Mechanism of formation of the A/B *cis* ring junction of ecdysteroids in *Polypodium vulgare*

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1. The fates of the 3 α -, 4 α - and 4 β -hydrogen atoms of cholesterol during formation of the A/B *cis* ring junction of ecdysteroids was investigated by administration of [4-¹⁴C, 3 α -³H]-, [4-¹⁴C, 4 α -³H]- and [4-¹⁴C, 4 β -³H]cholesterol species to the fern, *Polypodium vulgare*, and isolation of the 20-hydroxyecdysone formed in each case. 2. The ³H was retained in the ecdysteroid formed from each substrate. 3. Location of the ³H in the 20-hydroxyecdysone indicated that migration of ³H from the 3 α - and 4 β -positions to C-4 and C-5, respectively, had occurred, whereas the 4 α -³H atom was retained at C-4. 4. A possible mechanism for the formation of the A/B *cis* ring junction of ecdysteroids in *P. vulgare* is presented.

Most species of insect larvae investigated contain 20-hydroxyecdysone (I) as the major moulting hormone (ecdysteroid), which is frequently accompanied by smaller amounts of ecdysone (II) (Thompson *et al.*, 1973; Gilbert *et al.*, 1977). 20-Hydroxyecdysone is also the most widely occurring of the numerous compounds possessing insect-moulting-hormone activity that have been isolated from plants (Horn, 1971). Cholesterol can serve as a precursor of the C₂₇ ecdysteroids in both insects and plants (Rees, 1971). It is known that at least some modification of the nucleus precedes side chain hydroxylation.

In plants, 2 β ,3 β ,14 α -trihydroxy-5 β -cholest-7-en-6-one is incorporated into ecdysteroids (Tomita &

Sakurai, 1974), thus suggesting that as in insects, the A/B *cis* ring junction is introduced early in the ecdysteroid biosynthetic pathway. It has been assumed frequently that this A/B *cis* ring junction is formed by mere equilibration of a 6-oxo-5 α -H steroid. However, numerous plausible pathways could account for the transformation of a Δ^5 structure to give the 5 β stereochemistry in ecdysteroids (Rees, 1971). An analogous change during steroid hormone and bile acid formation in vertebrates involves the intermediacy of a 3-oxo- Δ^4 steroid (Samuels & Eik-Nes, 1968). Similarly, during the formation of cardenolides from cholesterol in the plant *Digitalis lanata*, introduction of 5 β stereochemistry involves oxidation at C-3 as an obli-

(I) R¹ = H, R² = OH

(II) R¹ = H, R² = H

(III) R¹ = Ac, R² = OH

(IV) R¹ = Ac, R² = H

(V) R = β -OH, α -H

(VI) R = O

gatory step (Caspi & Hornby, 1968). Although [4- ^{14}C]cholest-4-en-3-one is not incorporated significantly into 20-hydroxyecdysone in *Podocarpus elatus* seedlings (Sauer *et al.*, 1968), the possibility remains that a 3-oxo- Δ^4 grouping could be involved at a later stage in the pathway, e.g. after insertion of the Δ^7 bond or some of the hydroxyl groups.

The present paper reports investigations on the mechanism of formation of the A/B *cis* ring junction of ecdysteroids in the fern, *Polypodium vulgare*. [4- ^{14}C ,3 α - ^3H]-, [4- ^{14}C ,4 α - ^3H]- and [4- ^{14}C ,4 β - ^3H]cholesterol species were incorporated, in turn, into 20-hydroxyecdysone in this plant. Examination of the fate of the two C-4 hydrogen atoms of cholesterol would show whether either of these hydrogen atoms is removed during ecdysteroid biosynthesis, irrespective of the stage at which removal occurs. Similarly, investigation of the fate of the ^3H in [3 α - ^3H]cholesterol should reveal whether oxidation at C-3 occurs during formation of ecdysteroids. Part of this work has been reported previously in preliminary form (Lockley *et al.*, 1975). The development of improved chemical methods has now allowed more complete location of the tritium in the labelled 20-hydroxyecdysone.

