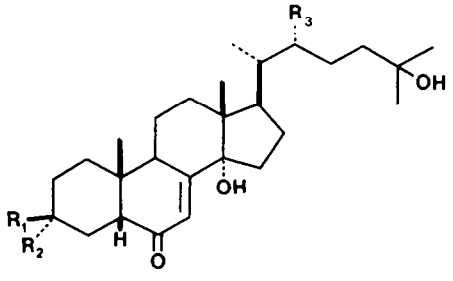
In the ^1H NMR spectrum, the two aromatic protons (δ = 7.5 ppm in D_2O) of reference adenine are clearly seen in the endogenous conjugate. As regards the ^{13}C NMR spectra (Table 3), a signal at δ = 68 ppm, a triplet assigned to carbon C-5' of ribose in reference AMP, is also present in the maternal conjugate (at δ = 67.5 ppm): this observation is of particular importance, because it shows that the carbon C-5' is engaged in a covalent binding with the phosphate group in the maternal conjugate; after hydrolysis of the purified maternal conjugate, the signal of C-5' is shifted upfield by 5 ppm (to δ = 62.5 ppm) which is explained by the cleavage of the C-5' ribose-phosphate bond (a chemical shift of 5 ppm is caused by the presence of a phosphate group). Thus, the predominant maternal conjugate is the 22-adenosine monophosphoric ester of 2-deoxyecdysone (**3**).

Phosphoesters are known to be unstable in the presence of amines;[19] this instability had a particular effect in our first purification procedures of **3**; indeed, initially we purified the maternal conjugate according to conventional techniques: after chromatography on silicagel, the ecdysteroid conjugate was purified by QAE-Sephadex ion exchange chromatography in 50 mM Tris-HCl buffer, pH 8, which was followed by reversed phase C8 liquid chromatography; the subsequent spectral analysis of the maternal conjugate isolated under these conditions indicated the presence of a 22-phospho-2-



(3)

deoxyecdysone (4) as a Tris salt. We believe that hydrolysis of the original adenosine-phosphate-2-deoxyecdysone (3) occurred during the process of drying the eluted fractions after ion exchange chromatography in the presence of Tris;[cf19] it is obvious that the phosphate group was not present as a salt during the first step of purification, i.e. silicagel column chromatography, which did not retain any conjugate as seen by RIA measurements: ecdysteroid phosphates would have been retained by silicagel as we will see below.



R_1	R_2	R_3	
H	OH	OH	3-Epi-2-deoxyecdysone (5)
H	OPO_3H_2	OH	3-Phospho-epi-2-deoxyecdysone (6)
OH	H	OPO_3H_2	22-Phospho-2-deoxyecdysone (4)
—O—		OH	3-Dehydro-2-deoxyecdysone (7)

Identification of the predominant maternal 2-deoxyecdysone conjugate in eggs at the end of blastokinesis of the embryo

The conjugate "C"-2-deoxyecdysone[11] appeared in our isolation process to be very polar and was difficult to elute from silicagel columns; we therefore based our purification procedure for this compound on C8 reversed phase chromatography, using a gradient of pure water to pure methanol as eluent. The presumed "C2"-2-deoxyecdysone conjugate was further purified by C8 reversed phase chromatography with an elution gradient from 20 mM Tris-HCl buffer, pH 7.5, to acetonitrile-Tris buffer (1:1); the final yield of the isolation, starting from 1.5 kg of lyophilyzed eggs, was 20 mg of pure presumed "C2"2-deoxyecdysone conjugate, which co-migrated on TLC with the "C2" conjugate group reported by Lagueux *et al.*[11]

The identification of the genin obtained after hydrolysis was based on ^{1}H NMR, ^{13}C NMR and mass spectrometry. The results were identical to those which we have reported in the foregoing section and demonstrated that the genin of the presumed "C2"-2-deoxyecdysone conjugate was actually 2-deoxyecdysone.

The non hydrolysed conjugate was subjected to microanalysis of phosphorus, ^{13}C NMR and ^{31}P NMR. The results of these analyses showed the presence of a phosphate group, the attachement site of which could be shown to be at C-22 (^{13}C NMR data). As all these data and the rationale of their interpretation are similar to those given in the above section, they will not be repeated here.

