The Stereochemistry of Hydrogen Elimination from C-7 during Biosynthesis of Ecdysones in Insects and Plants

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(Received 5 April 1973)

1. [7α - $^3\text{H}_1$]- and [7β - $^3\text{H}_1$]-Cholesterol were synthesized by a modified method. 2. The stereochemistry of Δ^7 -bond formation during ecdysone and ecdysterone biosynthesis in the insect, *Calliphora erythrocephala* and the plants, *Taxus baccata* and *Polypodium vulgare* was investigated by using [4 - ^{14}C , 7α - $^3\text{H}_1$]cholesterol and [4 - ^{14}C , 7β - $^3\text{H}_1$]cholesterol. 3. In each case, the 7β hydrogen was stereospecifically eliminated. 4. The possible significance of the results is discussed in relation to double-bond formation in other systems and the stage at which the Δ^7 bond is introduced during ecdysone biosynthesis.

The majority of insect species investigated to date contain ecdysterone (1) as the major moulting hormone, which is often accompanied by smaller amounts of ecdysone (2) (Rees, 1971; Nakanishi, 1971). Similarly, ecdysterone is the most widely occurring of the numerous insect moulting hormones (ecdysones) which have been characterized in plants (Horn, 1971). Although the complete pathways of ecdysone biosynthesis have not yet been elucidated (Thompson *et al.*, 1972; Rees & Goodwin, 1972), cholesterol is known to be a precursor in insects and plants (Karlson & Hoffmeister, 1963; Galbraith *et al.*, 1970; Sauer *et al.*, 1968; de Souza *et al.*, 1970).

The present paper reports investigations on the stereochemistry of introduction of the Δ^7 bond during moulting-hormone biosynthesis in the insect, *Calliphora erythrocephala* and the plants, *Taxus baccata* and *Polypodium vulgare*. The stereochemistry of double-bond introduction during cholesterol (Mulheirn & Ramm, 1972) and plant sterol biosynthesis (Goad & Goodwin, 1972) has been extensively investigated.

Double-bond formation in dehydrogenations is influenced by electron-withdrawing groups at positions α to the potential bond. In the presence of such groups removal of *trans* hydrogens occurs, whereas in their absence removal of *cis* hydrogens is observed [Wilton & Akhtar (1970) and references cited therein; Dewhurst & Akhtar (1967)]. Therefore it was of special interest to determine the stereochemistry of the Δ^7 -bond formation in ecdysones as they contain an electron-withdrawing group ($\text{C}=\text{O}$) at an α -position to the double bond.

The present study involves the use of [7α - $^3\text{H}_1$]-cholesterol and [7β - $^3\text{H}_1$]cholesterol. These compounds have previously been synthesized by Corey & Gregoriou (1959), but we now report a modified synthesis involving diaxial opening of a $6\beta,7\beta$ -epoxide.

Experimental

Plants

T. baccata seedlings and germinating rhizomes of the fern *P. vulgare* were obtained from Ness Botanical Gardens, Cheshire, U.K. The freshly germinating rhizomes were potted in soil and grown in a plant growth room for 4–6 weeks at 28°C.

Insects

Larvae of *C. erythrocephala* were reared at 25°C, and fed daily with fresh meat. Animals for experimental use were derived from eggs that were laid within a period of a few hours. Feeding experiments were carried out on unpupated larvae (approx. 7 days old) from synchronized batches in which 20% pupation had already occurred.

Chemicals

Lithium aluminium tritide was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Tritiated water and [4 - ^{14}C]cholesterol (60 $\mu\text{Ci}/\text{mmol}$) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive cholesterol (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was recrystallized before use. Synthetic ecdysone was kindly provided by Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. Ecdysterone was extracted from the rhizomes of *P. vulgare* and found to be identical in every respect with an authentic sample.

Thin-layer chromatography

T.l.c. was carried out as a routine on silica gel (0.75 mm, Kieselgel GF254; E. Merck A.-G.,

Darmstadt, Germany) with chloroform for development unless otherwise specified in the text. Cholesterol derivatives were eluted with diethyl ether and ecdysones were eluted with methanol. For analytical purposes, compounds were located by spraying with 3% (w/v) ceric sulphate in 10% (v/v) sulphuric acid solution and heating at 110°C for 15 min. After preparative t.l.c., compounds were visualized by examination under u.v. light or by a similar process after spraying with a 10% (w/v) solution of Rhodamine 6G in acetone.

Analytical methods

¹H N.m.r. spectra were determined for solutions in deuterated chloroform either at 60 MHz with a Perkin Elmer R12 spectrometer or at 100 MHz by the Physico-Chemical Measurements Unit, Harwell, Berks., U.K., with a Varian HA-100D spectrometer. Mass spectra were determined with A.E.I. M.S.12 and M.S.902 mass spectrometers.

