Mechanism of hydroxylation at C-2 during the biosynthesis of ecdysone in ovaries of the locust, *Schistocerca gregaria*

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1. The stereochemistry of hydroxylation at C-2 during the biosynthesis of ecdysone in the ovaries of *Schistocerca gregaria* was investigated by incorporation of [$1\alpha,2\alpha$ - $^3\text{H}(n)$]cholesterol in admixture with [4 - ^{14}C]cholesterol into oöcyte 2-deoxyecdysone and ecdysone conjugates in maturing adult female *S. gregaria*. 2. Extraction of the eggs followed by enzymic hydrolysis of the ecdysteroid conjugate fraction yielded free ecdysteroids, from which 2-deoxyecdysone and ecdysone were purified. 3. The $^3\text{H}/^{14}\text{C}$ ratios in the 2-deoxyecdysone and ecdysone were similar, suggesting that the 2α hydrogen of cholesterol was retained during hydroxylation at C-2. This was corroborated by oxidation at C-2 of the 3,22-diacetate derivative of the ecdysone, yielding the corresponding 2-oxo compound with removal of essentially all the ^3H originally present at the 2α position of cholesterol. 4. The results indicate that the 2β hydrogen of cholesterol has been eliminated during the hydroxylation at C-2. Thus, during ecdysone biosynthesis, hydroxylation at C-2 is direct and occurs with retention of configuration.

It has been demonstrated that during hydroxylation of several vertebrate steroids, there is direct replacement of a hydrogen atom with a hydroxy group and consequent retention of configuration about the hydroxylated carbon atom. A similar mechanism has been demonstrated during hydroxylation at C-22 in the formation of the insect moulting hormone, ecdysone (I), in insects (Greenwood & Rees, 1982). The present paper reports the mechanism of hydroxylation at C-2 during the biosynthesis of ecdysone (I). Part of this work has been presented previously in preliminary form (Rees *et al.*, 1981; Greenwood *et al.*, 1982).

Ovaries of numerous species of insects have been shown to synthesize appreciable quantities of insect moulting hormones (ecdysteroids), which in many cases are largely deposited in the developing oöcyte (for review see Hoffmann *et al.*, 1980). In the desert locust, *Schistocerca gregaria*, advantage has been taken of this comparatively large synthesis of ovarian hormone for obtaining a relatively high level of incorporation of radioactive cholesterol into ecdysteroids in maturing adult females (Dinan & Rees, 1981*b*). The ecdysteroids

in newly-laid eggs of *S. gregaria* are present primarily as phosphate conjugates (Isaac *et al.*, 1982), which can be hydrolysed by snail (*Helix pomatia*) gut enzymes to yield mainly ecdysone (I; Gande *et al.*, 1979), with lesser quantities of 2-deoxyecdysone (II) and traces of other ecdysteroids (Dinan & Rees, 1981*a*; Isaac *et al.*, 1981*a*).

The C-2 hydroxy of ecdysone has been shown by X-ray crystallography (Huber & Hoppe, 1965) and by n.m.r. studies (see Horn, 1971) to be in the equatorial 2β position. Although there is no convenient ecdysone precursor specifically labelled at C-2 available for the study of hydroxylation at that position, [$1\alpha,2\alpha$ - $^3\text{H}(n)$]cholesterol could be used for this investigation. Direct hydroxylation at C-2 with retention of configuration would result in retention of the C-2 label, which could then be removed on formation of a 2-oxoecdysone derivative.

