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INVESTIGATIONS OF THE CAPACITY OF SYNTHESIZING 3β-STEROLS IN MOLLUSCA—IV. THE BIOSYNTHESIS OF 3β-STEROLS IN SOME MESOGASTROPODS

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Abstract—1. The incorporation of sodium acetate-1-^{14}C into some classes of lipids in *Viviparus fasciatus* (Müller) and *Littorina littorea* (L.) is investigated.

2. It is demonstrated that these animals utilize the injected acetate for the biosynthesis of fatty acids and non-saponifiable lipids including squalene and 3β-sterols.

3. It is supposed that probably all mesogastropods that have retained the feeding habits of the Archaeogastropoda are able to synthesize 3β-sterols.

4. The sterol mixtures of these snails are investigated by means of gas chromatography on different stationary phases and the identity of the components is discussed.

INTRODUCTION

DATA concerning the sterol composition of snails belonging to the order Mesogastropoda are rather scanty. Kind & Herman (1948), who studied the sterols of *Littorina littorea*, demonstrated in them the presence of 85 per cent cholesterol and 9·6 per cent of a provitamin D, which they supposed to be identical with 7-dehydroclionasterol. Matsumoto & Tamura (1955) reported that in *Viviparus iaponicus* cholesterol was accompanied by brassicasterol, polypherasterol and γ-sitosterol, while on the ground of u.v. spectra they proved the presence of 6 per cent $\Delta^{5,7}$-sterols. Toyama (1958) showed the presence of 19·3 per cent $\Delta^{5,7}$-sterols in the sterol mixture of *V. histricus*. Tanaka & Toyama (1960), in studying *L. brevicula*, came to similar results as Kind & Herman (1948) in that they found cholesterol and 2·2 per cent $\Delta^{5,7}$-sterols.

The results of all these investigations agree in that the sterol mixtures occurring in mesogastropods are more complicated and contain more $\Delta^{5,7}$-sterols than those of the archeogastropods (Voogt, 1968b).

To the best of my knowledge, the origin of these sterols has never been investigated. As pointed out in a previous paper (Voogt, 1968b), the mesogastropods are of great interest in this respect as they are intermediate between the archaeogastropods, which are probably all able to synthesize 3β-sterols (Voogt, 1968b), and the neogastropods, which may all lack this capacity (Voogt, 1967b).

The present paper deals with the biosynthesis of 3β-sterols in *V. fasciatus* (Müller)* and *L. littorea* (L.)*, two mesogastropods which resemble the archeogastropods as they have retained the primitive deposit feeding or herbivorous habits which are characteristic for the latter order (Stone & Morton, 1958).

MATERIALS AND METHODS

The snails of the species *Viviparus fasciatus* (Müller) used in this investigation were collected in the neighbourhood of Utrecht, while those of *Littorina littorea* (L.) were obtained from the Netherlands Institute for Marine Research at Den Helder.

Seventy animals of the first species and 150 of the second one were each injected with 5 and 2·5 μc respectively of sodium acetate-1-^{14}C (Philips Duphar, specific activity 20 mc/mM) dissolved in 0·01 ml of water. The injections were applied into the body-part connecting the foot with the visceral pouch.

The animals were killed 95 and 66 hr, respectively, after injection. Lipids were extracted from them using the procedure described by Blight & Dyer (1959). The obtained total lipids were saponified in a solution of 1·5 N potassium hydroxide in 80% methanol for 6 hr under the usual conditions. The non-saponifiable lipids were extracted from the saponification mixtures with petroleum ether (b.p. 40–60°C), except in the case of *L. littorea*, as the non-saponifiable lipids were extracted here with diethyl ether. The extracts, after washing with water, were filtered over anhydrous sodium sulphate after which those containing the fatty acids were decolorized with acid-washed activated charcoal. The separation of the unsaponifiable lipids into three fractions, namely that containing squalene, that with sterols and the residue, was performed on columns of aluminium oxide (Merck) as described elsewhere (Voogt, 1967a, 1968b). The crude squalene fraction, enriched with carrier squalene, was purified by preparing the hexahydrochloride according to Loud & Bucher (1958). The 3β-sterols were isolated from the crude sterol fraction by preparing their digitonides (Vahouny *et al.*, 1960) which were decomposed by pyridine. The 3β-sterols were recrystallized from methanol until their specific radioactivities remained constant on further recrystallizations.

All radioactivities were determined with a Packard Tri-Carb Liquid Scintillation Spectrometer equipped with an automatic external standardization.

