Mechanism of hydroxylation at C-22 during the biosynthesis of ecdysteroids in the locust *Schistocerca gregaria*

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1. The fates of the 22-*pro-R* and 22-*pro-S* hydrogen atoms of cholesterol during the biosynthesis of ecdysteroids in the ovaries of *Schistocerca gregaria* were investigated. 2. Two stereospecifically labelled cholesterol species, obtained by incubating 3*R*,2*R*- and 3*R*,2*S*-[2-¹⁴C, 2-³H]mevalonic acid with rat liver preparations, were administered, in turn, to maturing adult female locusts and the radiolabelled ecdysteroid conjugates isolated from the eggs. Enzymic hydrolysis of the conjugates yielded free ecdysteroids, from which ecdysone was purified. 3. Derivative formation and oxidation at C-22 of both ecdysone samples indicated that the 22-*pro-R* and 22-*pro-S* hydrogen atoms of cholesterol were stereospecifically eliminated and retained respectively during ecdysteroid formation. This indicates that C-22 hydroxylation in ecdysone biosynthesis is direct and occurs with retention of configuration.

In hydroxylation reactions involving prochiral methylene groups, either direct replacement of the hydrogen atom from the position hydroxylated, or a Walden-type inversion with elimination of the hydrogen atom from the alternate position to the one hydroxylated, is possible. Direct hydroxylation with retention of configuration at the respective carbon atom is observed in many steroid hydroxylations (see, for example, Bergstrom *et al.*, 1958; Hayano *et al.*, 1958; Shimizu *et al.*, 1962; Dorfman & Ungar, 1965). However, there is little information on the stereochemistry of steroid hydroxylation in insects. Conversion of the insect moulting hormone, ecdysone (I), into 20-hydroxyecdysone (II) involves a direct hydroxylation at C-20, since the 20*R* configuration is retained. Also, hydroxylation of the ecdysteroid molecule at C-25 does not appear to involve hydration of a Δ^{24} bond (Rees *et al.*, 1981). The present paper concerns the mechanism of hydroxylation at C-22 during the formation of ecdysone (I) in the ovaries of *Schistocerca americana gregaria*. The hydroxy group at C-22 in ecdysone (I) has been shown to be in the *R* configuration by X-ray crystallographic analysis (Huber & Hoppe, 1965).

Cholesterol formed in rat liver by biosynthetic incorporation of either [2-¹⁴C, (2*R*)-2-³H]₁mevalonic acid (V) or [2-¹⁴C, (2*S*)-2-³H]₁mevalonic acid (VI) is labelled stereospecifically with ³H at C-22 as

shown in (XI) and (XII) respectively (Cornforth *et al.*, 1966; Caspi & Zander, 1969; Ramm & Caspi, 1969; Goad, 1970; Akhtar *et al.*, 1972). Therefore, in the present work, cholesterol samples labelled by rat liver enzymes from these mevalonic acid species (V) and (VI) as well as from [2-¹⁴C, 2-³H]₂mevalonic acid (IV) were incorporated into ecdysone (I) in the locust *S. gregaria*. After purification of the ecdysone (I) by formation of the 2,3-acetonide derivative, any remaining ³H at C-22 was located by production of 22-oxoecdysone-2,3-acetonide.

Comparatively large quantities of ecdysteroids are produced in ovaries of maturing adult females of *S. gregaria*, primarily as phosphate conjugates (Isaac

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et al., 1982), which are passed into the eggs (Gande *et al.*, 1979; Dinan & Rees, 1981a). These conjugates in newly-laid eggs can be hydrolysed by a crude enzyme preparation from *Helix pomatia* to yield primarily ecdysone (I), with lesser quantities of 2-deoxyecdysone (III) and 20-hydroxyecdysone (II) as well as traces of other ecdysteroids (Dinan & Rees, 1981a; Isaac *et al.*, 1981). Since good incorporation of radioactive cholesterol into ecdysteroids can be obtained in ovaries of maturing *S. gregaria*, this system was used for formation of ecdysone from the stereospecifically labelled cholesterol species. A preliminary account of this work has appeared (Rees *et al.*, 1981).