Experimental

Nomenclature

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

Chemicals

[4- ^{14}C]Cholesterol (54 Ci/mol) and NaB^3H_4 were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [4 α - ^3H]- and [4 β - ^3H]cholesterol samples were prepared by the method of Lockley *et al.* (1978) and contained 96 and 91%, respectively, of the ^3H in the expected positions. [3 α - ^3H]Cholesterol was prepared by Dr. I. F. Cook by reduction of cholest-5-en-3-one with NaB^3H_4 and purified by t.l.c. Samples of ecdysone and 20-hydroxyecdysone were generously given by Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand. Berberine sulphate was purchased from Sigma.

Thin-layer chromatography

Sterols were separated by t.l.c. on (i) silica gel (0.5 mm thick Kieselgel G; E. Merck A.G., Darmstadt, Germany) developed in chloroform and (ii) on 10% (w/w) AgNO_3 -impregnated silica gel (0.5 mm thick Kieselgel H) developed in chloroform/ethanol (97:3, v/v). After t.l.c., the sterol was detected under u.v. light (360 nm) by spraying with 0.05% berberine sulphate in methanol/acetone (1:1, v/v), and

was eluted from the gel with dry redistilled diethyl ether.

T.l.c. separation of ecdysteroids and their derivatives was carried out on silica gel (0.5 mm thick Kieselgel GF₂₅₄; E. Merck A.G., Darmstadt, Germany) developed as specified in the text. Compounds were detected under u.v. light and eluted thoroughly from the gel with chloroform/methanol (1:1, v/v).

Analytical methods

^1H .n.m.r. spectra were determined for solutions of ecdysteroid derivatives in (^2H)chloroform at 220 or 100 MHz on Varian HR-220 and HA 100 instruments, respectively, by the Physico-Chemical Measurements Unit, Harwell, Berks., U.K. or at 60 MHz on a Perkin-Elmer R12 instrument. Mass spectra were determined on A.E.I. M.S.902 and M.S.12 mass spectrometers.

Radiochemical methods

Radioactivity was measured on an Intertechnique three-channel scintillation spectrometer, model ABAC SL30. Samples were dissolved in 10 ml of a dioxan-based scintillation solution containing 15 g of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD)/litre and 100 g of naphthalene/litre. ^3H and ^{14}C radioactivities are quoted after corrections for background, counting efficiency and quenching had been applied.

Administration of labelled cholesterol

[4- ^{14}C]Cholesterol was mixed with the appropriate [^3H]cholesterol sample and purified by t.l.c. on silica gel. A portion of the purified cholesterol was removed, diluted with carrier non-radioactive cholesterol and recrystallized to constant specific radioactivity from chloroform/methanol to establish the $^3\text{H}/^{14}\text{C}$ radioactivity ratio for the substrate. The remaining cholesterol was then dissolved by sonication in ethanol (2 ml) containing 0.05% Tween 80.

Fronds of *Polypodium vulgare* sub-sp. *interjectum* (obtained from Ness Botanical Gardens, Cheshire, U.K.) were cut back to the rhizome, and were left until viable fronds (8–10 cm long) had appeared. The solutions of labelled cholesterol were applied to the leaves by using a glass capillary tube, twice weekly over a 4-week period. Approx. eight to twelve growing fronds were used per experiment. The entire plants were extracted 2 weeks after the final administration of substrate.

Extraction of plant material

The extraction of the plants treated with [4- ^{14}C , 4 α - $^3\text{H}_1$]cholesterol (13.5 μCi of ^{14}C ; 28.3 μCi of ^3H) is given as a typical procedure. The rhizomes and roots were washed free of soil and the whole plants (180 g) were cut into small pieces before maceration

in ethanol (1 litre). The slurry was then refluxed for 6 h, cooled, and filtered through glass wool. The solid residue was then re-extracted as above and the two ethanol extracts were combined and evaporated to dryness under vacuum.

