Mode of Formation of Cholesta-5,7-dien-3 β -ol from Cholest-5-en-3 β -ol by Larvae of *Calliphora erythrocephala*

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(Received 4 June 1975)

1. The conversion of cholest-5-en-3 β -ol (cholesterol) into cholesta-5,7-dien-3 β -ol by axenic *Calliphora erythrocephala* larvae was demonstrated. 2. The transformation is probably direct ($\Delta^5 \rightarrow \Delta^{5,7}$) and does not involve a Δ^0 intermediate ($\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$). 3. Δ^7 -bond formation involves the stereospecific elimination of the 7 β hydrogen atom. 4. The relative amounts of free and esterified sterols were determined in larvae grown on cholesterol as sole sterol source and on 5 α -cholestan-3 β -ol supplemented with minimal amounts of cholesterol. 5. The significance of the results is assessed in relation to the probable role of cholesta-5,7-dien-3 β -ol as an intermediate in the biosynthesis of ecdysones.

Insects cannot synthesize sterols *de novo* (Clayton, 1964), and hence have a dietary sterol requirement which is met by cholesterol in carnivorous insects such as *Calliphora erythrocephala*. Clark & Bloch (1959a) showed that the minimum amount of cholesterol required to facilitate growth of *Dermestes vulpinus* was 0.017% of the total diet. They also found that if the sterol source was supplemented with another 'sparing' sterol, the amount of cholesterol needed for growth would be decreased to only 3% of the total sterol. However, the 'sparing sterol' could not by itself satisfy the growth requirement of the insect, a minimal amount of cholesterol being always essential.

An important metabolic role of cholesterol is its function as a precursor of the moulting hormones, ecdysone (I) and ecdysterone (II) (Karlson & Hoffmeister, 1963; Galbraith *et al.*, 1970). Cholesta-5,7-dien-3 β -ol has been implicated as the first transformation product of cholesterol during biosynthesis of the ecdysones in *C. erythrocephala* (Galbraith *et al.*, 1970).

Formation of cholesta-5,7-dien-3 β -ol from cholesterol under axenic conditions has been reported for the cockroach *Blattella germanica* (Robbins *et al.*, 1964) and the housefly, *Musca domestica* (Monroe *et al.*, 1967).

After sterol analysis of insects, Martin & Carls (1968) suggested that the formation of cholesta-5,7-dien-3 β -ol ($\Delta^{5,7}$) from cholesterol (Δ^5) occurred via 5 α -cholestan-3 β -ol (Δ^0) and 5 α -cholest-7-en-3 β -ol (Δ^7), i.e. $\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$.

The conversion of 5 α -cholestan-3 β -ol into 5 α -cholest-7-en-3 β -ol ($\Delta^0 \rightarrow \Delta^7$) in cockroaches (Louloudes *et al.*, 1962; Clayton & Edwards, 1963), the conversion of the latter sterol into cholesta-5,7-

dien-3 β -ol ($\Delta^7 \rightarrow \Delta^{5,7}$) in *Drosophila pachae* (Goodnight & Kircher, 1971) and the saturation of the double bond in Δ^5 -sterols ($\Delta^5 \rightarrow \Delta^0$) in *Epilachna varivestis* (Svoboda *et al.*, 1974) have been demonstrated, but the overall pathway has not been demonstrated in a single insect species.

The transformation of 5 α -cholestan-3 β -ol into cholesterol ($\Delta^0 \rightarrow \Delta^5$) in *Thermobia domestica* (Thompson *et al.*, 1973) and of $\Delta^{5,7}$ -sterols into Δ^5 -sterols in several insect species (Clark & Bloch, 1959b; Svoboda *et al.*, 1972) has also been reported. The transformations which have been demonstrated in various insect species are thus:

The present paper reports investigations on the sterol nuclear double-bond transformations in one species of insect, *C. erythrocephala*, under axenic conditions, in an attempt to elucidate the mechanism of formation of cholesta-5,7-dien-3 β -ol. Under non-axenic conditions the contribution of associated microflora to the total sterol metabolism of the insect can be quite appreciable.

During the biosynthesis of ecdysterone (II) from cholesterol, the introduction of the Δ^7 -bond involves elimination of the 7 β -hydrogen atom (Cook *et al.*, 1973). We have now investigated the stereochemistry of the introduction of the Δ^7 -bond in the formation of cholesta-5,7-dien-3 β -ol from cholesterol.

We also report results on the relative amounts of constituent free and esterified sterols of *C. erythrocephala* grown on diets containing cholest-5-en-3 β -ol as sole sterol source and diets supplemented with a 'sparing sterol'. Part of this work has previously been reported in preliminary form (Johnson *et al.*, 1974).

