Formation of the A/B *cis* ring junction of ecdysteroids in the locust, *Schistocerca gregaria*

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1. The mechanism of formation of the A/B *cis* ring junction of ecdysteroids in the locust *Schistocerca gregaria*, was investigated by incorporation of [4-¹⁴C,3-³H], [4-¹⁴C,4-³H] and [4-¹⁴C,4β-³H]cholesterol into 20-hydroxyecdysone in fifth-instar larvae and into ecdysteroid conjugates in ovaries of maturing adult females. 2. In both systems there was retention of the 4-³H atom in the ecdysteroid and elimination of the 3-³- and 4β-³H atoms. 3. The ³H retained in the ecdysone formed from [4-³H]cholesterol in the ovarian system was probably located at C-4. The results are interpreted by postulating the involvement of a 3-oxo-Δ⁴ intermediate in ecdysteroid biosynthesis in insects.

The major moulting hormone (ecdysteroid) in most larval insect species investigated is 20-hydroxyecdysone (I) which is usually accompanied by smaller amounts of ecdysone (II; Thompson *et al.*, 1973; Gilbert *et al.*, 1977). During larval stages the prothoracic glands are a major (but not the exclusive) site of ecdysteroid synthesis (Gilbert *et al.*, 1977; Delbecq *et al.*, 1978 and references cited therein).

It has become apparent recently that ecdysteroids occur not only in the larval and pupal stages of insect development but also in maturing ovaries in females and in embryos (Hoffmann *et al.*, 1980). It has been shown in maturing adult females of the locust, *Locusta migratoria*, that ecdysteroids [primarily ecdysone (II) and 2-deoxyecdysone (III)] are synthesized in the ovarian follicle cells and passed into the oöplasm of the oocytes (Goltzené *et al.*, 1978). In another locust species, *Schistocerca gregaria*, which has been used in this work, the ecdysteroids in the newly laid eggs have been identified as ecdysone (II), with traces of 20-hydroxyecdysone (I; Gande & Morgan, 1979) and intermediate amounts of 3-deoxyecdysone (III; Dinan & Rees, 1980), all of which are present primarily as polar conjugates.

The incorporation of radioactive cholesterol into ecdysteroids has been demonstrated in several insect species. A characteristic feature of the ecdysteroids is the presence of an A/B *cis* ring junction (i.e. 5β-H), which is essential for biological activity; the corresponding 5-^α-compounds are practically hor-

monally inactive (Galbraith *et al.*, 1973). Formation of the A/B *cis* ring junction apparently occurs fairly early in the transformation of cholesterol into ecdysteroids. For example, 3β,14-^α-dihydroxy-5β-[3-³H]cholest-7-en-6-one is metabolized efficiently into ecdysteroid both *in vivo* in *Calliophora stygia* (Galbraith *et al.*, 1975) and *in vitro* in organ cultures of prothoracic glands of *Manduca sexta* (Bollenbacher *et al.*, 1977). Furthermore, 3β-hydroxy-5β-cholest-7-en-6-one has been isolated from ovaries of *Locusta migratoria* (Hetru *et al.*, 1978).

There are numerous plausible mechanisms that could account for the formation of the A/B *cis* ring junction of ecdysteroids (Rees, 1971). It has been assumed frequently that a 5β-ecdysteroid precursor is formed from the corresponding 5-^α-compound by enolization of a 6-oxo group. However, it would be difficult to explain adequately on this basis why numerous 6-oxo compounds with a 5-^α-H were hormonally inactive, whereas the corresponding ones with a 5β-H were active (Galbraith *et al.*, 1973). The analogous transformation of a Δ⁵ bond to give a 5β-H during steroid-hormone and bile-acid formation in animals involves the intermediacy of a 3-oxo-Δ⁴ steroid, the 5β-H being introduced during reduction of the Δ⁴ bond (Samuels & Eik-Nes, 1968). In the case of the formation of both bile acids and steroid hormones, introduction of an A/B *cis* ring junction follows at least some other change in the cholesterol molecule, e.g. introduction of a 7-^α-hydroxy group for bile acids.

To investigate the possible role of a Δ⁴ compound in ecdysteroid biosynthesis, [4-³H₁]- and [4β-³H₁]-

Abbreviation used: h.p.l.c., high-performance liquid chromatography.

cholesterol species were synthesized (Lockley *et al.*, 1978). Incorporation of these cholesterol species into ecdysteroids would demonstrate whether either of the two C-4 hydrogen atoms of cholesterol is removed, irrespective of the stage at which it occurs. Similarly, examination of the fate of the ^3H in [$3\alpha\text{-}^3\text{H}_1$]cholesterol should indicate whether oxidation at C-3 occurs during formation of ecdysteroids. We now report results on the incorporation of these three species of [^3H]cholesterol, in admixture with [$4\text{-}^{14}\text{C}$]cholesterol, into ecdysteroids in the locust, *Schistocerca gregaria*. Initially, the incorporations were carried out into 20-hydroxyecdysone (I) in fifth-instar larvae. Since this system did not provide clear-cut evidence for the fate of the $3\alpha\text{-}^3\text{H}$ atom of cholesterol, the incorporations were also carried out in maturing ovaries of adult females. The complicating factors that arise in the fifth-instar larvae are not apparent in the latter system.

