The Conversion of Cholest-5-en-3 β -ol into Cholest-7-en-3 β -ol by the Echinoderms *Asterias rubens* and *Solaster papposus*

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1. The echinoderms *Asterias rubens* and *Solaster papposus* (Class Asteroidea) metabolize injected [4-¹⁴C]cholest-5-en-3 β -ol to produce labelled 5 α -cholestan-3 β -ol and 5 α -cholest-7-en-3 β -ol. 2. Conversion of 5 α -[4-¹⁴C]cholestan-3 β -ol into 5 α -cholest-7-en-3 β -ol was demonstrated in *A. rubens*. 3. Incubations of *A. rubens* with [4-¹⁴C]cholest-4-en-3-one resulted in the production of labelled 5 α -cholestan-3-one, 5 α -cholestan-3 β -ol and 5 α -cholest-7-en-3 β -ol. 4. [4-¹⁴C]sitosterol was metabolized by *A. rubens* to give 5 α -stigmastan-3 β -ol and 5 α -stigmast-7-en-3 β -ol. 5. The significance of these results in relation to the presence of Δ^7 sterols in starfish is discussed.

Starfish (Phylum Echinodermata, Class Asteroidea) contain predominantly Δ^7 sterols (Bergmann, 1962; Gupta & Scheuer, 1968; Grossert *et al.*, 1973; Kobayashi *et al.*, 1973; Sheikh *et al.*, 1973; A. G. Smith *et al.*, 1973). This contrasts with most other animals which usually possess Δ^5 sterols. It has been established that the starfish *Asterias rubens* can convert mevalonic acid into 5 α -cholest-7-en-3 β -ol (I) by a route similar to that established for sterol biosynthesis in mammals (Smith & Goad, 1971a; Goad *et al.*, 1972; Smith & Goad, 1975). An alternative route for Δ^7 sterol production in starfish was demonstrated by Fagerlund & Idler (1960) who showed that *Pisaster ochraceus* could convert ingested cholest-5-en-3 β -ol (II) into 5 α -cholest-7-en-3 β -ol (I). As part of our investigations on sterol metabolism in the Phylum Echinodermata we have now examined this transformation in more detail by using the asteroids *Asterias rubens* and *Solaster papposus*. Part of the work has been briefly published (Smith & Goad, 1971b; Goad *et al.*, 1972).

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Experimental

Materials

Cholest-5-en-3 β -ol, 5 α -cholestan-3 β -ol and cholest-4-en-3-one were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. 5 α -cholestan-3-one and cholest-5-en-3-one were purchased from Steraloids Ltd., Croydon, Surrey, U.K. 5 α -stigmastan-3 β -yl acetate (m.p. 131–133°C) was a generous gift from Dr. H. H. Nicholas, Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Mo., U.S.A. 5 α -cholest-7-en-3 β -ol was prepared from cholesta-5,7-dien-3 β -ol by the method of Schenck *et al.* (1936). Cholest-4-en-3 β -ol [m.p. 131°C; literature value m.p. 132°C (Schoenheimer & Evans, 1936)] was obtained by the reduction of cholest-4-en-3-one with LiAlH₄ in dry diethyl ether. A mixture of 5 α -stigmast-7-en-3 β -ol (70%) and 5 α -stigmast-7-en-3 β -ol (30%) was isolated from lucerne leaves (Armarego *et al.*, 1973).

[4-¹⁴C]cholest-5-en-3 β -ol (50mCi/mmol) and [4-¹⁴C]sitosterol (50mCi/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. 5 α -[4-¹⁴C]cholestan-3 β -ol was prepared by the

hydrogenation of [4-¹⁴C]cholest-5-en-3 β -ol (20 μ Ci) with PtO₂ (5mg) and conc. H₂SO₄ (20 μ l) in ethyl acetate (4ml) for 3h (Hershberg *et al.*, 1951). The products of the reduction were treated with *m*-chloroperbenzoic acid in chloroform to epoxidize any unsaturated material and the 5 α -[4-¹⁴C]cholestan-3 β -ol (11 μ Ci) isolated by t.l.c. on silica gel developed with ethyl acetate-chloroform (7:13, v/v). No decrease in specific radioactivity was observed during repeated crystallization of a portion of the labelled material with carrier 5 α -cholestan-3 β -ol. [4-¹⁴C]-Cholest-4-en-3-one was prepared by an Oppenauer oxidation of [4-¹⁴C]cholest-5-en-3 β -ol (2.5mg, 7.7 mCi/mmol) by refluxing with cyclohexanone (0.6ml) and aluminium isopropoxide (10mg) in toluene (2ml) for 1.5h. The recovered [4-¹⁴C]cholest-4-en-3-one (54 μ Ci) had a g.l.c. retention time on 1% HiEFF 8B (Applied Science Laboratories, State College, Pa., U.S.A.) and mass spectrum identical with those of authentic cholest-4-en-3-one. T.l.c. on AgNO₃-impregnated silica gel developed three times with chloroform revealed no other labelled compounds and no decrease in specific radioactivity occurred after crystallization several times with carrier cholest-4-en-3-one.