Experimental

Nomenclature

The trivial names, ecdysone (I) and ecdysterone (II) have been used. The systematic names are: ecdysone, 2 β ,3 β ,14 α ,22*R*,25-pentahydroxy-5 β -cholest-7-en-6-one; ecdysterone, 2 β ,3 β ,14 α ,20,22*R*,25-hexahydroxy-5 β -cholest-7-en-6-one.

Chemicals

Cholesterol and 5 α -cholestan-3 β -ol were bought from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Cholesta-5,7-dien-3 β -ol was bought from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. 5 α -cholest-7-en-3 β -ol was prepared by hydrogenation of cholesta-5,7-dien-3 β -ol by using tris-(triphenylphosphin)rhodium chloride catalyst (Birch & Walker, 1966). All non-radioactive

sterols were recrystallized before use. [4-¹⁴C]-Cholesterol (60mCi/mmol) was bought from The Radiochemical Centre, Amersham, Bucks., U.K. 5 α -[4-¹⁴C]cholestan-3 β -ol was prepared by hydrogenation of [4-¹⁴C]cholesterol by using platinum dioxide catalyst (Hershberg *et al.*, 1951). [7 α -³H]-Cholesterol and [7 β -³H]cholesterol were prepared by Dr. I. F. Cook (Cook *et al.*, 1973). The purity of all radioactive substrates was checked before use by radio-g.l.c. and radioautography.

Wessons salt mix was bought from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Agar was bought from Difco Laboratories, Detroit, Mich., U.S.A. Fat-free casein was bought from Fisons, Loughborough, Leics., U.K. Reddichlor tablets were bought from Reddish Detergents, Cheadle, Cheshire, U.K.

4-Phenyl-1,2,4-triazoline-3,5-dione was bought from Fluka Chemical Co., Glossop, Derbyshire, U.K.

T.l.c.

T.l.c. was normally performed on silica gel (0.5mm-thick Kieselgel G; E. Merck A-G., Darmstadt, Germany); development was in chloroform unless otherwise specified in the text. Constituent

sterols were separated as acetates on 20% (w/v) AgNO₃-impregnated silica gel (0.5mm-thick Kiesel-gel H). Development was without a wick in benzene-hexane (1:1, v/v) to separate Δ^5 - (R_F 0.61) and $\Delta^{5,7}$ -sterols (R_F 0.12) and in chloroform (ethanol-free)-hexane (1:1, v/v) for separation of Δ^0 -(R_F 0.57), Δ^7 -(R_F 0.34), Δ^5 -(R_F 0.26) and $\Delta^{5,7}$ -(R_F 0.05) sterols.

Compounds were detected under u.v. light after t.l.c. by spraying with a 10% (w/v) solution of Rhodamine 6G in acetone. Elution was performed by using dry redistilled diethyl ether unless otherwise stated.

Analytical methods

¹H n.m.r. spectra were determined for solutions in deuterated chloroform either at 60MHz with a Perkin-Elmer R-12 spectrometer or at 100MHz 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. U.v. spectra were determined for solutions in spectroscopically pure ethanol. I.r. spectra were determined for compounds as chloroform films between NaCl discs. G.l.c. was performed on a Pye Series 104 gas chromatograph by using 1% QF-1 on Gas-Chrom Q (100/120 mesh), 1.5% OV-1 on Shimalite W (100/120 mesh) and 3% OV-17 on Gas-Chrom Q (100/120 mesh) columns. These columns were also used on a Pye-Panax radio-g.l.c. proportional counting system for radiochemical analysis.

Radiochemical methods

Radioactivity was measured on an Intertechnique three-channel scintillation spectrometer, model ABACSL40. Samples were dissolved in 10ml of a dioxan scintillation solution containing 15g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) and 100g of naphthalene per litre of dioxan. ³H and ¹⁴C radioactivities are quoted after corrections for background, counting efficiency and quenching had been applied.

Growth of insects

A defined medium slightly modified from that of Monroe (1962) was made up to contain (w/w) yeast RNA (1%), powdered cellulose (5%), Wessons salt mix (4%), agar (20%) and fat-free casein (70%). This medium was exhaustively extracted with acetone to remove endogenous sterol contaminants before the addition of a defined B-vitamin supplement (Monroe, 1962). A specific minimal sterol supplement containing both radioactive and non-radioactive sterols was then added to the medium (Table 1). The medium was then sterilized by autoclaving for 20 min at 15lb/in² (104kPa).

Eggs of *C. erythrocephala* were collected within a period of 2h and sterilized by shaking several times in 25ml of 0.1% chlorine solution, prepared by dissolving 1 Reddichlor tablet in 1.0 litre of water. They were then transferred to the defined medium and incubation was carried out at 30°C. Final-instar larvae were harvested 7 days after hatching. Any contamination was readily apparent and infected flasks were discarded.