The dried extract (15 g; 4.37×10^6 d.p.m. of ^{14}C) was then partitioned between n-hexane (300 ml) and methanol/water (7:3, v/v) (300 ml). Each phase was then re-partitioned against the complementary solvent and the combined hexane extracts and the combined methanol/water extracts were evaporated to dryness under vacuum. The residue (7.85 g; 5.44×10^5 d.p.m. of ^{14}C) from the methanol/water fraction was then partitioned between butan-1-ol (200 ml) and water (200 ml) and each phase was re-extracted with the complementary solvent. The combined butan-1-ol extracts (1.59 g; 1.01×10^5 d.p.m. of ^{14}C) were evaporated to dryness under vacuum. Sterols and ecdysteroids were isolated from the hexane and the butan-1-ol fractions, respectively.

Purification of recovered cholesterol

Sterols (3.59 mg; 2.42×10^5 d.p.m. of ^{14}C) were isolated from the hexane extract (1.98 g; 3.09×10^6 d.p.m. of ^{14}C) by repeated t.l.c. on silica gel and the Δ^5 sterols (including cholesterol) were then purified from the sterol band by t.l.c. on AgNO_3 -impregnated silica gel. The radioactive cholesterol band (2.01 mg; 1.43×10^5 d.p.m. of ^{14}C) was diluted with non-radioactive cholesterol (40 mg) and recrystallized from chloroform/methanol to constant specific radioactivity.

Purification of 20-hydroxyecdysone

After addition of 20-hydroxyecdysone (5 mg), the butanol extract was subjected to t.l.c. on silica gel with chloroform/ethanol (5:3, v/v) for development. The 20-hydroxyecdysone band was re-chromatographed on the same system to effect further purification (4.56 mg; 7.92×10^4 d.p.m. of ^{14}C).

The 20-hydroxyecdysone fraction was diluted further with non-radioactive material (25 mg) and the 2-acetate derivative was prepared by the method of Galbraith & Horn (1969). The 20-hydroxyecdysone 2-acetate (III; 10.41 mg; 1.91×10^4 d.p.m. of ^{14}C) was separated from the mixture of acetates by t.l.c. on silica gel with chloroform/methanol (9:1, v/v) for development. The derivative was characterized by mass spectrometry and then was recrystallized from methanol/water.

The 20-hydroxyecdysone 2-acetate (III) was then transformed into its 20,22-acetonide (20,22-O-isopropylidene) derivative (V) as follows. The 20-hydroxyecdysone 2-acetate (9.86 mg; 1.21×10^4 d.p.m. of ^{14}C) was dissolved in dry acetone (5 ml) and sufficient molybdophosphoric acid

(approx. 4 mg) was added to turn the solution green (Galbraith & Horn, 1969). The mixture was left for 30 min at room temperature and was then poured into butan-1-ol (50 ml), which was washed successively with saturated NaHCO_3 solution and water. The butanol layer was then evaporated to dryness under vacuum, azeotroping with benzene/ethanol. The 20,22-acetonide-20-hydroxyecdysone 2-acetate (V; 4.53 mg; 4.93×10^3 d.p.m. of ^{14}C) was isolated by t.l.c. on silica gel with chloroform/methanol (19:1, v/v) for development and was recrystallized from chloroform/diethyl ether: *m/e* 562 (M^+ , very weak), 544 ($\text{M}^+ - \text{H}_2\text{O}$), 526 ($\text{M}^+ - 2\text{H}_2\text{O}$), 510, 487 ($\text{M}^+ - \text{acetate} - \text{CH}_3$), 469 ($\text{M}^+ - \text{acetate} - \text{H}_2\text{O} - \text{CH}_3$), 451 ($\text{M}^+ - \text{acetate} - 2\text{H}_2\text{O} - \text{CH}_3$), 446, 405 ($\text{M}^+ - \text{side chain}$), 395, 387 ($405 - \text{H}_2\text{O}$), 345 ($387 - \text{acetate}$), 327 ($345 - \text{H}_2\text{O}$), 215, 213, 201 (side chain), 143, 125, 102; n.m.r. δ (p.p.m.) [^2H]chloroform, 220 MHz] 0.81 (s, C-18 methyl), 1.01 (s, C-19 methyl), 1.17 (s, C-21 methyl), 1.26 (s, C-26, C-27 methyls), 1.32 and 1.42 (acetonide methyls), 2.11 (s, acetoxy group), 3.00–3.20 (m, C-5 proton), 3.64–3.80 (m, C-22 proton), 4.09–4.16 (m, C-3 proton, W_1 7 Hz), 4.95–5.08 (m, C-2 proton, W_1 21 Hz), 5.85 (d, J 2 Hz, C-7 proton) (Lloyd-Jones *et al.*, 1973).