Gas chromatograms of the crude squalene fractions were made on a Becker gas chromatograph, Model 2300, with dual columns and flame ionization detection. The used glass column measured 120 × 0·4 cm i.d. and was packed with 2% SE-30 on Gas Chrom Q (80–100 mesh). The column temperature was 220°C and the gas flow 50 ml N_2/58 sec.

Gas chromatography of 3β-sterols was carried out on a Becker gas chromatograph, Model 1452, with the same equipment. Glass columns, 180 × 0·38 cm i.d. were used. The support, Chromosorb W (100–120 mesh), was acid washed and silanized before use. Different batches of this support were coated with 2% SE-30, 2% QF-10065 or 2% NPGS using the coating technique after Horning *et al.* (1959).

In addition a quantity of acid-washed support was coated in the same way with successively 1% PVP (Vanden Heuvel *et al.*, 1963; Knights, 1964) and 2% NPGS. The column temperatures were 200°C for the two columns with NPGS and that with QF-10065 and 210°C for SE-30. In all cases the gas flow was about 50 ml N_2/min. Chromatography was carried out with the trimethylsilyl ethers of the 3β-sterols which were prepared using the procedure of Hammond & Leach (1965).

The 3β-sterols were identified by comparing their relative retention times (relative to cholestane) with those of reference sterols on the different stationary phases. Furthermore, the steroid numbers (Vanden Heuvel & Horning, 1962; Hamilton *et al.*, 1963) of the reference sterols were determined for the four columns used and the steroid number contributions

* Scientific names after Mr. L. Butot, R.I.V.O.N., Zeist.

of the several functional groups were calculated. These data were applied to the unknown sterol mixtures of *Viviparus* and *Littorina* as it was then possible to predict the steroid number of nearly every sterol.

RESULTS

The quantities of the isolated lipid fractions and components are summarized in Table 1.

TABLE 1—QUANTITIES OF ISOLATED LIPID FRACTIONS AND COMPONENTS FROM *V. fasciatus* AND *L. littorea*

Lipid fraction	*V. fasciatus*	*L. littorea*
Total fresh wt. (mg)*	180,000·0	51,400·0
Total lipids (mg)	1290·2	2586·0
(% of fresh wt.)	0·72	5·03
Fatty acids (mg)	73·9	412·9
(% of fresh wt.)	0·04	0·80
Unsaponifiable lipids (mg)	179·5	361·5
(% of fresh wt.)	0·10	0·70
Crude squalene fraction (mg)	20·8	20·0
Crude sterol fraction (mg)	131·9	77·8
(% of fresh wt.)	0·07	0·15
3β-Sterols (mg)	130·6	21·7
(% of fresh wt.)	0·07	0·04

* All fresh weights are determined without the shells.

The crude squalene fractions were tested for the presence of squalene by gas chromatography on SE-30. In the chromatograms obtained for *Viviparus* twelve peaks were visible. Calculation of the proportional composition showed that squalene made up $8{\cdot}4 \pm 1{\cdot}2$ per cent of the crude squalene fraction which corresponds with 1·65 mg. After the addition of 6·6 mg carrier squalene to the total fraction the squalene hexahydrochloride was prepared. The radioactivity of this compound amounted to 149 dis/min per mg. So the radioactivity of the original (= native) squalene was about 1140 dis/min per mg. In the gas chromatograms of the crude squalene fraction of *Littorina* thirteen peaks were present. Squalene made up 2 per cent of the whole fraction, corresponding with 0·086 mg. Nine milligrams of carrier squalene were added. The radioactivity of the squalene hexahydrochloride amounted to $1{\cdot}5 \pm 0{\cdot}8$ dis/min per mg. So calculation of the radioactivity of the native squalene is senseless. The crude squalene fraction of *Littorina* was also subjected to thin-layer chromatography. However, no radioactivity could be detected on the squalene spot with a radiogram-scanner (Berthold, type L.B. 2721).

Though this investigation is concerned with the biosynthesis of sterols, the fatty acids have been isolated too. Their radioactivity has been measured to have

proof that the injected acetate has been metabolized by the animals. The radioactivities of the various fractions are summarized in Table 2.