Radiochemical methods

Radioactivity was measured on an Inter technique three channel scintillation spectrometer model ABACSL40. Samples were dissolved in 10 ml of a toluene scintillation solution containing 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 5 g of 2,5-diphenyloxazole per 1000 ml of toluene. Compounds found to be insoluble in toluene were dissolved in 10 ml of a dioxan scintillation solution containing 15 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (Butyl PBD) and 100 g of naphthalene per 1000 ml of dioxan. The ¹⁴C and ³H radioactivities are quoted after corrections for quenching and counting efficiency have been applied.

Nomenclature

Trivial names are often used. Systematic names are as follows: cholesterol, cholest-5-en-3β-ol; ecdysone, 2β,3β,14α,22*R*,25-pentahydroxy-5β-cholest-7-en-6-one; ecdysterone, 2β,3β,14α,20,22*R*,25-hexahydroxy-5β-cholest-7-en-6-one.

Synthesis of compounds

3β-Hydroxy-6β,7β-epoxy-5α-cholestane (3), 3β-acetoxy-5α-cholestan-6-one (6) and [5α,7β-³H₂]3β-acetoxy-7α-bromo-5α-cholestan-6-one (6) were prepared as described by Cory (1954), Corey & Snee (1956) and Corey & Gregorion (1959) respectively.

[7α-³H₁]3β,6β-Dihydroxy-5α-cholestane (8). 3β-Hydroxy-6β,7β-epoxy-5α-cholestane (3; 105 mg) dissolved in dry pyridine was treated with hexamethyldisilazane (1 ml) and trimethylsilyl chloride (1 ml). After 1 h at 20°C, the mixture was concentrated *in*

vacuo, the residue dissolved in dry diethyl ether (50 ml) and filtered through cottonwool. Concentration of the filtrate gave the gummy silyl ether (5; 107 mg). The crude silyl ether was then dissolved in dry tetrahydrofuran (2 ml) and added to a suspension of lithium aluminium tritride (2.3 mg; 5 mCi) in dry tetrahydrofuran (1 ml). After heating under reflux for 4 h the excess of reagent was destroyed with ethyl acetate and the reaction mixture worked up in the usual way with acid and by extracting with diethyl ether. Concentration of the dried extract gave a residue, which, after preparative t.l.c. [developed in chloroform-*n*-propanol (9:1, v/v)], yielded the diol (8; 20.1 mg; 2.06 mCi).

[7α-³H₁]6β-Hydroxy-5α-cholestan-3β-cathylate (9). The diol (8; 20.1 mg) was dissolved in dry pyridine (2.5 ml) and treated with ethyl chloroformate (150 μl). After 18 h at 20°C the reaction was terminated with water and the mixture extracted with diethyl ether in the usual manner. The resulting crude extract gave, after preparative t.l.c., the monocathylate (9; 20.7 mg; 1.82 mCi).

[7α-³H₁]Cholest-5-en-3β-cathylate (11). The monocathylate (9; 20.7 mg) dissolved in dry pyridine was treated with phosphoryl chloride (250 μl) and allowed to stand at room temperature for 14 h. The reaction mixture was heated for 1 h at 85°C, then poured into water and extracted with diethyl ether in the usual manner. The crude residue yielded, after preparative t.l.c., the olefin (12; 11.2 mg; 1.017 mCi).

[7α-³H₁]Cholest-5-en-3β-ol (12). The olefin (11; 11.2 mg) dissolved in methanol (10 ml) was treated with pyrogallol (0.1 g) and KOH (1 g) and then the reaction mixture was heated under reflux for 2 h. The cooled mixture was then poured into water and extracted with diethyl ether in the usual manner. The crude residue gave, after preparative t.l.c. (developed three times in chloroform), [7α-³H₁]cholesterol (12; 7.57 mg; 41.5 mCi/mmol).

[5α,7α-³H₁]6β,7β-Epoxy-5α-cholestan-3β-ol (4). The bromoketone (7; 159 mg; 25.88 mCi) dissolved in propan-2-ol (13 ml) was treated with a solution of sodium borohydride (0.1 g) in propan-2-ol (10 ml) and the mixture allowed to stand at room temperature for 4 h. A solution of KOH (1 g) in propan-2-ol (10 ml) was then added to the reaction mixture and the solution heated under reflux for 1 h. The mixture was then poured into water and the product extracted with diethyl ether in the usual manner. The resulting residue gave, after preparative t.l.c., the epoxide (4; 65 mg, 13.37 mCi).