The stereochemistry of hydrogen elimination during the introduction of the Δ^7 -bond in the transformation of cholesterol into cholesta-5,7-dien-3 β -ol was investigated by using [4-¹⁴C]- plus [7 α -³H]-cholest-5-en-3 β -ol as substrates (60mCi/mmol of ¹⁴C; 41.5mCi/mmol of ³H). The species were mixed and a small portion was removed for recrystallization to obtain an accurate initial ³H/¹⁴C ratio. The remainder was then added to the defined medium in the same way as previously stated (Table 3). A parallel experiment was performed with [4-¹⁴C]cholesterol plus [7 β -³H]cholesterol (35.4mCi/mmol of ³H). The feeding procedure was identical with that described above.

Extraction procedures

About 150 larvae were washed well with hexane to remove surface lipid contamination from the medium, then macerated and extracted in acetone. The total lipid (589mg, 5.15 $\times$ 10⁶ d.p.m. of ¹⁴C) was applied to an alumina column (acid-washed Brockmann grade III) and eluted with increasing percentages of ether in light petroleum. Sterol esters were obtained in the 2% (v/v) ether in light petroleum fraction (5.1mg, 3.50 $\times$ 10⁴ d.p.m. of ¹⁴C) and free sterol in the 25% ether in light petroleum fraction (45.6mg, 4.23 $\times$ 10⁶ d.p.m. of ¹⁴C). The free sterol was purified by t.l.c. and acetylated overnight by using acetic anhydride in pyridine. Sterol acetates (22.9mg, 3.17 $\times$ 10⁶ d.p.m. of ¹⁴C) were worked up in the usual manner, purified on a small alumina column and separated by t.l.c. on AgNO₃-silica gel. Weights and radioactivities presented in the extraction procedures were those obtained from a typical extraction.

Identification of sterol acetates was carried out by g.l.c. and mass spectrometry for all sterols, and additionally by u.v. spectrometry for cholesta-5,7-dien-3 β -yl acetate and n.m.r. spectrometry for 5 α -cholest-7-en-3 β -yl acetate. Radiochemical composition of sterol acetates was investigated by using radio-g.l.c. and substantiated by recrystallization from chloroform-methanol to constant specific radioactivity. Sterol esters from alumina-column fractionation were saponified in ethanolic 6% (w/v) KOH with 1% pyrogallol added as an anti-oxidant. The resulting free sterols were purified and investigated in the same manner as the constituent free sterols.

The relative proportions by mass of free and esterified sterols were determined by comparing the areas under peaks by using QF-1 and OV-17 columns, and for cholesta-5,7-dien-3 β -ol by the u.v. absorption at 282 nm.

Isolation procedures for sterols in the experiments for determination of the stereochemistry of Δ^7 -bond formation in cholesta-5,7-dien-3 β -ol were identical with those described above.

Preparation of derivatives

4-Phenyl-1,2,4-triazoline-3,5-dione 5,8 adduct of cholest-6-en-3-one (IV). Recrystallized cholesta-5,7-dien-3 β -yl acetate (12 mg) was saponified in 6% ethanolic KOH and worked up in the usual manner. The resulting free sterol (10 mg) was dissolved in dry acetone (0.6 ml) and a solution of 4-phenyl-1,2,4-triazoline-3,5-dione (5 mg) in dry acetone (0.3 ml) was added dropwise at 0°C until the reaction mixture just remained pink owing to the presence of unchanged reagent (Gillani & Trigg, 1966). The reaction mixture was evaporated to dryness under N₂ and the resulting solid was subjected to preparative t.l.c. on silica gel developed in chloroform-methanol (50:1, v/v), yielding the sterol adduct (III) (14.6 mg), which was eluted with chloroform; i.e., carbonyl doublet at 1675 and 1735 cm⁻¹; u.v., disappearance of $\lambda_{\max}$ at 282 nm. The adduct (14.5 mg) was dissolved in minimal dichloromethane and a suspension of chromium trioxide-pyridine complex in dichloromethane (1 ml) was added. The reaction was allowed to proceed with stirring at room temperature (20°C) for 4 h (Collins *et al.*, 1968). Preparative t.l.c. on silica gel developed in chloroform-methanol (50:1, v/v) gave the oxidized adduct (IV) (9.8 mg), which was eluted in chloroform; i.e., carbonyl triplet at 1675, 1685 and 1735 cm⁻¹; u.v., disappearance of $\lambda_{\max}$ at 282 nm; n.m.r., δ 7.4 [multiplet (m), *N*-phenyl], δ 6.18, 6.26, 6.52, 6.61 (quartet centred at δ 6.38, C-6/C-7).