TABLE 2—RADIOACTIVITY OF THE ISOLATED LIPID FRACTIONS AND COMPONENTS OF *V. fasciatus* AND *L. littorea* EXPRESSED IN CALCULATED dis/min per mg, AFTER ADMINISTRATION OF SODIUM-ACETATE-1-^{14}C

Lipid fraction	*V. fasciatus*	*L. littorea*
Total lipids	5340·2	6141·9
Fatty acids	7255·9	9087·8
Unsaponifiable lipids	987·6	7920·5
Crude squalene fraction	738·8	4517·8
Purified squalene hexahydrochloride (with carrier squalene)	149·0	1·5
Original squalene (calculated)	1140·0	—
Crude sterol fraction	576·9	2517·7
3β-Sterols	404·0	1656·2
3β-Sterols after three recrystallizations	388·9	1344·0
3β-Sterols after four recrystallizations	383·8	1220·8
3β-Sterols after five recrystallizations	384·1	1189·7

The results of the gas chromatography of the 3β-sterols of *Viviparus* and *Littorina* are summarized in Tables 3 and 4, respectively, while representative chromatograms are given in Figs. 1 and 2.

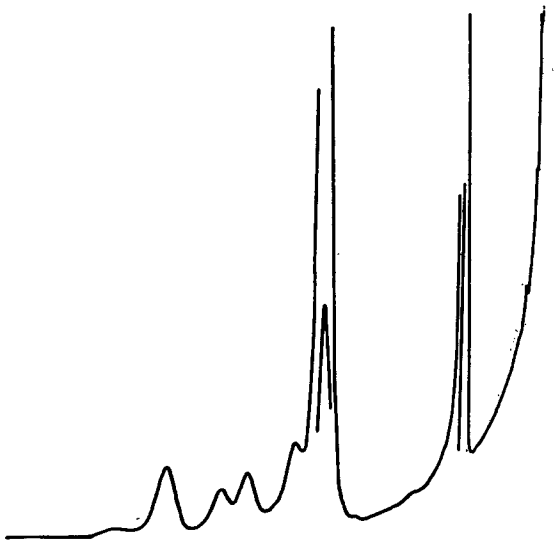

FIG. 1. 3β-Sterols of *V. fasciatus* on 2 per cent NPGS/PVP. Conditions as given in the text.

TABLE 3—RELATIVE RETENTION TIMES (R.R.T.) AND STEROID NUMBERS (S.N.) OF THE 3β-STEROLS OF *V. fasciatus* AND SOME REFERENCE STEROLS DETERMINED FOR DIFFERENT STATIONARY PHASES

Component	SE-30			NPGS/PVP			NPGS/DMCS			QF-10065		
	R.R.T.	S.N.	%	R.R.T.	S.N.	%	R.R.T.	S.N.	%	R.R.T.	S.N.	%
1										1·29	27·90	Trace
2				1·56	28·55	0·3	1·49	28·50	Trace	1·56	28·55	Trace
3	2·13	29·65	0·9	2·25	29·80	0·3				1·72	28·90	Trace
22-Dehydrocholesterol (calc.)*		29·60			29·80			29·75			28·90	
4	2·24	29·75	2·2							1·86	29·15	1·9
Δ^{22}-Cholestenol (calc.)		29·65			29·75			29·60			29·25	
5	2·53	30·30	71·5	2·65	30·35	67·2	2·39	30·23	71·5	2·12	29·65	78·6
Cholesterol	2·60	30·30		2·67	30·35		2·41	30·25		2·10	29·65	
6	2·86	30·78	5·6	2·99	30·80	8·6	2·65	30·63	5·2			
Brassicasterol (calc.)		30·60			30·80			30·60			29·90	
7										2·77	30·60	8·4
8	3·38	31·35	6·9	3·57	31·40	6·7	3·24	31·38	6·2			
Campesterol (calc.)		31·35			31·50			31·35			30·75	
Campesterol (determined)										2·83	30·75	
9	3·75	31·73	2·6	3·88	31·75	5·3	3·44	31·60	4·2			
Stigmasterol	3·74	31·60		3·94	31·75		3·42	31·60		2·97	30·90	
10							3·55	31·70	Trace			
Ergosterol	3·17	31·05		4·03	31·80		3·58	31·75		2·80	30·70	
24-Methylenecholesterol (calc.)		31·15			31·85			31·70			30·35	
11	4·39	32·28	12·1	4·54	32·25	10·4	4·04	32·20	11·6			
β-Sitosterol	4·57	32·30		4·49	32·30		4·00	32·15		3·62	31·65	
12				5·15	32·70	1·3	4·76	32·80	1·4	3·40	31·35	11·1
Fucosterol (calc.)		32·15			32·80			32·70				
Fucostrol (determined)										3·34	31·35	

* Explanation in text.