[5α,7β-³H₂]5α-Cholestan-3β,6β-diol (10). The epoxide (4) dissolved in dry tetrahydrofuran (15 ml) was added to a solution of lithium aluminium hydride (300 mg) in dry tetrahydrofuran (5 ml) and the mixture heated under reflux for 4 h. The excess of lithium aluminium hydride was decomposed with ethyl acetate and the mixture extracted with diethyl ether

in the usual manner. The crude residue gave, after preparative t.l.c., the diol (10; 52.3 mg; 10.84 mCi).

[7 β - 3 H]Cholest-5-en-3 β -ol (13). The diol (10; 52.3 mg), after undergoing the transformations described above in the conversion of the diol (8) into compound (12), gave crude [7 β - 3 H]cholesterol (22.4 mg; 2.06 mCi). Preparative t.l.c. of the crude cholesterol on silver nitrate-impregnated plates (10%) (developed twice in chloroform) yielded pure [7 β - 3 H₁]cholesterol (13; 19.1 mg; 35.4 mCi/mmol).

3 β -Acetoxycholest-5-en-7-one and 5 α -Hydroperoxycholest-6-en-3 β -ol. These were prepared by the methods of Wilton *et al.* (1968) and Nickon & Bagli (1961) respectively.

Feeding and extraction procedures

Insects. The tritiated cholesterol substrates (12) and (13) were separately mixed with [4- 14 C]cholesterol (30 μ Ci) and emulsified in water (1 ml) with

(1) R = OH

(2) R = H

(3) R₁ = R₂ = R₃ = H

(4) R₁ = H; R₂ = R₃ = 3 H

(5) R₁ = Si(CH₃)₃; R₂ = 3 H; R₃ = H

(6) R₁ = H; R₂ = H₂

(7) R₁ = 3 H; R₂ = α Br, β^3 H

(8) R₁ = R₃ = H; R₂ = α^3 H

(9) R₁ = —C(=O)—OEt ; R₂ = α^3 H; R₃ = H

(10) R₁ = H; R₂ = β^3 H; R₃ = 3 H

(11) R₁ = —C(=O)—OEt ; R₂ = α^3 H

(12) R₁ = H; R₂ = α^3 H

(13) R₁ = H; R₂ = β^3 H

(14) $R_1 = \beta OAc, \alpha H; R_2 = \beta-OH, \alpha H; R_3 = H$

(15) $R_1 = O; R_2 = \beta OAc, \alpha H; R_3 = H$

(16) $R_1 = \beta OAc, \alpha H; R_2 = O; R_3 = H$

(17) $R_1 = R_2 = \beta OAc, \alpha H; R_3 = H$

(18) R_1/R_2 , $\alpha H; R_3 = H$

(19) R_1/R_2 , $\alpha H; R_3 = Ac$

(20)

(21) $R_1 = R_2 = H$

(22) $R_1 = R_2 = Ac$

Tween 80 (1%) by sonication. The aqueous emulsion was injected into 200 larvae which were kept at 28°C for 12h. The insects were then homogenized in methanol (three times) followed by chloroform (three times) and the combined extracts concentrated *in vacuo*. Carrier ecdysone (5mg) and ecdysterone (5mg) were then added to the residue dissolved in methanol which was subjected to preparative t.l.c. [developed with chloroform-*n*-propanol (9:5, v/v)]. A non-polar radioactive band and radioactive bands co-chromatographing with ecdysone and ecdysterone were then eluted with methanol and the latter was purified further by formation of derivatives (Table 4) as described below.

The non-polar band was saponified with 10% (w/v) KOH in ethanol for 4h. The reaction mixture was

poured into water and after extraction with diethyl ether in the usual manner gave a gummy residue. Preparative t.l.c. of the residue yielded a band co-chromatographing with cholesterol, which after elution with diethyl ether and dilution with non-radioactive carrier material, was crystallized to constant specific radioactivity from chloroform-methanol (Table 4).

Plants. [4- ^{14}C]Cholesterol (20 μCi) and [3H]cholesterol (60–100 μCi) were mixed and a portion was taken for accurate radioactivity ratio determination. The remaining cholesterol was then dissolved in 80% (v/v) ethanol (2ml) containing 0.05% Tween 80. A portion (0.2ml) of this solution was administered twice weekly to the leaves of two young *T. baccata* seedlings or six young shoots of *P. vulgare* over a

period of 4 weeks. One week after the final feeding, the plant leaves were washed with hexane and the total plant was macerated and homogenized in methanol (500ml). The homogenate was then washed into a 2l flask and heated under reflux for 6h. After cooling overnight, the green methanolic solution was filtered through glass wool and the filtrate evaporated under reduced pressure to give a gummy solid. The residue was suspended in water (500ml), extracted with hexane (2 × 300ml) and the hexane extracts were combined and washed with water (75ml).