In two experiments (2a and 3a), carrier ecdysone was also added to the original butanol extract and this compound was eluted from the t.l.c. plates and was purified via the 2-acetate derivative (IV).

Location of ^3H in 20,22-acetonide-20-hydroxyecdysone 2-acetate (V)

Each reaction was first carried out on a larger scale on non-radioactive material and the product was characterized by physical methods.

Dimethyl sulphoxide/acetic anhydride oxidation. 20,22-Acetonide-20-hydroxyecdysone 2-acetate (approx. 2 mg) was dissolved in dimethyl sulphoxide (300 μl) and acetic anhydride (100 μl) was added (Albright & Goldman, 1967). The mixture was kept at room temperature for 18 h and then poured into diethyl ether (100 ml). The ethereal solution was washed successively with saturated NaHCO_3 solution and water and was then dried over anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The 20,22-acetonide-20-hydroxy-3-oxoecdysone 2-acetate (VI; yield 50–75%) was purified by t.l.c. on silica gel with chloroform/ethanol (19:1, v/v) for development and had the following physical characteristics: *m/e* 560 (M^+ , very weak), 469, 467 ($\text{M}^+ - 60 - \text{water} - \text{CH}_3$), 449, 403 [$\text{M}^+ - 157(\text{C}_{22} - \text{C}_{27})$], 386, 385 ($\text{M}^+ - 157 - \text{H}_2\text{O}$), 343 ($\text{M}^+ - 157 - \text{acetate}$), 201 [$\text{M}^+ - 359(\text{C}_1 - \text{C}_{19})$], 162, 161, 158, 149, 143 [$201 - 58(\text{C}_3\text{H}_6\text{O})$], 134, 133, 125 ($201 - 58 - \text{H}_2\text{O}$), 108, 102, 99; n.m.r. δ (p.p.m.) [^2H]chloroform, 60 MHz] 0.83 (s, C-18 methyl), 1.07 (s, C-19 methyl), 2.17 (s, acetoxy

group), 1.19 (s, C-21 methyl), 1.24 (s, C-26 and C-27 methyls), 1.34 and 1.41 (d, acetonide methyls), 5.87 (d, J 2 Hz, C-7 proton), 5.27–5.60 (1H, m, C-2 proton), 3.50–3.80 (m, C-22 proton) (cf. Lloyd-Jones *et al.*, 1973).

MnO₂ oxidation and equilibration. To 20,22-acetonide-20-hydroxyecdysone 2-acetate (approx. 2 mg) dissolved in acetonitrile (1 ml), activated MnO₂ (approx. 20 mg) was added (Attenburrow *et al.*, 1952) and the reaction mixture was left at room temperature. To facilitate removal of ³H by enolization, the MnO₂ was changed (by centrifugation and removal of the supernatant) twice daily over 5 days. The spent MnO₂ was washed with acetonitrile and the washings were kept and bulked with the final reaction mixture. The combined reaction mixture and washings were then worked up as in the case of the dimethyl sulphoxide oxidation to give 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI; yield, 41–84%). Samples of 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) prepared by using the dimethyl sulphoxide oxidation or by using MnO₂ were indistinguishable on the basis of their t.l.c., mass spectrometric and n.m.r. properties.

Equilibration at C-5 of 20,22-acetonide-20-hydroxyecdysone 2-acetate (V). 20,22-Acetonide-20-hydroxyecdysone 2-acetate (approx. 1 mg) was dissolved in 200 μ l of dioxan (dried over sodium/lead alloy and redistilled) and water (50 μ l) and conc. HCl (10 μ l) were added. The reaction mixture was left at room temperature for 2.5 h and then was azeotroped to dryness by using benzene/ethanol. The 20,22-acetonide-20-hydroxyecdysone 2-acetate (V; yield, 45–75%) was recovered from the products by t.l.c. on silica gel with chloroform/methanol (19:1,

v/v) for development and had mass and n.m.r. spectra similar to the starting material. Any corresponding 5 α -H compound formed in the reaction would have been removed during t.l.c.