From our results we conclude that "C2"-2-deoxyecdysone conjugate is the 22-phosphate ester of 2-deoxyecdysone (4). At the end of our isolation procedure, this phosphate of 2-deoxyecdysone was present as a salt, the cation of which was identified by GC–MS after trifluoracetylation,[20] as protonated tris(hydroxymethyl)-aminomethane (Tris) which was the basic constituent of our buffer system in the purification procedure; signals at δ = 62.65 ppm (s) and δ = 60.59 ppm (t) in our ^{13}C NMR spectra of 4 (signals not presented in Table 2) are the signals of Tris.

Isolation and identification of the major ecdysteroid metabolite in ageing eggs

From approx. 1.5 kg of postblastokinetic eggs we have isolated the "C3" conjugate of the non-identified ecdysteroid NI_2, according to the terminology of Lagueux *et al.*[11] This conjugate was difficult to eluate from silicagel column, as a consequence of its high polarity; we have therefore based our isolation procedure on repeated C8 reversed phase chromatography and isolated 25 mg of presumed "C3" conjugate of NI_2 which co-migrated on TLC with the "C3" conjugate group of Lagueux *et al.*[11]

Identification of NI_2

The following characteristics have been determined for 5: UV^{MeOH} nm: 243; IR^{KBr} cm^{-1}: 3340(OH), 1650(CO). The electron impact mass spectra included ions: *m/e* 449($M+H)^+$; 430(M^+-H_2O); 412(M^+-2H_2O); 394(M^+-3H_2O); 361($M^+-4H_2O\text{-}^{Me}$); 332{$M^+-(C_6H_{12}O_2)$}; 284{$M^+-C_8H_{18}O_2-H_2O$}; 99{$C_6H_{13}O_2-H_2O$}; these spectra are similar to those of 2-deoxyecdysone (1). The methyl signals of the ^{1}H NMR spectrum of 1, 2 and 5 are presented in Table 1; these signals are virtually identical for C-18, C-21, C-26 and C-27 methyls; the signal of C-19 methyl of 5 differs however from the corresponding signal of ecdysone (2) and 2-deoxyecdysone (1). The ^{1}H NMR of 5 shows a broad signal at δ = 4.15 of peak-width at half height of 20 Hz, indicative of an axial proton, to which we will come back below. From these physico-chemical characteristics, we conclude that 5 is a deoxyecdysone.

The final elucidation of the structure of 5 is essentially based on the comparison of the ^{13}C NMR data of 5 with those of ecdysone (2) and 2-deoxyecdysone (1) present in Table 2. It is obvious from this Table that the three compounds have identical chemical shifts for all carbon atoms except for those of the A ring. The quartet at δ = 24 ppm in the spectra of ecdysone (2) and 2-deoxyecdysone (1), which is assigned to carbon C-19, is

