Comp. Biochem. Physiol., 1972, *Vol.* 41**B**, *pp.* 831 *to* 841. *Pergamon Press. Printed in Great Britain*

INVESTIGATIONS OF THE CAPACITY OF SYNTHESIZING 3β-STEROLS IN MOLLUSCA—VI. THE BIOSYNTHESIS AND COMPOSITION OF 3β-STEROLS IN THE NEOGASTROPODS *PURPURA LAPILLUS* AND *MUREX BRANDARIS*

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(*Received* 7 *September* 1971)

Abstract—1. It is shown that *Purpura lapillus* and *Murex brandaris* are capable of synthesizing sterols from mevalonate and acetate. The biosynthesis of sterols from acetate proceeds only at a slow rate. This phenomenon is compared with that observed in *Natica cataena.*

2. The composition of the sterol mixtures of *P. lapillus* and *M. brandaris* is determined by means of gas–liquid chromatography.

3. In both animals cholesterol is the principal sterol making up 77 and 59 per cent of the total sterols respectively. The sterol composition of *Murex* differs rather strongly from that of other prosobranchs.

INTRODUCTION

MANY investigators have studied the sterols of neogastropod molluscs. Tsujimoto & Koyanagi (1934) reported that the sterols of *Rapana thomasiana* consisted of cholesterol and conchasterol. The latter sterol is identical with 24-methylenecholesterol (Idler & Fagerlund, 1955). Toyama *et al.* (1955b) showed that the sterols of the same species contained also 1·6 per cent of provitamin D. The sterols of *Urosalpinx cinereus* consisted of 90 per cent of cholesterol and 10 per cent of a non-identified $\Delta^{5,7}$ sterol (Kind & Goldberg, 1953). While Dorée (1909) reported the presence of only cholesterol in *Buccinum undatum*, Bock & Wetter (1938) demonstrated that also 7-dehydrocholesterol was present (making up even 27·5 per cent of the total sterols). Cholesterol is also the principal sterol of *Buccinum perryi* (Toyama & Tanaka, 1956) and *Hemifusus ternatanus* (Toyama *et al.*, 1955a). In *Nassa obsoleta* clionasterol was observed beside cholesterol (Kind *et al.*, 1948; Kind & Goldberg, 1953).

Summarizing one might say that cholesterol has been encountered repeatedly in neogastropods and is considered to be the principal sterol in these animals. Further, 24-methylenecholesterol and clionasterol have been observed as well as strongly varying amounts of $\Delta^{5,7}$ sterols, of which the latter have been identified only in one case.

In previous investigations it was found that the sterol compositions of Archaeogastropoda and Mesogastropoda were more complicated than the data in the literature suggested (Voogt, 1969, 1971a, b).

Hardly anything is known about the origin of the sterols in Neogastropoda. Kind & Goldberg (1953) concluded from the difference in sterol composition of *Urosalpinx cinereus* and the predated Bivalvia, that there was "indirect evidence" that *Urosalpinx* did synthesize its own sterols. However in *Buccinum undatum* no biosynthesis of sterols could be detected after injection of sodium acetate-1-^{14}C (Voogt, 1967). This paper deals with the composition and biosynthesis of sterols in *Purpura lapillus* (L.) and *Murex brandaris* L. (Mollusca, Gastropoda, Prosobranchia, Neogastropoda, Muricidae).

MATERIALS AND METHODS

Snails of the species *Purpura lapillus* were collected on old oyster-banks near Kattendijke (Zeeland), while the specimens of *Murex brandaris* were collected in the neighbourhood of the Laboratory for Marine Biology "Laboratoire Arago" at Banyuls-sur-mer, France. The animals were each injected with sodium acetate-1-^{14}C (NEN Chemicals) or with DL-mevalonic acid-2-^{14}C (DBED salt, NEN Chemicals) into the body-part connecting the foot with the visceral pouch and killed after some time. Then they were stored at −20°C until they were needed. Data about the experimental animals and the radioactive compounds injected are summarized in Table 1.

