Sterols and Other Unsaponifiable Substances in the Fats of Shell Fishes, Crustacea and Echinodermata. XI.

Sterols in the Fat of Clam

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Sterols in the fat of the clam, *Meretrix meretrix,* was studied in the 3rd report(1) of this series, and a sterol named meretristerol having m. p. 133—134.5°, and its acetate of m. p. 144—144.5° and the formula $C_{28}H_{46}O$ or $C_{29}H_{48}O$ was separated. But later in the 9th report(2) it was revealed that the meretristerol previously obtained was impure, since repeated recrystallizations of the steryl acetate gave a fraction of m. p. 149.5° which was believed to be pure meretristeryl acetate. Saponification of this acetate gave meretristerol of m. p. 144.5°. Meanwhile, Bergmann and Ottke(3) suggested that meretristerol is not a single sterol but a mixture of brassicasterol and chalinasterol.

With the purpose of obtaining pure meretristerol in a sufficient quantity to permit a further study, the sterol mixture was separated from the fat extracted from another lot of clams, and its acetate was subjected to repeated recrystallizations. There was obtained a fraction of m. p. 149°, but its melting point was raised somewhat promptly by further recrystallizations until a fraction of m. p. 173—174° was finally obtained in a very small quantity. Accordingly, it must be recognized that even the meretristerol described in the 9th report was not a pure single sterol. Also the fraction of high melting point, 173—174°, was not considered to be a single acetate from the results of the fractionation of its bromide.

The acetate mixture after removal of the high melting fraction was brominated in ether solution and the product was separated into the ether insoluble bromide (I) and the ether soluble bromide (II). The bromide I was then treated with chloroform-ethanol and ethanol, and there were obtained two fractions (Ia) and (Ib). The bromide II was treated with ethanol and a fraction melting at 120—123° was separated. Debromination of this fraction and

Table 1

	Sterol		Acetate		Benzoate		Acetate tetrabromide		Acetate dibromide	
	m. p., °C.	[α]°D	m. p., °C.	[α]°D	m. p., °C.	[α]°D	m. p., °C.	[α]°D	m. p., °C.	[α]°D
Sterol from bromide Ia	145	−63.8	151	−60.7	158	—	202	−43.8	215—216	—
Brassicasterol(4)	146	−61.1	152	−65	163	−35(5)	205	—	236—238	—
Sterol from bromide Ib	154	−53.3	146—147	−52.1	140	−21.7	192	−42.5	213—214	—
Poriferasterol(6)	155—156	−49.7	146.5—147	−53	139.5—140.5	−21.9	192	−43.5	211—212	−31
Sterol from fraction III	136—137	−39.3	137—138	−38.2	133—135	−21.2				
Clionasterol(7)	137.5—138.5	−37	137	−41.9	134.5—135	−16.8				
Sterol from fraction IV	142	−37.6	114	—	145	−15.8				
Cholesterol	148	−38	114	−43	146	−17				

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(1) Y. Toyama and M. Yajima, *J. Chem. Soc. Japan,* **64**, 878 (1943).

(2) M. Kita, I. Naka and Y. Toyama, *J. Chem. Soc. Japan, Pure Chem. Sect.,* **71**, 21 (1950).

(3) W. Bergmann and R. C. Ottke, *J. Org. Chem.,* **14**, 1085 (1949).

(4) E. Fernholz and H. E. Stavely, *J. Am. Chem. Soc.,* **61**, 142 (1939) ; **62**, 428 (1940).

(5) l. c., (3).

(6) F. R. Valentine, Jr. and W. Bergmann, *J. Org. Chem.,* **6**, 452 (1941); W. Bergmann and R. J. Feeney, ibid., **14**, 1078 (1949) ; W. Bergmann, F. H. McTigue, E. M. Low, W. M. Stokes and R. J. Feeney, ibid., **15**, 96 (1950).

(7) F. R. Valentine, Jr. and W. Bergmann, *J. Org. Chem.,* **6**, 452 (1941).

fractional crystallization of the debrominated product gave two fractions (III) and (IV). On examining each fraction, brassicasterol and poriferasterol were obtained from the bromides Ia and Ib respectively, while the fractions III and IV were found to contain clionasteryl acetate and cholesteryl acetate respectively. The properties of sterols obtained from each fraction are given in Table 1.

The occurrence of brassicasterol in the sterol components of Pelecypoda(8) had already been known, but poriferasterol had not yet been found in shell fish. Clionasterol was also found in Pelecypoda for the first time by the present study, though its occurrence in Gastropoda(9) had been known.

The acetate of m. p. 173—174° was brominated, the bromide separated into two fractions, and each fraction debrominated. The debromination products from each fraction had considerably lower melting points than the original acetate. Thus the original acetate was not regenerated by the debromination of the bromide. Accordingly the original acetate must have undergone isomerization or other changes in bromination and debromination cycle.