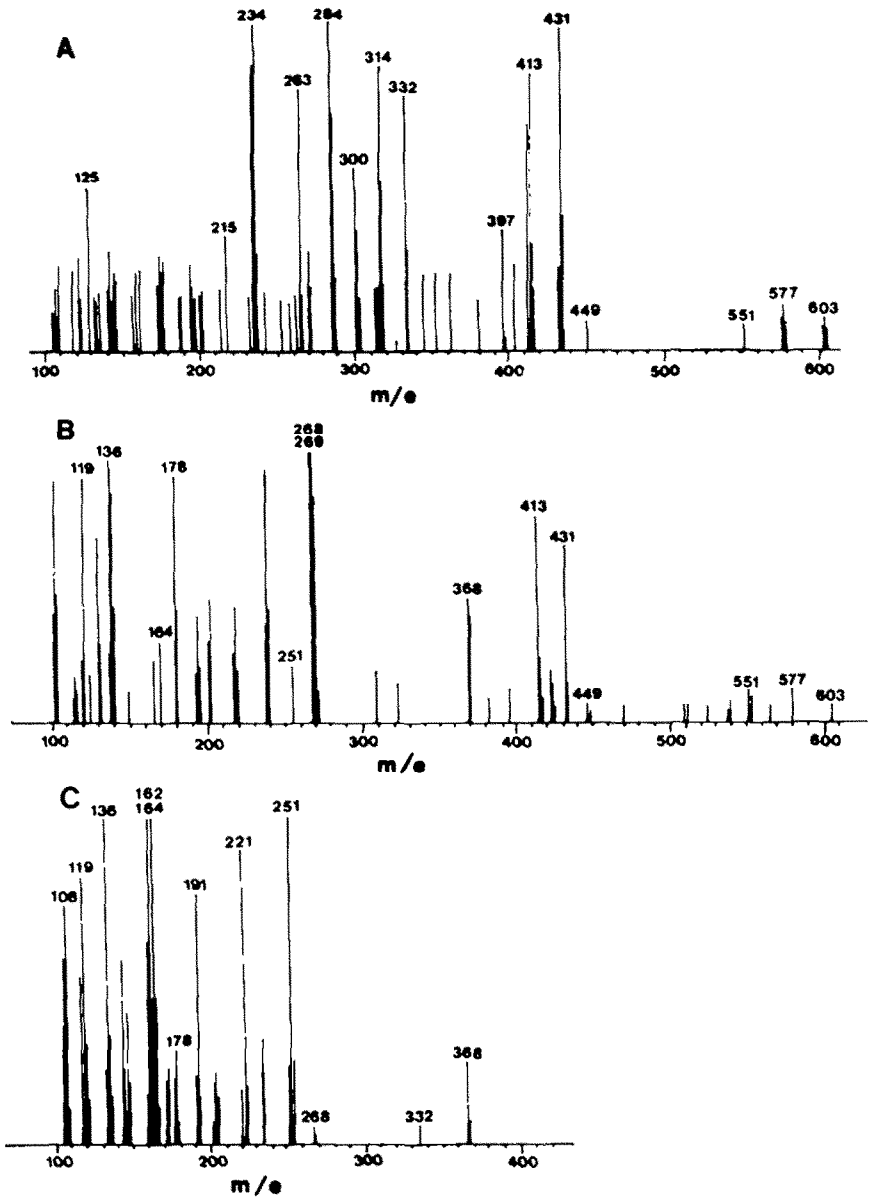


Fig. 1. Direct introduction (gold support) electron impact mass spectra of 2-deoxyecdysone (A) and "C1"-conjugate of 2-deoxyecdysone (B) from newly-laid eggs of *Locusta*. (C) gives mass spectrum of reference adenosine monophosphate in same conditions.

also recorded from **5**, showing that the A/B ring junction is *cis* (in case of a *trans* junction, the signal of C-19 would be in a higher field, close to 13 ppm.)[21] The signal of the carbon atom at C-3, at $\delta = 64$ ppm in 2-deoxyecdysone (**1**), is shifted downfield to $\delta = 69.30$ ppm: a downfield shift of 5 ppm can be explained by a change in the configuration of the hydroxyl group in C-3 between 2-deoxyecdysone (**1**) and **5**. It is well known that the signal of a carbon with an axial hydroxyl group lies at a higher field than that of a corresponding carbon with an equatorial hydroxyl substituent.[21]

Our inference that the change of configuration of a substituent in 5 has occured at C-3 is in agreement with the observation that the singlet signal at $\delta = 36.9$ ppm, attributed to carbon C-10 in the spectrum of 2-deoxyecdysone (**1**), is also present in the spectrum of **5**. It must be expected that in **5** the change of configuration of the hydroxyl from 3β-equatorial affects the signals of the carbons in α and β position of C-3. The empirical data[21] for monosubstituted cyclohexanes allow to calculate the expected chemical shifts for C-1, C-2, C-4 and C-5 in relation to the chemical shifts recorded for the carbons in 2-deoxyecdysone (**1**); these calculations indicate that the change from 3β-axial hydroxyl to 3α-equatorial hydroxyl will induce the following shifts: C-1: + 4 ppm; C-2: + 3 ppm; C-4: + 3 ppm; C-5: + 4 ppm. The predicted shifts are in good agreement with the observed values in Table 2. The same rationale leads to eliminate the possibility that other changes than those at C-3 differentiate 2-deoxyecdysone and **5**. The presence of a 3α equatorial hydroxyl group explains the observation in ^{1}H NMR of a broad signal at $\delta = 4.15$ indicative of an axial proton. As opposed to this observation, in 2-deoxyecdysone ^{1}H NMR spectra, the corresponding signal appears at