The original aqueous layer was then extracted with ethyl acetate (2 × 200ml) and the combined extracts washed with the above water washings. The original aqueous layer was then further extracted with butanol (4 × 200ml) and the extracts were washed with the above water washings. The respective extracts were evaporated to dryness and the distribution of ^{14}C radioactivity between each extract was determined (Table 2).

The butanol extract, after the addition of ecdysone (5mg) and ecdysterone (5mg), was subjected to preparative t.l.c. with chloroform-propan-2-ol (9:3, v/v) for development. A non-polar radioactive zone was obtained together with bands corresponding to ecdysone and ecdysterone (Table 3). The radioactive ecdysone and ecdysterone residues were purified further by crystallization and/or formation of the derivatives described below.

A small portion of the hexane extract (10^4 d.p.m.) was diluted with non-radioactive cholesterol (5mg) and purified by preparative t.l.c. on AgNO_3 -impregnated plates (10%). The cholesterol band was eluted with diethyl ether and after further dilution with non-radioactive cholesterol was crystallized to constant radioactivity ratio.

Transformation products of ecdysone and ecdysterone

General procedures. (a) Acetates and acetonides. Acetates and acetonides of ecdysones were prepared by the methods of Galbraith & Horn (1969). (b) Oxidation of ecdysones. Ecdysone (5mg) dissolved in acetone (5ml) was cooled in ice and treated with Jones reagent (Djerassi *et al.*, 1956) (0.1ml). After 1min the mixture was poured into water, extracted with ethyl acetate and the extract evaporated to dryness yielding a crude residue which was further purified by t.l.c.

Derivatives

2 β -Acetoxy-20,22-acetonideecdysterone (14). 2 β -Acetoxyecdysterone (12mg) on treatment with acetone-perchloric acid gave after preparative t.l.c. [developed in chloroform-*n*-propanol (9:1, v/v)], 2 β -acetoxy-20,22-acetonideecdysterone (14). The

acetoxyacetonide (14; m.p. 206–210°C) was found to be identical in every respect with the compound prepared by Lloyd-Jones *et al.* (1973).

2 β -Acetoxy-20,22-acetonide-3-oxoecdysterone (17). Oxidation of 2 β -acetoxy-20,22-acetonideecdysterone gave, after preparative t.l.c. [developed in chloroform-*n*-propanol (20:1, v/v)], 2 β -acetoxy-20,22-acetonide-3-oxoecdysterone (16) and 3 β -acetoxy-20,22-acetonide-2-oxoecdysterone (15) which could not be separated further by t.l.c. The mixture had identical spectroscopic properties to those reported earlier (Lloyd-Jones *et al.*, 1973).

2 β ,3 β -Acetonide-14 α -hydroxy-5 β -pregn-7-ene-6,20-dione (20). Oxidation of 2,3-acetonideecdysone (7.10mg) gave, after preparative t.l.c. developed with chloroform-*n*-propanol (20:1, v/v), 2 β ,3 β -acetonide-14 α -hydroxy-5 β -pregn-7-ene-6,20-dione (20) (8mg) as an oil. Proton magnetic resonance (p.m.r.) δ 0.62 (s, C-13 Me); 0.98 (s, C-10 Me); 1.32 and 1.49 (acetonide geminal methyls); 2.14 (s, C-21 Me); 4.1–4.4 (m, C-2H and C-3H); 5.79–5.88 (d, C-7H, J, 3Hz) m/e 402 (M^+ ; $\text{C}_{24}\text{H}_{34}\text{O}_5$; 30%); 387 ($\text{C}_{23}\text{H}_{31}\text{O}_5$; base peak), 345 [$\text{M}^+ - (58 + 18)$]; 73%].

2,3-Diacetoxy-20,22-acetonideecdysterone (17). Acetylation of 2-acetoxy-20,22-acetonideecdysterone (15; 5mg) gave, after preparative t.l.c. [developed in chloroform-*n*-propanol (20:1, v/v)], 2,3-diacetoxy-20,22-acetonideecdysterone (17) (10mg), p.m.r. δ 0.80 (s, C-13Me); 1.02 (s, C-10Me), 1.16 (s, C-20Me), 1.22 (s, C-25 geminal methyls); 1.32 and 1.40 (d, acetonide geminal methyls); 2.00 (s, 3-OAc); 2.12 (s, 2-OAc); 2.90–3.20 (m, C-5H); 3.58–3.74 (m, C-22H; $W_{\frac{1}{2}}$ 12Hz); 4.95–5.20 (m, C-2H); 5.28–5.44 (m, C-3H, $W_{\frac{1}{2}}$ 6Hz), 5.84–5.90 (d, C-7H, J 3Hz) m/e 604 (M^+ ; very weak); 447 ($\text{C}_{25}\text{H}_{35}\text{O}_7$; base peak), 345 ($\text{C}_{21}\text{H}_{29}\text{O}_4$; 40%); 327 ($\text{C}_{21}\text{H}_{27}\text{O}_3$; 37.5%), 201 ($\text{M}^+ - 403$; 12.5%).