Evidence has been presented that ovarian ecdysone synthesis in the locust, *Locusta migratoria*, may involve the hydroxylation sequence C-14, C-25, C-22 followed by C-2 (Hetru *et al.*, 1978, 1982). Although the conversion of 2-deoxyecdysone (II) into ecdysone (I) has not hitherto been demonstrated directly, the co-occurrence of both

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- (I) $R^1 = R^3 = R^4 = \text{OH}; R^2 = \text{H}$
 (II) $R^1 = R^2 = \text{H}; R^3 = R^4 = \text{OH}$
 (IV) $R^1 = R^2 = \text{H}; R^3 = R^4 = \text{OAc}$
 (V) $R^1 = R^3 = R^4 = \text{OAc}; R^2 = \text{H}$
 (VI) $R^1 = R^4 = \text{OAc}; R^2 = \text{H}; R^3 = \text{OH}$
 (VII) $R^1 = \text{OH}; R^2 = \text{H}; R^3 = R^4 = \text{OAc}$
 (VIII) $(R^1, R^2) = \text{O}; R^3 = R^4 = \text{OAc}$
 (IX) $R^1 = R^4 = \text{OAc}; R^2 = \text{H}; R^3 = \text{O}$

(III) $[1\alpha,2\alpha\text{-}^3\text{H}(n)]\text{Cholesterol}$
 (for amounts of ^3H at positions 1α , 2α and 1β , see the text).

these ecdysteroids and their mutual labelling from radioactive cholesterol in both *Schistocerca* and *Locusta* is consistent with 2-deoxyecdysone (II) being a penultimate intermediate in ecdysone biosynthesis (Dinan & Rees, 1981b; Hetru *et al.*, 1982).

In the present work, comparison of the $^3\text{H}/^{14}\text{C}$ ratio in 2-deoxyecdysone (II) and ecdysone (I) labelled maternally from $[4\text{-}^{14}\text{C}, 1\alpha, 2\alpha\text{-}^3\text{H}(n)]$ -cholesterol should indicate which of the two hydrogens at C-2 is eliminated during hydroxylation at that position. The ^3H remaining at C-2 in ecdysone (I) has been located by formation of the 3,22-diacetate derivative followed by oxidation at C-2 yielding the 2-oxo compound. Synthesis of the ecdysone 3,22-diacetate has been achieved by prior formation of the 2,3,22-triacetate followed by partial deacetylation.

Experimental

Nomenclature

The following trivial names are used: ecdysone (I), (22*R*)-2 β ,3 β ,14,22,25-pentahydroxy-5 β -cholest-7-en-6-one; 2-deoxyecdysone (II), (22*R*)-3 β ,14,22,25-tetrahydroxy-5 β -cholest-7-en-6-one.

Chemicals

Two batches of both $[4\text{-}^{14}\text{C}]\text{cholesterol}$ (50mCi/mmol and 56mCi/mmol) and $[1\alpha,2\alpha\text{-}^3\text{H}(n)]\text{cholesterol}$ (50Ci/mmol and 43Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and purified before use by t.l.c. (Dinan & Rees, 1981b). The distribution of ^3H label in the two different preparations of $[1\alpha,2\alpha\text{-}^3\text{H}(n)]\text{cholesterol}$ as determined by The Radiochemical Centre by ^3H n.m.r. is given in Table 1.

Authentic samples of ecdysone and 2-deoxyecdysone were gifts from Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand, and Professor E. Ohnishi, Nagoya University, Japan, respectively. Celite (80–120 mesh), purchased from BDH, was purified by washing with methanol containing 10% (v/v) concentrated HCl followed with water until neutral and then dried at 120°C.

Radiochemical methods

Samples for expt. 1 were radioassayed as described previously (Dinan & Rees, 1981b), whereas in expt. 2 the scintillation cocktail comprised 6g of Omnifluor (New England Nuclear) dissolved in 1.5l of toluene/Triton X-100 (2:1, v/v). Samples were assayed to less than 0.5% error in all channels.

Thin-layer chromatography

T.l.c. of cholesterol, ecdysteroids and their derivatives was carried out as described previously (Davies *et al.*, 1980).

