Sterols and Other Unsaponifiable Substances in the Lipids of Shell Fishes, Crustacea and Echinoderms. XII. Occurrence of Δ^7-Cholestenol as a Major Component of the Sterol Mixture of Chiton

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(Received July 11, 1953)

The first report[1] of this series recorded the results of a brief examination of the sterol mixture of chiton, *Liolophura japonica*, and several other aquatic invertebrates. In those experiments, the sterol mixture of the fatty substances extracted with ether from the chitons, caught around Sugashima Island, Mie Prefecture, and also the acetate of sterol mixture were subjected to several recrystallizations. The highest melting points of recrystallized products were 119~121°C. for the free sterol and 125~127°C. for the acetate, though neither melting points were yet constant.

In the present experiments, the chitons caught around Osaki-Shimojima Island in the Inland Sea of Seto were extracted with ether, the ether-extract was freed from the acetone-insoluble portion, and the unsaponifiable matter was separated from the acetone-soluble portion. The crystalline fraction of the unsaponifiable matter, consisting of crude sterols, was acetylated, and the acetate obtained was recrystallized from ethanol. Unlike the results described in the previous report, the acetate showed, after 4 recrystallizations, a constant melting point of 118~119°C. which was unaltered by further recrystallizations. The free sterol obtained by saponification of the acetate showed also a constant melting point of 122~122.5°C. Both free sterol and acetate were optically inactive, and a spectrophotometric examination indicated the absence of conjugated diene. They reacted very rapidly with the Liebermann-Burchard reagent and developed a blue-green color almost immediately. The acetate had a saponification value and a degree of unsaturation, determined by perbenzoic acid titration, both of which agreed closely with the calculated values for a C_{27}-stenol. These facts seem to suggest that the sterol obtained above is Δ^7-cholestenol (γ-cholestenol). In order to gain a further confirmation, the isomerization of Δ^7-cholestenol (γ-cholestenol)$\rightarrow\Delta^{8(14)}$-isomer ($\alpha$-cholestenol)$\rightarrow\Delta^{14}$-isomer ($\beta$-cholestenol) and the hydrogenation of the last named isomer to cholestanol were performed. As shown in

TABLE I

	Free sterol		Acetate		Benzoate	
	m.p., °C	$[\alpha]_D^{20}$	m.p., °C	$[\alpha]_D^{20}$	m.p., °C	$[\alpha]_D^{20}$
Δ^7-Cholestenol	122~122.5	0	118~119	0	158~159; 177	+6.9
„ [2]	122~123	0	118~119	0	157~158; 176	+7.14
„ [3]	125~126	+5.7	118~119	+1.5	—	—
„ [4]	122	+5.65	118	—	153~154	—
$\Delta^{8(14)}$-Cholestenol	118~120	—	77~78	+9.5	115~117; 144	—
„ [2]	119~120	+20.36	77~78	+9.46	115; 140	+8.53
Δ^{14}-Cholestenol	130~131	—	90~91	—	168~169	+31.5
„ [2]	130~131	+34	91~92	—	168	+32.54
„ [5]	131~133	+30	90~92	+22.8	172~174	+31
Cholestanol	—	—	104~105	+14.2	—	—
„ [6]	—	—	109~110	+11.5	—	—

1) Y. Toyama and F. Shibano, *J. Chem. Soc. Japan*, **64**, 323 (1943).
2) Fr. Schenck, K. Buchholz and O. Wiese, *Ber.*, **69**, 2696 (1936).
3) L. F. Fieser, *J. Am. Chem. Soc.*, **73**, 5007 (1951).
4) D. R. Idler and C. A. Baumann, *J. Biol. Chem.*, **195**, 623 (1952).
5) H. Wieland, F. Rath and W. Benend, *Ann.*, **548**, 19 (1941).
6) B. Heath-Brown, I. M. Heilbron and H. R. H. Jones, *J. Chem. Soc.*, **1940**, 1482.

