Sterol Biosynthesis in the Echinoderm *Asterias rubens*

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1. [2-¹⁴C]Mevalonic acid injected into the echinoderm *Asterias rubens* (Class Asteroidea) was effectively incorporated into the non-saponifiable lipid. 2. The most extensively labelled compounds were squalene and the 4,4-dimethyl sterols with much lower incorporations into the 4 α -monomethyl and 4-demethyl sterol fractions. 3. Labelled compounds identified were squalene, lanosterol, 4,4-dimethyl-5 α -cholesta-8,24-dien-3 β -ol, 4,4-dimethyl-5 α -cholesta-7,24-dien-3 β -ol, 4 α -methyl-5 α -cholesta-7,24-dien-3 β -ol and 4 α -methyl-5 α -cholest-7-en-3 β -ol; these are all intermediates in sterol biosynthesis. 4. The major sterol in *A. rubens*, 5 α -cholest-7-en-3 β -ol, was also labelled showing that this echinoderm is capable of sterol biosynthesis *de novo*. 5. No evidence was obtained for the incorporation of [2-¹⁴C]mevalonic acid into the C₂₈ and C₂₉ components of the 4-demethyl sterols or 9 β ,19-cyclopropane sterols found in *A. rubens* and it is assumed that these sterols are of dietary origin. 6. Another starfish *Henricia sanguinolenta* also incorporated [2-¹⁴C]mevalonic acid into squalene and lanosterol. 7. Various isolated tissues of *A. rubens* were all capable of incorporation of [2-¹⁴C]mevalonic acid into the non-saponifiable lipid. With the body-wall and stomach tissues radioactivity accumulated in squalene and the 4,4-dimethyl sterols, but with the gonads and pyloric caecae there was a more efficient incorporation of radioactivity into the 4-demethyl sterols, principally 5 α -cholest-7-en-3 β -ol.

The sterols of the Asteroidea (starfish) are typically Δ^7 compounds (Gupta & Scheuer, 1968; Grossert *et al.*, 1973; Kobayashi *et al.*, 1973; Sheikh *et al.*, 1973; Smith *et al.*, 1973) and it was suggested by Bergmann (1962) that they may be derived by metabolism of the predominantly Δ^5 sterols of the animals diet. This possibility gained support with the demonstration that starfish can convert administered cholest-5-en-3 β -ol into 5 α -cholest-7-en-3 β -ol (Fagerlund & Idler, 1960) with 5 α -cholestan-3 β -ol as a probable intermediate (Smith & Goad, 1971a; Goad *et al.*, 1972).

An investigation of the sterols of *Asterias rubens* showed that 5 α -cholest-7-en-3 β -ol was the major constituent in a complex mixture of C₂₆, C₂₇, C₂₈ and C₂₉ sterols (Smith *et al.*, 1973). In addition several C-4 methylated sterols (see Scheme 1) were characterized. Such compounds are intermediates in the conversion of squalene into cholesterol in mammals (Goad, 1970) and their presence in *A. rubens* therefore suggested that sterol biosynthesis *de novo* is proceeding in this starfish. The present investigation was therefore undertaken to examine this possibility; part of this work has been briefly reported (Smith & Goad, 1971b; Goad *et al.*, 1972).

Experimental

General methods

Melting points were determined with a Reichert hot-stage apparatus and are corrected. Radioactivity was assayed by liquid-scintillation counting as described by Lenton *et al.* (1971). Radioscanning of t.l.c. plates was accomplished with a Panax radio-scanner and radioautographs of t.l.c. plates were obtained by exposure to Kodak Kodirex X-ray film.

G.l.c. was performed as described by Lenton *et al.* (1971) with either 1% SE-30 or 3% OV-17 (Applied Science Laboratories, State College, Pa., U.S.A.) as the stationary phase. For measurements of radioactivity present in sterols after separation by g.l.c. the effluent was automatically subdivided (1:9) and samples were trapped at ambient temperature in glass capillary tubes which were changed at 1 min intervals. The trapped material was then washed into a counting vial with diethyl ether and the radioactivity was measured by liquid-scintillation counting in a Beckman LS200 instrument. Squalene samples were analysed for radioactivity by g.l.c. on a stainless-steel column (150cm $\times$ 8mm) packed with 3% OV-17 on Gaschrom Q (Applied Science Laboratories; 100–120 mesh). The column effluent was subdivided, one part going to the flame ionization detector and