High-performance liquid chromatography

Two types of h.p.l.c. systems were employed: (i) both adsorption (RAD-PAK Si, 10 μm) and reversed-phase (RAD-PAK C-18, 10 μm) columns were used with a radial compression module (model RCM-100, all from Waters Associates). Solvent compositions are described where appropriate. (ii) a Hypersil APS column (25cm $\times$ 4.6mm; Shandon) eluted with dichloroethane/methanol/propan-2-ol (95:3:2, by vol.) [see Dinan *et al.*, 1981]. A solvent flow rate of 2ml/min was used in all instances.

Analytical methods

Mass spectra were recorded on an A.E.I. MS 30 mass spectrometer (electron impact), and on a V.G. Micromass 70-70F mass spectrometer (chemical ionization using isobutane as the ionizing gas or fast atom bombardment with xenon). High resolution mass spectra were obtained with an A.E.I. MS 902 mass spectrometer. P.m.r. spectra were obtained in deuteriochloroform at 220MHz, 360MHz or 400MHz (Fourier transform).

Biosynthesis of ecdysteroids from [4-¹⁴C,1 α ,2 α -³H(n)]cholesterol

The rearing and maintenance of *Schistocerca gregaria* has been described previously (Dinan & Rees, 1981a).

In a typical experiment (expt. 2), [4-¹⁴C]cholesterol (30 μ Ci) and [1 α ,2 α -³H(n)]cholesterol (100 μ Ci) were mixed together and a small aliquot ($\frac{1}{40}$ th) was removed, diluted with carrier sterol and recrystallized to constant specific radioactivity from chloroform/methanol. The bulk of the [³H,¹⁴C]cholesterol was then injected into maturing adult female locusts and labelled ecdysteroids and cholesterol were isolated from the newly laid eggs as described (Dinan & Rees, 1981b).

Purification of labelled ecdysteroids

Ecdysone was separated from 2-deoxyecdysone either by chromatography on a 2g silicic acid column (expt. 1) as described previously (Dinan & Rees, 1981b) or by t.l.c. on silica gel plates developed twice in chloroform/95% ethanol (4:1, v/v) [expt. 2]. Both radioactive compounds were shown to be pure by h.p.l.c. of small aliquots on a RAD-PAK C-18 column eluted with methanol/water (11:9, v/v) with collection of fractions at 30s intervals for radioassay. Further portions of these compounds were purified by h.p.l.c.

Acetate derivatives of ecdysone were prepared according to Galbraith & Horn (1969) and were purified by silica gel t.l.c. using chloroform/95% ethanol (9:1, v/v) and reversed-phase h.p.l.c. using methanol/water (17:8, v/v) prior to radioassay (Table 1).

Quantification of tritium remaining at C-2 of ecdysone derived from [4-¹⁴C,1 α ,2 α -³H(n)]cholesterol

Tritium retained at C-2 in ecdysone formed from [4-¹⁴C,1 α ,2 α -³H(n)]cholesterol was located by the following transformations. The reactions were first carried out on non-radioactive material and the products were characterized by physical means. The purity of these unlabelled ecdysteroid derivatives separated by t.l.c. was monitored by h.p.l.c. on RAD-PAK Si (10 μ m), eluting isocratically with dichloroethane/propan-2-ol/methanol (60:5:1, by vol.). The chromatographic properties of the labelled compounds were identical with those of the characterized ecdysteroid derivatives.

Ecdysone 2,3,22-triacetate (V)

Ecdysone (40mg) was acetylated (18h, room temperature) by the method of Galbraith & Horn (1969) and the products of the reaction were separated by t.l.c. using chloroform/95% ethanol (9:1, v/v) for development. The major product, ecdysone-2,3,22-triacetate (V; R_F = 0.59), was eluted