δ = 4.12 ppm of peakwidth at half height of 12 Hz. From these data we may conclude that **5** (NI_2) is 3-epi 2-deoxyecdysone.

Identification of the non-hydrolysed conjugate "C3" of 3-epi 2-deoxyecdysone

The following characteristics were recorded for the non-hydrolysed conjugate of 3-epi 2-deoxyecdysone (**6**): UV^{MeOH}nm: 243; IR^{KBr} cm^{-1}: 3350(OH), 1650(CO), 1100–1050; **6** is highly polar. As a consequence of its high polarity, **6** is difficult to analyse by mass spectrometry; the mass spectrum after direct introduction on gold support[18] shows the fragments characteristics of **6**; the intensity of fragment *m/e* 449 is low in the spectrum of **6** as compared to that of **5**; in **5**, *m/e* 430 is the predominant fragment. Microanalysis of **6** shows the presence of phosphorus spectrum of **6**, a signal at δ = 1 ppm is observed relative to phosphoric acid, indicating the presence of a phosphate group. This result is in good agreement with the presence of an absorption band in the IR spectrum at 1050–1100 cm^{-1}. We can conclude from these data that **6** is a phosphate of **5**. The site of attachment of the phosphate moiety to the steroid can be determined through ^{13}C NMR (see Table 2). The attachment of a polar group (phosphate) to **5** is expected to induce a chemical shift of + 5 ppm for the signal of the carbon on which the attachment takes place;[22] if we compare the signals for **5** and **6** in Table 2, it is apparent that only C-3 shows the expected shift; the signal of C-3 is present in the form of a doublet in proton-noise decoupled spectra; the existence of this doublet is explained by the coupling effect due to the presence of $^{31}P(J_{P-O-C} = 6$ Hz). These various data are corroborated by the following observation: attachment of the phosphate at C-3 of **5** can be expected to shield in **6** carbons C-2 and C-4:[17] this is indeed the case, as seen in Table 2.

Finally, as was the case for 22-phospho-2-deoxyecdysone (**4**), in our extracts 3-phospho-3-epi 2-deoxyecdysone (**6**) was present as a salt, the cation of which was identified by GC–MS trifluoacetylation[20] as protonated tris (hydroxymethyl)-aminomethane (Tris).

Free ecdysteroids

From the same extracts, we have isolated several free ecdysteroids. Three are relevant for the present study and will be considered here.

The first of the free ecdysteroids was identified by mass spectrometry and NMR as 2-deoxyecdysone (**1**).

The second of these sterols was identified as 3-epi 2-deoxyecdysone (**5**) by the same methodology as that given above for the genin of the principal conjugate of ageing eggs; the data on which this identification is based will therefore not be repeated here. The ratio of unconjugated 3-epi 2-deoxyecdysone to the phosphate form (**6**) was in the range of 1–10 in the egg pool which we collected.

The third free ecdysteroid in these extracts was identified as 3-dehydro-2-deoxyecdysone (**7**) on the basis of gas liquid chromatography-mass spectrometry after trimethylsilylation (Fig. 2); the mass peak of the trimethyl silylated molecule was at *m/e* 663(M^+) corroborated by peaks at *m/e* 647(M^+ minus one methyl group), 572(M^+ minus one TMS–OH group), 557(M^+ minus one TMS-OH, minus one methyl group), 482(M^+ minus two TMS–OH groups), 372(M^+ minus side chain), 392(M^+ minus three TMS–OH groups), 282(M^+ minus side chain, minus one TMS–OH group). The peaks at *m/e* 171 and 131 are characteristic of the fragmentation of the side chain.[23]

This substance, which was a minor free ecdysteroid in our extracts of ageing eggs, was unstable during purification, and was not further characterized.