Experimental

The sources of chemicals and the radiochemical and t.l.c. methods were generally as described previously (Davies *et al.*, 1980). For analytical t.l.c. 0.25 mm-thick layers of silica gel were used.

Location of the label in the [$4\alpha\text{-}^3\text{H}$]- and [$4\beta\text{-}^3\text{H}$]cholesterol substrates used in this work (Lockley *et al.*, 1978) indicated that 96 and 85%, respectively, of the ^3H was in the expected positions. However, the latter value is probably an underestimate. Similarly, location of the label in the [$3\alpha\text{-}^3\text{H}$]cholesterol by Moffatt oxidation (Pfützner & Moffatt, 1965) revealed that 94% of the ^3H was at C-3.

Gas-liquid chromatography

A Pye 104 gas chromatograph was used, fitted with a flame ionization detector and a $2.1\text{ m} \times 6.5\text{ mm}$ diameter glass column packed with 3% OV-17 on GasChrom Q (100–200 mesh). For collection of effluent material a metal splitter was inserted between the end of the column and the detector, so that approx. 1 part in 10 passed to the detector, whereas the remainder was collected in glass capillary tubes at ambient temperature (20°C).

High-performance liquid chromatography

H.p.l.c. was carried out by using a Waters modified ALC-100 instrument fitted with a variable wavelength u.v. detector (Perkin–Elmer, model LC55). In this work the column ($25\text{ cm} \times 4.6\text{ mm}$ int. diameter packed with Hyperseil APS, $5\text{ }\mu\text{m}$; Shandon Southern Products, Runcorn, Cheshire, U.K.) was developed with dichloroethane/propan-2-ol/methanol (95:4:1, by vol.) at a flow rate of 4 ml/min. For radioassay of collected compounds,

the solvent was evaporated under N_2 and the samples were subjected to liquid-scintillation counting.

Insects

Schistocerca gregaria were reared on a diet of wheat seedlings and rolled oats and were kept under a 12 h 'day' period when the temperature was maintained at 35°C and a corresponding 12 h 'night' period at 28°C . Eggs were laid by mature female locusts into aluminium tubes containing a moist mixture of peat and sifted washed sand. Tubes containing eggs were incubated at 30°C for approx. 17 days after which time the young locusts hatched. Fifth-instar larvae were used in some experiments, the instar lasting for 8 days under the rearing conditions employed. In other experiments, locusts were synchronized at the adult moult and the maturing adult females were used.

Administration of substrates and extraction procedures

Each of the respective [^{14}C , ^3H]cholesterol substrates was purified by t.l.c. on silica gel and a portion was diluted with carrier cholesterol and recrystallized from chloroform/methanol to constant specific radioactivity. The remaining undiluted [^{14}C , ^3H]cholesterol was then emulsified by sonication in insect Ringer solution (Bodenstein, 1946; $60\text{ }\mu\text{l}/\mu\text{Ci}$ of ^{14}C) containing 3% (w/v) Tween 80. All three stereospecifically labelled [^{14}C , ^3H]cholesterol substrates were administered in turn to both fifth-instar and maturing adult female *S. gregaria*.

(a) *Fifth-instar S. gregaria*. The radioactive cholesterol substrates were administered to larvae of *S. gregaria* 4½ days into the fifth instar, a stage that precedes the increase in ecdysone titre. In a typical experiment, 12 larvae were each injected with $30\text{ }\mu\text{l}$ of the sterol emulsion into the ventral side of the abdomen between posterior sterna and were then kept for 24 h before being killed by freezing.

The extraction of the insects administered [$4\text{-}^{14}\text{C}$, $4\beta\text{-}^3\text{H}$]cholesterol ($6.5\text{ }\mu\text{Ci}$ of ^{14}C) is given as a typical procedure. Insects were macerated in methanol/water (7:3, v/v; 100 ml), the homogenate was centrifuged ($1000g$ for 10 min) and the residue was re-extracted successively with methanol ($3 \times 100\text{ ml}$) and chloroform (100 ml). The combined solvent extracts were evaporated to dryness under vacuum and the extract was partitioned between water (100 ml) and butan-1-ol (100 ml). The aqueous phase was re-extracted with butan-1-ol (100 ml) and then discarded. The combined butanol extracts were washed once with water (100 ml) and evaporated to dryness under vacuum. The residue was partitioned between methanol/water (7:3, v/v; 100 ml) and hexane (100 ml) and both phases were back-extracted with the other phase. Sterol and

20-hydroxyecdysone were purified from the combined hexane and the combined aqueous methanol fractions, respectively.

The hexane fraction was subjected to t.l.c. on silica gel developed in chloroform to give a sterol fraction, which was purified further by t.l.c. on AgNO_3 -impregnated silica gel developed with chloroform/ethanol (97:3, v/v). The material co-chromatographing with cholesterol (3.93×10^6 d.p.m. of ^{14}C) was diluted with non-radioactive carrier cholesterol and recrystallized from chloroform/methanol to constant specific radioactivity (Table 1).

Carrier 20-hydroxyecdysone (I) was added to the methanol/water (7:3, v/v) fraction, which was then fractionated by t.l.c. on silica gel GF₂₅₄ developed twice with chloroform/methanol (4:1, v/v). The 20-hydroxyecdysone band (9.7×10^3 d.p.m. of ^{14}C) was eluted thoroughly with chloroform/methanol (1:1, v/v) and was then purified further by formation of the 2-acetate (IV) and the 20,22-acetonide, 2-acetate (VII) derivatives, which were crystallized from methanol/water and chloroform/diethyl ether, respectively (Table 1).