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Scheme 1. Possible biosynthetic routes for the conversion of lanosterol (I) into 5 α -cholest-7-en-3 β -ol (X). All the compounds shown except for (II) and (IV) have been identified in *A. rubens* (Smith *et al.*, 1973).

nine parts passing through a combustion tube packed with copper oxide granules and maintained at 700°C. The CO₂ produced was monitored for radioactivity by using a Nuclear-Chicago proportional gas-flow counter (model 4998). The radioactivity and g.l.c. responses were recorded on a Honeywell two-pen recorder (model TP 496).

For t.l.c. the following systems were used. (a) The non-saponifiable lipid was separated into bands containing squalene (R_F 0.85), 4,4-dimethyl sterols (R_F 0.45), 4-monomethyl sterols (R_F 0.39) and 4-demethyl sterols (R_F 0.32) by t.l.c. on silica gel developed with ethanol-chloroform (1:49, v/v). (b) Squalene was purified by t.l.c. on silica gel developed with ethyl acetate-light petroleum (b.p. 60–80°C) (1:19, v/v). (c) Acetates of the 4,4-dimethyl and 4-monomethyl sterols were separated by t.l.c. on 10% (w/w) AgNO₃-impregnated silica gel developed with cyclohexane-benzene (1:1, v/v). (d) The 4-demethyl sterols were chromatographed on 10% (w/w) AgNO₃-impregnated silica gel developed twice with ethanol-chloroform (1:59, v/v). Compounds were located by spraying with Rhodamine 6G dissolved in acetone (Goad & Goodwin, 1966) and eluted from scraped bands with diethyl ether.

Radioactive squalene samples were purified after addition of carrier squalene (100mg) by formation of the thiourea adduct (Goad & Goodwin, 1966). Squalene hexahydrochloride was prepared as described by Goad & Goodwin (1966). The 4-demethyl sterols were purified by preparation of the sterol digitonides (Schoenheimer & Sperry, 1934) and the sterols were recovered by the method of Issidore *et al.* (1962). The hydroxylation of 4 α -methyl-5 α -cholest-7-en-3 β -yl acetate with OsO₄ and subsequent manipulations and purification were carried out as described by Rahman *et al.* (1970) and Smith *et al.* (1973).

Starfish were kept in aquaria held at 10–12°C. [2-¹⁴C]Mevalonic acid (10mCi/mmol, The Radiochemical Centre, Amersham, Bucks., U.K.) was dissolved in 0.2ml of water and injected into the body cavity at the base of one leg. During incubations the animals were kept at 10–12°C in approx. 6 litres of sea-water. After incubation the non-saponifiable lipid was obtained as described by Smith & Goad (1974).

Results

Specimens of *A. rubens* readily incorporated radioactivity from injected [2-¹⁴C]mevalonic acid into the non-saponifiable lipid. The efficiency of incorporation of [2-¹⁴C]mevalonic acid, however, varied considerably and ranged from 3% to as high as 40% in some experiments. It may be significant that a specimen incubated with [2-¹⁴C]mevalonic acid in July gave a higher incorporation than the animal used in November (Table 1). These variations may be attributable to several factors and clearly need careful verification under more strictly controlled conditions. However, such variations may perhaps result from the annual physiological cycles which occur in the starfish [see Boolootian (1966) for reviews on this topic].

Thin-layer radioscanning showed that the radioactivity in the non-saponifiable lipid was distributed principally between material chromatographing with squalene and the 4,4-dimethyl sterols. Although not very apparent from the thin-layer radioscan it was established by radioautography and preparative t.l.c. (Table 1) that the 4-monomethyl and 4-demethyl sterols were also labelled. However, the radioactivity co-chromatographing with the 4-demethyl sterols rarely exceeded 10% of the total radioactivity even with incubations of up to 1 week duration.

The material co-chromatographing on t.l.c. with

Table 1. Incorporation of radioactivity into the non-saponifiable lipids of *A. rubens* and *Henricia sanguinolenta* after injection of [2-¹⁴C]mevalonic acid

For experimental details see the text.