Experimental

Clams used in the present experiments were procured from the Shimono-isshiki Fishery Cooperation, Nagoya, in April and May, 1950. Thirty-six kg. of wet meat obtained from 150 kg. of clams was dried below 80° under vacuum, and the dried material (4.58 kg.) was reduced to powder, from which 462 g. of fat (10.1% of dried material) was extracted using trichloroethylene. Saponification of the fat and extraction of the soap solution with ether in the usual way yielded 83 g. of unsaponifiable substance (18.0% of fat), which was a mixture of crystalline solid and viscous oily liquid of dark reddish orange color.

Recrystallization of Steryl Acetate. — The unsaponifiable substance was recrystallized three times from ethanol, and there was obtained 27.1 g. of crystalline solid of m. p. 118—138°, which was acetylated by refluxing with acetic anhydride. The acetate (30.2 g.) was recrystallized five times from ethanol, once from acetone-ethanol, and twice from acetone, giving an acetate (4.5 g.) of m. p. 129—132°, $[\alpha]_D^{18}$ −40.3°, saponification value 131.1 and iodine value (pyridine sulfate dibromide method) 98.0. Further recrystallizations of this acetate were carried out as shown in Table 2.

Table 2

Recrystallized from	Acetate crystallized out		Acetate in mother liquor
	Yield, g.	m. p., °C.	m. p., °C.
Benzene-ethanol	3.0	132—135	130—132
Acetone	0.4	142—149	129—130(a)
〃	0.2	165—167	131—132
〃	0.12	173—174	155—158

(a) 2.6 g., $[\alpha]_D^{18}$ −41.5°, saponification value 128.3, iodine value 97.0. This fraction of the acetate from the mother liquor was subjected to further recrystallizations as shown below.

Benzene-ethanol	2.0	131—134	130—132
Acetone	1.2	136—141	128—132
Ether-ethanol	0.5	144—149	130—132
Acetone	0.33	145—149	132—134
〃	0.19	146—149	138—139
〃	0.16	148—149.5	142—143.5
〃	0.13	170—171	145—146
〃	0.11	173—174	145—149

Fractionation of Acetate Bromide.—Acetate contained in the mother liquors of recrystallizations was recovered, and two fractions (A) 4.9g., melting between 130° and 135°, and (B) 12 g., melting between 110° and 130°, were separated. Both fractions were brominated and bromides were fractionated in the following way. Fraction A was dissolved in 80 cc. of ether, and bromine in ether (5 g. in 20 cc.) was added dropwise under cooling at −10°. After keeping the solution at −10° for 20 hours, 1.4 g. of insoluble bromide (I) was filtered, washed twice with ether using 50 cc. each time, and then recrystallized four times from 20 cc. of chloroform-ethanol, by which there was obtained 0.12 g. of crystalline bromide (Ia). The chloroform-ethanol filtrate was added with ethanol, the precipitated bromide (0.2 g.) was filtered, and the filtrate was added with a further large quantity of ethanol, by which 0.8 g. of solid bromide (Ib) was precipitated. Fraction B yielded a very small amount of insoluble bromide by bromination in ether. The combined ether filtrate from fractions A and B was treated with sodium bisulfite solution for the removal of excess bromine, washed with water, dried over anhydrous sodium sulfate, and concentrated to a small volume by distilling ether. On adding ethanol to the concentrated ether solution, the bromide was fractionally precipitated, and there were obtained 7.4 g. of a fraction melting at 120—123° together with fractions of m. p. 175—180°, 110—114° and oily liquid.

Bromide Ia. — m. p. 202°, $[\alpha]^{18}_D$ −43.8°, Br 41.73% (calculated for $C_{30}H_{48}O_2Br_4$: Br, 42.04%). This bromide was debrominated with zinc and acetic acid. The debrominated product, after recrystallization from ethanol, had m. p. 151° and $[\alpha]^{18}_D$ −60.7°. It showed no depression of melting point when mixed with brassicasteryl acetate, m. p. 151—152°, prepared from rape oil in this laboratory. Saponification of the acetate gave

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(8) l. c., (3); T. Matsumoto and Y. Toyama, *J. Chem. Soc. Japan*, **65**, 258, 310 (1944).

(9) C. A. Kind and S. C. Herman, *J. Org. Chem.*, **13**, 867 (1948).

free sterol which, after recrystallization from ethanol, had m. p. 145° and $[\alpha]^{18}_{\mathrm{D}}$ $-63.8°$. Free sterol was added with benzoyl chloride in pyridine, kept for 24 hours at room temperature, and then water was added. The precipitated benzoate was washed with water and then recrystallized four times from acetone. It melted at 158°. A portion of the bromide Ia was partially debrominated by potassium iodide.(10) The product, dibromide, melted at 215—216° after recrystallizing several times from ethanol.