General methods

Starfish were maintained in aquaria of aerated seawater maintained at 10–12°C. Radioactive sterols were emulsified in 0.2ml of 5% (w/v) aq. Tween 80 and injected into the body cavity at the base of one leg. After incubation the non-saponifiable lipid was extracted as described by Smith & Goad (1974).

G.l.c. and radioassay methods were performed as described by Lenton *et al.* (1971) and Smith & Goad (1974b).

4-Demethyl sterols were obtained from the non-saponifiable lipid by t.l.c. on silica gel developed with ethanol-chloroform (1:49, v/v) (Smith & Goad, 1975). The 4-demethyl sterols were separated according to the number and position of double bonds by t.l.c. on 10% (w/w) AgNO₃-impregnated silica gel developed three times with ethanol-chloroform (1:49, v/v). 5 α -Cholestan-3 β -ol and 5 α -cholest-7-en-3 β -ol virtually co-chromatograph (*R_F* 0.34) in this system but do separate from cholest-5-en-3 β -ol (*R_F* 0.26).

5 α -Cholestan-3 β -ol, 5 α -cholest-7-en-3 β -ol and cholest-5-en-3 β -ol were separated by epoxidation of the latter two compounds by treatment of the mixture with *m*-chloroperbenzoic acid (1.1 molar equivalent) in chloroform for 3h (Fieser & Fieser, 1967). T.l.c. on silica gel developed with ethyl acetate-chloroform (7:13, v/v) gave a good separation of unchanged 5 α -cholestan-3 β -ol (*R_F* 0.65), 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (*R_F* 0.52) and 5 α ,6 α -epoxy-cholestan-3 β -ol (*R_F* 0.45). 7 α ,8 α -Epoxy-5 α -cholestan-3 β -ol

(m.p. 131–134°C) was examined by mass spectrometry: *m/e* 403 (*M*⁺), 387, 384, 369, 247. Acetylation gave a monoacetate [m.p. 89–92°C; literature value 86–93°C, (Fieser & Goto, 1960)], mass spectrum: *m/e* 444 (*M*⁺), 426, 411, 384, 366, 253. The n.m.r. spectra of both compounds showed an absorption integrating for one proton at δ 3.27 p.p.m. which was assigned to the C-7 proton adjacent to the epoxide (Bhacca & Williams, 1964).

It was found that 4 β ,5 β -epoxycholestan-3 β -ol [m.p. 95–96°C; literature value 90°C (Plattner *et al.*, 1948)] co-chromatographed with 5 α -cholestan-3 β -ol on the above t.l.c. system for the separation of sterol epoxides. To separate these compounds the mixture was reduced by refluxing with LiAlH₄ in diethyl ether which converted the epoxysterol into cholestane-3 β ,5 β -diol [m.p. 146–148°C; literature value 149°C (Plattner *et al.*, 1948)]; mass spectrum: *m/e* 404 (*M*⁺); i.r. 3450 cm⁻¹. This compound separated (*R_F* 0.37) from 5 α -cholestan-3 β -ol (*R_F* 0.60) by t.l.c. on silica gel developed with ethylacetate-chloroform (7:13, v/v).