DISCUSSION

The results which we have presented in this paper call for a certain number of comments:

(1) The physico-chemical data demonstrated first of all that the major ovarian ecdysteroid recovered from

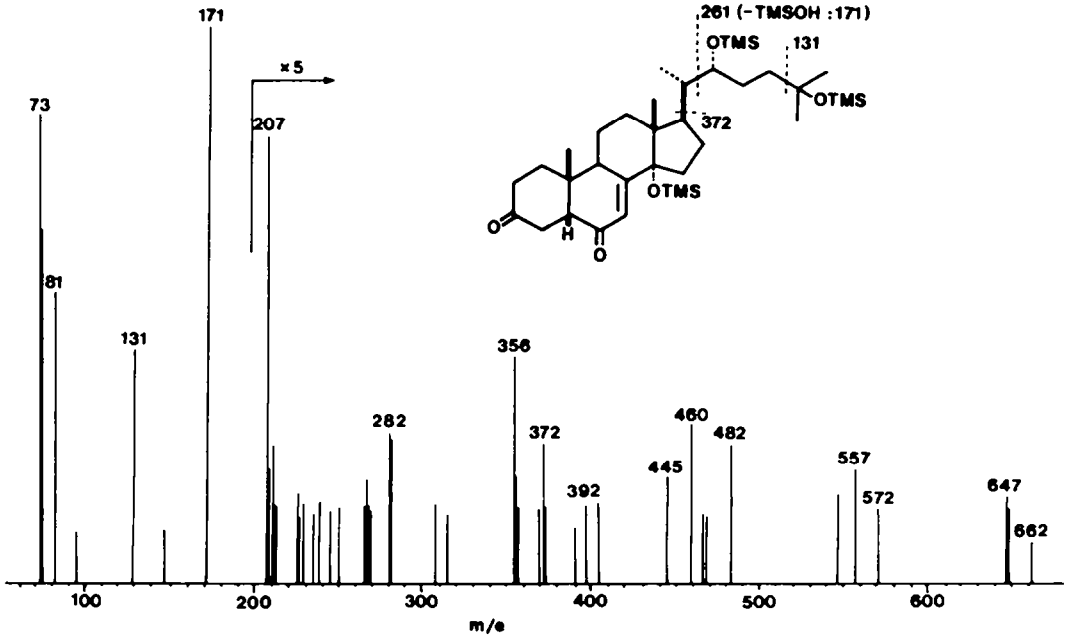


Fig. 2. Mass spectrum of trimethylsilylated **7**. Gas liquid chromatography-electron impact mass spectrometry.

newly-laid eggs is the 22-adenosine-monophosphoric ester of 2-deoxyecdysone. This result was unexpected, inasmuch as covalent binding of a nucleotide to a steroid hormone has hitherto not been reported from a biological system.

(2) Lagueux *et al.*[11] had noted that the concentration of the predominant maternal conjugate decreases dramatically shortly after blastokinesis of the embryo, concomitantly to the increase of the "C2"-deoxyecdysone conjugate. This process occurs at a time of intense degradatation of vitellin to which the conjugated ecdysteroids are reportedly bound.[24] Our results provide an explanation for the above observation: as vitellin is being degraded in the eggs, the maternal conjugate, that is the 22-adenosinemonophosphoric ester of 2-deoxyecdysone, becomes free in the egg and undergoes than either: (a) a partial hydrolysis between C-5′ of ribose and the phosphate or (b) a complete hydrolysis between adenosinemonophosphate and 2-deoxyecdysone, which is later followed by phosphorylation of the C-22 hydroxyl group of 2-deoxyecdysone; although we cannot yet decide between these two alternatives, they both have same end result in the context of this study: a replacement of the large amounts of 22-adenosinemonophosphoric ester by high quantities of 22-phospho-2-deoxyecdysone in the eggs.