3 β -Acetoxy-8-hydroxy-5 α -cholestan-7-one (VI). The cholesta-5,7-dien-3 β -yl acetate (60 mg) was hydrogenated by using tris(triphenylphosphin)-rhodium chloride catalyst (Birch & Walker, 1966) to give 5 α -cholest-7-en-3 β -yl acetate (33.7 mg) after purification by t.l.c. on AgNO₃-silica gel. The 5 α -cholest-7-en-3 β -yl acetate (30 mg) and OsO₄ (30 mg) in diethyl ether (0.5 ml) plus a little pyridine (0.05 ml) were stirred at room temperature for 80 h (Canonica *et al.*, 1968). The reaction mixture was evaporated to dryness with care and redissolved in 3 ml of pyridine. After addition of sodium metabisulphite (3 g), water was added until a homogeneous single phase was obtained. The mixture was stirred for 1 h and then extracted thoroughly with chloroform (4 $\times$ 10 ml). The combined chloroform extracts were washed with water, dried over anhydrous Na₂SO₄, and purified by

preparative t.l.c. on silica gel developed in chloroform-methanol (20:1, v/v), yielding 7,8-dihydroxy-5 α -cholestan-3 β -yl acetate (V) (22.5 mg); n.m.r. δ 0.82 [singlet (s), C-18/C-19]; δ 4.15 (m, W₁ = 20 Hz, C-7); δ 4.69 (m, C-3); *m/e* 444 (*M*⁺-H₂O, 50%), 426 (*M*⁺-2H₂O, 36%), 411 (*M*⁺-2H₂O-CH₃, 20%), 384 (*M*⁺-acetate-H₂O, base peak), 366 (*M*⁺-acetate-2H₂O, 62%), 351 (*M*⁺-acetate-H₂O-side chain, 18%); *M*⁺ was not detectable.

7,8-Dihydroxy-5 α -cholestan-3 β -yl acetate (20 mg) dissolved in pyridine (0.5 ml) was added to chromium trioxide (20 mg) in pyridine (0.5 ml) and the mixture stirred at room temperature for 24 h (Canonica *et al.*, 1968). 3 β -Acetoxy-8-hydroxy-5 α -cholestan-7-one (10.1 mg) was obtained by preparative t.l.c. on silica gel developed in chloroform-methanol (20:1, v/v): n.m.r., δ 0.81 (s, C-18/C-19); δ 4.75 (m, C-3); *m/e* 460 (*M*⁺, very weak), 442 (*M*⁺-H₂O; 58%), 427 (*M*⁺-H₂O-CH₃, 11%), 400 (*M*⁺-acetate, weak), 382 (*M*⁺-acetate-H₂O, base peak), 329 (*M*⁺-H₂O-side chain, 28%), 287 (*M*⁺-side chain-acetate, 18%).

Results and Discussion

Transformations of nuclear double bonds

The present results (Table 1, Expt. 1) demonstrate the overall conversion of cholesterol into cholesta-5,7-dien-3 β -ol in *C. erythrocephala* under axenic conditions. The only sterols detected as acetates in this experiment were: (i) cholesterol acetate, *m/e* 428 (*M*⁺, weak); *m/e* 368 (*M*⁺-acetate, base peak); *m/e* 353 (*M*⁺-acetate-CH₃, 14%); *m/e* 255 (*M*⁺-acetate-side chain, 13%); *m/e* 213 (*M*⁺-acetate-side chain-ring D, 8%); (ii) cholesta-5,7-dien-3 β -yl acetate, *m/e* 426 (*M*⁺, very weak); *m/e* 366 (*M*⁺-acetate, base peak); *m/e* 351 (*M*⁺-acetate-CH₃, 10%); *m/e* 253 (*M*⁺-acetate-side chain, 12%); *m/e* 211 (*M*⁺-acetate-side chain-ring D, 7%); peaks were also observed at *m/e* 158 (31%), 143 (24%) and 128 (weak), characteristic of $\Delta^{5,7}$ -steryl acetates (Galli & Maroni, 1967); u.v., $\lambda_{\max}$ at 272, 282, 294 nm. No detectable mass or radioactivity was associated with 5 α -cholestan-3 β -yl acetate or 5 α -cholest-7-en-3 β -yl acetate, indicating that the conversion of cholesterol into cholesta-5,7-dien-3 β -ol may occur directly. Further evidence for this was furnished by the second experiment (Table 1, Expt. 2), in which larvae were reared under 'sparing' conditions. 5 α -Cholestan-3 β -ol was used as a 'sparing sterol', with a minimal amount of cholesterol as well as [4-¹⁴C]cholesterol also present. It was hoped in this way to observe the initial metabolism of the 'sparing sterol' and to increase incorporation of [4-¹⁴C]-cholesterol. Once again radioactivity was only detected in cholesterol acetate and cholesta-5,7-dien-3 β -yl acetate. However, appreciable masses of 5 α -cholestan-3 β -yl acetate and 5 α -cholest-7-en-3 β -yl

Table 1. *Distribution of radioactivity among individual components in the free sterols from Calliphora erythrocephala grown under different conditions*

For details see the text. —, Denotes negligible radioactivity.