Results

[4-¹⁴C, 3 α -³H]-, [4-¹⁴C, 4 α -³H]- and [4-¹⁴C, 4 β -³H]-cholesterol samples were administered separately to three batches of *Polypodium vulgare* plants. The plants were extracted and unmetabolized cholesterol and the ecdysteroids (ecdysone and 20-hydroxyecdysone) were purified as described in the Experimental section. The incorporations of ¹⁴C into 20-hydroxyecdysone were within the range 0.030–0.37%.

The ³H/¹⁴C ratios and specific radioactivities during recrystallization of cholesterol and derivatives of the ecdysteroids are given in Tables 1–3. For all three substrates, the ³H/¹⁴C atomic ratios of the purified 20,22-acetonide-20-hydroxyecdysone 2-acetate (or ecdysteroid 2-acetates) and of the recovered cholesterol were similar to the ³H/¹⁴C ratio of 1:1 for the administered cholesterol. Thus, there is retention of the 3 α -, 4 α -, and 4 β -³H atoms of cholesterol in ecdysteroids.

The ³H in the purified 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) samples was then located by means of the three chemical reactions summarized in Table 4. When the 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) derived from [4-¹⁴C, 4 β -³H]cholesterol was oxidized to the corresponding 3-oxo compound (VI), without equilibration, by using the dimethyl sulphoxide/acetic anhydride oxidation, there was no loss of ³H (Table

Table 1. ³H/¹⁴C ratios and specific radioactivities of cholesterol and derivatives of the biosynthesized ecdysteroids isolated from *P. vulgare* after administration of [4-¹⁴C, 3 α -³H]cholesterol

For further details see the text.

Substance	Recrystallization	Expt. ... (1a)			(1b)		
		[¹⁴ C]Cholesterol administered (μ Ci) ... 20			27.2		
		Specific radioactivity of ¹⁴ C (d.p.m./mg)	³ H/ ¹⁴ C radioactivity ratio	³ H/ ¹⁴ C atomic ratio*	Specific radioactivity of ¹⁴ C (d.p.m./mg)	³ H/ ¹⁴ C radioactivity ratio	³ H/ ¹⁴ C atomic ratio*
Administered cholesterol	1	11982	4.34	4.33	12304	1.13	1.11
	2	11955	4.38		14501	1.10	
	3	12311	4.28		13072	1.11	
Recovered cholesterol	1	17670	4.53	4.38	998	1.04	1.04
	2	17751	4.28		991	1.03	
	3	18099	4.35		1005	1.04	
20,22-Acetonide-20-hydroxyecdysone 2-acetate (V)	1	721	4.86	4.76	215	1.07	1.09
	2	717	4.55		207	1.10	
	3	673	4.87				

* Based on the average ³H/¹⁴C ratio of the administered cholesterol.

Table 2. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of cholesterol and derivatives of the biosynthesized ecdysteroids isolated from *P. vulgare* after administration of $[4\text{-}^{14}\text{C}, 4\alpha\text{-}^3\text{H}]\text{cholesterol}$
For further details see the text.

Substance	Recrystallization	Expt. ... (2a)			(2b)		
		$[^{14}\text{C}]\text{Cholesterol}$ administered (μCi) ... 20			13.5		
		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	409	2.27	1:1	6380	1.87	1:1
	2	458	2.28		5083	2.07	
	3	469	2.26		4824	2.14	
	4				4628	2.13	
	5				5156	2.04	
Recovered cholesterol	1	2236	2.46	1.09:1	3278	2.09	1.00:1
	2	2189	2.55		3480	2.07	
	3	2244	2.48		3685	2.09	
	4				3610	2.14	
Ecdysone 2-acetate	1	129	2.38	1.05:1			
	2	103	2.39				
	3	114	2.38				
	4	115	2.38				
20-Hydroxyecdysone	1	122	2.08	0.91:1			
2-acetate (III)	2	126	2.08				
20,22-Acetonide-20-Hydroxyecdysone 2-acetate (V)	1				1026	1.93	0.93:1
	2				1096	1.97	

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

Table 3. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of cholesterol and derivatives of the biosynthesized ecdysteroids isolated from *P. vulgare* after administration of $[4\text{-}^{14}\text{C}, 4\beta\text{-}^3\text{H}]\text{cholesterol}$
For further details see the text.