and recrystallized from ethyl acetate/hexane to produce 35mg (69% yield) of material having the following properties: the p.m.r. spectrum included signals at δ (p.p.m.) [(²H)chloroform, 220MHz], 2.02 (3H, s, C-2 acetate methyl), 2.07 (3H, s, acetate methyl), 2.12 (3H, s, acetate methyl), 4.92 (1H, m, w_1 = 18Hz, C-22 β H), 5.10 (1H, m, w_1 = 22Hz, C-2 α H), 5.38 (1H, m, w_1 = 7Hz, C-3 α H); cf. Takemoto *et al.* (1968); m/z (chemical ionization, isobutane), 613 ([M + C₃H₅ (isobutane fragment) - H₂O]⁺, <1%), 597 ([M + C₃H₇ (isobutane fragment) - 2H₂O]⁺, 2%), 595 ([M + C₃H₅ - 2H₂O]⁺, 2%), 573 ([M + 1 - H₂O]⁺, 7%), 555 ([M + 1 - 2H₂O]⁺, 38%), 537 (3%), 535 (2%), 513 (20%), 497 (42%), 495 (65%), 471 (2%), 453 (17%), 437 (19%), 435 (17%), 411 (7%), 393 (25%), 385 (21%), 384 (21%), 375 (10%), 109 (43%), 99 (21%), 81 (24%), 69 (33%), 61 (100%).

Ecdysone 3,22-diacetate (VII)

Partial deacetylation of ecdysone-2,3,22-triacetate (V) was carried out by a modification of the method of Galbraith & Horn (1969) for the corresponding 20-hydroxyecdysone derivative. Aqueous potassium bicarbonate (3%, w/v, 930 μ l) was added to a solution of ecdysone 2,3,22-triacetate (V; 40.5mg) dissolved in methanol (3.6ml) and left for 115min at room temperature. The reaction was stopped by neutralization with acetic acid followed by addition of water and removal of the methanol by rotary evaporation. The aqueous mixture was extracted with ethyl acetate (4 $\times$) and the combined ethyl acetate fractions were washed, evaporated to dryness and coated onto acid-washed Celite (~1g) from ethanol. The resulting dry powder was applied to a 30g silicic acid column (Mallinckrodt CC-7; 5% deactivated) prepared in chloroform. The column was developed sequentially with chloroform (100ml); chloroform/ethanol (25:1, v/v) (150ml), which eluted unreacted ecdysone 2,3,22-triacetate (V, 22.9mg); chloroform/ethanol (19:1, v/v) (150ml), which eluted the ecdysone diacetate fraction (8.3mg); chloroform/ethanol (9:1, v/v) (200ml) and finally methanol, which eluted ecdysone 22-monoacetate (4.8mg).

The ecdysone diacetate fraction was subjected to preparative t.l.c. on silica gel, with chloroform/95% ethanol (9:1, v/v) for development. By eluting and rechromatographing the lower band, ecdysone 2,22-diacetate and ecdysone 3,22-diacetate (R_F = 0.28) were separated from their putative 5 α -epimers (R_F = 0.31). Ecdysone 3,22-diacetate (VII) was then separated from ecdysone 2,22-diacetate (VI) by reversed-phase h.p.l.c. [RAD-PAK C-18, isocratic elution with methanol/water (13:7, v/v), k' = 4.40, 7.00, respectively]. The 3,22-diacetate derivative (VII, 0.46mg) had the following characteristics: i.r. (KBr disc, cm⁻¹)

1650 α,β -unsaturated C=O, 1725 acetate C=O; u.v. (methanol) $\lambda_{\max.} = 243$ nm; m/z (chemical ionization, isobutane), 591 ($[M + C_3H_7$ (isobutane fragment)] $^+$, <1%), 589 ($[M + C_3H_5$ (isobutane fragment)] $^+$, <1%), 573 ($[M + C_3H_7 - H_2O]^+$, 5%), 571 ($[M + C_3H_5 - H_2O]^+$, <1%), 555 ($[M + C_3H_7 - 2H_2O]^+$, 5%), 549 ($[M + 1]^+$, <1%), 531 ($[M + 1 - H_2O]^+$, 22%), 513 (26%), 495 (25%), 489 (6%), 471 (41%), 453 (82%), 435 (14%), 429 (5%), 411 (37%), 395 (28%), 393 (72%), 377 (11%), 375 (11%), 342 (15%), 283 (17%), 109 (30%), 99 (100%), 81 (20%).