Transformation products of 20-hydroxyecdysone. Acetates and acetonides of ecdysteroids were prepared by the methods of Galbraith & Horn (1969); see also Davies *et al.* (1980). The 20-hydroxyecdysone (I) was transformed successively into the 2-acetate (IV) and the 20,22-acetonide, 2-acetate (VII) derivatives. In one experiment, the latter derivative was transformed into the 3-oxo derivative (VIII; Table 2) by using (i) dimethyl sulphoxide/acetic anhydride oxidation (non-equilibrating conditions) and (ii) MnO_2 oxidation for 18 h (equilibrating conditions) (Davies *et al.*, 1980).

(b) *Maturing adult female S. gregaria.* The

stereospecific [^{14}C , ^3H]cholesterols were injected into the abdomen of maturing female locusts (30 μl /insect) between the 8th and 9th sterna 10–12 days after the adult moult, which is a period well before the large increase in ecdysteroid production. The injected insects were mixed with an equal number of mature adult male insects and kept under normal conditions. Egg pods were collected within 12 h of being laid and were frozen (-20°C) until extracted. In one experiment (with [4- ^{14}C , 4 α - ^3H]cholesterol) the mature ovaries were dissected from the insects rather than allowing eggs to be laid.

Extraction of the eggs from the [4- ^{14}C , 4 β - ^3H]cholesterol incorporation experiment is given as a typical procedure. The eggs (or ovaries) were cleaned, weighed (4.83 g), and extracted by maceration successively with methanol (25 ml), 70% (v/v) aqueous methanol (2×25 ml), methanol (25 ml) and dichloromethane/methanol (1:1, v/v; 2×25 ml). The first four extracts (methanol and aqueous methanol) were combined and evaporated to dryness at room temperature. The residue was partitioned between 25 ml each of hexane and 70% (v/v) aqueous methanol, and the hexane phase was combined with the original dichloromethane/methanol extracts. Thus, apolar (0.9 μCi of ^{14}C) and polar (0.12 μCi of ^{14}C) fractions were obtained, from which sterols and ecdysteroids respectively were isolated.

Separation of sterols. The apolar fraction was diluted with a small amount (approx. 50–100 μg) of unlabelled carrier 7-dehydrocholesterol, subjected to t.l.c. on silica gel with chloroform for development and the free sterol band (0.76 μCi of ^{14}C) was eluted. The free sterols were further fractionated by t.l.c. on AgNO_3 /silica gel developed with chloroform/ethanol (97:3, v/v), and the bands corresponding to

cholesterol ($0.71\mu\text{Ci}$ of ^{14}C) and 7-dehydrocholesterol ($0.04\mu\text{Ci}$ of ^{14}C) were eluted. Both bands were subjected to g.l.c. on OV-17 with collection of fractions every minute for radioassay. The radioactivity in the 7-dehydrocholesterol ($\Delta^{5,7}$) band co-chromatographed with the authentic material and the label in the band corresponding to cholesterol was eluted in the cholesterol peak, with an insignificant amount being associated with the 5α -cholest-7-en- 3β -ol peak. Any possible 5α -cholestan- 3β -ol (which co-chromatographs with cholesterol on OV-17) would have been removed during the $\text{AgNO}_3/\text{silica}$ gel t.l.c. step. Therefore, the cholesterol band from the $\text{AgNO}_3/\text{silica}$ gel t.l.c. was diluted with unlabelled cholesterol (40mg) and recrystallized from methanol/chloroform to constant specific radioactivity.

Separation of ecdysteroids. The majority of the radioactive ecdysteroid in the 'polar' fraction is in the form of 'polar conjugates' with a very small percentage being present in the free form (Dinan & Rees, 1980). The free and conjugated ecdysteroids were then separated by chromatography on a silicic acid column as follows. The 'polar' fraction was dissolved in methanol to which Celite (approx. 0.2g, previously washed with methanol and chloroform; Koch-Light) had been added and the methanol was evaporated under N_2 . The Celite was then applied to a 2g column of silicic acid (Kieselgel 60, 70–230 mesh; Merck, Darmstadt, Germany) which was developed successively with the following solvents (20ml each): chloroform, chloroform/methanol (7:3, v/v), chloroform/methanol (1:4, v/v), and methanol. Free and conjugated ecdysteroids were eluted in the second and third fractions, respectively.

The ecdysteroid conjugate fraction was dissolved in 2ml of 0.1M-triethanolamine/NaOH buffer, pH 7.0, and treated with 2ml of a hydrolysing enzyme solution in the same buffer [2mg of aryl sulphatase (30 units/mg; contaminated with β -glucuronidase, 300 units/mg) from *Helix pomatia* and 2mg of acid phosphatase (2 units/mg); both enzymes from Sigma (London) Chemical Co.] for 24h at 37°C . The reaction was stopped by the addition of ethanol (10ml), approx. $100\mu\text{g}$ of unlabelled ecdysone was added and the precipitated protein was extracted with methanol ($3 \times 10\text{ml}$). The extract ($0.112\mu\text{Ci}$ of ^{14}C) was evaporated to dryness, reabsorbed onto Celite and chromatographed as before on a column (2g) of silicic acid. The majority of the radioactivity was eluted in the chloroform/methanol (7:3, v/v) (free ecdysteroid) fraction (108600 d.p.m. of ^{14}C). Analysis of a portion of this fraction by t.l.c. [Kieselgel GF₂₅₄ developed with chloroform/methanol (4:1, v/v)] and scanning for radioactivity showed the presence of two major peaks, accounting for 75–90% of the total radioactivity, the larger peak co-chromato-

graphing with ecdysone (II) and the smaller peak corresponding to 2-deoxyeecdysone (III). Sometimes a small peak corresponding to 20-hydroxyecdysone (I) was observed with extracts of newly-laid eggs.