Materials and methods

Nomenclature

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

hydroxy-5 β -cholest-7-en-6-one; 2-deoxyecdysone (III), (22*R*)-3 β ,14,22,25-tetrahydroxy-5 β -cholest-7-en-6-one.

Chemicals

DL-[2- ${}^{14}\text{C}$]Mevalonolactone (sp. radioactivity 10.3 Ci/mol), DL-[2- ${}^3\text{H}_2$]mevalonolactone (93 Ci/mol), [3*R*(2*S*)-2- ${}^3\text{H}_1$ + 3*S*(2*R*)-2- ${}^3\text{H}_1$]-mevalonolactone (250 Ci/mol) and [3*R*(2*R*)-2- ${}^3\text{H}_1$ + 3*S*(2*S*)-2- ${}^3\text{H}_1$]-mevalonolactone (175 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Authentic samples of ecdysone were given by Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand. Cholesterol and squalene were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Berberine sulphate and mevalonic acid lactone (mevalonolactone) were purchased from The Sigma Chemical Co., Poole, Dorset, U.K.

T.l.c.

The radiochemical purity of portions of the mevalonolactones mixed with carrier mevalono-

lactone (10 μ g) was ascertained by t.l.c. on silica gel (0.25 mm thick, Kieselgel G; E. Merck A.-G., Darmstadt, Germany). Plates were developed in toluene/ethanol (1:1, v/v) and scanned for radioactivity by using a Panax RTLS-1A instrument fitted with a P7900A ratemeter. In all cases the labelled mevalonolactones were >90% pure.

Sterols were separated by t.l.c. on (i) silica gel (0.5 mm thick, Kieselgel G) developed in chloroform, and on (ii) reversed-phase silica gel [0.25 mm thick, Kieselguhr G plates immersed in a solution of 5% (v/v) paraffin in hexane and air-dried] developed in paraffin-saturated acetone/water (4:1, v/v). Sterols and squalene were eluted from the supports with dry redistilled diethyl ether.

Preparative separations of ecdysteroids were performed on silica gel (0.5 mm thick Kieselgel GF₂₅₄) developed twice in chloroform/ethanol (4:1, v/v). For ecdysone derivatives, chloroform/ethanol mixtures (9:1, v/v and 12:1, v/v) were used for development. Ecdysteroids and derivatives were detected under u.v. light and recovered by elution with chloroform/methanol (1:1, v/v).

Non-u.v.-absorbing compounds were detected on developed plates sprayed with a 1% (w/v) solution of berberine sulphate in acetone/methanol (1:1, v/v), by viewing under u.v. light (350 nm).

High-performance liquid chromatography

A Waters modified ALC/100 liquid chromatograph fitted with an M6000A pumping system was used, connected in line to a refractometer model R401. Samples were introduced on to the column via a U6K valve injector. High-performance liquid chromatography of the labelled mevalonolactones (+carrier) was performed by using a μ -Porasil column (30 cm $\times$ 4.0 mm int. diameter; Waters Associates, Northwich, Cheshire, U.K.) eluted with toluene/chloroform (1:1, v/v) at 4 ml/min. Fractions (2 ml) were collected, evaporated to dryness under N₂ and radioassayed. All labelled mevalonolactones were >90% pure.

G.l.c.

A Pye series 104 gas chromatograph fitted with a flame-ionization detector was used. The effluent from a 2.1 m $\times$ 4 mm int. diameter glass column packed with 3% OV-1 on Gas Chrom Q (100–120 mesh) was passed into a stream splitter so that 10% of the emerging gas passed into the detector and the remainder was collected at ambient temperature in glass capillary tubes, which were changed at 1 min intervals. The tubes were broken into scintillation vials and radioassayed. With a column temperature of 268°C and a carrier gas (N₂) flow rate of 43 ml/min, cholesterol had a retention time of 21 min.