The 3,22-diacetate derivative was stored desiccated at -20°C whenever possible. Reversed-phase h.p.l.c. fractions were quickly evaporated to dryness to minimize the probability of acetate exchange between the C-3 and C-2 positions. Upon Fourier transform n.m.r. of the 3,22-diacetate in C^2HCl_3 , acyl migration readily occurred yielding predominantly the 2,22-diacetate derivative (see preparation of IX). Acyl migration (Haines, 1976) has been reported previously for ecdysteroid derivatives (Lloyd-Jones *et al.*, 1973; Isaac *et al.*, 1981b).

2-Oxo-ecdysone 3,22-diacetate (VIII)

For oxidation of ecdysone 3,22-diacetate (VII) at C-2, the method of Huang *et al.* (1976) was adapted to minimize conditions for exchange of C-1 and C-5 protons with the medium by keto/enol tautomerism. A solution of dry redistilled dimethyl sulphoxide (71 μl) in dry redistilled dichloromethane (400 μl) was cooled to below -70°C in a stoppered test-tube by immersion in liquid N_2 . Trifluoroacetic anhydride (105 μl) was injected slowly via a Teflon septum with mixing. After 10 min at low temperature, ecdysone 3,22-diacetate (2 mg) dissolved in dichloromethane (200 μl) was added slowly to the mixture, which was then shaken intermittently over 30 min. Triethylamine (210 μl) was then added slowly, the mixture was brought to room temperature quickly and immediately partitioned between water and dichloromethane. The dichloromethane phase was evaporated to dryness and the residue fractionated by t.l.c., with chloroform/95% ethanol (14:1, v/v) for development. A trace of remaining diacetate (R_F 0.12) was clearly separated from an intense band (R_F 0.33) of 2-oxo-ecdysone 3,22-diacetate: u.v. (methanol) $\lambda_{\max.} = 243$ nm; i.r. (KBr disc, cm^{-1}) 1660 (α/β unsaturated C=O), 1720 (C=O, enhanced 1.28 times in relative peak height relative to ecdysone 3,22-diacetate); m/z (electron impact), 546.3181 (M^+ , <1%, $\text{C}_{31}\text{H}_{46}\text{O}_8$ requires 546.3192), 528 ($[M - H_2O]^+$, 1%), 510 (1%), 486 (2%), 468 (4%), 453 (5%), 450 (3%), 426 (4%), 408 (4%), 398 (9%), 340 (9%), 290 (22%), 289 (18%), 248 (13%), 161 (27%), 109 (90%), 99 (76%), 81 (100%). The p.m.r.

spectrum [^2H]chloroform, 400 MHz Fourier transform] of the non-radioactive material indicated that it contained appreciable 3-oxo-ecdysone 2,22-diacetate (IX) (formed from the 2,22-diacetate arising by acyl migration) in addition to 2-oxo-ecdysone 3,22-diacetate (VIII): δ (p.p.m.) 4.89 (m, $w_1 = 18$ Hz, C-22H), 5.09 (m, $w_1 = 22$ Hz, C-3H of VIII) and 5.51 (m, $w_1 = 22$ Hz, C-2H of IX) (cf. spectrum of IX below). In the radioactive experiment, the ecdysone 3,22-diacetate was immediately oxidized following purification to minimize acyl migration.