The chloroform/methanol (7:3, v/v) fraction was dissolved in chloroform/methanol (19:1, v/v) and chromatographed on a column (2g) of silicic acid for separation of the ecdysone (II) and 2-deoxyeecdysone (III) derived from the conjugates. The ecdysteroids were eluted with the following solvents and fractions (5ml) were collected: 20ml of chloroform/methanol (19:1, v/v) (fractions 1–4); 30ml of chloroform/methanol (23:2, v/v) (fractions 5–10); 20ml of chloroform/methanol (17:3, v/v) (fractions 11–14). Ecdysone (50680 d.p.m. of ^{14}C) and 2-deoxyeecdysone (27500 d.p.m. of ^{14}C) were eluted in fractions 11–13 and 7–9, respectively, and both compounds were purified further by t.l.c. and by h.p.l.c. on a Hypersil APS column. In an initial experiment, the radioactive ecdysone was characterized further by co-chromatography on a reversed-phase h.p.l.c. column with authentic material and by transformation into the 2,3-acetonide derivative (VI) followed by t.l.c.

Location of ^3H in ecdysone derived from [^{14}C , $4\alpha\text{-}^3\text{H}$]cholesterol

The ^3H retained in the ecdysone formed from the [^{14}C , $4\alpha\text{-}^3\text{H}$]cholesterol was located by the following transformations. The reactions were first carried out on non-radioactive material and the products were characterized by physical methods.

5α -Epimer of ecdysone. A portion (3800 d.p.m. of ^{14}C) of the radioactive ecdysone (II) was diluted with unlabelled ecdysone (10mg) and dissolved in 20ml of 0.6% K_2CO_3 in 90% (v/v) aqueous methanol and the mixture was left for 30min before refluxing it for a further 30min (Mori *et al.*, 1968). After cooling, the reaction mixture was partitioned between butanol (50ml) and water (50ml), the aqueous phase was re-extracted with butanol (50ml) and the combined butanol extracts were washed with water (50ml). The butanol was evaporated to dryness and the C-5 epimers of ecdysone were separated by t.l.c. with a continuous-elution solvent system [chloroform/ethanol (6:1, v/v) developed for 2h]. Two major u.v.-absorbing bands were obtained, the lower and upper ones corresponding to ecdysone (750 d.p.m. of ^{14}C) and the 5α -epimer of ecdysone (362 d.p.m. of ^{14}C) respectively. The 5α -epimer had the following characteristics: m/e 446.3028 ($M^+ - \text{H}_2\text{O}$, 2%, requires m/e 446.3032), 431.2796 ($M^+ - \text{H}_2\text{O} - \text{CH}_3$, 2%, requires m/e 431.2797), 428.2921 ($M^+ - 2\text{H}_2\text{O}$, 9%, requires m/e 428.2926), 413.2690 ($M^+ - 2\text{H}_2\text{O} - \text{CH}_3$, 6%, requires m/e 413.2691), 410.2809 ($M^+ - 3\text{H}_2\text{O}$, 4%, requires m/e 410.2820), 395.2579 ($M^+ - 3\text{H}_2\text{O} - \text{CH}_3$, 3%, requires m/e 395.2586),

348.2306 ($M^+ - [C_{22} - C_{27}]$, 8%, requires m/e 348.2301), 330.2183 ($348 - H_2O$, 18%, requires m/e 330.2195), 315.1970 ($330 - CH_3$, 10%, requires m/e 315.1960), 300.1722 ($M^+ - \text{side chain} - H_2O$, 26%, requires m/e 300.1725), 285.1494 ($300 - CH_3$, 9%, requires m/e 285.1490), 99.0810 ($C_6H_{11}O$, 100%, requires m/e 99.0810); n.m.r. δ (p.p.m.) ($C_5^2H_5N$, 220MHz) 0.64 (3H, s, C-18 methyl), 1.42 (3H, s, C-19 methyl), 1.31 (3H, d, J 6.4 Hz, C-21 methyl), 1.42 (6H, s, C-26, C-27). The downfield shift of 0.34 p.p.m. in the C-19 methyl signal compared with ecdysone ($\delta = 1.08$ for the C-19 methyl) lends credence to the 5α -configuration of the compound (Furlenmeier *et al.*, 1966).