Radiochemical methods

Radioactivity was measured on an Inter technique three-channel liquid-scintillation spectrometer, model ABAC SL30, to an accuracy of 1%. Samples were dissolved in 10 ml of a dioxan-based scintillation cocktail containing 15 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole and 100 g of scintillation-grade naphthalene/litre of dioxan. ³H and ¹⁴C radioactivities are quoted after corrections for background, counting efficiency and quenching had been applied. For radioassay of cholesterol fractions collected from g.l.c., 5 ml of a toluene-based scintillation solution [8 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/litre of toluene] was used.

Analytical methods

Mass spectra were recorded on a V.G. Micro-mass 70-70F mass spectrometer in the electron-impact mode, whereas high-resolution mass spectra were obtained with a V.G. Micro-mass ZAB-1F mass spectrometer.

¹H N.m.r. spectra were obtained in [²H]chloroform at 220 MHz on a Perkin–Elmer R-34 n.m.r. spectrometer.

I.r. spectra were recorded for samples in KBr discs or as solid films on NaCl discs with a Perkin–Elmer Infracord model 237 spectrometer.

Biosynthesis of [¹⁴C,³H]cholesterol labelled with ³H at C-22

[2-¹⁴C, 2-³H₂]Mevalonolactone was used in an initial experiment for the biosynthesis and purification of cholesterol and subsequent conversion into ecdysteroids before using stereospecific [³H]-mevalonolactone species. Incorporation of this mevalonolactone into cholesterol is given as a typical procedure. The method of Bucher & McGarahan (1956) as modified by Popják (1969) was used with certain changes for the incorporation of mevalonic acid into cholesterol. Two young female albino rats derived from the CFHB strain (100–125 g) were starved for 24 h, killed and their livers removed quickly and chilled on ice. Subsequent manipulations were performed at 0–4°C. The tissue was sliced with scissors and phosphate buffer (0.1 M, pH 7.5) containing 5 mM-MgCl₂ and 30 mM-nicotinamide was added (0.25 ml/g) before homogenization in a loose-fitting Teflon/glass (Potter–Elvehjem) homogenizer (15 full passes at 250 rev./min). The thick homogenate produced was centrifuged at 750 g for 20 min (Sorvall RC-2B). The supernatant was passed through four layers of cotton gauze to remove fat and re-centrifuged at 10000 g for 30 min. The resulting supernatant (S₁₀ postmitochondrial fraction; 16 ml) was decanted through gauze and added to a 50 ml conical flask

containing the labelled mevalonolactones ($4\mu\text{Ci}$ of DL-[2- ^{14}C]mevalonolactone + $21\mu\text{Ci}$ of DL-[2- $^3\text{H}_2$]mevalonolactone) dissolved in 2 ml of the homogenization buffer supplemented with 3 mM-ATP, 1 mM-NADP⁺ and 3 mM-glucose-6-phosphate (final concentrations).

The mixture was incubated for 4 h at 37°C in a reciprocating water bath, with the addition of ATP, NADP⁺ and glucose 6-phosphate being repeated after 1 and 2 h. The incubation was stopped by addition of ethanolic KOH to produce a final volume of 50 ml of 6% KOH (w/v) in 50% ethanol (v/v). Pyrogallol (0.5 g) was added and the saponification mixture was refluxed for 30 min and left to cool overnight at 4°C.

The non-saponifiable fraction was extracted into diethyl ether ($\times 4$) and the combined diethyl ether extracts washed with water until no longer alkaline. The diethyl ether extract was rotary-evaporated to dryness (21.8 mg, $0.86\mu\text{Ci}$ of ^{14}C) and carrier squalene (2 mg) was added. The mixture was applied to preparative silica-gel t.l.c. plates alongside appropriate markers. After development of the plates with chloroform, the radioactive bands co-chromatographing with cholesterol (8.1 mg, $0.53\mu\text{Ci}$ of ^{14}C) and squalene (1.3 mg, $0.074\mu\text{Ci}$ of ^{14}C) were recovered.