TABLE 1—DATA ABOUT THE ORIGIN AND TREATMENT OF *P. lapillus* AND *M. brandaris* EXAMINED FOR THEIR CAPACITY OF SYNTHESIZING STEROLS

	P. lapillus		*M. brandaris*	
	I	II	I	II
No. of animals	70	64	45	43
Place and date of collecting	Kattendijke 26.4.1967	Kattendijke 26.4.1967	Banyuls-sur-mer 15.6.1967	Banyuls-sur-mer 15.6.1967
Compound injected and specific activity ηC_i	Sodium acetate -1-^{14}C, 21·6 mc/mM	DL-mevalonic acid-2-^{14}C, (DBED salt), 1·64 mc/mM	Sodium acetate -1-^{14}C, 20 mc/mM	DL-mevalonic acid-2-^{14}C (DBED salt), 1·64 mc/mM
Dosage administered to each animal (μC_i)	3·5	0·5	5	0·25
"Incubation time" (hr)*	8	120	72	72

* Time between the moment of injection and sacrificing the animals.

The animals were cut into pieces and hydrolysed with 1·5 N potassium hydroxide in 80% methanol. The unsaponifiable and saponifiable lipids were isolated from the saponification mixture in the usual way. The unsaponifiable lipids were separated into three fractions by means of chromatography on columns of aluminium oxide (Merck) as described previously (Voogt, 1970). This yielded a crude squalene fraction, a crude sterol fraction and

a remaining fraction. The 3β-sterols were isolated from the crude sterol fraction by means of digitonin. Further purification of the sterols was performed by recrystallizing them from methanol. All radioactivities were measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3315.

The 3β-sterols were analysed on a Becker gas chromatograph, Model 1452, equipped with dual columns and flame ionization detection. The glass columns (180 × 0·38 cm i.d.) were silanized before use by rinsing them with a solution of 5% DMCS in toluene. Application of the stationary phases SE-30 and NPGS, and preparation of trimethylsilylethers were performed as described previously (Voogt, 1971c). Sterols were identified by means of their steroid numbers, using androstane and cholestane or cholestane and cholesterylvalerate as internal standards. Steroid numbers were calculated from the formula

$$SN = 27 + 5{\cdot}84 \frac{\log rrt_s}{\log rrt_{cv}} \quad \text{(SE-30)}$$

or

$$SN = 27 + 8{\cdot}63 \frac{\log rrt_s}{\log rrt_{cv}} \quad \text{(NPGS)}.$$

In these formulae SN stands for the steroid number of a sterol, while rrt_s and rrt_{cv} stand for retention time relative to that of cholestane of this sterol and cholesterylvalerate respectively.

RESULTS

The quantities of the isolated lipid fractions are given in Table 2.

The specific radioactivities are determined for a number of the lipid fractions. The results are summarized in Table 3.

TABLE 2—QUANTITIES OF THE ISOLATED LIPID FRACTIONS FROM *P. lapillus* AND *M. brandaris*

	P. lapillus		*M. brandaris*	
Lipid fraction	I	II	I	II
Total fresh wt. (mg)*	209,800	131,500	410,000	443,000
Unsaponifiable lipids (mg)	431·6	423·8	3327·0	1844·2
(% of fresh wt.)	0·21	0·32	0·81	0·42
Crude squalene fraction (mg)	66·1	17·3	17·6	33·5
Crude sterol fraction (mg)	135·5	305·0	766·9	648·0
(% of fresh wt.)	0·06	0·23	0·19	0·15
3β-sterols (mg)	117·2	271·5	583·4	450·3
(% of fresh wt.)	0·06	0·21	0·14	0·10

* Fresh weights were determined without the shells.

A small quantity of the sterols of the two species was hydrogenated and then acetylated. These sterylacetates were analysed by means of gas–liquid chromatography and representative chromatograms are shown in Figs. 1 and 2. The compositions are given in Table 4.