Expt. no.	Sterol composition of diet	Radioactivity in sterols (% of total)			
		5 α -Cholestan-3 β -ol	Cholesterol	5 α -Cholest-7-en-3 β -ol	Cholesta-5,7-dien-3 β -ol
1	0.02% Cholesterol, [4- ¹⁴ C]-cholesterol (15 μ Ci; 60 mCi/mmol)	—	86	—	14
2	0.02% 5 α -Cholestan-3 β -ol, 0.006% cholesterol, [4- ¹⁴ C]-cholesterol (20 μ Ci; 60 mCi/mmol)	—	90	—	10
3	0.2% 5 α -Cholestan-3 β -ol, 0.006% cholesterol, 5 α -[4- ¹⁴ C]cholestan-3 β -ol (10 μ Ci)	84	—	16	—

Table 2. *Relative retention times of sterols and sterol acetates isolated from C. erythrocephala on various g.l.c. columns*

Substance	Retention time on g.l.c. column (relative to 5 α -cholestan-3 β -ol)		
	QF 1	OV 1	OV 17
5 α -Cholestan-3 β -ol	1.00	1.00	1.00
Cholesterol	0.92	1.00	1.00
5 α -Cholest-7-en-3 β -ol	1.00	1.08	1.20
Cholesta-5,7-dien-3 β -ol	1.00	1.08	1.20
5 α -Cholestan-3 β -yl acetate	1.26	1.27	1.36
Cholest-5-en-3 β -yl acetate	1.18	1.33	1.35
5 α -Cholest-7-en-3 β -yl acetate	1.27	1.50	1.59
Cholesta-5,7-dien-3 β -yl acetate	1.32	1.47	1.58

acetate were detected; 5 α -cholestan-3 β -yl acetate: *m/e* 430 (M^+ , 80%), 415 (M^+ -CH₃, 12%), 370 (M^+ -acetate, 72%), 355 (M^+ -acetate-CH₃, 32%), 275 (M^+ -side chain-ring D, 44%), 257 (M^+ -acetate-side chain, 6%), 215 (M^+ -acetate-side chain-ring D, base peak); 5 α -cholest-7-en-3 β -yl acetate; n.m.r., δ 0.54 (s, C-18); δ 0.82 (s, C-19) (Scallen & Krueger, 1968); *m/e* 428 (M^+ , base peak), 413 (M^+ -CH₃, 20%), 368 (M^+ -acetate, 11%), 353 (M^+ -acetate-CH₃, 9%), 315 (M^+ -side chain, 10%), 255 (M^+ -acetate-side chain, 50%), 213 (M^+ -acetate-side chain-ring D, 26%). All sterols were also identified by g.l.c.; the retention times (relative to 5 α -cholestan-3 β -ol) of constituent sterols and sterol acetates are summarized in Table 2. The occurrence of the 5 α -cholest-7-en-3 β -ol suggests that the transformation of 5 α -cholestan-3 β -ol into

5 α -cholest-7-en-3 β -ol was occurring, and the lack of radioactivity associated with both these sterols again suggests that the transformation of cholesterol into cholesta-5,7-dien-3 β -ol occurs directly.

The transformation of 5 α -cholestan-3 β -ol into 5 α -cholest-7-en-3 β -ol was confirmed by the results of the third experiment (Table 1, Expt. 3) in which larvae were again grown under 'sparing' conditions but with the addition of 5 α -[4-¹⁴C]cholestan-3 β -ol. In this experiment appreciable radioactivity was found associated with 5 α -cholest-7-en-3 β -yl acetate, but none was detectable in cholesterol acetate nor in cholesta-5,7-dien-3 β -yl acetate, indicating that the Δ^7 -sterol is not further metabolized into the $\Delta^{5,7}$ -sterol. From the combined results, it seems probable that in *C. erythrocephala* the conversion of cholesterol into cholesta-5,7-dien-3 β -ol occurs directly and not via 5 α -cholestan-3 β -ol and 5 α -cholest-7-en-3 β -ol. However, the possibility of anatomical compartmentation of parts of the pathway cannot be discounted. For example, it is conceivable that formation of cholesta-5,7-dien-3 β -ol occurs very rapidly only in a specific organ with no detectable accumulation of intermediates, whereas the $\Delta^0 \rightarrow \Delta^7$ transformation occurs more generally.

The significance of the large conversion of 5 α -cholestan-3 β -ol into 5 α -cholest-7-en-3 β -ol is uncertain. It is possible that some 5 α -cholestan-3 β -ol must be converted into 5 α -cholest-7-en-3 β -ol to fulfil its 'sparing' function. 5 α -Cholest-7-en-3 β -ol has all the suggested characteristics (Clayton, 1964) necessary for specific membrane roles, including a nuclear double bond, which 5 α -cholestan-3 β -ol lacks. It is also evident that the presence of a 'sparing sterol' does not significantly affect the overall conversion of cholesterol into cholesta-5,7-dien-3 β -ol.