The cholesterol band was then subjected to preparative reversed-phase t.l.c. on paraffin-coated silica-gel t.l.c. plates to remove desmosterol. Paraffin eluted with the cholesterol was removed by chromatography of the material dissolved in light petroleum (b.p. 40–60°C) on alumina (5 g, acid-washed, Brockmann grade III). The column was developed sequentially with the following solvents (50 ml each); (i) light petroleum (which removed paraffin), (ii) 6% (v/v) diethyl ether in light petroleum; (iii) 30% (v/v) diethyl ether in light petroleum, which eluted the cholesterol (4.0 mg, $0.43\mu\text{Ci}$ of ^{14}C). All three labelled cholesterol species (X, XI and XII) appeared homogeneous (>95%) on t.l.c. and g.l.c. analyses. Portions of each purified cholesterol were diluted with carrier cholesterol and recrystallized to constant specific radioactivity from chloroform/methanol (Table 1).

Purification of labelled squalene species

Squalene recovered from the initial t.l.c. of the non-saponifiable fraction was rechromatographed on silica-gel t.l.c. plates, developed with hexane and the squalene band eluted. Carrier squalene (30 mg) was then added to the labelled material and the thiourea adduct was prepared. To the squalene dissolved in butan-1-ol (0.5 ml) was added a saturated solution of thiourea in methanol (2.5 ml) and the mixture was left at 4°C for 10 h. Crystals of the adduct were recovered by filtration, washed with cold methanol on a glass sinter and the squalene was

regenerated by the addition of water (5 ml). The free squalene was extracted from the thiourea solution with diethyl ether and evaporated to dryness. The adduct was formed several times, taking small portions of the regenerated squalene for radioassay (Table 1).

Biosynthesis of ecdysteroids from [^{14}C , ^3H]cholesterols

S. gregaria were maintained as described previously (Dinan & Rees, 1981a).

Labelled cholesterol were injected into locusts as emulsions in Tween 80 (one part of cholesterol to 10 parts of Tween) in insect Ringer solution (Bodenstein, 1946) to yield 5% (w/w) Tween solutions. Sonication (3×10 s bursts on ice in a Kerry KS-200 ultrasonication bath) was necessary for complete suspension.

Adult female locusts (15–20) were injected with the radioactive cholesterol and the egg pods were collected as described previously (Dinan & Rees, 1981b).

Purification of [^{14}C , ^3H]ecdysteroids

The method of Dinan & Rees (1981a) was used with modifications. Egg pods (11 g) from 15 injected locusts were macerated in methanol (25 ml) with a Polytron homogenizer. After centrifugation of the resulting brei, the supernatant was decanted and the residue re-extracted successively with methanol, then twice with 70% (v/v) methanol/water, followed by methanol again, each time centrifuging to allow removal of the supernatant. The combined supernatant fractions were evaporated to dryness under vacuum using butan-1-ol as an azeotrope to remove water. The dry extract was partitioned between hexane and 70% (v/v) methanol/water to separate apolar lipids from ecdysteroid material. The residue from the aqueous methanol phase was adsorbed on to Celite (Koch-Light Laboratories; prewashed in chloroform and methanol, and dried, 0.5 g) from methanol by rotary evaporation.