Ecdysone 2,22-diacetate (V). Another portion (8750 d.p.m. of ^{14}C) of the labelled ecdysone (II) was diluted with carrier ecdysone (4 mg) and acetylated in pyridine (300 μ l) and acetic anhydride (150 μ l) for 1 h at room temperature. The reaction was stopped by addition of excess ice and the reaction mixture was left for a further 30 min before azeotroping the solvent to dryness. The products were separated by t.l.c. on Kieselgel GF₂₅₄ with chloroform/ethanol (9:1, v/v) for development and the ecdysone 2,22-diacetate (V; R_F 0.22) as well as ecdysone (R_F 0.04) and ecdysone-2-acetate (R_F 0.16) were eluted. This acetylation procedure was repeated on the mixed ecdysone and its 2-acetate derivative to maximize the yield of the 2,22-diacetate (final yield 43%, 3730 d.p.m. of ^{14}C): u.v. $\lambda_{\max}$ (ethanol) 243 nm; m/e 530 ($M^+ - H_2O$, 4%), 512 ($M^+ - 2H_2O$, 9%), 494 ($M^+ - 3H_2O$, 16%), 470 ($M^+ - \text{acetate} - H_2O$, 13%), 452 ($470 - H_2O$, 25%), 410 ($452 - \text{acetate}$, 11%), 342 ($M^+ - \text{side chain}$, 75%), 300 ($342 - \text{acetate}$, 25%), 99 ($C_6H_{11}O$, 61%) and 81 ($99 - H_2O$, 100%; n.m.r. δ (p.p.m.) $C_5^2H_5N$, 220MHz) 0.79 (3H, s, C-18 methyl), 1.12 (3H, s, C-19 methyl), 1.18 (3H, d, J 7 Hz, C-21 methyl), 1.41 (6H, s, C-26 and C-27 methyls), 2.00 (3H, s, acetate methyl), 2.12 (3H, s, acetate methyl). The two signals for acetate methyls indicate the presence of two acetate groups and the upfield shift of 0.12 p.p.m. in the position of the C-21 doublet as compared with ecdysone is consistent with the location of one of these acetate groups at C-22.

3-Oxoecdysone 2,22-diacetate (IX). The labelled ecdysone 2,22-diacetate (V; 3730 d.p.m. of ^{14}C , approx. 2 mg) was subjected to dimethyl sulphoxide oxidation by dissolving in dimethyl sulphoxide (300 μ l) to which was added acetic anhydride (100 μ l) and the mixture was left at room temperature for 16 h (Albright & Goldman, 1967). The mixture was poured into diethyl ether (50 ml) which was then washed well with water (5×50 ml) and the ether was evaporated to dryness under reduced pressure. Separation of the products by t.l.c. on Kieselgel GF₂₅₄ plates developed with chloroform/ethanol (14:1, v/v) yielded 3-oxoecdysone 2,22-

diacetate [IX; R_F 0.40, cf. R_F ecdysone 2,22-diacetate (V) 0.11] as the major product (1370 d.p.m. of ^{14}C , 40% yield): u.v. $\lambda_{\max}$ (ethanol) 243 nm; i.r. $\nu_{\max}$ (NaCl disc) 1670 (α, β -unsaturated C=O) and 1740 (C=O) cm^{-1} ; m/e 546 (M^+ , <1%), 528 ($M^+ - H_2O$, <1%), 510 ($M^+ - 2H_2O$, <1%), 468 ($M^+ - 2H_2O - \text{acetate}$, 2%), 450 ($468 - H_2O$, 6%), 340 ($M^+ - \text{side chain} - H_2O$, 9%), 282 (42%), 109 (56%), 99 ($C_6H_{11}O$, 24%), 81 (C_6H_9 , 71%), 69 (100%).

Equilibration of 3-oxoecdysone 2,22-diacetate (IX). The labelled 3-oxoecdysone 2,22-diacetate (IX; approx. 1 mg, 1230 d.p.m. of ^{14}C) was dissolved in acetonitrile (1 ml, h.p.l.c. grade), activated MnO_2 (10 mg, Attenburrow *et al.*, 1952) was added and the reaction mixture was stirred at room temperature.

The MnO_2 was changed seven times over a period of 4 days by centrifugation of the reaction mixture and washing the pellet of MnO_2 with acetonitrile. The combined supernatants were evaporated to dryness and the treatment with MnO_2 was repeated. After eight treatments with MnO_2 , the 3-oxoecdysone 2,22-diacetate (IX) was separated on t.l.c. with chloroform/ethanol (95:5, v/v) for development (yield 3%).

Results

Fifth-instar *S. gregaria*

[4- ^{14}C , 3 α - 3H]-, [4- ^{14}C , 4 α - 3H]-, and [4- ^{14}C , 4 β - 3H] cholesterol species were administered, in turn, to fifth-instar locusts, *Schistocerca gregaria*, and the radioactive 20-hydroxyecdysone (I) formed was purified by transformation into 20,22-acetonide-20-hydroxyecdysone 2-acetate (VII). In the case of each substrate, the $^3H/^{14}C$ atomic ratio of the recovered cholesterol was similar to that of the administered cholesterol (Table 1). The $^3H/^{14}C$ atomic ratios (Table 1) for the 20,22-acetonide-20-hydroxyecdysone 2-acetate (VII) samples derived from [4- ^{14}C , 4 α - 3H]- and [4- ^{14}C , 4 β - 3H] cholesterol indicate that the 4 α - and 4 β -hydrogen atoms of cholesterol are retained and eliminated, respectively, during ecdysteroid formation. The 20,22-acetonide-20-hydroxyecdysone 2-acetate (VII) derived from [4- ^{14}C , 3 α - 3H] cholesterol shows extensive loss of 3H ($^3H/^{14}C$ atomic ratio 0.22:1). Similar results for the incorporation of all three cholesterol substrates into 20-hydroxyecdysone (I) were obtained in a previous duplicate experiment. In that case, the small amount of 3H remaining in the 20,22-acetonide-20-hydroxyecdysone 2-acetate (VII) derived from [4- ^{14}C , 3 α - 3H] cholesterol was located by oxidation to the corresponding 3-oxo compound (VIII) by using the dimethyl sulphoxide/acetic anhydride and MnO_2 (18 h only) methods (Table 2). Both samples of 3-oxo compound had $^3H/^{14}C$ ratios

Table 1. $^3\text{H}/^{14}\text{C}$ ratios of cholesterol and derivatives of the biosynthesised ecdysteroids isolated from fifth-instar *Schistocerca gregaria* after administration of stereospecifically-labelled [^{14}C , ^3H] cholesterol substrates

For further details see the text.