	<i>A. rubens</i>		<i>H. sanguinolenta</i>
	Expt. 1	Expt. 2	
Weight of animal (g)	47.2	30.0	30.6
Month	November	July	July
Incubation time (h)	41	41	17
[2- ¹⁴ C]Mevalonic acid injected (μ Ci)	23.0	5.0	5.0
Percentage incorporation into non-saponifiable lipid	3	23	17
¹⁴ C radioactivity (d.p.m.) in:			
Squalene	43 000	84 000	287 000
4,4-Dimethyl sterols	568 600	481 000	299 000
4-Monomethyl sterols	96 000	103 000	74 000
4-Demethyl sterols	62 000	69 000	7 000

squalene was examined by radio-g.l.c.; only one radioactive peak was observed and this was eluted with the squalene mass peak. Portions of the squalene samples were diluted with carrier squalene and purified through the formation of the thiourea adduct (Expt. 1, 139 d.p.m./ μ mol; Expt. 2, 429 d.p.m./ μ mol) and subsequently by conversion into squalene hexahydrochloride which was repeatedly recrystallized. The specific radioactivities remained essentially constant throughout these procedures (Expt. 1, 115, 123, 105, 109, 116 d.p.m./ μ mol; Expt. 2, 429, 413, 421, 381, 345, 338 d.p.m./ μ mol) thus establishing the biosynthesis of squalene by *A. rubens*. During the initial purification of the squalene samples by t.l.c. a small amount of radioactivity (<5%) was detected which chromatographed with squalene-2,3-oxide, but this was not investigated further.

The radioactive 4,4-dimethyl sterol fraction was diluted with a mixture of lanosterol (I, lanosta-8,24-dien-3 β -ol) and 24,25-dihydrolanosterol (II, lanost-8-en-3 β -ol) and the mixture acetylated and subjected to t.l.c. on AgNO₃-impregnated silica gel. Radio-scanning and radioautography revealed three radio-

progressive loss of radioactivity (initial specific radioactivity 235 d.p.m./mg; specific radioactivity after ten crystallizations 9 d.p.m./mg). In our previous examination of the 4,4-dimethyl sterols of *A. rubens*, 24,25-dihydrolanosterol (II) was not detected (Smith *et al.*, 1973). However, two other compounds present were identified as cycloartanol (XI), which gives an acetate with the same R_F value as 24,25-dihydrolanosteryl acetate, and 4,4-dimethyl-5 α -cholest-7-en-3 β -ol (VI) which gives a slightly more polar acetate. Since cycloartanol is generally regarded to be of plant origin and because no incorporation of mevalonic acid into cycloartenol was detected in *A. rubens* (see below) it is tentatively concluded that the labelled compound of R_F 0.36 was the acetate of 4,4-dimethyl-5 α -cholest-7-en-3 β -ol (VI).

The second band, of intermediate polarity on the AgNO₃-impregnated silica gel-t.l.c. system, contained about 20% of the radioactivity in the 4,4-dimethyl sterol fraction and had an R_F 0.30 corresponding to the acetates of lanosterol (I) and cycloartenol (XII), two compounds previously identified in *A. rubens* (Smith *et al.*, 1973). However, the third and most heavily labelled band (R_F 0.26) did not

actively labelled bands. The least polar band (R_F 0.36), which was also the most weakly labelled (<10% of the total radioactivity), did not chromatograph exactly with the added carrier 24,25-dihydrolanosteryl acetate (R_F 0.39). Its non-identity with this compound was confirmed by elution and addition of more carrier 24,25-dihydrolanosteryl acetate; repeated recrystallization was accompanied by a

completely separate from the band of R_F 0.30. Smith *et al.* (1973) showed that this third band contains the acetates of 4,4-dimethyl-5 α -cholesta-8,24-dien-3 β -ol (III) and 4,4-dimethyl-5 α -cholesta-7,24-dien-3 β -ol (V). Consequently when the lanosteryl acetate-containing band (R_F 0.30) was obtained by preparative t.l.c. it was contaminated with a portion of the more-polar radioactive band (R_F 0.26). This

Table 2. Crystallization to constant specific radioactivity of the labelled lanosteryl acetate samples obtained from *A. rubens* and *H. sanguinolenta* after incubation with [2-¹⁴C]mevalonic acid

Details are given in the text. The lanosteryl acetate was crystallized from chloroform-methanol (1:20, v/v). Specific radioactivities are given as d.p.m./mg.