Table 3—Radioactivity in some of the isolated lipid fractions from *P. lapillus* and *M. brandaris* after the injection of sodium acetate-1-^{14}C (A) or DL-mevalonic acid-2-^{14}C (M), expressed in dpm/mg

	P. lapillus		*M. brandaris*	
Lipid fractions	I (A)	II (M)	I (A)	II (M)
Fatty acids	1308·8	364·4	2300·8	37·9
Unsaponifiable lipids	724·5	4571·3	707·7	397·5
Crude squalene fraction	682·1	5583·1	63·4	791·5
Crude sterol fraction	84·4	1212·7	56·1	189·4
3β-sterols	33·5	1006·5	17·2	145·8
3β-sterols after three recrystallizations	16·5	866·5	10·4	
3β-sterols after four recrystallizations	18·0		12·4	
3β-sterols after five recrystallizations	17·9	406·5		94·7
3β-sterols after six recrystallizations		369·5		75·1
3β-sterols after seven recrystallizations		331·8		65·8
3β-sterols after eight recrystallizations				62·3

Gas chromatography of the sterols as their trimethylsilylethers on the stationary phases SE-30 and NPGS yielded more details about the sterol compositions. Representative chromatograms of *M. brandaris* are shown in Figs. 3 and 4.

Nine peaks were present in the chromatograms of *Murex* performed on SE-30 and eleven in those performed on NPGS. The corresponding chromatograms of *P. lapillus* both showed nine peaks. The identity and percentage composition of the sterols of *Murex* are given at the bottom of Figs. 3 and 4. As no chromatograms of *Purpura* are depicted, the tentative identification of the several peaks is given in Table 5.

Table 4—Proportional composition according to the number of carbon-atoms of the sterols of *P. lapillus* and *M. brandaris*

Species	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}
P. lapillus	1·3	89·5	7·9	1·2	
M. brandaris	0·4	61·6	15·4	20·3	2·4

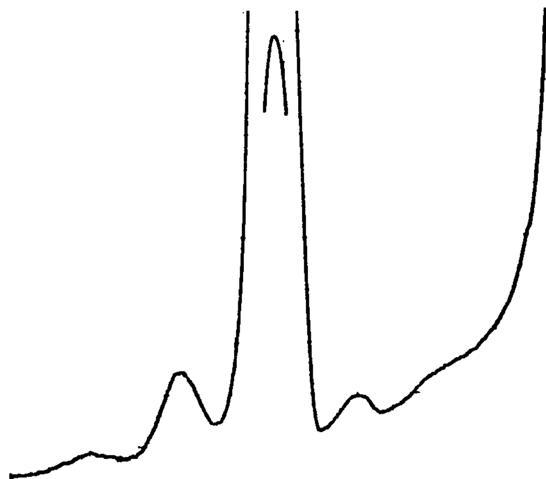

FIG. 1. Chromatogram of the hydrogenated sterylacetates of *P. lapillus*.

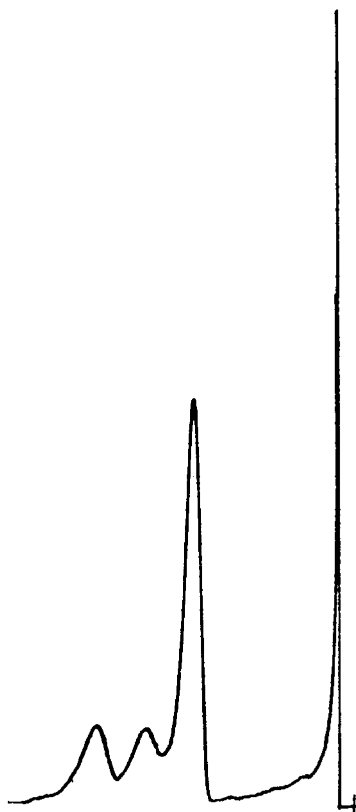

FIG. 2. Chromatogram of the hydrogenated sterylacetates of *M. brandaris*.