Results

A specimen of *A. rubens* was incubated with [4-¹⁴C]cholest-5-en-3 β -ol (5 μ Ci) for 41h and the non-saponifiable lipids were isolated (98mg, 1.9 μ Ci). T.l.c. on silica gel revealed that only the 4-demethyl sterols were labelled. T.l.c. of the 4-demethyl sterols (8.8 $\times$ 10⁵ d.p.m.) on AgNO₃-impregnated silica gel demonstrated a radioactive band corresponding to unchanged [4-¹⁴C]cholest-5-en-3 β -ol (*R_F* 0.23) and a second labelled band (5.1mg; 1.56 $\times$ 10⁵ d.p.m.) that chromatographed with 5 α -cholest-7-en-3 β -ol (I) and 5 α -cholestan-3 β -ol (III) which co-chromatograph (*R_F* 0.33). Crystallization from chloroform-methanol of a portion (7.81 $\times$ 10⁴ d.p.m.) of this material with added 5 α -cholest-7-en-3 β -ol (I) (140mg) gave a constant specific radioactivity after an 85% decrease (543, 233, 194, 150, 104, 90, 100, 88, 77, 80 and 81 d.p.m./mg for successive crystallizations). This result indicated that although 5 α -cholest-7-en-3 β -ol (I) appeared to be a metabolite of cholest-5-en-3 β -ol (II) in starfish, as reported by Fagerlund & Idler (1960) there was a second more highly labelled metabolite. G.l.c. analysis of the labelled mixture confirmed that part of the radioactivity chromatographed with 5 α -cholest-7-en-3 β -ol (I). However, the main radioactive peak had a shorter retention-time which corresponded to 5 α -cholestan-3 β -ol (III). Since cholest-5-en-3 β -ol (II) has a very similar retention-time it was necessary to show that the radioactivity was not due to contaminating unchanged [4-¹⁴C]cholest-5-en-3 β -ol. A portion (8.9 $\times$ 10³ d.p.m.) of the labelled material was therefore mixed with cholest-5-en-3 β -ol (II, 50mg)

which was then purified via formation of the dibromide (Fieser, 1955). This procedure would be expected to destroy any labelled 5 α -cholest-7-en-3 β -ol (I), but would have left labelled 5 α -cholestan-3 β -ol (III) in the recovered cholest-5-en-3 β -ol (II). Crystallization of the cholest-5-en-3 β -ol (II) three times from methanol resulted in the specific radioactivity decreasing to 14% of the initial value so indicating that the bulk, if not all, of the radioactivity was in a compound other than unchanged [4- 14 C]cholest-5-en-3 β -ol. The mother liquors from the above crystallizations were added to 5 α -cholestan-3 β -ol (III, 20mg) and this compound was purified by t.l.c. on AgNO₃-impregnated silica

gel. The radioactivity was recovered in the 5 α -cholestan-3 β -ol (III) and was retained during crystallization (98, 86, 112 and 102 d.p.m./mg for successive crystallizations) thus providing evidence that 5 α -cholestan-3 β -ol (III) was a metabolite of cholest-5-en-3 β -ol (II) in *A. rubens*.

To confirm further the identification of 5 α -cholestan-3 β -ol (III), specimens of *A. rubens* and *S. papposus* were incubated with [4- 14 C]cholest-5-en-3 β -ol. The 4-demethylsterols were isolated and treated with *m*-chloroperbenzoic acid to epoxidize unsaturated sterols. T.l.c. gave a good separation of 5 α ,6 α -epoxycholestan-3 β -ol (IV) 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (V) and 5 α -cholestan-3 β -ol (III). The

sterols recovered from *A. rubens* contained radioactivity which co-chromatographed with 5 α -cholestan-3 β -ol (III, 55%) and 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (V, 24%; derived from 5 α -cholest-7-en-3 β -ol, I) as well as with 5 α ,6 α -epoxycholestan-3 β -ol (IV; derived from unmetabolized [4-¹⁴C]cholest-5-en-3 β -ol, II). Similar results were obtained with the labelled sterols from *S. papposus*. The radioactive t.l.c. bands were eluted and crystallized to constant specific radioactivity (Table 1) thus confirming the production of both 5 α -cholest-7-en-3 β -ol (I) and 5 α -cholestan-3 β -ol (III) from ingested cholest-5-en-3 β -ol (II) in the two starfish.

To establish that 5 α -cholestan-3 β -ol (III) can act as a precursor of 5 α -cholest-7-en-3 β -ol (I) in *A. rubens* two specimens were incubated for 70h with 5 α -[4-¹⁴C]cholestan-3 β -ol (2.75 μ Ci). The 4-demethyl sterols were isolated (109mg; 1.56 μ Ci), a portion (1.73 $\times 10^6$ d.p.m.) was epoxidized and the mixture was separated by t.l.c. Two radioactive bands were detected corresponding to 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (V, 18%) and unmetabolized 5 α -cholestan-3 β -ol (III, 82%). The 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (43mg, 2.01 $\times 10^5$ d.p.m.) was obtained by preparative t.l.c. and a portion (8.5mg) was diluted with carrier material (58mg) and crystallized to constant specific radioactivity (602, 643, 638, 671, 690 and 645 d.p.m./mg for successive crystallizations).