Substance	Recrystallization	Expt. ... (3a)			(3b)		
		$[^{14}\text{C}]\text{Cholesterol}$ administered (μCi) ... 20			13.8		
		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	30039	1.70	1:1	12646	1.50	1:1
	2	31392	1.63		14030	1.50	
	3	30382	1.67		13285	1.52	
	4				11396	1.50	
	5				12014	1.46	
Recovered cholesterol	1	27190	1.81	1.09:1	5033	1.61	1.04:1
	2	27423	1.87		5193	1.63	
	3	26466	1.88		5169	1.65	
	4				5601	1.57	
	5				5038	1.54	
Ecdysone 2-acetate (IV)	1	608	1.66	0.99:1			
	2	463	1.67				
	3	432	1.70				
20,22-Acetonide-20-hydroxyecdysone 2-acetate (V)	1	673	1.65	0.98:1	2079	1.55	1.03:1
	2	771	1.62		2126	1.44	
	3	744	1.67		2117	1.60	

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

Table 4. Location of ^3H in 20,22-acetonide 2-acetate derivatives of 20-hydroxyecdysone samples isolated from *P. vulgare* plants after administration of [^{14}C , ^3H]cholesterol

Portions of each of the purified 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) samples were treated as follows: (i) converted into 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) under non-equilibrating conditions by using the dimethyl sulphoxide/acetic anhydride oxidation, (ii) converted into the 3-oxo derivative under equilibrating conditions by using MnO_2 , and (iii) subjected to acid-catalysed equilibration. In each case the product was purified by t.l.c. and assayed for radioactivity. For further details see the text.

Derivative	Substrate	Expt.	$^3\text{H}/^{14}\text{C}$ atomic ratios*					
			[4- ^{14}C , 3 α - ^3H]-Cholesterol		[4- ^{14}C , 4 α - ^3H]-Cholesterol		[4- ^{14}C , 4 β - ^3H]-Cholesterol	
			(1a)	(1b)	(2b)		(3a)	(3b)
Initial purified 20,22-acetonide-20-hydroxyecdysone 2-acetate (V)			1.09:1	0.98:1	0.93:1		0.98:1	1.03:1
20,22-Acetonide-20-hydroxy-3-oxoecdysone 2-acetate (VI; from dimethyl sulphoxide oxidation)			1.14:1	1.23:1	1.03:1			1.04:1
20,22-Acetonide-20-hydroxyecdysone 2-acetate (V; after acid-catalysed equilibration)				0.95:1	1.00:1			0.41:1
20,22-Acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI; from MnO_2 oxidation)			0.45:1	0.29:1	0.54:1		0.61:1	0.56:1

* Based on the average $^3\text{H}/^{14}\text{C}$ ratio of the administered cholesterol (see Table 1).

Table 5. Loss of ^3H with time during exposure of 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (prepared from 20-hydroxyecdysone formed from [4- ^{14}C , 4 β - ^3H]cholesterol in *P. vulgare*) to the MnO_2 oxidation reaction conditions

Substance	$^3\text{H}/^{14}\text{C}$ atomic ratio*
Initial 20,22-acetonide-20-hydroxyecdysone 2-acetate (V)	0.98:1
20,22-Acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) after:	
(a) MnO_2 oxidation for 2 h	0.92:1
(b) MnO_2 oxidation for 21 h	0.84:1
(c) MnO_2 oxidation for 48 h	0.75:1
(d) re-subjection of sample (c) to the MnO_2 oxidation reaction conditions	0.61:1

* Based on the $^3\text{H}/^{14}\text{C}$ radioactivity ratio of the administered cholesterol = 1.67.