Table 5—Relative retention times (RRT), steroid numbers (SN) and proportional composition of the sterols of *P. lapillus* and the calculated steroid numbers of some suitable sterols determined for two different stationary phases

SE-30				NPGS			
No.	RRT	SN	%	No.	RRT	SN	%
1.	1·19	27·60	Trace				
2.	1·54	28·48	1·0	1.	1·55	29·67	1·2
3.	1·69	28·80	0·2				
4.	2·25	29·80	3·9	2.	2·35	30·15	3·5
	22-Dehydrocholesterol (trans)	29·80			22-Dehydrocholesterol (trans)	30·09	
5.	2·53	30·18	76·9	3.	2·52	30·42	76·1
	Cholesterol	30·18			Cholesterol	30·36	
6.	2·79	30·52	13·1	4.	2·86	30·84	4·6
	Brassicasterol	30·58			Brassicasterol	30·83	
				5.	3·08	31·27	4·0
	Desmosterol	30·50			Desmosterol	31·13	
	7-Dehydrocholesterol	30·56					
7.	3·33	31·12	2·1	6.	3·44	31·45	6·8
	Campesterol	31·19			Campesterol	31·43	
					7-Dehydrocholesterol	31·38	
8.	3·67	31·48	0·5	7.	3·75	31·80	2·1
	Stigmasterol	31·50			Stigmasterol	31·71	
9.	4·31	32·00	0·5	8.	4·26	32·27	0·9
	β-Sitosterol	32·04			β-Sitosterol	32·29	
				9.	4·48	32·45	0·9

DISCUSSION

Table 3 shows that *Purpura* and *Murex* are able to synthesize saponifiable and unsaponifiable lipids from both acetate and mevalonate. Starting from acetate the saponifiable lipids are labelled much higher than the unsaponifiable ones. As could be expected the situation is reversed when mevalonate is the precursor.

With the exception of *Murex* I all crude squalene fractions are labelled high; this is especially the case, starting from mevalonate. Squalene which might be present in the crude squalene fractions from the two experiments with *Purpura* was converted to squalenedodecabromide according to Mackenna *et al.* (1950). From *Purpura* I 23·08 mg of the bromide was obtained. After several washings with cold ether the specific activity amounted to 152 dpm/mg and after an additional washing to 170 dpm/mg. So the total radioactivity present in squalene amounted to about 4700 dpm.

From the crude squalene fraction of *Purpura* II no precipitate of squalenedodecabromide could be obtained. After several washings the material which might

be present was rinsed into a counting vial and radioactivity was determined. This amounted to 369 dpm and possibly should be ascribed to residues of the crude squalene fraction other than squalene. Thus starting from acetate the crude squalene fraction is labelled to a lesser degree, but contains more squalene than when mevalonate is used as the precursor.

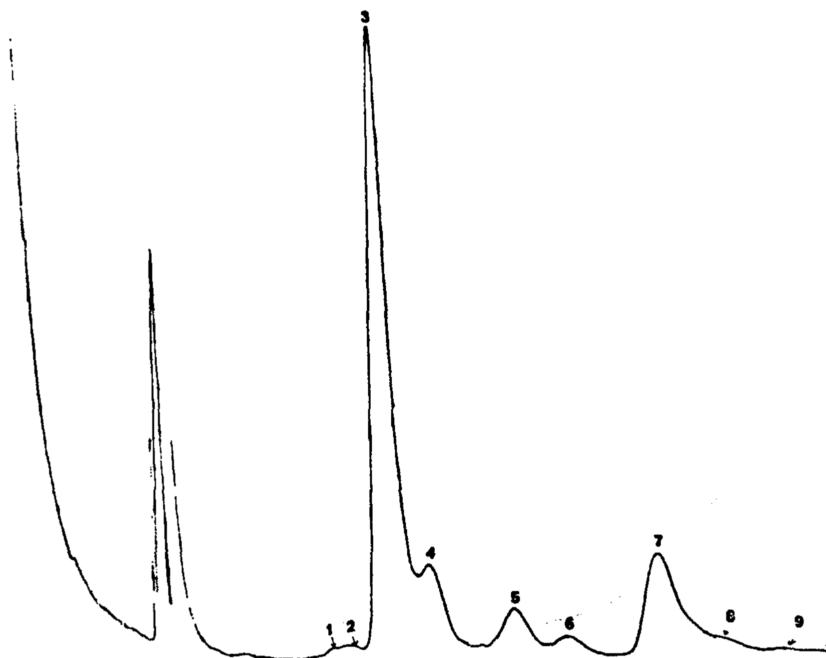

FIG. 3. Chromatogram of the TMS derivatives of the sterols of *M. brandaris* after GLC separation on SE-30. The percentage composition and the steroid number of each sterol is given. The calculated steroid numbers of the suitable sterols are given in parentheses.