The extract (on Celite) was applied to a 4 g silicic acid column (Kieselgel 60; 70–230 mesh) packed as a slurry in chloroform and containing a shallow bed of acid-washed sand. The column was developed sequentially with the following solvents (60 ml each): chloroform (which eluted any residual non-polar material); 30% (v/v) methanol in chloroform (which eluted free ecdysteroids); 80% (v/v) methanol in chloroform (which eluted conjugated ecdysteroids). The conjugate fraction [eluted by 80% (v/v) methanol in chloroform] was evaporated to dryness under vacuum, redissolved in water (1 ml) and transferred to a test tube. To this was added an enzyme solution consisting of 2 mg of arylsulphatase (*Helix pomatia*, type H-1; Sigma) and 2 mg of acid phosphatase (ex potato, type IV; Sigma) in 1 ml

Table 1. Incorporation of [^3H , ^{14}C]mevalonic acids into squalene and cholesterol in rat liver and subsequent incorporation of the cholesterol samples into ecdysone in *Schistocerca gregaria*
For further details see the text.

Substrate	Recrystallization	(3R)-[2- ^{14}C , 2, 3- $^3\text{H}_2$]- Mevalonic acid (IV)		(3R)-[2- ^{14}C , (2R)-2- $^3\text{H}_1$]Mevalonic acid (V)			(3R)-[2- ^{14}C , (2S)-2- $^3\text{H}_1$]Mevalonic acid (VI)		
		$^3\text{H}/^{14}\text{C}$ 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 †
Squalene	1			5555	3.51		260	3.65	
	2†			4833	3.33		254	3.64	
	3			6526	3.44		254	3.79	
	4			5154	3.30		202	3.62	
	5			4089	3.23	6:6	247	3.50	6:6
Cholesterol	—	5.10	8:5		3.13		—	2.37	
	1			3124	3.08		2457	2.38	
	2			3154	3.05		2704	2.41	
	3			3125	3.20		2685	2.41	
	4			3298	3.11		2348	2.34	
Ecdysone Ecdysone-2,3- acetonide	5			3160	3.15	5:5	2661	2.40	3:5
	—	3.60	5.65:5		1.95		—	2.21	
	—	3.74	5.87:5						3:10:5 2.80:5
	1			1088	1.83		523	2.05	
	2			1164	1.83		647	1.94	
22-Oxoecdysone- 2,3-acetonide	3			1006	1.80	2.78:5 2.91:5	628	1.94	2.72:5 2.46:5
	—	3.08	4.83:5						
	1			842	1.78		651	1.49	
	2			750	1.85		781	1.38	
	3			815	1.79	2.78:5 2.91:5	504	1.51	2.02:5 1.82:5

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

† Based on the $^3\text{H}/^{14}\text{C}$ ratio of squalene.

‡ Squalene was purified via the thiourea adduct.

of triethanolamine hydrochloride/NaOH buffer (0.2 M, pH 7.0). The mixture was incubated for 24 h at 37°C. Protein was then precipitated with ethanol and the soluble fraction obtained after centrifugation and washing the pellet twice with ethanol was adsorbed on to Celite (0.5 g) and applied to a silicic acid column as described above. In this instance 2 g of the support and 20 ml of the eluting solvents were used. Ecdysteroids released by hydrolysis of the conjugated fraction now eluted with 30% (v/v) methanol in chloroform. Ecdysone was separated from other ecdysteroids by t.l.c. on silica gel by developing twice with chloroform/ethanol (4:1, v/v).

To locate residual ^3H label at C-22 in ecdysone, 22-oxoeecdysone-2,3-acetonide was prepared. Derivatives were characterized initially by using unlabelled ecdysone. Labelled ecdysone samples were diluted with carrier material before derivative formation and the products were recrystallized to constant specific radioactivity from ethyl acetate/hexane (Table 1).