(3) In eggs containing postblastokinetic embryos, three free 2-deoxyecdysteroids are observed which were identified in this study as 2-deoxyecdysone, 3-dehydro and 3-epi 2-deoxyecdysone. This observation leaves no doubt that 2-deoxyecdysone resulting from the hydrolysis of the maternal conjugate (either after one single or two hydrolytic steps) is metabolized first by oxidation to 3-dehydro-2-deoxyecdysone, which is then reduced to the 3α-epimer of 2-deoxyecdysone. The latter substance, as shown by our results, accumulates under the form a 3-monophosphoric ester in the eggs.

Epimerisation of the hydroxyl group at C-3 ecdysteroids has been reported in various insect systems as an inactivation procedure. It has been postulated that 3-epimerisation proceeded via 3-dehydro compounds; this appears to be actually the case in *locusta* eggs as illustrated by the simultaneous presence of 3-dehydro and 3-epi compounds.

(4) The sequence of events leading from the maternal 22-adenosine monophosphoric ester of 2-deoxyecdysone to the 3-phosphoric ester of 3-epi-2-deoxyecdysone is the major ecdysteroid pathway, in quantitative terms in egg of *locusta*; this observation means that, at least in this biological model, 2-deoxyecdysone cannot merely be considered as a precursor molecule for ecdysone. Our observations do not of course eliminate the possibility that some 2-deoxyecdysone of maternal origin is converted to ecdysone during embryonic development, but this is obviously not the fate for the majority of these molecules.

(5) Finally, the picture which has evolved in this study of the origin and fate of deoxyecdysteroids in ovaries and eggs of the migratory locust *Locusta migratoria*, is probably valid for several other insect species as well. Indeed, Ohnishi *et al.* have reported[4] that ovaries and eggs of *Bombyx mori* contain large amounts of conjugated 2-deoxyecdysone, an observation also made in *Schistocerca gregaria* ovaries and eggs.[10] In the latter species, Isaac *et al.*[25] have recently reported the presence in newly-laid eggs of 22-phosphate of 2-deoxyecdysone. Whether the absence of adenosine in this substance in newly-laid eggs reflects a species difference between *Schistocerca* and *Locusta* or a difference in methodology, is not known at present. The same group has isolated 3-epi-2-deoxyecdysone from ageing eggs of *Schistocerca*[26] where they find this ecdysteroid to be present in the free and conjugated forms. This result is consistent with our observations in *Locusta*, where 3-epi-2-deoxyecdysone represents, in the form of a 3-phosphoric ester, the major ecdysteroid metabolite at the end of embryonic development.

The present paper has focused its attention on the 2-deoxyecdysone conjugate of maternal origin and its fate during embryonic development in *Locusta*; although maternal 2-deoxyecdysone conjugates are predominant among ovarian ecdysteroids in this species, we also find large amounts of conjugates of ecdysone, the structure and metabolic destiny of which will be reported in a next paper. The hormonal relationship between the female and its offspring which is demonstrated by these sudies on insects was undocumented so far in oviparous animals; this biological model appears as extremely challenging for future research in the fields of chemistry, developmental biology and endocrinology.

EXPERIMENTAL

Locusta migratoria migratorioides were reared in *gregaria* at a day temp. of 28–30°C, falling to 25° at night. Electric light bulbs inside the cages allowed temperature gradients up to 38°. Day conditions lasted from 7 a.m. to 7 p.m. Eggs were collected immediately after egg-laying and kept in an incubator at 33°.

All solvents and reagents were of analytical grade and were used without further purification.

Ecdysone was purchased from SIMES, Milano. 2-Deoxyecdysone was a gift of Dr D. H. S. Horn, CSIRO, Melbourne. Adenosine 5′-phosphate (disodium salt) was purchased from SIGMA.

For TLC, precoated silicagel HF 254 plates, 0.25 mm thick were purchased from Merck. For HPLC analysis, a Waters Associates M 6000 A pump was used and ecdysteroids were monitored by UV absortion at 254 nm with a Waters Associates M 440 spectrophotometer; separations were performed on reversed phase HPLC prepacked columns (LiChroprep RP-18, 10 μm column, 250 × 4 mm Meck; flow rate: 1 ml/min).