1.	0·9%	22-Cis-cholesta-5,22-dien-3β-ol	29·66 (29·67)
2.	1·1%	22-Trans-cholesta-5,22-dien-3β-ol	29·82 (29·80)
3.	58·9%	Cholesterol	30·18 (30·18)
4.	10·0%	Brassicasterol	30·53 (30·58) or 7-dehydro-cholesterol (30·56)
5.	6·2%	Campesterol	31·17 (31·19) or 24-methylene-cholesterol (31·01)
6.	2·8%	Stigmasterol	31·50 (31·50)
7.	16·4%	β-Sitosterol	32·05 (32·04)
8.	3·5%	C_{29} sterol	32·30
9.	1·0%	Unknown	32·65

When acetate is used as the precursor the specific activity of the sterols is low but significant. Sterols from the experiments with mevalonate are labelled higher. It should be remarked that the dose of mevalonate per fresh weight (μC_i) in *Murex*

is about one-tenth of that in *Purpura* II. This may explain the rather low specific activity in the sterols of *Murex* II.

From the foregoing it may be concluded that the two representatives of the Muricidae investigated are able to synthesize sterols from mevalonate as well as from acetate. In a previous paper (Voogt, 1967) it was reported that no biosynthesis of sterols could be detected in *B. undatum* after the injection of acetate.

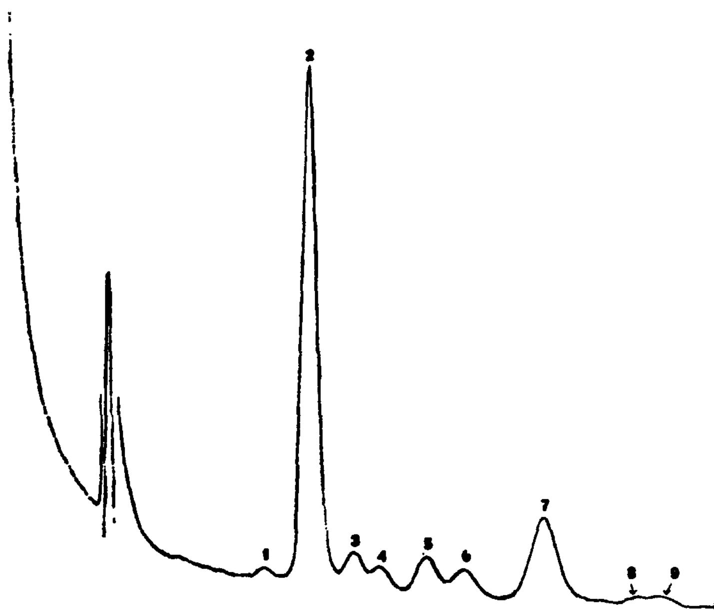

FIG. 4. Chromatogram of the TMS derivatives of the sterols of *M. brandaris* after GLC separation on NPGS. The percentage composition and the steroid number of each sterol is given. The calculated steroid numbers of the suitable sterols are given in parentheses.