Bromide Ib.—m. p. 192° (with decomp.), $[\alpha]^{18}_{\mathrm{D}}$ $-42.5°$, Br 41.46% (calculated for $C_{31}H_{50}O_2Br_4$: Br, 41.33%). This bromide was debrominated with zinc and acetic acid. The product, after recrystallization from ethanol, had m. p. 146—147°, $[\alpha]^{18}_{\mathrm{D}}$ $-52.1°$ and saponification value 124.7 (calculated for $C_{31}H_{50}O_2$ 123.4). Saponification of the acetate yielded free sterol which, after recrystallization from ethanol, had m. p. 154° and $[\alpha]^{18}_{\mathrm{D}}$ $-53.3°$. Benzoylation of free sterol gave a benzoate of m. p. 140°, $[\alpha]^{18}_{\mathrm{D}}$ $-21.7°$ and saponification value 110.5 (calculated for $C_{36}H_{52}O_2$ 108.6). Dibromide obtained by partial debromination of the bromide Ib had m. p. 213—214° and Br 27.24% (calculated for $C_{31}H_{50}O_2Br_2$: Br, 26.05 %).

Sterol from Fraction III. — The ether soluble bromide of m. p. 120—123° was debrominated with potassium iodide in ethanol, whereby the debromination occurred nearly quantitatively yielding a product which had less than 0.1% of bromine. Accordingly, the bromide of m. p. 120—123° is considered to contain only a minute amount, if any, of compound having bromine in its side chain. On debromination of this bromide with zinc and acetic acid, there was obtained an acetate of m. p. 131° and $[\alpha]^{17}_{\mathrm{D}}$ $-38.2°$. Recrystallizing twice from acetone, it had m. p. 137—138° and $[\alpha]^{17}_{\mathrm{D}}$ $-38.2°$. The melting point was unaltered by further recrystallization. Saponification of this acetate and recrystallization of the product from acetone yielded a free sterol of m. p. 136—137° and $[\alpha]^{17}_{\mathrm{D}}$ $-39.3°$. The benzoate prepared from this sterol had m. p. 133—135°, $[\alpha]^{17}_{\mathrm{D}}$ $-21.2°$ and saponification value 108.3 (calculated for $C_{36}H_{54}O_2$ 108.1). On solidifying the melted benzoate, it developed a blue coloration at 130—110° as is the case with clionasteryl benzoate(11).

Sterol from Fraction IV.—The acetate contained in the acetone mother liquors of recrystallizations of the acetate fraction III was saponified, the resulting free sterol was benzoylated, and the benzoate was recrystallized three times from acetone. The product had m. p. 145° and $[\alpha]^{17}_{\mathrm{D}}$ $-15.8°$. Saponification of the benzoate yielded free sterol having m. p. 142° and $[\alpha]^{17}_{\mathrm{D}}$ $-37.6°$ after recrystallization from ethanol. The acetate prepared from this sterol had m. p. 114° after recrystallization from ethanol.

The Acetate of m. p. 173—174°. — The high melting acetate obtained by the final recrystallization recorded in Table 2 had $[\alpha]^{17}_{\mathrm{D}}$ $-62.5°$ and saponifacation value 123.9. The bromination product of this acetate was separated into two fractions, m. p. 172—174° (with decomp.) and m. p. 115—125°, by means of ethanol. The fraction of m. p. 172—174° (with decomp.) had Br 38.71%. Debromination of this fraction with zinc and acetic acid yielded a product which had m. p. 143° after three recrystallizations from ethanol. The fraction of m. p. 115—125° had Br 26.10%. Debromination of this fraction yielded a product which had m. p. 136° after two recrystallizations from ethanol.

Summary

(1) Steryl acetate obtained from clam fat was recrystallized from several different solvents, and it was found that the acetate recorded in the previous report as pure meretristeryl acetate, m. p. 149.5°, was not a pure single compound. Final recrystallization gave an acetate of m. p. 173—174°, though in a very small yield.

(2) Acetate mixture after removal of the fraction of m. p. 173—174° was fractionated by way of bromide, and it was found that brassicasterol, poriferasterol, clionasterol and cholesterol are present among the sterol components of clam fat.

(3) Bromination of the acetate of m. p. 173—174° and debromination of the bromination product yielded acetate having considerably lower melting points than the original acetate. Accordingly, there must have occurred isomerization or other changes in bromination and debromination cycle.

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(10) E. Fernholz and H. E. Stavely, *J. Am. Chem. Soc.*, **62**, 428 (1940).

(11) l. c., (7).