Radioimmunoassay (RIA) of ecdysteroids was performed after de Reggi *et al.*[27] RIA was performed either directly on aliquots of the eluted fractions or after enzymatic hydrolysis by *Helix pomatia* enzyme preparation (Industrie Biologique Francaise, Clichy, France) in 50 mM acetate buffer, pH 5.3, at 37° for 18 hr.

IR spectra were recorded on a Perkin–Elmer 171 apparatus and UV spectra on a Beckman 31 spectrophotometer.

NMR spectra were recorded in the following conditions: (a) ^{1}H NMR: 250 MHz; internal standards: tetramethylsilane or methanol; Cameca instruments; (b) ^{13}C NMR: 62.8 MHz; internal standards: tetramethylsilane or methanol; Cameca instruments; (c) ^{31}P NMR: 36.43 MHz; external standard: phosphoric acid; Bruker instrument. Gated decoupled spectra were run with a pulsing time of 5 μsec and a recovery time of 1 sec.

For MS analysis two experimental approaches were used: (a) the samples were directly introduced into the ionisation chamber of a double focussing Thomson THN 208 B (Mattauch–Herzog geometry) apparatus on gold support;[18] ionisation energy was 70 eV and source temperature was between 200 and 260°; (b) the samples were subjected to trifluoracetylation, followed by gas chromatography (2m × 2mm glass column filled with 1% OV 101) and electron impact mass spectrometry either on a Finnigan 4000 GC/MS instrument equipped with INCOS data system or on a LKB 9000 S mass spectrometer coupled to a gas chromatograph (Varian 1200; 3m × 2mm glass column filled with 1% OV 101); ionisation energy was 70 eV and source temperatures were between 200 and 260°C.

The microanalysis was performed by Service Central des Microanalyses du CNRS, Lyon, France.

Extraction of ecdysteroids

Eggs were lyophilyzed and pooled until sufficient mass was available. This pool was extracted with various solvents: hexane, chloroform, methanol and aqueous methanol (60%). The hexane and chloroform phases were discarded as they contained little or no detectable ecdysteroid immunoreactivity: the methanol phases were pooled and subjected to repeated reversed phase C8 liquid chromatography (LiChroprep RP-8, 40–63 μm, prepacked column, 410 × 25 mm, Merck; elution gradient from 20 mM Tris-HCl buffer, pH7.5, to acetonitrile-Tris biffer, (1:1), followed by rechromatography in 30% aqueous methanol on the same RP-8 column.

In our initial extraction procedures, the pooled methanol phases were subjected to silicagel column chromatography (column of 50 cm × 4 cm, filled with 70–230 mesh Kieselgel 60, Merck; elution gradient from pure methanol to water); this step was later abandoned as the elution of the polar conjugates from the silicagel column was not found to be satisfactory.

Starting from the recent report of Lagueux *et al.*[24] that the maternal conjugates are bound to vitellin in newly-laid eggs, we have extracted this major protein by homogenization of newly-laid eggs in ice-cold 20 mM Tris-HCl buffer, pH7.4, containing 0.4 M NaCl; the debris were removed by low speed centrifugation and vitellin present in the supernatant was precipitated by addition of ten volumes of ice-cold distilled water to precipitate egg yolk proteins.[28] After low-speed centrifugation, the protein precipitate, consisting predominantly of vitellin, was subjected to ecdysteroid extraction by 50% aqueous methanol; this aqueous methanol extract was dried under vacuum and partitioned between hexane and 50% aqueous methanol. The methanol phase of this partition was subjected to repeated reversed phase C8 column chromatography (LiChroprep RP-8, 40–63 μm, prepacked column, 410 × 25 mm, Merck; elution gradient from water to pure methanol).

During all extraction and purification procedures, the presence of ecdysteroids was monitored by UV absorption at 245 nm and by ecdysteroid radioimmunoassay of aliquots of the eluted fractions subjected to enzymatic hydrolysis; ecdysteroid-containing fractions were dried under vacuum and repeatedly rechromatographed in the same conditions.

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