Table 3. Relative proportions of constituent free and esterified sterols present in *C. erythrocephala* larvae grown on diets of different sterol composition

Constituent sterols	Diet containing 0.2% 5 α -cholestan-3 β -ol plus 0.006% cholesterol			Diet containing 0.02% cholesterol		
	Diet	Free sterol	Esterified sterol	Diet	Free sterol	Esterified sterol
5 α -Cholestan-3 β -ol	97	48	53	—*	—	—
Cholesterol	3	34	14	100	91	90
5 α -Cholest-7-en-3 β -ol	—	16	33	—	—	—
Cholesta-5,7-dien-3 β -ol	—	2	—	—	9	10

* Denotes non-detectable mass.

Table 4. $^3\text{H}/^{14}\text{C}$ ratios and specific radioactivities of sterols isolated from *C. erythrocephala* larvae after administration of [4- ^{14}C ,7 α - ^3H]- and [4- ^{14}C ,7 β - ^3H]-cholesterol in a diet containing 0.2% 5 α -cholestan-3 β -ol plus 0.006% cholesterolThe $^3\text{H}/^{14}\text{C}$ atomic ratios are based on the $^3\text{H}/^{14}\text{C}$ radioactivity ratio of the initial cholesterol.

Substance	Substrate ...	Recrystallizations	[4- ^{14}C ,7 α - ^3H]Cholesterol			[4- ^{14}C ,7 β - ^3H]Cholesterol		
			$^3\text{H}/^{14}\text{C}$ 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)
Administered cholesterol	1	1	4.666	1:1	2180	4.18	1:1	5970
	2	2	4.481		2150	4.07		5820
	3	3	4.413		2240	4.13		5860
Recovered cholesteryl acetate	1	1	4.374	0.98:1	12280	3.97	0.96:1	29100
	2	2	4.251	0.96:1	12990	3.85	0.93:1	28810
	3	3	4.311	0.97:1	12870	3.86	0.93:1	28650
Recovered cholesta-5,7-dien-3 β -yl acetate	1	1	4.234	0.95:1	383	1.63	0.39:1	3960
	2	2	3.889	0.87:1	389	1.60	0.39:1	3870
	3	3	3.929	0.88:1	373	1.63	0.39:1	3840

The relative percentages of free and esterified sterols were determined in final-instar larvae of *C. erythrocephala* grown either on diets containing cholest-5-en-3 β -ol as the sole sterol, or on diets supplemented with a 'sparing sterol', 5 α -cholestan-3 β -ol, plus minimal cholest-5-en-3 β -ol (Table 3). The proportion of cholest-5-en-3 β -ol to other sterols in the insects is much higher than in the diet for larvae grown on a defined medium not containing cholest-5-en-3 β -ol as the sole sterol source. This suggests a preferential uptake of cholest-5-en-3 β -ol from the diet or a preferential secretion of other sterols. In general, the sterol composition of the esters reflects results obtained for free sterols. However, when 5 α -cholestan-3 β -ol is present in the diet, there seems to be a significantly larger percentage of 5 α -cholest-7-en-3 β -ol in the esters at the expense of cholesterol. It is possible that some 5 α -cholest-7-en-3 β -ol is esterified for storage and/or transport for membrane

synthesis in place of cholesterol. This would allow the latter to be used for metabolic processes such as the formation of moulting hormones.

Stereochemistry of hydrogen elimination from C-7 during formation of cholesta-5,7-dien-3 β -ol

[4- ^{14}C ,7 α - ^3H]Cholesterol and [4- ^{14}C ,7 β - ^3H]cholesterol were separately administered to insects. The insects were extracted and the sterols isolated. Portions of administered cholesterol (control), recovered cholesterol acetate and recovered cholesta-5,7-dien-3 β -yl acetate were recrystallized to constant specific radioactivity (Table 4).

Comparison of the $^3\text{H}/^{14}\text{C}$ atomic ratios of the recovered cholesta-5,7-dien-3 β -yl acetate from the two parallel experiments shows extensive but incomplete stereospecific elimination of the 7 β hydrogen atom during introduction of the Δ^7 -bond. An

explanation must be sought for the significant retention of the 7 β - ^3H atom in the cholesta-5,7-dien-3 β -yl acetate ($^3\text{H}/^{14}\text{C}$, 0.39:1).

Cook *et al.* (1973) furnished evidence that during ecdysone biosynthesis in the fern *Polypodium vulgare*, an appreciable amount of the ^3H from [7β - ^3H]cholesterol probably enters a compartmentalized pool of NADPH and is re-incorporated at C-3 during alcohol dehydrogenase action at C-3. To investigate whether an analogous transfer to the C-3 position of cholesta-5,7-dien-3 β -ol occurs, we prepared the 3-oxo derivative of cholesta-5,7-dien-3 β -ol. Double-bond rearrangement during oxidation was prevented by formation of a 5,8 adduct with 4-phenyl-1,2,4-triazoline-3,5-dione before oxidation. The derivative was too unstable to allow recrystallization, and redevelopment on silica-gel t.l.c. was used to effect further purification. However, in this case there was no loss of ^3H from the cholesta-5,7-dien-3 β -ol on oxidation at C-3 (Table 5), indicating that the ^3H retained must be located elsewhere.