By analogy with 5 α -cholestan-3 β -ol and bile acid biosynthesis in mammals (Danielsson & Tchen, 1968) it seemed likely that cholest-4-en-3-one (VI) might be an intermediate in the conversion of cholest-5-en-3 β -ol (II) into 5 α -cholestan-3 β -ol (III) in the starfish. To test the possible involvement of

cholest-4-en-3-one (VI) a specimen of *A. rubens* was incubated for 65h with [4-¹⁴C]cholest-4-en-3-one (5 μ Ci) and the non-saponifiable lipids (105mg, 1.67 μ Ci) were examined by t.l.c. Three radioactive regions were observed with R_F values corresponding to 5 α -cholestan-3-one (VII, R_F 0.70, 4%), cholest-4-en-3-one (VI, R_F 0.56, 16%) and 3 β -hydroxy sterol (R_F 0.33, 80%). These bands were recovered by preparative t.l.c. and a portion (1.01 $\times 10^4$ d.p.m.) of the 5 α -cholestan-3-one band was diluted with carrier 5 α -cholestan-3-one (VII, 39mg) and crystallized to constant specific radioactivity (258, 223, 239, 248 and 241 d.p.m./mg for successive crystallizations). Crystallization of a second portion of this radioactive band with carrier cholest-5-en-3-one, with which it chromatographs on the t.l.c. system, gave a progressive drop in the specific radioactivity showing that little if any isomerization of cholest-4-en-3-one into cholest-5-en-3-one had occurred during the incubation. The 3 β -hydroxy sterol fraction (1.6 $\times 10^6$ d.p.m.) showed only one radioactive peak after t.l.c. on AgNO₃-impregnated silica gel which corresponded to the 5 α -cholestan-3 β -ol and 5 α -cholest-7-en-3 β -ol band. Since cholest-4-en-3 β -ol (VIII) also co-chromatographs with these two sterols on this t.l.c. system the possibility existed that cholest-4-en-3-one (VI) was reduced by the starfish to give cholest-4-en-3 β -ol (VIII). To examine this possibility a portion (7.3 $\times 10^5$ d.p.m.) of the 3 β -hydroxy sterol mixture was diluted with 5 α -cholestan-3 β -ol (18mg), 5 α -cholest-7-en-3 β -ol (51mg) and cholest-4-en-3 β -ol (40mg) and the mixture epoxidized. Analysis of the products by t.l.c. showed two radioactive peaks corresponding in R_F values to 5 α -cholestan-3 β -ol (III) and 4 β ,5 β -epoxycholestan-3 β -ol (IX) which co-chromatograph, and to 7 α ,8 α -epoxycholestan-3 β -ol (V) respectively. The recovered 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol (39mg, 9.2 $\times 10^4$ d.p.m.), after a further t.l.c. purification, was crystallized to constant specific radioactivity (2337, 2248, 2353, 2274 and 2352 d.p.m./mg for successive crystallizations).

A sample (1.1 $\times 10^5$ d.p.m.) of the radioactive material chromatographing with 5 α -cholestan-3 β -ol (III) and 4 β ,5 β -epoxycholestan-3 β -ol (IX) was refluxed with lithium aluminium hydride in diethyl ether to convert the latter compound into cholestane-3 β ,5 β -diol (X). T.l.c. of the products resulted in the recovery of 97% of the radioactivity in 5 α -cholestan-3 β -ol (III, 6.5 $\times 10^4$ d.p.m.) which was crystallized to constant specific radioactivity with carrier 5 α -cholestan-3 β -ol (100mg; 609, 571, 574, 592 and 579 d.p.m./mg for successive crystallizations). The cholestane-3 β ,5 β -diol (X, 1.65 $\times 10^3$ d.p.m.) contained only 3% of the recovered radioactivity demonstrating that very little of the injected [4-¹⁴C]cholest-4-en-3-one had been reduced by *A. rubens* to give cholest-4-en-3 β -ol (VIII).

Table 1. Crystallizations to constant specific radioactivity of the 5 α -cholestan-3 β -ol and 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol obtained from *A. rubens* and *S. papposus* after incubation with [4-¹⁴C]cholest-5-en-3 β -ol

Specimens of *A. rubens* and *S. papposus* were each injected with [4-¹⁴C]cholest-5-en-3 β -ol (10 μ Ci) and the animals incubated in aquaria at 10–12°C. The non-saponifiable lipids were extracted from the *A. rubens* after 7 days and from the *S. papposus* after 9 days. The sterols were isolated by t.l.c., epoxidized and 5 α -cholestan-3 β -ol and 7 α ,8 α -epoxy-5 α -cholestan-3 β -ol separated by preparative t.l.c. as described in the Experimental section. After addition of carrier material these two compounds were crystallized several times from methanol. Specific radioactivities are in d.p.m./mg.