No. of recrystallization	<i>A. rubens</i>			<i>H. sanguinolenta</i>
	Expt. 1	Expt. 2	Expt. 3	
Initial value	1710	1523	1129	519
1	1275	1258	1083	459
2	1090	1062	1077	450
3	1012	1019	1109	432
4	866	911	1091	440
5	807	832		
6	688	777		
7	675	761		
8	641	712		
9	657	756		

resulted in an initial large fall in specific radioactivity on crystallization with carrier lanosteryl acetate before constant specific radioactivity was achieved (Table 2).

To establish conclusively that lanosterol (I) was labelled the 4,4-dimethyl sterols obtained from the incubation of a third animal with [2-¹⁴C]mevalonic acid (Expt. 3) were acetylated and subjected to preparative t.l.c. on AgNO₃-impregnated silica gel as described for the previous experiments. However, only the upper two-thirds of the band which chromatographed with lanosteryl acetate was eluted and this material was then subjected to repeated re-chromatography to free it from the more-polar radioactive compounds. A portion of the purified fraction was then diluted with more carrier lanosteryl acetate and crystallized to constant specific radioactivity with virtually no decrease from the initial value (Table 3). As a further check for lanosterol (I) biosynthesis and also to investigate the possible labelling of cycloartenol (XII) a portion of the labelled material was mixed with carrier lanosteryl acetate and cycloartenyl acetate and the mixture was examined by g.l.c. Approx. 97% of the recovered radioactivity chromatographed with the lanosteryl acetate mass peak, but no significant amount of radioactivity was associated with the cycloartenyl acetate. These results therefore established that *A. rubens* is capable of squalene cyclization to give lanosterol (I), the normal sterol precursor in animals (Goad, 1970). The starfish does not apparently produce cycloartenol (XII), the sterol precursor found in plants (Goad & Goodwin, 1972).

The most-polar labelled fraction obtained from the t.l.c. separation on AgNO₃-impregnated silica gel was also analysed by g.l.c. after admixture with the corresponding carrier material previously isolated from *A. rubens* (Smith *et al.*, 1973). Only two labelled

compounds were detected which chromatographed with the mass peaks of the acetates of 4,4-dimethyl-5 α -cholesta-8,24-dien-3 β -ol (III) and 4,4-dimethyl-5 α -cholesta-7,24-dien-3 β -ol (V) with the former compound being the more highly labelled.

The constituent 4-monomethyl sterols of *A. rubens* have been identified as 4 α -methyl-5 α -cholesta-7,24-dien-3 β -ol (VII), 4 α -methyl-5 α -cholest-7-en-3 β -ol (VIII) and 31-norcycloartanol (XIII) (Smith *et al.*, 1973). To investigate the labelling of these compounds the radioactive 4-monomethyl sterols (from Expts. 1 and 2) were mixed with unlabelled 4-monomethyl sterol mixture obtained from *A. rubens* and acetylated. Separation by t.l.c. on AgNO₃-impregnated silica gel followed by radioscanning and radioautography revealed that 4 α -methyl-5 α -cholesta-7,24-dien-3 β -yl acetate was the most extensively labelled component of the mixture. Preparative t.l.c. of portions of the labelled 4-monomethyl sterol acetates yielded 4 α -methyl-5 α -cholesta-7,24-dien-3 β -yl acetate (Expt. 1, 16500 d.p.m.; Expt. 2, 26400 d.p.m.) and a co-chromatographing mixture of 4 α -methyl-5 α -cholest-7-en-3 β -yl acetate and 31-norcycloartanyl acetate (Expt. 1, 10500 d.p.m.; Expt. 2, 6900 d.p.m.). Owing to a lack of carrier material the former labelled compound could not be further characterized, but the latter mixture was treated with OsO₄, which gave the 7,8-dihydroxy derivative of 4 α -methyl-5 α -cholest-7-en-3 β -yl acetate (Rahman *et al.*, 1970; Smith *et al.*, 1973), but left the 31-norcycloartanyl acetate unchanged. After conversion of the 3 β -acetoxy derivatives into the corresponding 3 β -hydroxy compounds by treatment with lithium aluminium hydride in diethyl ether the 31-norcycloartanol (XIII; Expt. 1, 398 d.p.m.; Expt. 2, 286 d.p.m.) and 4 α -methyl-5 α -cholestane-3 β ,7 α ,8 α -triol (Expt. 1, 5500 d.p.m.; Expt. 2, 4000 d.p.m.) were separated by t.l.c. on silica gel. The retention of radioactivity in the triol

