[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CONNECTICUT]

STEROLS OF MARINE MOLLUSKS. III.[1] FURTHER OBSERVATIONS ON COMPONENT STEROLS[2]

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In a recent review of the sterols of marine invertebrates W. Bergmann has drawn attention to the quantitative differences in the component sterols of mollusks (1). Although only a relatively small number of species in this phylum has been investigated, it is already apparent that cholesterol is invariably the major component sterol in gastropods and that the pelecypods may be expected to show a diversity in major component sterols. These differences have been emphasized in previous communications from this laboratory (2, 3). The identification of cholesterol as a major component sterol in gastropods and a minor sterol in pelecypods usually presents no difficulties but the identification of other sterols in mollusks is far from satisfactory. In only a few cases (3, 4) have sterols of orders C_{28}, C_{29} been characterized.

The present study provides data on the component sterols of two gastropods and a pelecypod. The results strengthen the original hypothesis of Bergmann and Low (5) regarding the occurrence of cholesterol as a major component sterol in gastropods and in addition provide indirect evidence of the biosynthesis of cholesterol by a member of this class. Although there seems little doubt that some marine invertebrates possess mechanisms for the synthesis of their component sterols, inadequate information regarding the feeding habits of many species and a complete lack of knowledge of the sterols of plankton, which make up a major part of their diets, leave unanswered the question of sterol biosynthesis by invertebrates in general. The oyster-drill, *Urosalpinx cinereus*, proves an almost ideal species for an investigation of the question of biosynthesis in mollusks since it feeds exclusively on oysters and other bivalves whose characteristic sterol is the C_{28} chalina-(ostrea) sterol.

The sterol fraction of *Urosalpinx* has been shown in the present study to contain 90% cholesterol which was obtained by purification through the acetate dibromide. The absence of any detectable amounts of sterols of orders C_{28}, C_{29} seem to preclude the possibility that dietary sterols are utilized by this species. The remaining 10% consists of an unidentified $\Delta^{5,7}$-sterol. The original sterol mixture gave a strong Tortelli-Jaffe reaction and, when examined spectrophotometrically, exhibited absorption maxima at 272, 282, and 293 mμ. The estimation of the % of $\Delta^{5,7}$-sterol is based on the spectrophotometric data using the extinction coefficient of ergosterol. Confirmation of this value is evidenced by the fact that the original mixture showed unsaturation corresponding to 1.2 double bonds, which agrees with the calculated value for a

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mixture of 90% cholesterol and 10% of a triunsaturated component. Other pertinent data on the composition of *Urosalpinx* are given in Table I.

In a previous communication (2) it was suggested that the gastropod, *Nassa obsoleta*, might contain in addition to cholesterol a small percentage of clionasterol. This suggestion was made on the basis of the identification of the latter as a minor component of the sterol fraction of a related species, *Littorina littorea* (3). Clionasterol has now been identified in *Nassa* by fractionation of the mixed acetate dibromides with subsequent debromination of the soluble clionasterol adduct to yield clionasterol acetate, and by saponification of the more soluble clionasterol benzoate to yield clionasterol.

TABLE I

COMPOSITION OF *Urosalpinx cinereus*

	MEAT AND SHELL, %	FAT, %	UNSAP., %
Meat and shell	100		
Fat	0.64	100	
Unsap. matter	.078	12.1	100
Sterol	.039	6.2	51

TABLE II

COMPOSITION OF *Anomia simplex*

	ORGANIC MATTER, %	FAT, %	UNSAP. MATTER, %
Organic matter	100		
Fat	16	100	
Unsap. matter	0.98	6.1	100
Sterol	.29	1.8	29

The bivalve, *Anomia simplex*, was of interest partly because it is not attacked by the oyster-drill, *Urosalpinx*. The composition of the air-dried animal is given in Table II. The crude sterol, examined spectrophotometrically, contained no more than 1% $\Delta^{5,7}$-sterol. The properties of the acetate; m.p. 137–138°, $[\alpha]_D$ −45.7°, and the benzoate, m.p. 147–148° (clear) 154°, $[\alpha_D]$ −17°, indicated a possible identity with chalinasterol (6). Titration of the acetate with perbenzoic acid however showed unsaturation corresponding to 1.71 double bonds. Bromination of the acetate yielded a high-melting (184–186°) tetrabromide, which is probably a mixture of the C_{24} epimers, chalinasterol and brassicasterol, both of which have been previously isolated from bivalves. A small amount of cholesterol acetate dibromide (m.p. 115–116°) was obtained from the mother liquor of the bromination mixture.