2,3-Acetonide-22,25-diacetoxyecdysone (22) and 25-Acetoxy-2,3:20,22-diacetoxiecdysterone (19). (a) Acetylation of 2,3-acetonideecdysone (21) (10mg) in acetic anhydride-pyridine for 72h at 37°C gave, after preparative t.l.c. [developed with chloroform-*n*-propanol (20:1, v/v)] 2,3-acetonide-22,25-diacetoxyecdysone (22; 4mg) P.m.r. δ 0.66 (s, C-13Me), 0.97 (s, C-10Me), 1.3, 1.4 and 1.46 (t, C-21, C-25, and acetonide methyls), 1.95 and 2.02 (s; OAc) 4.1–4.4 (m, C-2 and C-3H), 4.8–5.0 (m, C-22H), 5.78–5.84 (d, J 1Hz) m/e 588 (M^+ , $\text{C}_{34}\text{H}_{52}\text{O}_8$, very weak), 528 ($\text{C}_{32}\text{H}_{48}\text{O}_6$, 20%), 387 ($\text{C}_{24}\text{H}_{35}\text{O}_4$, base peak), 341 ($\text{C}_{22}\text{H}_{29}\text{O}_3$, 36%).

(b) Acetylation of 2,3:20,22-diacetonideecdysterone (18) as described above gave 25-acetoxy-2,3:20,22-diacetonideecdysterone (19). P.m.r. δ 0.80 (s, C-13Me), 0.98 (s, C-10Me), 1.24 and 1.34 (d, C-25 and C-21 methyls) 1.4, 1.43, 1.46 and 1.48 (s, acetonide methyls). 1.98 (s, OAc), 2.65–2.95 (M, C-5H, $W_{\frac{1}{2}}$ 8Hz) 3.5–3.7 (m, C-22H $W_{\frac{1}{2}}$ 7Hz), 4.0–4.38 (m, C-2H and C-3H), 7.8–8.4 (d, J 1Hz, C-7H). m/e 602 (M^+ ,

$C_{35}H_{54}O_8$, 3%), 542 ($C_{33}H_{50}O_6$, 18%), 403 ($C_{24}H_{35}O_5$, base peak).

Results and Discussion

[7 α - 3H]- and [7 β - 3H]-Cholesterol species were prepared and the tritium content at C-7 was determined by conversion into 3 β -acetoxycholest-5-en-7-one (Table 1). Since transformation of cholesterol into 5 α -hydroperoxycholest-6-en-3 β -ol is known specifically to remove the 7 α -H (Nickon & Bagli, 1961), this reaction was used to confirm the stereochemistry of the C-7 tritium in the synthesized cholesterol substrate (Table 1). The [7 α - 3H]- and [7 β - 3H]-cholesterol were independently mixed with [4- ^{14}C]cholesterol before administration to the insect, *C. erythrocephala* and the plants, *T. baccata* and *P. vulgare*. These tissues were then extracted, fractionated (Tables 2 and 3) and the ecdysones purified

further by crystallization and derivative formation.

When samples of [7 α - 3H]- and [7 β - 3H]-cholesterol prepared by Corey & Gregoriou (1959) were subjected to stereospecific location of the C-7 deuterium by formation of 5 α -hydroperoxycholest-6-en-3 β -ol, 8.5% and 95% retention of deuterium was observed in the two samples respectively (Nickon & Bagli, 1961). In the present work, transformation of the [7 α - 3H]- and [7 β - 3H]-cholesterol species, prepared by diaxial opening of the 6 β ,7 β -epoxides (3 and 4), into 3 β -acetoxycholest-5-en-7-one resulted in retention of 13% ($^3H/^{14}C$, 0.13:1) and 2% ($^3H/^{14}C$, 0.02:1) of the tritium respectively (Table 1). This indicates that 2% and 13% of the tritium label in the [7 β - 3H]- and [7 α - 3H]-cholesterol respectively is at positions other than C-7. The removal of practically all of the tritium label at C-7 in the [7 α - 3H]-cholesterol on conversion into the 5 α -hydroperoxycholest-6-en-3 β -ol ($^3H/^{14}C$, 0.14:1) confirms that the latter is

Table 1. $^3H/^{14}C$ Radioactivity ratios and specific radioactivities of cholesterol derivatives obtained during location of the tritium label within the synthesized [7 α - 3H]- and [7 β - 3H]-cholesterol, which had been mixed with [4- ^{14}C]-cholesterol (60 mCi/mmol)

For details see the text.