4, Expt. 3b). This demonstrates that the ^3H in that sample of 20-hydroxyecdysone derivative is not located at C-3. When another portion of this 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) was subjected to acid-catalysed equilibration, 59% of the ^3H in that compound was removed ($^3\text{H}/^{14}\text{C}$ atomic ratio = 0.41:1; Table 4). This result indicates that the ^3H derived from [4 β - ^3H]cholesterol is probably located at C-5 in 20-hydroxyecdysone and is removed by equilibration of the 6-oxo group. When the third portion of 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) from Expt. (3b) was subjected to MnO_2 oxidation under the conditions

described in the Experimental section, there was extensive loss of ^3H from the resulting 3-oxo compound ($^3\text{H}/^{14}\text{C}$ atomic ratio of 0.56:1). Thus it is apparent that, under the conditions of the MnO_2 oxidation used in the present work, slow equilibration of the 6-oxo group occurs.

This conclusion was substantiated as follows. The 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) from Expt. 3(a) was oxidized to the corresponding 3-oxo compound (VI) by using active MnO_2 as described in the Experimental section, except that in this case portions of the reaction mixture were removed after varying periods of time (2, 21 and 48 h) and the 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) was purified by t.l.c. and assayed for radioactivity. Comparison of the $^3\text{H}/^{14}\text{C}$ atomic ratio (Table 5) in the initial 20,22-acetonide-20-hydroxyecdysone 2-acetate ($^3\text{H}/^{14}\text{C}$ = 0.98:1) and in the sample of 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) isolated after 2 h ($^3\text{H}/^{14}\text{C}$ = 0.92:1), shows that there is only a small loss of ^3H . However, there is increasing loss of ^3H from the 3-oxo compound with time, the loss of ^3H continuing even after 48 h. Furthermore, when the sample of 20,22-acetonide-20-hydroxy-3-oxo-ecdysone 2-acetate (VI) isolated after 48 h of reaction ($^3\text{H}/^{14}\text{C}$ atomic ratio = 0.75:1) was resubjected to the MnO_2 oxidation conditions, and then re-isolated, further loss of ^3H occurred ($^3\text{H}/^{14}\text{C}$ atomic ratio = 0.61:1). These results indicate that, under the conditions of the MnO_2 oxidation used, there is a slow exchange of the C-5 hydrogen atom by equilibration of the 6-oxo group. By analogy, it would be expected that equilibration of the 3-oxo

group would occur during prolonged exposure to the conditions of the MnO_2 oxidation reaction.

It is apparent (Table 4) that the 20,22-acetonide-20-hydroxyecdysone 2-acetate (V) samples derived from the $[3\alpha\text{-}^3\text{H}]$ - or $[4\alpha\text{-}^3\text{H}]$ cholesterol species (Expts. 1a, 1b and 2b) do not lose appreciable ^3H from C-5 (by acid-catalysed equilibration) or from C-3 (by dimethyl sulphoxide/acetic anhydride oxidation). For Expt. 1(b), an apparently anomalous slight increase in $^3\text{H}/^{14}\text{C}$ ratio occurred during the dimethyl sulphoxide oxidation; the reason for this is obscure. When these two species of 20,22-acetonide-20-hydroxyecdysone 2-acetate (from Expts. 1a, 1b and 2b) were subjected to prolonged MnO_2 oxidation, there was a marked loss of ^3H ($^3\text{H}/^{14}\text{C}$ atomic ratios of 0.45:1, 0.29:1 and 0.54:1, respectively; Table 4). Since there is no ^3H at C-5 in these samples, the loss of ^3H almost certainly occurred from C-4 by equilibration of the 3-oxo group formed in the reaction. It is unlikely that the ^3H was removed from C-2, since enolization of the 3-oxo group to that position is much less favoured than to C-4, on account of the presence of a hydroxyl group at C-2.