Substance	Amount of ^{14}C (μCi)	Substrate ...	[4- ^{14}C , 3 α - ^3H]Cholesterol					[4- ^{14}C , 4 α - ^3H]Cholesterol					[4- ^{14}C , 4 β - ^3H]Cholesterol				
			5.6					6.8					6.5				
Administered cholesterol		Recrystallization	Specific radioactivity of ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radioactivity ratio		$^3\text{H}/^{14}\text{C}$ atomic ratio*		Specific radioactivity of ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radioactivity ratio		$^3\text{H}/^{14}\text{C}$ atomic ratio*		Specific radioactivity of ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radioactivity ratio		$^3\text{H}/^{14}\text{C}$ atomic ratio*	
Administered cholesterol		1	150937	1.01				80321	1.51				60208	1.17			
		2	156814	1.24				120891	1.52				60560	1.18			
		3	157143	1.21	1.20	1:1		89104	1.48	1.47	1:1		58935	1.20	1.20	1:1	
		4	185400	1.17				92069	1.46				61180	1.18			
		5	168345	1.19				104822	1.48				60216	1.25			
Recovered cholesterol		1	43799	1.20				15573	1.37				95182	1.07			
		2	42497	1.22	1.23	1.02:1		15700	1.35	1.37	0.93:1		105267	1.05	1.05	0.88:1	
		3	40213	1.26				16264	1.40				104186	1.03			
20-Hydroxyecdysone 2-acetate (IV)		1		0.91		0.76:1			1.52		1.03:1			0.83		0.69:1	
20,22-Acetonide-20-hydroxyecdysone 2-acetate (VII)		1	406	0.28				232	2.57				233	0.00			
		2	391	0.28	0.27	0.22:1		242	1.62	1.72	1.17:1		245	0.02	0.01	0.01:1	
		3	373	0.24				252	1.82				253	0.00			

* Based on the $^3\text{H}/^{14}\text{C}$ ratio of the administered cholesterol.

similar to those of the original 20,22-acetonide-20-hydroxyecdysone 2-acetate (VII). This indicates that the residual ^3H in the ecdysteroid is not located at C-3. During the comparatively short exposure (18 h) to the MnO_2 oxidation conditions employed in this instance, little equilibration of the 3-oxo group would be expected (see Davies *et al.*, 1980).

Table 2. $^3\text{H}/^{14}\text{C}$ ratios of cholesterol and derivatives of the biosynthesized ecdysteroids isolated from fifth-instar *Schistocerca gregaria* after administration of [$4\text{-}^{14}\text{C}$, $3\alpha\text{-}^3\text{H}$]cholesterol

Substance	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio
Administered cholesterol	3.59	1:1
Recovered cholesterol	3.65	1.02:1
20-Hydroxyecdysone	0.85	0.24:1
2-acetate (IV)		
20,22-Acetonide-20-hydroxy- ecdysone 2-acetate (VII)	0.82	0.23:1
20,22-Acetonide-3-oxo-20- hydroxyecdysone		
2-acetate(VIII)		
(i) from dimethyl sulph- oxide oxidation	0.83	0.23:1
(ii) from MnO_2 oxidation (18 h only)	0.79	0.22:1

Two possibilities exist for the extensive removal of the $3\alpha\text{-}^3\text{H}$ of cholesterol in the biosynthesized 20-hydroxyecdysone (I). If the ecdysteroid biosynthetic pathway involved obligatory formation of a 3-oxo- Δ^4 intermediate, the $3\alpha\text{-}^3\text{H}$ would be completely removed. This ^3H could enter a small compartmentalized pool of NAD(P)^+ and be partly reintroduced somewhere in the ecdysteroid molecule during some subsequent reductive process. Alternatively, a 3-oxo intermediate might not be involved, in which case the $3\alpha\text{-}^3\text{H}$ would be retained during the biosynthesis of the 20-hydroxyecdysone (I). However, extensive elimination of this ^3H could occur subsequently during reversible formation of the corresponding 3-dehydroecdysteroid (Karlson & Koolman, 1973). In this case, the remaining ^3H should be located at C-3. However, the data indicating that the residual ^3H in the 20-hydroxyecdysone formed from [$3\alpha\text{-}^3\text{H}$]cholesterol is not located at C-3 suggests that the elimination of ^3H from C-3 is not due to ecdysone oxidase-catalysed formation of 3-dehydroecdysteroid. To eliminate further this latter possibility, the same three cholesterol substrates were incorporated *in vivo* into ecdysone in maturing female ovaries of *S. gregaria*. It has not been possible to detect the reversible formation of 3-dehydroecdysone in this system, where the majority of the hormone is passed directly

Table 3. $^3\text{H}/^{14}\text{C}$ ratios of cholesterol and ecdysteroids and their subsequent chemical transformation products isolated from *Schistocerca gregaria* oöcytes after administration of stereospecifically labelled [^3H , ^{14}C]cholesterol substrates to maturing females

For further details see the text.