EXPERIMENTAL[3]

Melting points were taken with Anschütz total immersion thermometers. Optical rotations were taken in a 2-decimeter tube. The analyses were carried out by the Laboratory of Microchemistry, Teaneck, New Jersey.

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Urosalpinx cinereus

Isolation of the sterol mixture. The specimens were air-dried at 80° and meat and shell pulverized in a power grinder. The dried material (38 kg.) was extracted with acetone and ether in five-liter modified Soxhlet extractors (1). The acetone-ether-soluble oil weighed 245 g. Saponification of the fatty material by the method previously described (2) yielded 29.5 g. of unsaponifiable matter. The unsaponifiable matter was treated repeatedly with hot methanol which dissolved all but a small amount of dark brown resinous material. The methanol solution deposited a light brown crystalline solid on cooling. Recrystallization from methanol yielded 15 g. of white crystalline sterol mixture melting at 135–139°. The melting point could not be raised by recrystallization. The product, which gave strong Liebermann-Burchard and Tortelli-Jaffe reactions, was acetylated to give an acetate melting at 120–122°, and which showed unsaturation corresponding to 1.2 double bonds on titration with perbenzoic acid.

Cholesterol acetate dibromide. The impure steryl acetate (4.1 g.) was dissolved in 15 ml. of anhydrous ether and 30 ml. of a 10% solution of bromine in glacial acetic acid was added. The mixture was allowed to stand at 5° for six hours and the precipitate of acetate bromide was filtered, washed with glacial acetic acid, and recrystallized from ether-methanol. The product weighed 3.6 g. and melted at 116–117°.

Anal. Calc'd for $C_{29}H_{48}Br_2O_2$: C, 59.2; H, 8.2; Br, 27.2.
Found: C, 59.5; H, 8.5; Br, 28.04.

Debromination of the mother liquor with zinc and acetic acid yielded a small amount of a red oil which could not be crystallized.

Cholesteryl acetate. The acetate dibromide was debrominated by Schoenheimer's procedure (7). Recrystallization from ether-methanol yielded an acetate which melted at 112°; $[\alpha]_D^{25}$ −39° (50.6 mg. in 4.98 ml. of chloroform; α −0.79°). The acetate gave no depression in melting point when mixed with cholesterol acetate.

Saponification of the acetate gave cholesterol, m.p. 146–148°; $[\alpha]_D^{25}$ −37.5° (53.8 mg. in 4.98 ml. of chloroform; α −0.81°).

Cholesteryl benzoate. The sterol, purified through the acetate dibromide, was treated with benzoyl chloride in pyridine and the product was crystallized from ethyl acetate. The benzoate melted to an opalescent liquid at 148° and cleared at 179°. A mixture melting point with cholesteryl benzoate showed no depression. $[\alpha]_D^{25}$ −14.6° (80.9 mg. in 5.01 ml. of chloroform; α −0.47°).

Anal. Calc'd for $C_{34}H_{50}O_2$: C, 83.21; H, 10.27.
Found: C, 83.25; H, 10.60.

Nassa obsoleta

From 17.4 kilograms of air-dried material (meat and shell) there was obtained 4.5 g. of impure sterol which melted at 140–142°. The sterol fraction was benzoylated and the benzoate mixture was separated into two fractions on the basis of solubility in absolute ethanol. Repeated recrystallization of these fractions from absolute ethanol however failed to yield constant melting products.