Substrate ...	[4- ^{14}C ,7 α - 3H]Cholesterol			[4- ^{14}C ,7 β - 3H]Cholesterol		
	$^3H/^{14}C$ radioactivity ratio	$^3H/^{14}C$ atomic ratio*	Specific radioactivity ^{14}C (d.p.m./mg)	$^3H/^{14}C$ radioactivity ratio	$^3H/^{14}C$ atomic ratio*	Specific radioactivity ^{14}C (d.p.m./mg)
Derivatives						
Initial cholesterol acetate	6.01	1:1	—	8.37	1:1	—
3 β -Acetoxycholest-5-en-7-one	0.75	0.12:1	7815	0.13	0.015:1	5370
	0.76	0.13:1	7915	0.14	0.017:1	5480
	0.75	0.12:1	7850	0.14	0.017:1	5450
Initial cholesterol	2.46	1:1	—	7.63	1:1	—
5 α -Hydroperoxycholest-6-en-3 β -ol	0.38	0.15:1	4127	7.19	0.94:1	10 272
	0.36	0.14:1	4135	7.18	0.94:1	10 305
	0.36	0.14:1	4136	7.18	0.94:1	10 341

* Based on the average $^3H/^{14}C$ radioactivity ratio of the initial cholesterol (or acetate).

Table 2. Distribution of radioactivity within the crude extracts obtained from *T. baccata* and *P. vulgare* administered with [4- ^{14}C]cholesterol (20 μCi) plus [7- 3H]cholesterol (60–100 μCi)

For details see the text.

Plant species ...	Radioactivity (μCi of ^{14}C)		
	<i>T. baccata</i>		<i>P. vulgare</i>
Cholesterol administered ...	[4- ^{14}C ,7 α - 3H] (66 μCi of 3H)	[4- ^{14}C ,7 β - 3H] (67 μCi of 3H)	[4- ^{14}C ,7 β - 3H] (65 μCi of 3H)
Leaf washings	6.8	10.9	9
Hexane extract	1.48	3.1	2.0
Ethyl acetate extract	0.74	0.07	—
Butanol extract	2.03	0.2	4

Table 3. Fractionation of the polar extracts from *T. baccata*, *P. vulgare* (butanol extracts) and *C. erythrocephala* (methanol extract) by preparative t.l.c. developed with chloroform–*n*-propanol (9:5, v/v)

For further details see the text.

Species ...	10 ⁻⁵ × ¹⁴ C radioactivity (d.p.m.)				
	<i>T. baccata</i>		<i>P. vulgare</i>	<i>C. erythrocephala</i>	
	Cholesterol ...	...	...	...	...
	[4- ¹⁴ C,7α- ³ H]	[4- ¹⁴ C,7β- ³ H]	[4- ¹⁴ C,7β- ³ H]	[4- ¹⁴ C,7α- ³ H]	[4- ¹⁴ C,7β- ³ H]
Fraction					
Non-polar	1.60	1.9	2.3	—	—
Ecdysone	—	—	1.22	0.157	0.22
Ecdysterone	0.50	0.30	4.4	0.28	0.32

formed in a stereospecific manner and that a negligible isotope effect operates during this transformation. Similarly, transformation of the [7β-³H]cholesterol gave a hydroperoxide which had a ³H/¹⁴C ratio of 0.94:1. This indicates that 4% of the tritium label in the synthesized [7β-³H]cholesterol is located at the 7α-position.

In both plants and insects, the ³H/¹⁴C ratio of the administered and recovered cholesterol were in close agreement (Tables 4 and 5). In all cases the actual radioactivity in the ecdysones after purification via derivative formation was appreciably lower than estimated in the moulting hormone purified only by t.l.c. Results in Table 4 indicate that *P. vulgare* is a far superior system than *T. baccata* for the transformation of cholesterol into ecdysones. The ³H/¹⁴C isotopic ratios recorded in Table 4 demonstrate clearly that in the insect, *C. erythrocephala* there is stereospecific removal of the 7β-hydrogen and retention of the 7α-hydrogen of cholesterol during formation of the Δ⁷ bond in the ecdysones. The small retention of isotope (³H/¹⁴C, 0.10:1) when [7β-³H]cholesterol is the substrate is probably due mainly to the presence of a small amount of tritium in the 7α position of the administered cholesterol (estimated to be 4%) as discussed previously. There is also the possibility of traces of a C-7 saturated impurity in the crystallized ecdysone derivatives.