Table 1, the respective products obtained in these experiments showed the same melting points and specific rotations as those recorded previously for the corresponding compounds, excepting that the hydrogenation product had a little lower melting point and higher specific rotation than cholestanol, possibly due to the contamination of unaltered Δ^{14}-cholestenol.

From sterol-free conponents of unsaponifiable matter, a small amount of saturated alcohols, possibly chimyl and batyl alcohols, was separated.

Δ^{7}-Cholestenol had been known as the reduction product of 7-dehydrocholesterol, but its occurrence among natural sterols was not known until quite recently. In 1951 Fieser[7] found this sterol, named lathosterol by him, as a companion sterol in various samples of crude cholesterol. Also Idler and Baumann[8] found a relatively large amount of this sterol in skin sterols of an albino rat, but the isolation of this sterol from skin sterols was attained by chromatography of the azoyl ester of sterol mixture, while repeated recrystallizations of the acetate of the sterol mixture were not efficient to concentrate this sterol. In contrast to these findings, it may be noted that only several recrystallizations of the acetate of sterol mixture of the chitons were sufficient to yield a pure Δ^{7}-cholestenol in the present experiments.

Since the acetate of sterol mixture of the chitons caught around Sugashima Island, as described in the previous report, did not yield an acetate of constant melting point, the authors have procured another lot of chitons caught at the same locality, and re-examined the sterol mixture from this lot of chitons. On repeatedly recrystallizing the acetate of sterol mixture, the melting point of the acetate became higher than that of Δ^{7}-cholestenyl acetate, as was the case with the previous experiments. The sterol mixture showed no ultraviolet absorption maximum, but it was positive for the Tortelli-Jaffé test, and formed a blue-green color very rapidly in the Liebermann-Burchard reaction. These facts seem to permit a presumption that the sterol mixture of the chitons caught around Sugashima Island contains Δ^{7}-cholestenol together with other sterol, and that the acetate of additional sterol is less soluble in ethanol and has a higher melting point than the acetate of Δ^{7}-cholestenol, so that repeated recrystallizations of the acetate mixture result in the concentration of the acetate of additional sterols with a higher melting point. Whether such a striking difference between the chitons from Sugashima Island and from Osaki-Shimojima Island in the content of Δ^{7}-cholestenol is a general case caused by the variation in the catching locality or a special case caused by some other factors, remains unknown.

7) l. c., (3).
8) l. c., (4).

Experimental

The chitons used in these experiments were caught around Osaki-Shimojima Island in the Inland Sea of Seto in August, 1952. Air dried chitons (4.98 kg.) were cracked to small pieces and lipid was extracted with ether, yielding 1.05% of lipid which was a dark reddish orange viscous liquid. The lipid (52.0 g.) was refluxed with 450 cc. of acetone, and the mixture was cooled and filtered, by which 42.0 g. of acetone-solubble oil with the following constants was obtained: d_4^{20} 0.9332, n_D^{20} 1.4784, acid value 110.2, saponification value 184.4, iodine value (pyridine sulfate dibromide method) 138.6, unsaponifiable matter 18.77%.

Unsaponifiable Matter—The acetone-soluble oil (36.8 g.) was saponified, and unsaponifiable matter was extracted with ether from the diluted soap solution. The unsaponifiable matter (7.2 g.) was an orange-yellow solid, and melted at 48~50°C. to a turbid liquid and at 92°C. to a clear liquid. The sterol content in the unsaponifiable matter was found to be 44.86% by the digitonide method. Recrystallization of the unsaponifiable matter from 250 cc. of methanol gave a crude sterol mixture (3.2 g.) of m.p. 113~118°C. It showed no characteristic absorption maximum in the region of 230~310 mμ. The acetate (3.1 g.), m.p. 112.5~114.5°C., prepared by refluxing the crude sterol mixture with acetic anhydride for one hour, was recrystallized from ethanol. After 4 recrystallizations, the melting point was raised to 118~119°C., which was unaltered by further recrystallizations from acetone and ether-acetone (1:2). The total yield of the acetate of m.p. 118~119°C. was 1.34 g. Further 0.94 g. of the acetate melting somewhat less sharply at 115~118.5°C. was separated from the mother liquor of recrystallizations. The remainder of the acetate recovered from the mother liquor showed a lower melting point.