Preparation of ecdysone-2,3-acetonide

Ecdysone-2,3-acetonide was prepared by the method of Galbraith & Horn (1969), purified by t.l.c. with chloroform/ethanol (9:1, v/v) as solvent, and after recrystallization had the following properties: ^1H n.m.r. δ (p.p.m.) (^{12}H chloroform, 220 MHz) 0.68 (3 H, s, C-18 methyl), 0.94 (3 H, d, $J = 9$ Hz, C-21 methyl), 0.98 (3 H, s, C-19 methyl), 1.24 (2×3 H, s, C-26/C-27 methyls), 1.33 (3 H, s, acetonide methyl), 1.49 (3 H, s, acetonide methyl), 2.82 (1 H, m, $w_1 = 24$ Hz, C-9 proton), 3.68 (1 H, m, $w_1 = 18$ Hz, C-22 proton), 4.28 (2 H, m, $w_1 = 13$ Hz, C-2/C-3 proton), 5.83 (1 H, m, $w_1 = 4$ Hz, C-7 proton); i.r. ν_{max} (solid film on NaCl disc) 1640 cm^{-1} (α,β -unsaturated C=O); m/z 504 (M^+ , 1.9%), 489 ($[M-\text{CH}_3]^+$, 1.8%), 486 ($[M-\text{H}_2\text{O}]^+$, 8%), 471 ($[M-\text{H}_2\text{O}-\text{CH}_3]^+$, 6%), 468 ($[M-2\text{H}_2\text{O}]^+$, 12%), 453 ($[M-2\text{H}_2\text{O}-\text{CH}_3]^+$, 4%), 388 ($[M-(\text{C}-22 \rightarrow \text{C}-27) + \text{H}]^+$, 7%), 341 ($[M-\text{H}_2\text{O}-(\text{C}-20 \rightarrow \text{C}-27)]^+$), 340 ($[M-\text{H}_2\text{O}-(\text{C}-20 \rightarrow \text{C}-27)-\text{H}]^+$, 11%), 319 (10%), 165 (17%), 126 ($[(\text{C}-20 \rightarrow \text{C}-27)-\text{H}_2\text{O}-\text{H}]^+$, 21%), 109 (27%), 99 ($[(\text{C}-22 \rightarrow \text{C}-27)-\text{H}_2\text{O}]^+$, 100%), 93 (25%), 81 ($[(\text{C}-22 \rightarrow \text{C}-27)-2\text{H}_2\text{O}]^+$, 62%).

Preparation of 22-oxoeecdysone-2,3-acetonide

Activated MnO_2 (300 mg; Attenburrow *et al.*, 1952) was added to ecdysone-2,3-acetonide (200 mg) dissolved in acetonitrile (20 ml) and the reaction stirred at room temperature overnight. The MnO_2 was removed by centrifugation and the evaporated supernatant subjected to t.l.c. on silica gel, developing with chloroform/ethanol (12:1, v/v). A single product (>60% yield) was obtained and on recrystallization from ethyl acetate/hexane had the

following properties: ^1H n.m.r. δ (p.p.m.) (^{12}H -chloroform, 220 MHz) 0.67 (3 H, s, C-18 methyl), 0.98 (3 H, s, C-19 methyl), 1.13 (3 H, d, $J = 7$ Hz, C-21 methyl), 1.23 (2×3 H, s, C-26/C-27 methyls), 1.33 (3 H, s, acetonide methyl), 1.50 (3 H, s, acetonide methyl), 2.65 (1 H, broad, m, C-20) (identified on decoupling C-21 methyl), 2.88 (1 H, m, $w_1 = 24$ Hz, C-9 proton), 4.30 (2 H, m, $w_1 = 13$ Hz, C-2/C-3 proton), 5.85 (1 H, m, $w_1 = 4$ Hz, C-7 proton); i.r. ν_{max} (KBr disc) 1640 cm^{-1} (α,β -unsaturated C=O) and 1710 cm^{-1} (C=O); m/z 502 (M^+ , 1.5%), 487 ($[M-\text{CH}_3]^+$, 4%), 484.3187 ($[M-\text{H}_2\text{O}]^+$, 19%), $\text{C}_{30}\text{H}_{44}\text{O}_5$ requires 484.3191), 469 ($[M-\text{H}_2\text{O}-\text{CH}_3]^+$, 8%), 466 ($[M-2\text{H}_2\text{O}]^+$, 10%), 451 ($[M-2\text{H}_2\text{O}-\text{CH}_3]^+$, 7%), 387 ($[M-(\text{C}-22 \rightarrow \text{C}-27)]^+$, 5%), 341 ($[M-\text{H}_2\text{O}-(\text{C}-20 \rightarrow \text{C}-27)]^+$), 340 ($[M-\text{H}_2\text{O}-(\text{C}-20 \rightarrow \text{C}-27)-\text{H}]^+$, 22%), 152 (86%), 126 ($[(\text{C}-20 \rightarrow \text{C}-27)-\text{H}_2\text{O} + \text{H}]^+$, 100%), 109 (34%), 97 ($[(\text{C}-22 \rightarrow \text{C}-27)-\text{H}_2\text{O}]^+$, 53%), 93 (C_7H_9 , 27%), 81 (27%).