3-Oxo-ecdysone 2,22-diacetate (IX)

3-Oxo-ecdysone 2,22-diacetate (IX) was formed via ecdysone 2,22-diacetate (VI) from the [$^{14}\text{C}, ^3\text{H}$]ecdysone to check for any possible anomalous losses of ^3H during the oxidation procedure. Partial acetylation (1 h) of ecdysone was carried out according to the method of Davies *et al.* (1981). The major product, ecdysone 2,22-diacetate (VI, 9 mg), was purified by t.l.c. and had the following characteristics: m/z (chemical ionization, isobutane) 591 ($[M + C_3H_7$ (isobutane fragment)] $^+$, <1%), 589 ($[M + C_3H_5$ (isobutane fragment)] $^+$, 1%), 573 ($[M + C_3H_7 - H_2O]^+$, 4%), 571 ($[M + C_3H_5 - H_2O]^+$, <1%), 555 ($[M + C_3H_7 - 2H_2O]^+$, <1%), 549 ($[M + 1]^+$, <1%), 531 ($[M + 1 - H_2O]^+$, 12%), 513 (20%), 495 (19%), 489 (12%), 471 (37%), 453 (48%), 435 (18%), 429 (11%), 411 (42%), 395 (21%), 393 (55%), 375 (11%), 109 (25%), 99 (100%), 81 (15%); the p.m.r. spectrum included signals at δ (p.p.m.) [^2H]chloroform, 220 MHz], 2.04 (3H, s, acetate methyl 2.09 (3H, s, acetate methyl), 4.13 (1H, m, $w_1 = 9$ Hz, C-3 α H), 4.89 (1H, m, $w_1 = 18$ Hz, C-22H), 5.02 (1H, m, $w_1 = 22$ Hz, C-2 α H); cf. Galbraith & Horn (1969). The ecdysone 2,22-diacetate (VI) is readily distinguished from ecdysone 3,22-diacetate (VII) by the p.m.r. signals for the 2 α - and 3 α -protons, which occur at δ 4.05 ($w_1 = 21$ Hz) and δ 5.24 ($w_1 = 7$ Hz), respectively, in VII.

Oxidation of ecdysone 2,22-diacetate (VI) under conditions identical with those used for oxidation of the corresponding 3,22-diacetate yielded 3-oxo-ecdysone 2,22-diacetate (IX): m/z (negative ion fast atom bombardment) 545 [$M - H$] $^-$; the p.m.r. spectrum included signals at δ (p.p.m.) [^2H]chloroform, 400 MHz] 2.06 and 2.18 (6H, s, acetate methyls), 4.89 (1H, m, $w_1 = 17$ Hz, C-22H), 5.50 (1H, m, $w_1 = 22$ Hz, C-2 α H).

Results and discussion

Biosynthesis of ecdysteroids from [$4\text{-}^{14}\text{C}$, $1\alpha, 2\alpha\text{-}^3\text{H}_1(\text{n})$]cholesterol

[$4\text{-}^{14}\text{C}$, $1\alpha, 2\alpha\text{-}^3\text{H}_1(\text{n})$]cholesterol (III) was incorporated into the conjugates of 2-deoxyecdysone (II)

Table 1. $^3\text{H}/^{14}\text{C}$ radioactivity ratios of cholesterol and ecdysteroids and their subsequent chemical transformation products isolated from *Schistocerca gregaria* eggs after administration of $[4\text{-}^{14}\text{C}, 1\alpha, 2\alpha\text{-}^3\text{H}(n)]$ cholesterol to maturing females
For further details see the text.