We next considered the possibility that the residual ^3H in the cholesta-5,7-dien-3 β -ol formed from [7β - ^3H]cholesterol might be located at C-7. This possibility was investigated by the transformation of the cholesta-5,7-dien-3 β -yl acetate formed from [7β - ^3H]cholesterol into 3 β -acetoxy-8 β -hydroxy-5 α -cholestan-7-one (see Table 6). The results show a total loss of ^3H from cholesta-5,7-dien-3 β -ol after oxidation at C-7, thus locating the residual ^3H at that position. Two possible explanations may be considered for this retention of ^3H .

Stereospecific location of the ^3H in the two substrates by chemical reaction indicated that the [7α - ^3H]- and [7β - ^3H]-cholesterol samples were contaminated with approx. 1% of [7β - ^3H]- and 4% of [7α - ^3H]-cholesterol respectively (Cook *et al.*, 1973). With [7β - ^3H]cholesterol as substrate, there may be a large primary-isotope effect discriminating in favour of the [7α - ^3H]cholesterol impurity being metabolized (with retention of ^3H). Thus the product may be enriched with molecules originating from the impurity at the expense of molecules derived from the

[7β - ^3H]cholesterol. This would give some retention of ^3H in the resulting cholesta-5,7-dien-3 β -ol.

Alternatively, the ^3H retention in the cholesta-5,7-dien-3 β -ol formed from [7β - ^3H]cholesterol might be explained by the possibility that production of cholesta-5,7-dien-3 β -ol is reversible, as has been shown in various other insects (Clark & Bloch, 1959*b*; Thompson *et al.*, 1973). The analogous transformation of cholesta-5,7-dien-3 β -ol into cholesterol in rat liver involves hydride addition at the 7 α position of the sterol (Caspi *et al.*, 1968). If this were the case in *C. erythrocephala*, the retention of ^3H in the cholesta-5,7-dien-3 β -ol formed from [7β - ^3H]cholesterol could be accounted for as shown in Scheme 1. The 7 β - ^3H eliminated from the cholesterol could enter a compartmentalized pool of NADPH and be re-introduced at the 7 α position. This ^3H would then be retained in any subsequent forward reaction involving elimination of the 7 β hydrogen atom. For this recycling scheme to be feasible, it is imperative that the NADP ^3H pool be compartmentalized, otherwise the isotopic abundance of ^3H would be too low for re-incorporation. This recycling scheme might also

Table 5. $^3\text{H}/^{14}\text{C}$ ratios of the cholesta-5,7-dien-3 β -ol biosynthesized from [4 - ^{14}C , 7β - ^3H]cholesterol and the derivatives involved in oxidation at C-3

The $^3\text{H}/^{14}\text{C}$ atomic ratios are based on the $^3\text{H}/^{14}\text{C}$ radioactivity ratio of the initial cholesterol.

Substance	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio
Cholesta-5,7-dien-3 β -ol after saponification of acetate	1.59	0.39:1
4-Phenyl-1,2,4-triazoline-3,5-dione adduct of cholest-6-en-3 β -ol	1.67	0.40:1
4-Phenyl-1,2,4-triazoline-3,5-dione adduct of cholest-6-en-3-one	1.48	0.36:1

Table 6. $^3\text{H}/^{14}\text{C}$ ratios of derivatives involved in the oxidation at C-7 of cholesta-5,7-dien-3 β -ol biosynthesized from [4 - ^{14}C , 7β - ^3H]cholesterol

The $^3\text{H}/^{14}\text{C}$ atomic ratios are based on the $^3\text{H}/^{14}\text{C}$ radioactivity ratio of the initial cholesterol.

Derivative	Recrystallizations	$^3\text{H}/^{14}\text{C}$ radioactivity ratio	$^3\text{H}/^{14}\text{C}$ atomic ratio	^{14}C specific radioactivity (d.p.m./mg)
5 α -Cholest-7-en-3 β -yl acetate	—	1.49	0.36:1	
7 β ,8 β -Dihydroxy-5 α -cholestan-3 β -yl acetate	—	1.57	0.38:1	
3 β -Acetoxy-8 β -hydroxy-5 α -cholestan-7-one	1	0.03	0.01:1	567
	2	0.03	0.01:1	545
	3	0.00	0.00:1	547

Scheme 1. Possible mechanism for the re-introduction of ^3H at the C-7 position from compartmentalized NADP^3H

account for the slight loss of ^3H during formation of cholesta-5,7-dien-3 β -ol ($^3\text{H}/^{14}\text{C}$, 0.88:1) from [7 α - ^3H]cholesterol. In this case, the loss of ^3H is less than the retention of ^3H in the experiment with [7 β - ^3H]cholesterol as substrate, since the complicating net isotope effects operating during recycling in the two cases might be different.