Results and discussion

Transformation of [$^{14}\text{C},^3\text{H}$]mevalonic acids to cholesterol

All ^3H and ^{14}C atoms from C-2-labelled mevalonic acid species are retained on formation of squalene and lanosterol in rat liver. During the subsequent formation of cholesterol, both ^3H and ^{14}C are eliminated to yield the expected $^3\text{H}/^{14}\text{C}$ atomic ratios of 5:5 for cholesterol (XI) derived from [$2\text{-}^{14}\text{C},(2R)\text{-}2\text{-}^3\text{H}_1$]mevalonic acid (V) and 3:5 for cholesterol (XII) obtained from [$2\text{-}^{14}\text{C},(2S)\text{-}2\text{-}^3\text{H}_1$]mevalonic acid (VI) (Goad, 1970). However, comparison of these expected values with the observed ratios in Table 1 indicates an increase in the $^3\text{H}/^{14}\text{C}$ atomic ratio (i.e. 3:5 $\rightarrow$ 3.33:5) in cholesterol (XII) derived from [(2S)-2- $^3\text{H}_1$]mevalonic acid (VI) and a concomitant decrease in the ratio of the other cholesterol (XI) (i.e. 5:5 $\rightarrow$ 4.79:5). Analogous slight anomalies in the labelling pattern of sterols formed from [$2\text{-}^3\text{H}$]mevalonic acids have been observed previously (Smith *et al.*, 1968; Bimpson *et al.*, 1969) and are interpreted as being indicative of partial randomization of ^3H label during the reversible isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase reaction (Goad, 1970). The reversibility of this isomerase reaction also leads to some elimination of ^3H from C-2 of mevalonic acid. However, the latter effect is not detected in the present work since the $^3\text{H}/^{14}\text{C}$ ratios of isoprenoids are not compared with that of the initial mevalonic acid. To minimize the randomization of ^3H , a comparatively short incubation time (4 h) was chosen. Owing to this randomization a small but significant proportion of the isoprenoid units formed from [(2R)-2- $^3\text{H}_1$]-

mevalonic acid (V) will have the labelling pattern of those derived from [(2*S*)-2- ${}^3\text{H}_1$]mevalonic acid (VI) and vice versa. The effect of this randomization is not apparent in squalene, since there is no loss of ${}^3\text{H}$ during its formation. On subsequent formation of cholesterol (XI; theoretical ${}^3\text{H}/{}^{14}\text{C}$ atomic ratio = 5:5) derived from [2- ${}^{14}\text{C}$, (2*R*)-2- ${}^3\text{H}_1$]mevalonic acid (V) some cholesterol will be labelled as if it were derived from the 2*S* anomer (VI) (theoretical ${}^3\text{H}/{}^{14}\text{C}$ atomic ratio = 3:5) and vice versa. This explains the low observed ratio for the cholesterol formed from [2- ${}^{14}\text{C}$, (2*R*)-2- ${}^3\text{H}_1$]mevalonic acid (Table 1) and the correspondingly high ratio for that formed from [2- ${}^{14}\text{C}$, (2*S*)-2- ${}^3\text{H}_1$]mevalonic acid (Table 1). Therefore, atomic ratios of ecdysone and derivatives have been based on both squalene and cholesterol, the latter being preferred.