Substance	Distribution of ^3H label in substrate	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	
		Expt. 1 1α (42%), 2α (45%), elsewhere (13%)	Expt. 2 1α (46.4%), 1β (16.8%), 2α (36.8%)
Administered cholesterol		1.72	2.46
Recovered cholesterol		1.68	
Conjugated ecdysteroids		1.73	
2-Deoxyecdysone (II; ex silicic acid column)		1.81	
2-Deoxyecdysone (II; ex t.l.c.)			2.30
2-Deoxyecdysone (II; ex h.p.l.c.)		1.85†	2.19*
2-Deoxyecdysone 3,22-diacetate (IV; ex h.p.l.c.)			2.28†
Ecdysone (I; ex silicic acid column)		1.73	
Ecdysone (I; ex t.l.c.)			2.09
Ecdysone (I; ex h.p.l.c.)		1.68†	1.99*
Ecdysone 2,3,22-triacetate (V; ex h.p.l.c.)			1.98†
Ecdysone 2,3,22,25-tetra-acetate (ex h.p.l.c.)			1.91†
Ecdysone (2,3),22-diacetate (ex t.l.c.)			1.97
Ecdysone 3,22-diacetate (VII, ex h.p.l.c.)			1.90†
2-Oxoecdysone 3,22-diacetate (VIII; ex h.p.l.c.)			1.17†
Water fraction from preparation of (VIII)			15.3
Ecdysone 2,22-diacetate (VI; ex h.p.l.c.)			1.95†
3-Oxoecdysone 2,22-diacetate (IX; ex h.p.l.c.)			1.86†
Water fraction from preparation of (IX)			1.87

* RAD-PAK C-18 column eluted with methanol/water (11:9, v/v).

† Hypersil APS column.

‡ RAD-PAK C-18 column eluted with methanol/water (17:8, v/v).

and ecdysone (I) in maturing adult female *S. gregaria*. After extraction of the newly-laid eggs and enzymic hydrolysis of the conjugates, the free ecdysteroids released were purified. The percentage incorporations of ^{14}C from the initial cholesterol into ecdysone (I) and 2-deoxyecdysone (II) were in the range of 0.3–0.5% and 0.1–0.3%, respectively. The $^3\text{H}/^{14}\text{C}$ ratios of the ecdysteroids and their derivatives are shown in Table 1.

Based on analysis by ^3H n.m.r. the distribution of ^3H in the $[1\alpha, 2\alpha\text{-}^3\text{H}(n)]$ cholesterol used in experiment 1 was: 1α (42%), 2α (45%), and other positions (13%). Thus, should the 2α -hydrogen be eliminated during introduction of the 2β -hydroxy of ecdysone, 45% of the ^3H originally in cholesterol should be removed. In expt. 1, the recovered cholesterol and 2-deoxyecdysone (II), both had approximately the same $^3\text{H}/^{14}\text{C}$ ratio as the initial cholesterol. The ecdysone also maintained the $^3\text{H}/^{14}\text{C}$ ratio of the administered cholesterol, indicating that the $2\alpha\text{-}^3\text{H}$ is retained during the hydroxylation of 2-deoxyecdysone to yield ecdysone.

Similarly, the $[1\alpha, 2\alpha\text{-}^3\text{H}_1]$ cholesterol used in experiment 2 had ^3H localized exclusively in the 1α (46.4%), 1β (16.8%), and 2α (36.8%) positions, with none at 2β . During incorporation of this substrate

in admixture with $[4\text{-}^{14}\text{C}]$ cholesterol into 2-deoxyecdysone (II) and ecdysone (I), no dramatic loss of ^3H was observed (Table 1). Nevertheless, there was some apparent unexplained loss of ^3H label during formation of both 2-deoxyecdysone (II; $^3\text{H}/^{14}\text{C}$ ratio = 2.27; 7% loss of ^3H from cholesterol) and ecdysone (I; $^3\text{H}/^{14}\text{C}$ ratio = 1.99; 19% loss of ^3H from cholesterol). However, had the 2α hydrogen been removed during C-2 hydroxylation a fall of 36.8% in the $^3\text{H}/^{14}\text{C}$ ratio of ecdysone compared with 2-deoxyecdysone would be expected on the basis that 36.8% of the ^3H in the substrate is at the 2α position. In fact, the $^3\text{H}/^{14}\text{C}$ ratio for ecdysone is only 12% lower than that of 2-deoxyecdysone, indicating that the $2\alpha\text{-}^3\text{H}$ of cholesterol is retained during C-2 hydroxylation. To substantiate this, the amount of ^3H remaining at C-2 in the ecdysone produced in expt. 2 was determined.