provided strong evidence for the biosynthesis of 4 α -methyl-5 α -cholest-7-en-3 β -ol (VIII) by *A. rubens*, whereas the small amount of radioactivity associated with the 31-norcychoartanol (XIII) indicated that this sterol is not produced *de novo* by the animal but is of dietary origin.

The 4-demethyl sterols of *A. rubens* are a mixture of mono- and di-unsaturated C₂₆, C₂₇, C₂₈ and C₂₉ sterols with 5 α -cholest-7-en-3 β -ol (X) as the most abundant component (Smith *et al.*, 1973). In the past, some investigations designed to establish the sterol-biosynthesizing capacity of invertebrates have relied only on the crystallization of labelled sterol mixtures to constant specific radioactivity. If the specific radioactivity attained a constant value this was judged to indicate that sterol biosynthesis was operative with the resulting incorporation of the labelled precursor. On the other hand a continuous decrease in specific radioactivity was regarded as evidence that sterol biosynthesis had probably not occurred (Voogt, 1969; Nomura *et al.*, 1969; Salaque *et al.*, 1966). However, if this technique is to give valid results with a complex sterol mixture one of two criteria apparently need to be satisfied. Either all the components of the sterol mixture must be labelled and be of approximately the same specific radioactivity, or alternatively, if only one sterol in the mixture is radioactive then it must co-crystallize with the other sterols and not be lost by fractional crystallization. The fact that a particular sterol is the major component of a mixture is no guarantee that it will remain so after repeated crystallizations. Thus a continuous loss of radioactivity during crystallization of a sterol mixture may result from the removal of the labelled component and does not necessarily prove the absence of sterol labelling; it may merely indicate that only one or two sterols are labelled in the mixture. These points were illustrated by the results obtained in the present work. The labelled 4-demethyl sterols obtained after the injection of *A. rubens* with [2-¹⁴C]-mevalonic acid (Expt. 1) were diluted with the unlabelled *A. rubens* sterols and the mixture was purified by formation of the digitonides and regeneration of the sterol. This purification proceeded without any change in the composition of the sterol mixture, but the specific radioactivity dropped by about 50% from 265 d.p.m./mg to 137 d.p.m./mg thus indicating the presence of label in either an unstable sterol or in compounds other than 3 β -hydroxy sterols. When the labelled sterol mixture was recrystallized from chloroform-methanol the specific radioactivity fell continuously to a very low value (137, 126, 105, 92, 67, 60, 44, 40, 17 and 10 d.p.m./mg for successive crystallizations) and this was accompanied by a rise in the melting point of the mixture from an initial value of 126–129°C to a final value of 161–165°C. G.l.c. analysis of the mother liquors from each crystallization revealed that the proportion

of 5 α -cholest-7-en-3 β -ol fell continuously from an initial value of 60% to only 5% after the ninth recrystallization. However, the proportion of (24*S*)-24-methylcholesta-7,22-dien-3 β -ol increased from 24% of the initial mixture to 68% after the final crystallization thus accounting for the observed change in melting point. Although this result shows that the C-24 alkylated sterols remaining in the mixture after recrystallization were essentially unlabelled it does not exclude the possibility that radioactivity was present in 5 α -cholest-7-en-3 β -ol (X) or some other sterol lost from the original mixture during recrystallization. This result alone therefore does not constitute proof that *A. rubens* is incapable of 4-demethyl sterol biosynthesis.