1.	0·8%	22-Cis-cholesta-5,22-dien-3β-ol	29·82 (29·92)
		Not shown in this figure (1b) trace amount of 22-trans-cholesta-5,22-dien-3β-ol	30·14 (30·09)
2.	58·7%	Cholesterol	30·42 (30·36)
3.	3·9%	Brassicasterol	30·84 (30·83)
4.	3·0%	Desmosterol	31·11 (31·13)
5.	6·0%	Campesterol	31·43 (31·44) or 7-dehydrocholesterol (31·38)
6.	5·2%	Stigmasterol	31·80 (31·72) or 24-methylenecholesterol (31·70)
7.	16·1%	β-Sitosterol	32·29 (32·29)
8.	2·0%	C_{29} sterol	32·81
9.	2·0%	C_{29} sterol	32·90 (not visible in all chromatograms)
10.	2·4%	C_{30} sterol	33·29 (not visible in this chromatogram)

As the way of living and the feeding habit of *Buccinum* is similar to that of the animals under discussion, it might be interesting to investigate some more representatives of the Buccinidae, because the low specific activities of the sterols in the experiments with acetate suggest that the biosynthesis of these compounds proceeds only at a slow rate. Perhaps this is also reflected in the rather high specific activities of the crude squalene fractions. Finally it should be pointed out that the results obtained here are fully comparable with those obtained for *Natica cataena* (Voogt, 1971b), a carnivorous mesogastropod. The discussion about the slight sterol biosynthesis given for *Natica* may apply here too.

Table 4 shows a rather large difference in the sterol composition of *Purpura* and *Murex*. Whereas *Purpura* shows good agreement with the sterol composition of other prosobranchs (Voogt, 1971a), *Murex* is quite different, due to its high content of C_{29} sterols. As the content of C_{28} sterols is also rather high (comparable to that of *Crepidula* and *Natica*) the content of C_{27} sterols is low of course. In addition, about 2·5 per cent of C_{30} sterols was present.

Most sterols could be identified with the aid of their steroid numbers. The probable proportional composition is given in Table 6.

TABLE 6—PROPORTIONAL COMPOSITION OF THE STEROL MIXTURES OF *M. brandaris* AND *P. lapillus*

Sterol C_{26} sterol(s)	*M. brandaris*	*P. lapillus*
22-Cis-dehydrocholesterol	0·8	1·2 (two components)
22-Trans-dehydrocholesterol	Trace	3·9
Cholesterol	59	77
Desmosterol	3	4
7-Dehydrocholesterol	3	4·5
Brassicasterol	4	4·5
Campesterol	3	2
24-Methylenecholesterol	2	
β-Sitosterol	16	0·5
Stigmasterol	3	1
Unknown (C_{29})	2 (two components)	Trace
Unknown (C_{30})	2·4	

The occurrence of C_{26} sterols was reported for the first time by Wainai *et al.* (1964). Recently the structure of one of these sterols has been elucidated by Idler *et al.* (1970). The identity of the C_{26} sterols encountered in *Purpura* and *Murex* has not been determined.

Whereas in *Purpura* only 22-trans-cholesta-5,22-dien-3β-ol could be detected, in *Murex* 22-cis-cholesta-5,22-dien-3β-ol was also present. Recently the latter component has been found in scallop too (Idler & Wiseman, 1971).

The presence of 24-methylenecholesterol in *Murex* is not quite certain, but its presence cannot be excluded and would account for the different quantities of "stigmasterol" found on the two stationary phases. The high content of β-sitosterol in *Murex* is obvious.

Some of the C_{29} sterols could not be identified. So in the chromatograms of *Murex* on SE-30 the place of the peak with steroid number 32·30 can hardly be determined exactly. It may even be possible that it is a double peak. Notable is the absence of fucosterol.

Concluding, one may say that the sterols encountered in Neogastropoda, according to data in the literature, have also been found in this investigation, but, as could be expected, several others were present too.

Acknowledgements—The author is indebted for gifts of sterols to Professor P. J. Scheuer, University of Hawaii, for 24-methylene-cholesterol; to Dr. M. J. Thompson, Agricultural Research Service, Beltsville, Maryland, for brassicasterol, 22-cis- and 22-trans-cholesta-5,22-dien-3β-ol; and to W. Wientjens, Central Laboratory TNO, Delft, for desmosterol and campesterol.

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Key Word Index—Sterol biosynthesis in Molluscs; gas–liquid chromatography of sterols; sterol composition of some neogastropods; *Murex brandaris*; *Purpura lapillus*.