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STEROLS OF MARINE MOLLUSKS. I. THE PRESENCE OF CHOLESTEROL IN TWO GASTROPODS[1]

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Recent investigations of the sterols of marine invertebrate animals have provided valuable information regarding the types of structures one may expect to find in the naturally occurring members of this class of compounds. Of particular significance is the occurrence of such C_{28} sterols as stellasterol and stellastenol (1), neospongosterol (2), and chalinasterol (3). The latter compound is likely to prove of considerable importance in studies of the comparative biochemistry of marine life, since evidence has been offered to indicate that chalinasterol is identical with ostreasterol isolated by Bergmann from bivalves in 1934 (4). Bergmann and Low (5) have called attention to the apparent quantitative differences in sterol content within the phylum *Mollusca*. They have postulated that the gastropods may be characterized by the presence of cholesterol as the principal sterol, whereas the pelecypods may be expected to contain ostreasterol-like compounds.

We have investigated the sterol fraction of two gastropods, the *Nassa obsoleta* common to the New England coast, and the *Nerita peleronta* obtainable from the coastal waters of Florida, Bermuda, and the West Indies. In both cases the isolation of cholesterol as the principal sterol supports the suggestion offered by Bergmann and Low.

The specimens of *Nassa obsoleta* were obtained from the Marine Biological Laboratory at Woods Hole, Massachusetts, and were found to contain 0.27% of nonsaponifiable matter. When this material was treated with hot methanol and cooled to room temperature, a hydrocarbon fraction was precipitated. This fraction upon crystallization from methanol yielded a product melting at 59–60°, which we presume to be heptacosane. When the filtrate was cooled to 5°, a second precipitate was obtained which gave typical color reactions for sterols. The crude sterol fraction was crystallized repeatedly from ethanol, giving a product melting at 132–139°. Attempted purification through the digitonide yielded a material of somewhat higher melting point (138–141°). When this material was acetylated and treated with bromine in glacial acetic acid, an acetate dibromide was precipitated, m.p. 110–112°, which gave no depression with cholesteryl acetate dibromide. The small yield obtained by the bromination procedure, and the fact that several samples decomposed on standing, led to attempts to separate the sterol mixture by fractional crystallization of a derivative. After unsuccessful efforts to fractionate either the steryl bromides or the acetates, advantage was taken of the insolubility of cholesteryl benzoate. Benzoylation of the mixture gave a product the bulk of which proved to be very difficultly soluble in 95% ethyl alcohol. Repeated crystallizations from absolute

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alcohol yielded cholesteryl benzoate as the least soluble fraction. The filtrates contained a benzoate melting at 130–132°, which could not be identified due to lack of material. An investigation now in progress in this laboratory suggests that this lower-melting benzoate may be clionasteryl benzoate (m.p. 134–135°). The presence of clionasterol has not been demonstrated in mollusks as far as the authors are aware.

Specimens of the *Nerita peleronta*, commonly known as the "bleeding tooth", were obtained from Bermuda through the courtesy of Prof. Werner Bergmann of Yale University. The tissue contains about three and one-half per cent of acetone-ether soluble material. The nonsaponifiable matter consists of about ten per cent sterol, identified as cholesterol through the preparation of the acetate and benzoate. No evidence was found for the presence of other sterols in the Nerita.

EXPERIMENTAL

Nassa obsoleta[2]

Isolation of the nonsaponifiable matter. The specimens of this mollusk had been collected over a period of several weeks and preserved in ethyl alcohol. The material, weighing 15.6 kg., was placed in a mechanical grinder and shell and tissue were ground together. The finely ground mass was filtered and without further drying was divided into four batches of approximately 4 kilograms each. All batches were dehydrated by extraction with acetone in a Soxhlet apparatus and then exhaustively extracted with ether. The solvents were removed and the extracts saponified. The nonsaponifiable fraction was obtained as a yellow-brown crystalline solid weighing 42.5 g. or 0.27% of the wet weight.

Separation of the sterol fraction. Ten grams of the nonsaponifiable matter was dissolved in 50 cc. of boiling methanol. On cooling to room temperature, a light yellow mass of amorphous material precipitated (Fraction A). This was filtered and the filtrate cooled at 5° for 12 hours, during which time a white crystalline precipitate had formed. This was also filtered and will be referred to as Fraction B. Fraction B gave positive Liebermann-Burchard and Salkowski tests whereas these tests were negative when applied to Fraction A. The melting points of Fractions A and B were 52–60° and 118–131° respectively.

The mother liquor remaining after the removal of the above fractions contained a dark brown oil which could not be crystallized. Attempts to purify this material by distillation and chromatographic adsorption were unsuccessful, and investigation of this mixture has been temporarily abandoned.

Fraction A. This material, constituting about 5% of the nonsaponifiable matter, was crystallized twice from methanol and five times from acetone until the melting point had reached a constant value of 59.6–60.4°. It was optically inactive and gave negative tests for unsaturation. Heptacosane is reported as melting at 59.2° (6).

Anal. Calc'd for $C_{27}H_{56}$: C, 85.17; H, 14.83.
Found: C, 84.85; H, 14.99.