Discussion

The results of initial labelling experiments (Expts. 1a, 2a and 3a) demonstrated that no loss of ^3H occurred from the 3α -, 4α - and 4β -positions of cholesterol during transformation into 20-hydroxyecdysone in *P. vulgare*, and that the 4β -hydrogen atom of cholesterol was probably located at C-4 or C-5 in the ecdysteroid. The availability of improved chemical reactions has now allowed more complete location of the label in different samples of 20-

hydroxyecdysone produced in *P. vulgare* by administration of $^{14}\text{C}, ^3\text{H}$ -labelled cholesterol substrates. These results indicate that, during 20-hydroxyecdysone formation in *P. vulgare*, the 4β -hydrogen atom of cholesterol migrates to C-5, whereas the 3α - and 4α -hydrogen atoms of cholesterol are both located at C-4 in the ecdysteroid.

Previous work showed that, when 20-hydroxyecdysone was labelled by administration of $[4\text{-}^{14}\text{C}, 3\alpha\text{-}^3\text{H}]$ cholesterol to *Taxus baccata* and the 20,22-acetonide-20-hydroxyecdysone 2-acetate was oxidized at C-3 with Jones reagent (Djerassi *et al.*, 1956) (which also caused some acyl migration from C-2 to C-3), 53% of the ^3H was eliminated (Lloyd-Jones *et al.*, 1973). In view of the present results with *Polypodium vulgare*, this elimination of ^3H during the location reaction most probably occurred from C-4 by some equilibration of the 3-oxo group, rather than from C-3 during formation of the oxo group as suggested originally.

On the basis of the present results, a possible mechanism for formation of the A/B *cis* ring junction of ecdysteroids is presented in Scheme 1. According to this scheme, epoxidation of the Δ^5 bond of cholesterol (or 7-dehydrocholesterol) occurs; the $5\alpha,6\alpha$ -epoxide would be favoured on steric considerations. It is envisaged that proton attack (e.g. from a protonated group on the enzyme active site) on the C-5—O bond could initiate a series of concomitant 1,2- (Wagner—Meerwein) hydride migrations (from the 4β - and the 5β -position and from the 3α - to the 4α -position), which terminate with elimination of a proton from the 3β -hydroxy group and formation of a 3-oxo function. During this process the 4α -hydrogen atom of cholesterol is displaced to the 4β -position in the ecdysteroid.

Scheme 1. Possible mechanism of formation of the 6-oxo- 5β -H grouping in ecdysteroids in *Polypodium vulgare*

Reduction of the 3-oxo group and oxidation of the 6 α -hydroxy function would then give the 3 β -hydroxy-6-oxo-5 β -H grouping of ecdysteroids. Formation of a distinct 5 α ,6 α -epoxide is not a prerequisite for this mechanism; attack of any positively charged group at C-6 in the Δ^5 (or $\Delta^{5,7}$) compound could initiate the Wagner–Meerwein shifts. The attacking species could well be a protonated oxygen function.

Although the results for the fates of the 3 α -, 4 α - and 4 β - 3 H atoms of cholesterol are consistent with the operation of a mechanism involving 1,2 shifts (e.g. Scheme 1), the possibility cannot be discounted that the 3 α - and 4 β - 3 H atoms are eliminated during formation of a 3-oxo- Δ^4 steroid intermediate, with subsequent re-incorporation of these 3 H atoms at C-4 and C-5 respectively, during reduction of the Δ^4 bond. However, such an explanation is unlikely to be tenable since all the 3 H removed initially would have to be re-incorporated.

It has been shown in *Podocarpus elatus* that neither [4- 14 C]cholesterol-5 β ,6 β -epoxide nor the corresponding 5 α ,6 α -epoxide is incorporated into 20-hydroxyecdysone (Joly *et al.*, 1969). However, it is possible that, during ecdysteroid biosynthesis, the epoxide could be introduced at a later stage, e.g. after insertion of the Δ^7 bond or some of the hydroxyl groups. In fact, in *Polypodium vulgare*, cholest-7-en-3 β -ol is incorporated more efficiently than cholesterol into 20-hydroxyecdysone (Boid, 1975). This result could be explained if 7-dehydrocholesterol was an obligatory precursor of ecdysteroids and if 5 α -cholest-7-en-3 β -ol was transformed more efficiently than cholesterol into 7-dehydrocholesterol. In insects, there is evidence suggesting that 7-dehydrocholesterol may be an early intermediate in the transformation of cholesterol into ecdysteroids (Horn *et al.*, 1974; Johnson & Rees, 1977).

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