Fractionation via the acetate bromides. The less-soluble fraction of the mixed benzoates was saponified and the free sterol acetylated. The acetates were brominated with 10% bromine in glacial acetic acid. The precipitate of cholesterol acetate dibromide (2) was removed. A stream of nitrogen was passed through the mother liquor and a second crop of crystals m.p. 108–112° was removed. The filtrate was debrominated by refluxing with zinc dust for six hours. The acetate was recrystallized from ether-methanol and chloroform-methanol and melted at 130–131°. $[\alpha]_D^{25}$ −42.2° (49.5 mg. in 5.01 ml. of chloroform, α −0.83°). Clionasterol acetate $[\alpha]_D^{25}$ −41.9° is reported to melt at 137° (8).

Clionasterol. The more-soluble fraction of the benzoate mixture was combined with material obtained from the ethanol mother liquors and saponified. The resulting sterol was recrystallized twice from methanol and twice from ethanol, m.p. 137–139°. $[\alpha]_D^{25}$ −33.6° (37.4 mg. in 5.01 ml. of chloroform, α −0.50°). A mixture melting point with authentic clionasterol showed no depression.

Anal. Calc'd for $C_{29}H_{50}O$: C, 83.99; H, 12.15.
Found: C, 84.00; H, 12.40.

Anomia simplex

From 185 g. of air-dried tissue there was obtained 30.1 g. of acetone-ether soluble fat and 1.82 g. of unsaponfiable matter. The unsaponifiable material was taken up in hot methanol and on cooling a crop of tan crystals was deposited. Concentration of the methanol solution yielded a second crop of crystals which when combined with the first crop weighed 530 mg. Treatment of the mother liquor with digitonin gave no insoluble digitonide.

Steryl acetate. The acetate was prepared by the usual method and after recrystallization from ether-methanol melted at 137–138°; $[\alpha]_D^{25}$ −45.7°. (70.8 mg. in 5.01 ml. of chloroform, α −1.29°). Titration with perbenzoic acid showed unsaturation corresponding to 1.71 double bonds.

Anal. Calc'd for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98.
Found: C, 81.12; H, 10.63.

Steryl benzoate. A sample of the crude sterol was benzoylated and the benzoate was crystallized from ethyl acetate-methanol. The product melted to an opalescent liquid at 147–148°, displayed a blue-green color at 152.4°, and cleared at 154°. The color reappeared momentarily on cooling to 153°; $[\alpha]_D^{25}$ −17.2° (100.7 mg. in 4.98 ml. of chloroform, α −0.69°). Perbenzoic acid titration showed unsaturation corresponding to 1.55 double bonds.

Anal. Calc'd for $C_{35}H_{50}O_2$: C, 83.61; H, 10.01.
Found: C, 83.74; H, 10.03.

Acetate tetrabromide. The steryl acetate (90 mg.) was dissolved in one ml. of ether and 2 ml. of 5% bromine in glacial acetic acid was added. The crystalline bromide that separated after 24 hours was washed with ether and recrystallized from chloroform-methanol; m.p. 184–186° with decomposition.

Anal. Calc'd for $C_{30}H_{48}Br_4O_2$: C, 47.38; H, 6.36.
Found: C, 47.45; H, 6.45.

Addition of methanol to the ether washings yielded a small amount of cholesterol acetate dibromide, m.p. 115–116°.

SUMMARY

The gastropod mollusk, *Urosalpinx cinereus*, contains cholesterol as the major component sterol. The sterol fraction also contains about 10% of an unidentified $\Delta^{5,7}$-sterol. The cholesterol was purified through the acetate dibromide and identified by the preparation of characteristic esters.

The gastropod, *Nassa obsoleta*, has been shown to contain clionasterol in addition to the cholesterol previously identified. Clionasterol acetate was obtained by fractionation of the acetate dibromides and the free sterol was obtained by saponification of the alcohol-soluble fraction of the mixed benzoates.

The pelecypod, *Anomia simplex*, contains only a trace of cholesterol. The major portion of the sterol fraction consists of a mixture of C_{28} sterols. It has been suggested that these are the difficultly separable chalinasterol and brassicasterol.

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