TABLE 4—RELATIVE RETENTION TIMES AND STEROID NUMBERS OF THE 3β-STEROLS OF *L. littorea* AND SOME REFERENCE STEROLS DETERMINED FOR DIFFERENT STATIONARY PHASES

Component	S.E.-30			NPGS/PVP			NPGS/DMCS			QF-10065		
	R.R.T.	S.N.	%	R.R.T.	S.N.	%	R.R.T.	S.N.	%	R.R.T.	S.N.	%
1										1·31	27·90	1·4
2							1·60	28·75	0·4	1·58	28·55	0·3
3	2·30	29·85	Trace							1·92	29·25	0·7
Δ^{22}-Cholesterol (calc.)		29·65			29·75			29·60			29·25	
4	2·55	30·23	60·4	2·61	30·35	64·5	2·34	30·18	66·8	2·15	29·65	65·0
Cholesterol	2·60	30·30		2·67	30·35		2·41	30·25		2·10	29·65	
5				3·03	30·95	5·2	2·62	30·60	4·4	2·32	29·90	20·5
Brassicasterol (calc.)		30·60			30·80			30·60			29·90	
6	2·78	30·54	25·0	3·29	31·25	15·6	3·09	31·25	19·0			
Desmosterol (calc.)		30·55			31·30			31·25				
Desmosterol (determined)										2·29	30·00	
7										2·83	30·60	5·8
8				3·68	31·65	4·3						
9	3·35	31·15	14·5	3·88	31·85	4·8	3·48	31·63	3·1			
24-Methylenecholesterol (calc.)		31·15			31·85			31·70			30·35	
10				4·52	32·35	3·3	3·95	32·15	3·9			
β-Sitosterol	4·57	32·30		4·49	32·30		4·00	32·15		3·62	31·65	
11	4·48	32·15	5·1	5·17	32·85	2·2	4·64	32·70	2·4	3·44	31·35	6·4
Fucosterol (calc.)		32·15			32·80			32·70				
Fucosterol (determined)										3·34	31·35	

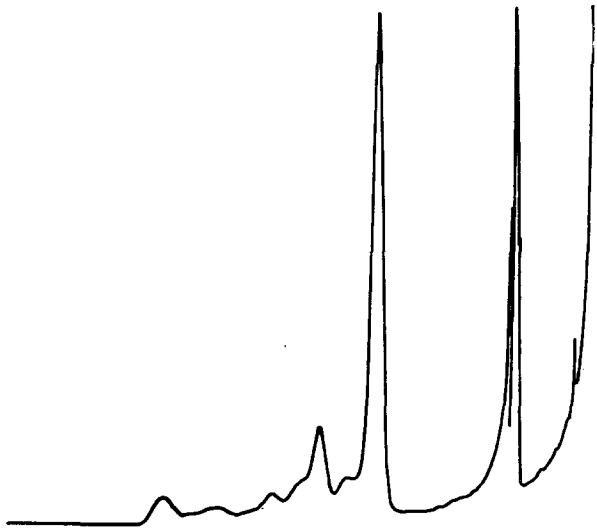

FIG. 2. 3β-Sterols of *L. littorea* on 2 per cent NPGS/PVP. Conditions as given in text.

DISCUSSION

Table 1 shows the content of total lipids in the animals investigated. These amounts are rather high, especially for *Littorina*, and it should be remarked that these substances of both species and the unsaponifiable lipids of *Littorina* were difficult to get entirely "dry". They remained sticky even after several days in a vacuum desiccator. As they retained the same weight however and the unsaponifiable lipids make up 14 per cent of the total lipids in both cases, these values must be correct.

It is striking that the lipid content of the fresh-water inhabitant *Viviparus* is much lower than that of the marine *Littorina*. This is in agreement with what has been discussed in a previous paper (Voogt, 1968a).

The composition of the unsaponifiable lipids in both species seems to be quite different, as in *Viviparus* these lipids consist mainly of 3β-sterols, whereas in *Littorina* they contain only 6 per cent 3β-sterols.

The difference is still more striking if we compare the weight of the crude sterol fraction with that of the 3β-sterols. It is unlikely that the low yield of 3β-sterols in *Littorina* is due to the extraction or purification procedure.

In another experiment with *Littorina* 181·4 mg of unsaponifiable lipids were obtained from 63,700 mg fresh weight. These unsaponifiable lipids gave 76·7 mg of crude sterol fraction and 6·4 mg of 3β-sterols.

With regard to the calculation of the radioactivity in the native squalene of *Viviparus*, it should be borne in mind that there are numerous sources of errors in this kind of determination. So the number of 1140 dis/min per mg has no absolute value but permits only to conclude that the native squalene is labelled with C^{14}.

Of course the same holds for *Littorina*. In this case the content of squalene is very low (perhaps this is related to the low content of 3β-sterols) and it is not possible to draw any conclusion about the labelling of this substance.