No. of recrystallizations	7 α ,8 α -Epoxy-5 α -cholestan-3 β -ol		5 α -Cholestan-3 β -ol	
	<i>A. rubens</i>	<i>S. papposus</i>	<i>A. rubens</i>	<i>S. papposus</i>
Initial value	4537	656	2681	1227
1	4491	722	2410	1230
2	4558	699	2524	1211
3	4720	664	2564	1269
4	4885	655	2534	1240

A. rubens contains a number of Δ^7 C₂₈ and C₂₉ sterols (A. G. Smith *et al.*, 1973), which are not produced by synthesis *de novo* in the animal but are most probably derived from the diet (Smith & Goad, 1974b). Since the dietary sterols most commonly encountered by *A. rubens* are probably Δ^5 compounds it was decided to demonstrate that Δ^5 sterols which are alkylated at C-24 can also be converted into the Δ^7 analogues by the starfish. *A. rubens* was therefore incubated for 25h with [4-¹⁴C]sitosterol (XI, 2 μ Ci) and the recovered 4-demethylsterols (3.7×10^5 d.p.m.) purified by repeated t.l.c. on AgNO₃-impregnated silica gel to give a fraction (7.3×10^4 d.p.m.) containing the saturated and Δ^7 sterols which was free of unchanged [4-¹⁴C]sitosterol. G.l.c. of this material showed that the major peak of radioactivity had a retention-time corresponding to 5 α -stigmastan-3 β -ol (XII) and a minor radioactive peak that chromatographed with 5 α -stigmast-7-en-3 β -ol (XIII). There was no evidence of dealkylation to produce C₂₇ sterols. To confirm the presence of labelled 5 α -stigmastan-3 β -ol (XII) and 5 α -stigmast-7-en-3 β -ol (XIII) a mixture of 5 α -stigmasta-7,22-dien-3 β -ol and 5 α -stigmast-7-en-3 β -ol (1mg) was added to the radioactive sterol and the mixture epoxidized. T.l.c. of the mixture showed two major peaks of radioactivity corresponding to 7 α ,8 α -epoxy-5 α -stigmastan-3 β -ol (15%) and 5 α -stigmastan-3 β -ol (79%). The latter compound was recovered and acetylated (1.34×10^4 d.p.m.), mixed

with carrier 5 α -stigmastan-3 β -yl acetate (28mg) and crystallized to constant specific radioactivity (494, 457, 438, 459 and 438 d.p.m./mg for successive crystallizations).

Discussion

The results presented in this paper establish that *A. rubens* and *S. papposus* can metabolize cholest-5-en-3 β -ol (II) to 5 α -cholest-7-en-3 β -ol (I) by a pathway involving the intermediacy of cholest-4-en-3-one (VI), 5 α -cholestan-3-one (VII) and 5 α -cholestan-3 β -ol (III). The reaction sequence for the production of 5 α -cholestan-3 β -ol (III) is the same as that present in mammals, whereas the conversion of 5 α -cholestan-3 β -ol (III) into 5 α -cholest-7-en-3 β -ol (I) has been reported in insects (Louloudes *et al.*, 1962; Clayton & Edwards, 1963). Additional evidence for the production of 3-oxosteroid intermediates in the conversion of cholest-5-en-3 β -ol (II) into 5 α -cholestan-3 β -ol (III) has been presented (Smith *et al.*, 1972). The observation that sitosterol (stigmast-5-en-3 β -ol, XI) is metabolized in a similar manner to produce 5 α -stigmastan-3 β -ol (XII) and 5 α -stigmast-7-en-3 β -ol (XIII) strongly suggests that the various Δ^7 C₂₆, C₂₈ and C₂₉ sterols of the starfish arise by modification of the appropriate dietary Δ^5 sterols since the animal is apparently only capable of C₂₇ sterol biosynthesis *de novo* (Smith & Goad, 1975).

The sterol sulphates of *A. rubens* contain cholest-5-en-3 β -ol (II), 5 α -cholest-7-en-3 β -ol (I) and 5 α -cholestan-3 β -ol (III) (Goodfellow & Goad, 1973). It seems probable that the sulphated 5 α -cholestan-3 β -ol (III) is derived by metabolism of dietary cholest-5-en-3 β -ol (II) as outlined above. The incorporation by *A. rubens* of the injected [4-¹⁴C]-cholest-5-en-3 β -ol and its metabolites into the corresponding sulphates and also possibly into asterosaponins (D. H. S. Smith *et al.*, 1973; Ikegami *et al.*, 1973; Sheikh *et al.*, 1972) might explain the failure to recover all the injected radioactivity in the non-saponifiable lipid. In this context Voogt & Schoenmakers (1973) have reported extensive catabolism of [26-¹⁴C]cholest-5-en-3 β -ol by *A. rubens*.

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