To investigate more rigorously the possibility that 5 α -cholest-7-en-3 β -ol (X) was labelled a sample of the radioactive 4-demethyl sterols obtained from Expt. 3 was mixed with an excess of pure carrier 5 α -cholest-7-en-3 β -ol (X) and the mixture recrystallized several times. In this case there was no loss of the 5 α -cholest-7-en-3 β -ol from the mixture and after an initial large fall the specific radioactivity soon became constant (518, 184, 164, 159, 150 and 156 d.p.m./mg). When another portion of the labelled sterol was examined by t.l.c. on AgNO₃-impregnated silica gel the major band of radioactivity corresponded to 5 α -cholest-7-en-3 β -ol (X; *R_F* 0.53) but there were a number of more-polar radioactive bands, one of which chromatographed with 5 α -cholesta-7,24-dien-3 β -ol (IX, *R_F* 0.43). The labelled 5 α -cholest-7-en-3 β -ol (X), obtained by preparative t.l.c. on AgNO₃-impregnated silica gel, crystallized to constant specific radioactivity without any significant decrease from the initial value (128, 124, 120, 120 and 120 d.p.m./mg). These crystallizations therefore provided very good evidence that 5 α -cholest-7-en-3 β -ol (X) was labelled but to confirm further this fact, a sample of the radioactive 4-demethyl sterol mixture was examined by g.l.c. The major radioactive peak co-chromatographed with 5 α -cholest-7-en-3 β -ol (X) showing that this compound was labelled. However, a relatively high background radioactivity across the remainder of the chromatogram indicated the presence of other minor labelled compounds and also possibly the presence of a compound which was unstable under the g.l.c. conditions used. When the Δ^7 sterols were first purified by t.l.c. on AgNO₃-impregnated silica gel it was found that the background radioactivity during g.l.c. analysis was very low and the only radioactive peak observed had a retention time the same as the 5 α -cholest-7-en-3 β -ol (X) mass peak. It is therefore concluded that *A. rubens* has at least a limited capacity for the synthesis of 5 α -cholest-7-en-3 β -ol (X) from [2-¹⁴C]mevalonate.

Various tissues of *A. rubens* were investigated to determine their ability to metabolize [2-¹⁴C]mevalonic

Table 3. Incorporation of [2-¹⁴C]mevalonic acid into non-saponifiable lipid by various tissues of *A. rubens*

A specimen of *A. rubens* was dissected to provide stomach, gonads, pyloric caecae and body-wall and these tissues were incubated for 20h at 20°C with [2-¹⁴C]mevalonic acid (2.0 μCi) dissolved in 5.0 ml of 0.1 M-sodium phosphate buffer, pH 7.8. At the termination of the incubation the non-saponifiable lipid was isolated and separated by t.l.c. on silica gel as described in the text.

	Body-wall	Stomach	Gonads	Pyloric caecae
Total weight of tissue from animal (g)	30.4	0.8	1.3	4.7
Weight of tissue for incubation (g)	1.3	0.8	1.3	0.9
Weight of non-saponifiable lipid (mg)	1.2	2.1	1.7	1.3
Percentage incorporation of ¹⁴ C into non-saponifiable lipid	9.5	18.0	3.1	10.3
Percentage distribution of radioactivity recovered in:				
Squalene	54.6	13.5	20.5	8.8
4,4-Dimethyl sterols	37.0	48.4	26.6	32.7
4-Monomethyl sterols	6.6	31.8	29.4	37.6
4-Demethyl sterols	1.8	6.3	23.5	20.9

acid and thus provide some preliminary information on their relative contributions to the production of sterols in the whole animal. An animal was dissected to provide samples of the pyloric caecae, gonads, stomach and body-wall and the tissues were incubated with [2-¹⁴C]mevalonic acid. T.l.c. and subsequent radioautography revealed very different distributions of radioactivity in the non-saponifiable lipids recovered from the four tissues (Table 3). The body-wall tissue gave a radioactive pattern similar to that observed in a whole animal with the squalene and 4,4-dimethyl sterols the most highly labelled but with little radioactivity in the 4-demethyl sterols. Similarly the stomach tissue gave a low incorporation of [2-¹⁴C]mevalonic acid into the 4-demethyl sterols, but there was an appreciable incorporation into the 4-monomethyl sterols. By contrast both the gonads and the pyloric caecae were far more efficient in the production of labelled 4-demethyl sterols. Examination of the labelled 4-demethyl sterols by t.l.c. on AgNO₃-impregnated silica gel established that with the body-wall, stomach and gonads about 35–40% of the radioactivity co-chromatographed with 5α-cholest-7-en-3β-ol (X), whereas with the pyloric caecae 55% was associated with this sterol. A portion of the purified sterol from the pyloric caecae incubation was crystallized with added carrier 5α-cholest-7-en-3β-ol (X) to constant specific radioactivity (274, 310, 260, 246 and 261 d.p.m./mg for successive crystallizations). The remainder was analysed by g.l.c. (Fig. 1) and the major radioactive peak coincided with the mass peak of 5α-cholest-7-en-3β-ol (X) thus establishing the synthesis *de novo* of this sterol. Analysis of the sterol mixtures from the four tissues by g.l.c. showed that in the stomach and body wall 5α-cholest-7-en-3β-ol (X) constituted 35 and 43% respectively of the total mixture of C₂₆, C₂₇, C₂₈ and C₂₉ sterols. In the gonads and pyloric caecae this sterol (X) accounted for 55 and 58% respectively of the