Substrate	... [$4\text{-}^{14}\text{C}$, $3\alpha\text{-}^3\text{H}$]Cholesterol		... [$4\text{-}^{14}\text{C}$, $4\alpha\text{-}^3\text{H}$]Cholesterol		... [$4\text{-}^{14}\text{C}$, $4\beta\text{-}^3\text{H}$]Cholesterol	
	Amount of ^{14}C (μCi) ... 4.2		9.0		9.0	
	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*
Administered cholesterol	3.14	1:1	1.51	1:1	0.65	1:1
Recovered cholesterol	3.05	0.97:1	1.51	1:1	0.65	1:1
7-Dehydrocholesterol	—		1.73	1.14:1	0.61	0.94:1
Conjugates fraction	0.28	0.09:1	1.52	1.01:1	0.00	0:1
Ecdysone (II) from conjugates (ex. t.l.c.)	0.02	0.01:1	1.61	1.07:1	0.05	0.08:1
2-Deoxyecdysone (III) from conjugates (ex. t.l.c.)	0.14	0.04:1	1.60	1.06:1	0.13	0.20:1
Ecdysone-2,3-acetonide (VI)	0.02	0.01:1				
Ecdysone (ex. h.p.l.c.)	0.04	0.01:1	1.78	1.18:1	0.12	0.18:1
2-Deoxyecdysone (ex. h.p.l.c.)	0.00	0:1			0.03	0.05:1
5α -Epimer of ecdysone			1.84	1.22:1		
Ecdysone 2,22-diacetate (V)			1.66	1.10:1		
3-Oxoecdysone 2,22-diacetate (IX)						
(i) from dimethyl sulphoxide oxidation			1.72	1.14:1		
(ii) after prolonged exposure to MnO_2			1.05†	0.69:1†		

* Based on the $^3\text{H}/^{14}\text{C}$ ratio of the administered cholesterol.

† Indicative only, due to very low radioactivity.

into the oöcyte from the follicle cells and does not circulate in the haemolymph.

Mature female S. gregaria

The three labelled cholesterol substrates were administered to maturing adult female *S. gregaria* and the eggs were collected within 12 h of being deposited. The newly laid eggs or mature ovaries were extracted, the extract was subjected to solvent partition and column chromatography, and the polar ecdysteroid conjugate fraction was hydrolysed enzymically to yield primarily ecdysone (II) and 2-deoxyecdysone (III), which were purified by t.l.c., h.p.l.c. and derivative formation. The percentage incorporation (0.41–0.90%) of the administered cholesterol into ecdysone and 2-deoxyecdysone in this system was appreciably higher than that into 20-hydroxyecdysone (0.04–0.12%) in fifth-instar *S. gregaria*.

In this biosynthetic system there is essentially complete removal of the ^3H from $[3\alpha\text{-}^3\text{H}]$ - and $[4\beta\text{-}^3\text{H}]$ cholesterol and complete retention of ^3H from the $[4\alpha\text{-}^3\text{H}]$ cholesterol during incorporation into ecdysone and 2-deoxyecdysone (Table 3). The ^3H retained in the ecdysone formed from $[4\alpha\text{-}^3\text{H}]$ cholesterol was then located. Transformation of this ecdysone (II) into its 5α -epimer occurred without loss of ^3H (Table 3) demonstrating that the ^3H is not located at C-5. Similarly, there was no elimination of ^3H (Table 3) during oxidation of the ecdysone 2,22-diacetate (V) to its 3-oxo derivative (IX) with the dimethyl sulphoxide oxidation (i.e. under non-equilibrating conditions). This demonstrates that the ^3H retained is not located at C-3. However, when this 3-oxoecdysone 2,22-diacetate (IX) sample ($^3\text{H}/^{14}\text{C}$ atomic ratio 1.14:1) was subjected to prolonged exposure to MnO_2 (equilibrating conditions), the $^3\text{H}/^{14}\text{C}$ atomic ratio in the recovered 3-oxoecdysone 2,22-diacetate (IX) was markedly lower (0.69:1, Table 1). This latter ratio can only be taken as indicative, since the recovery of radioactivity in the 3-oxoecdysone 2,22-diacetate (IX) in this case after equilibration was very low. However, this result suggests that the ^3H retained in the ecdysone formed from $[4\text{-}^{14}\text{C}, 4\alpha\text{-}^3\text{H}]$ cholesterol is located at C-4 and is eliminated partly from the 3-oxoecdysone 2,22-diacetate (IX) during prolonged exposure to MnO_2 in acetonitrile by slow equilibration of the 3-oxo group (see Davies *et al.*, 1980).

In summary, during biosynthesis of ecdysone in maturing ovaries of *S. gregaria*, there is retention (probably at C-4) of the 4α -hydrogen atom of cholesterol and elimination of the 3α - and 4β -hydrogen atoms. Since there is no evidence for the reversible formation of 3-dehydroecdysone in this ovarian system, the 3α -hydrogen atom must be eliminated during the biosynthesis of ecdysone and not subsequently in a side reaction.