In the case of the plants examined (Table 5) there is again a retention of the 7α-hydrogen of cholesterol in the isolated ecdysones, which emphasizes the stereospecificity of the desaturation. Unexpectedly, there is also retention of a significant amount of the 7β-hydrogen (*T. baccata* ³H/¹⁴C, 0.60:1; *P. vulgare*, ecdysterone ³H/¹⁴C, 0.42:1, ecdysone ³H/¹⁴C, 0.45:1) and one must look for an additional explanation to those already discussed for the slight retention of label observed in the ecdysones biosynthesized in *C. erythrocephala* from the same substrate.

We considered the possibility that C-7 tritium removed entered an NADPH pool, and was then re-

introduced into ecdysones during alcohol dehydrogenase enzyme action at C-3 or other secondary hydroxyl groups within the ecdysone molecule. The former possibility was checked by formation of 20,22-acetonide-2-acetoxy-3-oxoecdysterone (³H/¹⁴C, 0.16:1) which could not be chromatographically separated from 20,22-acetonide-3-acetoxy-2-oxoecdysterone (25%) arising from acyl migration under the reaction conditions employed (Lloyd-Jones *et al.*, 1973). Allowing for the acyl migration the corrected ³H/¹⁴C ratio for the 3-oxo derivative is 0.07:1. The latter possibility was ruled out since conversion of 2,3-acetonidecdysone into 2β,3β-acetonid-14α-hydroxy-5β-pregn-7-en-6,22-dione (³H/¹⁴C, 0.46:1) occurred without an appreciable change in ³H/¹⁴C ratio (Table 6). These results indicate that an appreciable amount of the tritium from C-7 in the case of *P. vulgare*, and probably *T. baccata*, enters a compartmentalized NADPH pool and is reincorporated at C-3 during non-specific dehydrogenase enzyme action. Analogous transfer of label to C-3 of steroids has been observed in the metabolism of steroid hormones in rats (Cronholm, 1972) and in the microbial transformation of cholesterol into coprostanol (Björkhem & Gustafsson, 1971). The result indicated that the NADPH formed during dehydrogenation at C-7 and C-8 is closely related to the coenzyme pool involved in alcohol dehydrogenase activity at C-3 and corroborates our earlier observation that some degree of non-specific dehydrogenase activity occurs at C-3 during ecdysone biosynthesis in *T. baccata* (Lloyd-Jones *et al.*, 1973). However, it should be emphasized that despite the above purification procedure the retention of label could still be attributed to a co-chromatographing impurity.

The overall results in *C. erythrocephala*, *T. baccata* and *P. vulgare* clearly demonstrate that introduction of the Δ⁷ bond during moulting hormone biosynthesis occurs by removal of the 7β- and by inference the 8β-hydrogens. The same stereochemistry has been observed during the transformation of cholesterol

Table 4. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of cholesterol and derivatives of the biosynthesized ecdysone and ecdysterone isolated from *C. erythrocephala* after administration of $[4-^{14}\text{C}, 7\alpha-^3\text{H}]$ - and $[4-^{14}\text{C}, 7\beta-^3\text{H}]$ -cholesterol

For further details see the text.

Substrate ...	Substance	Recrystal- lizations	$[4-^{14}\text{C}, 7\alpha-^3\text{H}]\text{Cholesterol}$				$[4-^{14}\text{C}, 7\beta-^3\text{H}]\text{Cholesterol}$			
			$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radioactivity ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radioactivity ^{14}C (d.p.m./mg)		
Administered cholesterol		1	3.31		2715	4.60		10542		
		2	3.34	1:1	2721	4.62	1:1	10579		
		3	3.32		2717	4.61		10515		
Recovered cholesterol		1	3.33		1910	4.59		8741		
		2	3.31	1:1	1907	4.60	1:1	8775		
		3	3.32		1904	4.61		8795		
2,3:20,22-Diacetonidecdysterone		1	3.13	0.94:1	93	0.49	0.11:1	304		
		2	3.11	0.94:1	96	0.47	0.10:1	294		
		3	3.14	0.94:1	97	0.45	0.098:1	298		
2,3-Acetonidecdysone		1	3.17	0.96:1	211	0.52	0.11:1	232		
		2	3.12	0.94:1	207	0.50	0.11:1	242		
		3	3.14	0.94:1	209	0.49	0.10:1	237		
2,5-Acetoxy-2,3:20,22-diacetonid- ecdysterone			3.10	0.93:1		0.48	0.10:1			

* Based on the average $^3\text{H}/^{14}\text{C}$ ratios obtained for the administered cholesterol.