Quantification of ^3H remaining at C-2 of ecdysone

^3H at C-2 in ecdysone was located by transformation of ecdysone 3,22-diacetate (VII) [prepared by partial deacetylation of ecdysone 2,3,22-triacetate (V)] into 2-oxoecdysone-3,22-diacetate (VIII). Galbraith & Horn (1969) showed that the C-2 acetate group of 20-hydroxyecdysone 2,3,22-triacetate could be readily hydrolysed, although

not selectively, to yield the 3,22-diacetate derivative in low yield. Ecdysone 2,3,22-triacetate behaved in an analogous manner in the current study. For the preparation of the ecdysone 3,22-diacetate, deacetylation of ecdysone 2,3,22-triacetate provided better yields than direct acetylation, although considerable levels of 5 α -ecdysteroids resulted from the controlled hydrolysis owing to the alkaline conditions used. During the equilibration of ecdysteroids possessing the 2 β ,3 β ,6-oxo,7-ene functionalities under mildly acidic or basic conditions, the 5 β configuration (A/B *cis* ring junction) is favoured. For ecdysone, the 5 α /5 β ratio is 1:4 (Mori *et al.*, 1968), the 5 α epimer being thermodynamically more unstable due to the steric interaction between the axial C-2 hydroxy and the axial C-19 methyl. Depending on the substituents at C-2 and C-3, the ratio of 5 α /5 β epimers changes considerably as evidenced by an approximate 1:1 ratio for ecdysone 3,22-diacetate prepared in the present work by deacetylation of the triacetate derivative. Chromatography on silica gel t.l.c. conveniently separated the 5 α /5 β epimers, but reversed-phase h.p.l.c. was required to separate ecdysone 2,22-diacetate from ecdysone 3,22-diacetate.

Oxidation of ecdysone 3,22-diacetate at C-2 was undertaken using dimethyl sulphoxide/trifluoroacetic anhydride oxidation at low temperature (Huang *et al.*, 1976). Conditions were chosen to minimize contact with alkali to prevent possible enolization of the 2-oxo group, thereby removing ^3H at C-1. Even so, some equilibration of the C-5 proton was suggested from the presence of another u.v. absorbing band chromatographing just ahead ($R_F = 0.37$) of 2-oxo-ecdysone 3,22-diacetate ($R_F = 0.33$) on t.l.c. Although this putative 5 α epimer was in approximately equal amounts to the required product, it could be reasonably expected that the C-5 hydrogen atom is considerably more acidic than hydrogen at C-1 on the basis of relief of strain in ring B of the steroid nucleus. Therefore, no extensive equilibration would be expected to occur at C-1.

Oxidation of the labelled ecdysone 3,22-diacetate ($^3\text{H}/^{14}\text{C} = 1.90$) derived from expt. 2 yielded 2-oxoeecdysone 3,22-diacetate ($^3\text{H}/^{14}\text{C} = 1.17$) with elimination of 38.4% of the ^3H . It is likely that this ^3H was almost exclusively removed from C-2 under the oxidation conditions used, with negligible loss of ^3H from C-1 by enolization of the 2-oxo group produced. This ^3H eliminated from C-2 accounts for all the ^3H originally present at the 2 α -position (36.8%) in the initial [$1\alpha,2\alpha$ - $^3\text{H}(n)$]cholesterol (Table 1). During the oxidation of ecdysone 2,22-diacetate (VI) under identical conditions, yielding 3-oxoeecdysone 2,22-diacetate (IX), carried out for control purposes, there was no significant

decrease in the $^3\text{H}/^{14}\text{C}$ ratio (Table 1) as expected.

In conclusion, the results taken together indicate that the 2 α -hydrogen of cholesterol is retained during 2 β -hydroxylation in the formation of ecdysone. Thus, the mechanism of hydroxylation at C-2 involves direct replacement of the 2 β -hydrogen with retention of configuration at C-2. This is in agreement with the mechanism of other steroid hydroxylations which have been studied (Holland, 1982).

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