The possibility that introduction of the Δ^7 -bond was non-stereospecific can be discounted, as this should lead to approximately equivalent losses of ^3H from both the [7 α - ^3H]- and the [7 β - ^3H]-cholesterol substrates.

The overall results in *C. erythrocephala* clearly demonstrate that introduction of the Δ^7 -bond during cholesta-5,7-dien-3 β -ol formation from cholesterol involves removal of the 7 β - and, of necessity, the 8 β -hydrogen atoms. This is consistent with the possible intermediacy of cholesta-5,7-dien-3 β -ol in ecdysone biosynthesis, since the same stereochemistry has been previously observed during ecdysone formation from cholest-5-en-3 β -ol (Cook *et al.*, 1973).

We thank the Medical Research Council for a research studentship to P. J., Mr. J. Ireland for mass spectra and Mrs. Y. J. Shenton for maintenance of insects. We are most grateful to Dr. W. E. Robbins and colleagues, Agricultural Research Centre, Beltsville, Md., U.S.A. and Dr. L. Levenbook, N.I.H., Bethesda, Md., U.S.A., for invaluable advice about the aseptic rearing of insects.

References

- Birch, A. J. & Walker, K. A. M. (1966) *J. Chem. Soc. C* 1894–1896
- Canonica, L., Fiecchi, A., Kienle, G. M. & Scala, A. (1968) *Steroids* **11**, 287–298
- Caspi, E., Grieg, J. B., Ramm, P. J. & Varma, K. R. (1968) *Tetrahedron Lett.* 3829–3832
- Clark, A. J. & Bloch, K. (1959a) *J. Biol. Chem.* **234**, 2583–2588
- Clark, A. J. & Bloch, K. (1959b) *J. Biol. Chem.* **234**, 2589–2594
- Clayton, R. B. (1964) *J. Lipid Res.* **5**, 3–19
- Clayton, R. B. & Edwards, A. M. (1963) *J. Biol. Chem.* **238**, 1966–1972
- Collins, J. C., Hess, W. W. & Frank, F. J. (1968) *Tetrahedron Lett.* 3363–3366
- Cook, I. F., Lloyd-Jones, J. G., Rees, H. H. & Goodwin, T. W. (1973) *Biochem. J.* **136**, 135–145
- Galbraith, M. N., Horn, D. H. S., Middleton, E. J. & Thomson, J. A. (1970) *J. Chem. Soc. Chem. Commun.* 179–180
- Galli, G. & Maroni, S. (1967) *Steroids* **10**, 189–197
- Gibbons, G. F., Goad, L. J. & Goodwin, T. W. (1968) *J. Chem. Soc. Chem. Commun.* 1212–1214
- Giliani, S. S. H. & Triggle, D. J. (1966) *J. Org. Chem.* **31**, 2397–2398
- Goodnight, K. C. & Kircher, H. W. (1971) *Lipids* **6**, 166–169
- Hershberg, E. B., Oliveto, E., Rubin, M., Staendle, H. & Kuhlen, L. (1951) *J. Am. Chem. Soc.* **73**, 1144–1145
- Johnson, P., Rees, H. H. & Goodwin, T. W. (1974) *Biochem. Soc. Trans.* **2**, 1062–1065

- Karlson, P. & Hoffmeister, H. (1963) *Hoppe Seyler's Z. Physiol. Chem.* **331**, 298–300
- Louloudes, S. J., Thompson, M. J., Monroe, R. E. & Robbins, W. E. (1962) *Biochem. Biophys. Res. Commun.* **8**, 104–106
- Martin, M. M. & Carls, G. A. (1968) *Lipids* **3**, 256–259
- Monroe, R. E. (1962) *Ann. Entomol. Soc. Am.* **55**, 140
- Monroe, R. E., Hopkins, T. L. & Walder, S. A. (1967) *J. Insect Physiol.* **13**, 219–233
- Robbins, W. E., Thompson, M. J., Kaplanis, J. N. & Shortino, T. J. (1964) *Steroids* **4**, 635–644
- Scallen, T. J. & Krueger, W. (1968) *J. Lipid Res.* **9**, 120–128
- Svoboda, J. A., Robbins, W. E., Cohen, C. F. & Shortino, T. J. (1972) in *Insect and Mite Nutrition* (Rodriguez, T. G., ed.), pp. 505–516, North-Holland, Amsterdam
- Svoboda, J. A., Thompson, M. J., Elden, T. C. & Robbins, W. E. (1974) *Lipids* **9**, 752–755
- Thompson, M. J., Kaplanis, J. N., Robbins, W. E. & Svoboda, J. A. (1973) *Adv. Lipid Res.* **11**, 219–265