Table 2 shows that all the isolated lipid fractions, with exception of the squalene hexahydrochloride of *Littorina*, possess a high specific radioactivity. From this it may be concluded that the investigated animals are able to synthesize fatty acids and 3β-sterols from acetate. This could be expected on the ground of the results found for some archeogastropods (Voogt, 1968b) and the close relationship between the animals investigated and these archeogastropods. So it seems reasonable to enlarge the supposition that all archeogastropods may possess the capacity of synthesizing 3β-sterols to those mesogastropods that have retained the feeding habits of the former order. In this connexion the labelling of squalene is important as it suggests that, as in vertebrates, squalene is an intermediate in the biosynthesis of sterols.

In Table 3 the results of gas chromatography of the sterols of *Viviparus* are listed. As can be seen, the QF-10065 column gives a good resolution in the first part of the chromatogram, but later a less good one. So on this column the first four components are clearly visible, but component 6 is not separated from component 5.

Components 8, 9 and 10 seem to be absent and a component 7, which could not be identified, is present. In all probability this peak 7 is not a separate substance but represents the missing peaks mentioned above. What has been remarked about components 5 and 6 holds also for the components 11 and 12. If the supposition that peak 7 does not represent one single substance is right, eleven sterols have been distinguished.

Components 1 and 2, present only in trace amounts, are identified as C-26 sterols. The presence of this type of sterol has been pointed out by Wainai *et al.* (1964). Perhaps the first one is a $\Delta^{5,22}$-sterol.

The third component could be identified almost with certainty as 22-dehydrocholesterol. Probably it makes up less than 1 per cent of the total sterols.

The fourth component has been tentatively identified as Δ^{22}-cholesterol and makes up about 2 per cent.

About component 5, cholesterol, there could not be any doubt. It is the main sterol in this animal (about 70 per cent).

Component 6 has been identified as brassicasterol after comparison with stigmasterol on SE-30. It is present in an amount of about 5 per cent. Together components 5 and 6 make up 77·1, 75·8, 76·7 and 78·6 per cent on the four different columns.

Component 8 (more than 6 per cent) has been identified as campesterol.

Component 9 could be identified as stigmasterol and may make up about 4 per cent.

The identification of component 10 is not very reliable. A small peak was only visible in chromatograms obtained from the column pre-treated with DMCS and coated with NPGS. As can be seen from Table 3, the steroid numbers of the

suitable sterols, ergosterol and 24-methylenecholesterol, are nearly the same on the first three columns, the fourth column could have given the decision but has failed to do so. As no yellow colour appeared on storage of the TMS ethers it is more probable that this component is 24-methylenecholesterol. Components 11 and 12, identified as β-sitosterol and fucosterol, make up about 10 and 1 per cent, respectively.

Striking is the total absence of $\Delta^{5,7}$-sterols in the gas chromatograms, while several investigators have reported their presence on the grounds of u.v. spectra. It may be possible that they are oxidized during column chromatography and isolation.

There was no reason to accept the presence of γ-sitosterol which is indeed difficult to separate from β-sitosterol.

What has been remarked about the results of gas chromatography on QF-10065 for *Viviparus* holds also for *Littorina*.

The composition of the sterol mixture is rather similar to that of *Viviparus*. There are some quantitative differences, but the principal differences are the absence of 22-dehydrocholesterol, campesterol, and stigmasterol and the presence of a large amount of desmosterol.

Component 8 could not be identified, but on the ground of calculation it might be chondrillasterol. The steroid number of chondrillasterol on QF-10065 should be 30·60 (component 7 ?) and on SE-30 it should be 31·15. The latter should explain the high percentage of 24-methylenecholesterol for the SE-30 column. No $\Delta^{5,7}$-sterols could be detected.

In Table 5 the sterols of *Viviparus* and *Littorina* are listed.

TABLE 5—REVIEW OF THE 3β-STEROLS IN *V. fasciatus* AND *L. littorea*

Component No.	Identity	*Viviparus* (%)	*Littorina* (%)
1	C-26	Trace	1
2	C-26	Trace	0·3
3	22-Dehydrocholesterol	< 1	Absent
4	Δ^{22}-Cholestenol (?)	2	< 1
5	Cholesterol	70	64
6	Brassicasterol	5	5
7	Desmosterol	Absent	20
8	Chondrillasterol (?)	Absent	4
9	Campesterol	< 6	Absent
10	Stigmasterol	4	Absent
11	24-Methylenecholesterol	Trace	4
12	β-Sitosterol	10	3
13	Fucosterol	1	2

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Key Word Index—Sterol synthesis in molluscs; molluscs; 3β-sterols; *Viviparus fasciatus*; *Littorina littorea*; squalene.