Fig. 1. G.l.c. analysis of the labelled 4-demethyl sterol fraction obtained from an incubation of the pyloric caecae of *A. rubens* with [2-¹⁴C]mevalonic acid

The pyloric caecae were incubated with [2-¹⁴C]mevalonic acid and the non-saponifiable lipid was extracted as described in the text. The 4-demethyl sterols (32500 d.p.m.) were obtained by t.l.c. on silica gel and then subjected to t.l.c. on AgNO₃-impregnated silica gel to give a fraction containing 5α-cholest-7-en-3β-ol (18000 d.p.m.). A portion of this material was then analysed on g.l.c. (3% OV-17) with sample trapping at 1 min intervals.

total which perhaps reflects the greater ability of these two tissues to synthesize C₂₇ sterols.

The ability of another starfish species, *H. sanguinolenta*, to produce sterols *de novo* was investigated briefly. This animal incorporated [2-¹⁴C]mevalonic acid efficiently into the non-saponifiable lipid;

squalene and the 4,4-dimethyl sterols were the most highly labelled compounds (Table 1). The squalene was characterized by the production of the thiourea adduct (790 d.p.m./ μ mol) and squalene hexahydrochloride (800, 836, 832, 859, 859 d.p.m./ μ mol for successive crystallizations) and by g.l.c. radioassay. Acetylation of the 4,4-dimethyl sterols and t.l.c. on AgNO₃-impregnated silica gel gave two radioactive bands. The more-polar material co-chromatographed with lanosteryl acetate and crystallized to constant specific radioactivity with added lanosteryl acetate (Table 2). The less-polar material co-chromatographed with 24,25-dihydrolanosteryl acetate and also crystallized to constant specific radioactivity with carrier 24,25-dihydrolanosteryl acetate (637, 649, 624, 647 and 631 d.p.m./mg for successive crystallizations). However, this material was not investigated further and its identification is regarded as tentative. The labelled 4-monomethyl sterols and 4-demethyl sterols were not examined.

Discussion

The results obtained in this study show unequivocally that the starfish *A. rubens* has the ability to biosynthesize sterols from mevalonic acid. Evidence for the incorporation of [1-¹⁴C]acetate into the sterols of *A. rubens* and other starfish species has also been presented by Voogt (1973). In mammalian tissues a wide range of intermediates have been identified between lanosterol and cholesterol (Schroepfer *et al.*, 1972). The many possible intermediates arise by non-specificity in the order of the reduction of the Δ^{24} bond, elimination of the methyl groups from C-4 and C-14 and the isomerization of the Δ^8 bond to the Δ^5 position. In *A. rubens* the relative concentrations of the various sterol intermediates (Smith *et al.*, 1973) and the extent to which they were labelled from mevalonate suggests that in the major pathway the C-14 and C-4 demethylation steps and the $\Delta^8 \rightarrow \Delta^7$ isomerization reaction tend to precede the reduction of the Δ^{24} bond (Scheme 1). However, it is apparent from the identification of sterols (VI) and (VII) in *A. rubens* that the Δ^{24} sterol reductase is sufficiently non-specific to lead to minor divergent pathways as shown in Scheme 1. The tentative identification of labelled 24,25-dihydrolanosterol (II) in *H. sanguinolenta* after incubation with [2-¹⁴C]-mevalonic acid indicates that the Δ^{24} sterol reductase may be relatively more active in this asteroid species.