Fraction B. The crude material (m.p. 118–131°) weighed 0.950 g. or 9.5% of the nonsaponifiable matter. Repeated crystallizations from methanol gave 255 mg. of product melting at 132–139°. This material was dissolved in ethanol, 75 cc. of a 1% solution of digitonin was added, and the mixture heated to boiling. After standing at room temperature for 24 hours, the mixture was again heated to boiling and allowed to stand overnight. The digitonide was filtered, washed with cold ethanol, and dried. Eight hundred fifty-six milligrams was obtained, indicating the presence of 214 mg. of sterol. The digitonide was

[2] The authors wish to acknowledge the cooperation of Prof. John S. Rankin of the Department of Zoology, University of Connecticut in obtaining this material.

split by treatment with pyridine according to the method of Bergmann (7) and, after recrystallization from methanol, 151 mg. of sterol was obtained melting at 138–141°; $(\alpha)_D^{24}$ −32.6° (44.2 mg. in 3 cc. of chloroform gave an α reading of −0.48°).

Preparation of the acetate bromide. The acetate of the impure sterol (m.p. 138–141°) was prepared in the usual manner, and melted at 121–123°. The acetate was brominated by the method of Windaus and Hauth (8) and a small yield of insoluble acetate dibromide was obtained. This material melted at 110–112° and gave no depression when mixed with cholesteryl acetate dibromide.

Anal. Calc'd for $C_{29}H_{46}Br_2O_2$: Br, 27.16.
Found: Br, 26.68.

Debromination of the acetate with zinc and acetic acid yielded cholesteryl acetate, m.p. 112–114°, which gave no depression of the melting point when mixed with authentic material.

Preparation of the benzoate. Four hundred fifty-six milligrams of sterol, prepared through the digitonide, was converted to the benzoate by treatment with benzoyl chloride in pyridine. The product was recrystallized from 95% ethyl alcohol, in which it was difficultly soluble. Repeated crystallizations from absolute alcohol, in which the material was more soluble, afforded two fractions. The less soluble fraction, representing about 80% of the mixture, melted to a turbid liquid at 145–147° and cleared at 175°; $(\alpha)_D^{26}$ −17.70° (26.4 mg. in 3 cc. of chloroform gave an α reading of −0.15°). When mixed with cholesteryl benzoate no depression of the melting point was observed.

Anal. Calc'd for $C_{34}H_{50}O_2$: C, 83.21; H, 10.27.
Found: C, 82.63; H, 10.60.

Saponification of the benzoate gave cholesterol, m.p. 145–146°; $(\alpha)_D^{26}$ −38.2° (21.2 mg. in 3 cc. of chloroform gave an α reading of −0.27°). There was no depression of the melting point when this material was mixed with authentic cholesterol.

The more soluble fraction of the benzoate mixture melted at 130–132°. Lack of material made identification of this fraction impossible.

Nerita peleronta

Soxhlet extraction of 533 g. of this mollusk with acetone and ether in the manner previously described yielded 18 g. (3.4%) of a dark brown viscous oil. The oil was saponified with 50 cc. of alcoholic potassium hydroxide, and the nonsaponifiable matter consisted of 3.2 g. of a light brown gummy mass mixed with large plate-like crystals. The mixture was treated repeatedly with boiling methanol, in which the gum was very slightly soluble. On cooling, the combined methanol solutions deposited a crystalline precipitate weighing 0.675 g. and melting at 139–143°. Ten crystallizations from methanol yielded 0.332 g. of sterol melting at 146–147° which gave no depression of the melting point when mixed with cholesterol; $(\alpha)_D^{24}$ −39.4° (28.1 mg. in 3 cc. of chloroform gave an α reading of −0.37°).

Anal. Calc'd for $C_{27}H_{46}O$: C, 83.87; H, 11.99.
Found: C, 83.86; H, 12.02.

Acetylation of the sterol with acetic anhydride gave cholesteryl acetate, m.p. 113–114°. No depression of the melting point was observed when mixed with authentic material.

Cholesteryl benzoate. One hundred milligrams of sterol was converted to the benzoate by treatment with benzoyl chloride in pyridine. The product displayed the characteristic behavior of cholesteryl benzoate, melting to a turbid liquid at 146° and clearing at 177–178°. A mixed melting point with authentic cholesteryl benzoate showed no depression; $(\alpha)_D^{26}$ −16.1° (36.4 mg. in 3 cc. of chloroform gave an α reading of −0.19°).

Anal. Calc'd for $C_{34}H_{50}O_2$: C, 83.21; H, 10.27.
Found: C, 82.98; H, 10.50.

SUMMARY

The nonsaponifiable matter of two gastropod mollusks, *Nassa obsoleta* and *Nerita peleronta*, has been investigated and cholesterol has been identified as the

principal sterol present in these animals. The presence of cholesterol in both cases has been established by comparison of the properties of the free sterol and several derivatives.

In addition, the nonsaponifiable matter of the *Nassa obsoleta* has been shown to contain considerable quantities of a hydrocarbon mixture, the major component of which is heptacosane.

The results of this investigation may be interpreted as supporting the suggestion that the sterols of mollusks are probably independent of the diet and that the gastropods contain cholesterol as the principal sterol.

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REFERENCES

(1) BERGMANN AND STANSBURY, *J. Org. Chem.*, **9**, 281 (1944).
(2) BERGMANN, GOULD, AND LOW, *J. Org. Chem.*, **10**, 570 (1945).
(3) BERGMANN, SCHEDL, AND LOW, *J. Org. Chem.*, **10**, 587 (1945).
(4) BERGMANN, *J. Biol. Chem.*, **104**, 317, (1934).
(5) BERGMANN AND LOW, *J. Org. Chem.*, **12**, 67 (1947).
(6) PIPER, CHIBNALL, HOPKINS, POLLARD, SMITH, AND WILLIAMS, *Biochem. J.*, **25** 2072 (1931).
(7) BERGMANN, *J. Biol. Chem.*, **132**, 471 (1940).
(8) WINDAUS AND HAUTH, *Ber.*, **39**, 4378 (1906).