Conversion of cholesterol into ecdysone

During the transformation of cholesterol (XI) formed from [2- ${}^{14}\text{C}$, (2*R*)-2- ${}^3\text{H}_1$]mevalonic acid (V) into ecdysone (XIV), the ${}^3\text{H}/{}^{14}\text{C}$ atomic ratio (based on that expected for cholesterol) decreases from 5:5 to 3.11:5 (Table 1). One of the ${}^3\text{H}$ atoms eliminated is located at the 7 β -position of cholesterol, since it has been demonstrated that this hydrogen is stereospecifically removed during introduction of the Δ^7 bond in ecdysteroids (Cook *et al.*, 1973). That the other ${}^3\text{H}$ atom eliminated during ecdysone formation from the cholesterol (XI) is lost from C-22 is indicated by the fact that the ${}^3\text{H}/{}^{14}\text{C}$ ratio of the corresponding ecdysone-2,3-acetonide derivative (XVII) does not change during oxidation at C-22 to 22-oxoecdysone-2,3-acetonide (XX) (Table 1). Since cholesterol (XI) formed from [2- ${}^{14}\text{C}$, (2*R*)-

2-³H₁]mevalonic acid (V) is labelled at the 22-*pro-R* position, this hydrogen atom is eliminated on introduction of the 22-hydroxy group of ecdysone.

When cholesterol (XII) formed from [2-¹⁴C,(2*S*)-2-³H₁]mevalonic acid (VI) is incorporated into ecdysone (XV), the ³H/¹⁴C atomic ratio (based on that expected for cholesterol) does not change appreciably (3.00:5 → 2.80:5). That a ³H atom is located at C-22 in this ecdysone sample is indicated by the loss of one ³H atom on transformation to the 22-oxoecdysone-2,3-acetonide derivative (XXI) (³H/¹⁴C atomic ratio, 1.82:5; Table 1). Thus, the 22-*pro-S* hydrogen atom of cholesterol (XII) is retained during ecdysone formation.

During the biosynthesis of ecdysone from cholesterol (XI) labelled from [2-¹⁴C,(2*R*)-2-³H₁]mevalonic acid (V), the actual ³H/¹⁴C atomic ratio in ecdysone (3.11:5) would be expected to be higher than the theoretical value (3.0:5), since the cholesterol contaminant labelled from [2-¹⁴C,(2*S*)-2-³H₁]mevalonic acid (VI) arising by randomization of label will not have lost any ³H during this transformation. By using the same argument, a correspondingly lower ³H/¹⁴C ratio than theoretical would be expected in ecdysone formed from the other mevalonic acid species, which indeed is observed (Table 1). However, the radiochemical purity of both ecdysone species (from t.l.c.) before acetonide formation must remain in doubt, as significant but unaccounted-for decreases in ³H/¹⁴C ratios of both ecdysteroid samples on acetonide formation were observed.

Although the experiment with [2-¹⁴C,2-³H₂]mevalonic acid (IV) does not provide information on the mechanism of hydroxylation at C-22 in ecdysone, the results (Table 1) demonstrate that transformation of the cholesterol (X) formed from this mevalonic acid into ecdysone involves elimination of two ³H atoms, one of which is removed from C-22; the other ³H atom is eliminated from C-7. This is in agreement with the combined results for the stereospecifically labelled [2-³H₁]mevalonic acid species.

In conclusion, the present results show clearly that the 22-*pro-S* and 22-*pro-R* hydrogen atoms of cholesterol are retained and eliminated respectively during ecdysone formation. Therefore, the mechanism of hydroxylation at C-22 involves direct replacement of the 22-*pro-R* hydrogen atom with retention of configuration about C-22.

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