Discussion

During the biosynthesis of 20-hydroxyecdysone from stereospecifically labelled $[^3\text{H}]$ cholesterol species in fifth-instar *S. gregaria* in the present work, the 4α - and 4β - ^3H atoms of cholesterol are retained and eliminated, respectively, whereas there is extensive but incomplete elimination of the 3α - ^3H atom [approx. 20% retention at position(s) other than C-3]. Essentially similar results have been obtained previously in *Calliphora erythrocephala* where up to 30% retention of the 3α - ^3H atom of cholesterol was observed (Lockley *et al.*, 1975; W. J. S. Lockley, unpublished work). However, it was not possible to deduce from results in these systems whether the 3α - ^3H was eliminated during biosynthesis of ecdysteroids or subsequently during their metabolism. The incorporation of the $[^3\text{H}]$ -cholesterol species into ecdysteroids in maturing ovaries of *S. gregaria* (where reversible formation of 3-dehydroecdysteroids cannot be detected) has now indicated that the 3α -hydrogen atom is eliminated during biosynthesis of the ecdysteroids rather than subsequently in a side reaction.

The present results are probably best explained by postulating the involvement of a 3-oxo- Δ^4 intermediate in the formation of the A/B *cis* ring junction (i.e. $5\beta\text{-H}$) of ecdysteroids, at least in the *S. gregaria* ovarian system; other possibilities cannot be excluded. This probably holds also for ecdysteroid synthesis in larval systems, since it appears that, whereas the later stages of ecdysone synthesis may differ somewhat in different species (King, 1972) and also between synthesis in larval and ovarian systems, the early stages are probably similar. For example, $3\beta,14\alpha$ -dihydroxy- 5β -cholest-7-en-6-one is a probable intermediate in both *Locusta* ovaries (Hetru *et al.*, 1978) and during larval synthesis of ecdysteroids in *Calliphora erythrocephala* (Galbraith *et al.*, 1975) and *Manduca sexta* (Bollenbacher *et al.*, 1977).

If a 3-oxo- Δ^4 steroid is an obligatory intermediate at some stage in ecdysteroid biosynthesis, the retention of up to 30% of the ^3H during incorporation of $[3\alpha\text{-}^3\text{H}]$ cholesterol in larvae is best explained by transfer of the 3α - ^3H to a small compartmentalized pool of NAD(P)^+ by 3β -hydroxysteroid dehydrogenase action during formation of the 3-oxo group, with reintroduction of some of this ^3H during a subsequent reductive step requiring NAD(P)H . The results for the fifth-instar locusts indicate that this ^3H is re-incorporated at a position other than C-3 in the ecdysteroid. There are several precedents in the case of steroid metabolism in rats and microbes for analogous efficient incorporation of ^3H (or ^2H) at certain positions (particularly C-3) within steroids from labelled, compartmentalized NAD(P)^+ pools (Cronholm, 1972; Björkhem & Gustafsson, 1971). Further-

more, during 20-hydroxyecdysone formation in the fern, *Polypodium vulgare*, there was significant transfer of label, presumably via NAD(P)⁺, from the 7 β -position of cholesterol to the 3 α -position of the hormone (Cook *et al.*, 1973).

The stereochemistry of hydrogen elimination from C-4 of cholesterol (i.e. 4 β) during ecdysteroid formation is the same as that observed during production of a 3-oxo- Δ^4 steroid intermediate in the formation of steroid hormones and bile acids in vertebrates. In such vertebrate systems there is apparently intramolecular transfer of some of the 4 β -H to the C-6 position, during isomerization of the Δ^5 bond to the Δ^4 position (Murota *et al.*, 1971). If such a transfer did occur to any extent during ecdysteroid synthesis the ³H would be removed during subsequent introduction of the 6-oxo group.

It has been shown that, in prothoracic glands from *Bombyx mori*, cholesterol is converted into 6-oxo-5 α -cholestan-3 β -ol, which is further transformed into ecdysone (Sakurai *et al.*, 1977). By analogy with vertebrate systems, a 3-oxo- Δ^4 intermediate would presumably be formed by oxidation at C-3 followed by isomerization of the Δ^5 bond to the Δ^4 position. This could not occur with 6-oxo-5 α -cholestan-3 β -ol, which lacks a Δ^5 bond. Therefore, it is difficult at present to envisage the mutual intermediacy of a 3-oxo- Δ^4 steroid and 6-oxo-5 α -cholestan-3 β -ol.

A recent report (Kinnear *et al.*, 1979) that certain putative Δ^4 and 3-oxo- Δ^4 ecdysteroid precursors are not incorporated into 20-hydroxyecdysone does not preclude the involvement of a 3-oxo- Δ^4 intermediate, since the structural features in the rest of the molecule may be of critical importance.

During the biosynthesis of 20-hydroxyecdysone in the fern, *Polypodium vulgare*, the 3 α - and 4 β -hydrogen atoms of cholesterol apparently migrate to the C-4 and C-5 positions, respectively (Davies *et al.*, 1980). The reason for the observed differences in the fates of the 3 α - and 4 β -hydrogen atoms of cholesterol during ecdysteroid synthesis in the plant and insect systems is obscure. It may be related to the fact that insects lack the ability to synthesise sterols *de novo* whereas plants possess this capability. Consequently, it is possible that in the latter system the ecdysteroid synthetic pathway could branch off from the normal sterol pathway before the Δ^5 stage.

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