Table 5. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of the steroids isolated from plants after administration of $[4\text{-}^{14}\text{C}, 7\alpha\text{-}^3\text{H}]$ - and $[4\text{-}^{14}\text{C}, 7\beta\text{-}^3\text{H}]$ -cholesterol, and their subsequent chemical transformation products

For further details see the text.											
Plant species ...		<i>T. baccata</i>						<i>P. vulgare</i>			
Substrate ...		$[4\text{-}^{14}\text{C}, 7\alpha\text{-}^3\text{H}]\text{Cholesterol}$			$[4\text{-}^{14}\text{C}, 7\beta\text{-}^3\text{H}]\text{Cholesterol}$			$[4\text{-}^{14}\text{C}, 7\beta\text{-}^3\text{H}]\text{Cholesterol}$			
		$^3\text{H}/^{14}\text{C}$ radio-activity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radio-activity ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radio-activity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radio-activity ^{14}C (d.p.m./mg)	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radio-activity ^{14}C (d.p.m./mg)	
Substance	Recrystallizations	1	4.96	1234	2.93		3577	3.25		2974	
		2	5.06	1:1	2.89	1:1	3652	3.3	1:1	2857	
		3	4.97	1188	2.96		3571	3.29		3068	
Cholesterol administered		1	4.97	142	2.96		1072	3.33		74	
		2	5.05	1:1	2.90	1:1	1088	3.26	1:1	75	
		3	5.15	134	2.92		1072	3.4		78	
Cholesterol recovered		1	4.3	40	1.8	0.61:1	47	1.37	0.42:1	1363	
		2	4.7	35.6	1.72	0.59:1	46	1.37	0.42:1	1324	
		3	4.3	38	1.75	0.60:1	46	1.37	0.42:1	1384	
20,22-Acetonide-2-acetoxycdysterone		1	4.2	—	1.67	0.57:1	—	—	—	—	
20,22-Acetonide-2,3-diacetoxycdysterone											
20,22-Acetonide-2-acetoxycdysterone											
3-oxoecdysterone											

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

Table 6. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of ecdysone, isolated from *P. vulgare* administered with $[4\text{-}^{14}\text{C}, 7\beta\text{-}^3\text{H}]\text{cholesterol}$, and its subsequent chemical transformation products

For further details see the text.				
Substance	Recrystallization	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio*	Specific radioactivity ^{14}C (d.p.m./mg)
Ecdysone	1	1.46	0.44:1	774
	2	1.42	0.43:1	731
2,3-Acetonideecdysone	1	1.5	0.46:1	274
	2	1.4	0.43:1	270
	3	1.52	0.46:1	304
$2\beta,3\beta$ -Acetonide- 14α -hydroxy- 5β -pregn-7-ene-6,20-dione	1	1.53	0.46:1	—

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

Scheme 1. Possible mechanism involving successive *trans* processes for the *cis*-elimination of hydrogens during double-bond formation Scheme 2. Possible molecular oxygen mechanisms for the *cis*-elimination of hydrogen during double-bond formation

into cholesta-5,7,22-trien- 3β -ol in *Tetrahymena pyriformis* (Zander & Caspi, 1970; Wilton & Akhtar, 1970; Bimpson *et al.*, 1969) and during conversion of cholestanol into cholest-5 α -7-en- 3β -ol in a cockroach (Clayton & Edwards, 1963). Although formation of all these Δ^7 bonds involves removal of the *cis* orientated $7\beta,8\beta$ -hydrogens, the process does not neces-

sarily have to be a *cis* dehydrogenation. Possible mechanisms, involving two successive *trans* processes (Scheme 1) (Zander & Caspi, 1970) or alternatively molecular oxygen (Scheme 2), have been suggested for the *cis*-elimination of hydrogens during double-bond formation (Wilton & Akhtar, 1970).

As suggested in the introduction, the stereo-

chemistry of double-bond formation may be dependent on the presence of an electron-withdrawing substituent at position α to the potential double bond. If this generalization holds, the removal of the $7\beta,8\beta$ -hydrogens observed during ecdysone biosynthesis would be consistent with the introduction of the Δ^7 bond before formation of the 6-oxo group.

We thank the Science Research Council for financial assistance, Mr. J. K. Hulme, Ness Botanical Gardens for *Taxus baccata* and *Polypodium vulgare*, and Miss J. M. Griffiths for skilled technical assistance.

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