In mammals cholest-5-en-3 β -ol is the principal sterol which is produced via the $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ sterol sequence (Goad, 1970) and such a pathway apparently operates in one class of echinoderms, the Echinoidea (Voogt, 1972; Smith & Goad, 1974). In the present work no evidence was found for the biosynthesis of $\Delta^{5,7}$ or Δ^5 sterols and it appears that *A. rubens* may lack the sterol C-5, C-6 dehydrogenase

system thus leading to the accumulation of 5 α -cholest-7-en-3 β -ol (X). This suggests that the cholest-5-en-3 β -ol found as the sulphate in the tissues of *A. rubens* (Björkman *et al.*, 1972; Goodfellow & Goad, 1973) is of dietary origin, but this point warrants further detailed study.

Examination of the 4-demethyl sterols indicated that only C₂₇ sterols were labelled. It thus appears that the various Δ^7 C₂₆, C₂₈, and C₂₉ sterols in *A. rubens* are of dietary origin. They are probably derived by transformation of the corresponding Δ^5 compounds (Fagerlund & Idler, 1960; Smith & Goad, 1971a; Goad *et al.*, 1972; Smith & Goad, 1975), which can be anticipated to predominate in the largely molluscan diet of *A. rubens* (Feder & Christensen, 1966; Idler & Wiseman, 1972). Similarly the absence of significant amounts of radioactivity in cycloartanol (XI), cycloartenol (XII) and 31-norcycloartanol (XIII) in the *A. rubens* incubations with [2-¹⁴C]mevalonic acid suggests that these sterols are also obtained in the diet and accumulated to some extent in the animals tissues. These three sterols would be expected to occur as sterol precursors in algae (Goad & Goodwin, 1972) and must reach *A. rubens* via the marine food chain. It is also possible that cycloartanol (XI) and 31-norcycloartanol (XIII) might be post-absorption metabolites of ingested cycloartenol (XII) if *A. rubens* can demethylate at C-4 and reduce the Δ^{24} bond but cannot open the 9 β ,19-cyclopropane ring. It has been reported (Gibbons *et al.*, 1971) that an animal tissue (rat liver) is incapable of opening the 9 β ,19-cyclopropane ring of cycloartenol (XII).

The relative contributions to the total body pool of 5 α -cholest-7-en-3 β -ol (X) by synthesis *de novo* and by the production of this sterol from dietary cholest-5-en-3 β -ol remains to be established. In the present work mevalonic acid was used, but this compound enters the biosynthetic sequence after the principal control point known to operate in mammalian tissues, namely the 3 β -hydroxy-3 β -methylglutaryl-CoA to mevalonic acid step (Rodwell *et al.*, 1973). Voogt (1973) found that [1-¹⁴C]acetate was incorporated into sterols by *A. rubens* and other starfish, but the amounts incorporated were low. It will therefore be interesting to determine what role 3 β -hydroxy-3 β -methylglutaryl-CoA reductase plays in the control of sterol synthesis in echinoderms. The accumulation of radioactivity from [2-¹⁴C]mevalonic acid in squalene and lanosterol in *A. rubens* indicates that a second rate-limiting step may occur between lanosterol and 4-demethyl sterols and warrants further study.

The investigations with the different tissues of *A. rubens* established that although all the tissues examined were capable of squalene biosynthesis from [2-¹⁴C]mevalonate the rate at which squalene was metabolized to sterol apparently varied. The ac-

cumulation of label in squalene and 4,4-dimethyl sterols in the body-wall, the major tissue, reflected the labelling pattern observed in the whole animal. In the gonads and pyloric caecae, however, there was a considerable incorporation of radioactivity into the 4-demethyl sterols. The gonads and pyloric caecae contain a higher proportion of 5 α -cholest-7-en-3 β -ol (X) in the 4-demethyl sterols than either the body-wall or the stomach and this could result from their relatively greater efficiency of C₂₇ sterol biosynthesis *de novo*. Also the pyloric caecae and gonads undergo seasonal changes related to reproduction and therefore may have greater demands for sterols which are met by synthesis *de novo*.

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