The methanol filtrate from the initial recrystallization of the unsaponifiable matter was concentrated, and a solid deposit (0.67 g.) of m.p. 53~56°C. was separated and recrystallized from acetone, by which a crystalline solid (0.44 g.) of m.p. 61~63°C. was obtained. Since this solid was still contaminated with a minute amount of sterol, the latter was removed by digitonin. The sterol-free solid, after recrystallization from acetone, had m.p. 62.5~63.5°C., iodine value 0.8 and acetyl value 263.2 (calculated for chimyl alcohol 280.1; for batyl alcohol 261.8).

Acetate of m.p. 118~119°C.—This acetate showed saponification value 130.7 (calculated for $C_{29}H_{48}O_2$ 130.8) and number of ethylenic bond, determined by perbenzoic acid titration, 1.05.* It showed a positive Tortelli-Jaffé reaction, and developed a blue-green color very rapidly in the Liebermann-Burchard reaction. Free sterol obtained by saponification of the acetate showed, after recrystallization from ethanol, m.p. 122~122.5°C. and $[\alpha]_D^{12}=0$ (in chloroform). Benzoate was prepared by allowing free sterol to react with benzoyl chloride in pyridine; m.p. 158—159°C. (turbid), 176~177°C. (clear), $[\alpha]_D^{22}=+6.9°$.

$\Delta^{8(14)}$-Cholestenol—The acetate (0.65 g.) described above was dissolved in 30 cc. of acetic acid to which was added platinum black previously saturated with hydrogen. The solution was agitated in an atmosphere of hydrogen. No hydrogen absorption occured. After 3 hours, the solution was poured into 500 cc. of water, and was extracted with ether. The ether-extract, recrystallized from ethanol, gave $\Delta^{8(14)}$-cholestenyl acetate in lustrous leaflets; yield 0.56 g., m.p. 77~78°C., $[\alpha]_D^{10}=+9.5°$. Free stenol, after recrystallization from ethanol, melted at 118.5~119.5°C., and was positive for the Tortelli-Jaffé reaction. Benzoate prepared from free stenol had m.p. 115~117°C. (turbid) and 144°C. (clear).

Δ^{14}-Cholestenol — $\Delta^{8(14)}$-Cholestenyl benzoate (0.21 g.) was dissolved in 8 cc. of anhydrous chloroform, and dry hydrogen chloride was passed into the solution under cooling with ice for 2.5 hours. The solution was then washed successively with sodium bicarbonate solution and water, dried over anhydrous sodium sulfate, and the chloroform was distilled off. The residue contained no chlorine, and recrystallized from ethanol in lustrous needles; yield 0.13 g., m.p. 168~169°C., $[\alpha]_D^{22}=+31.5°$. Free stenol was obtained by saponification of the benzoate, and recrystallized from ethanol in fine needles; m.p. 130~131°C. Acetate was prepared from free stenol and recrystallized from acetone-ethanol (2:1) in fine needles; m.p. 89.5~91°C. It was negative for the Tortelli-Jaffé reaction.

Cholestanyl Acetate—Δ^{14}-Cholestenyl acetate (0.03 g.) was dissolved in 10 cc. of ether-acetic acid (1:1), and hydrogenated in the presence of platinum black as catalyst by stirring the solution in an atomosphere of hydrogen for 4 hours at room temperature. The hydrogenation product was extracted with ether from the solution diluted with water. After recrystallization from ethanol, it formed fine needles; yield 0.02 g., m.p. 104~105°C., $[\alpha]_D^{22}=+14.2°$. The final product still showed a violet color in the Liebermann-Burchard reaction.

Sterol Mixture of Chitons from Sugashima Island—Air dried material of the chitons (400 g.)* caught around Sugashima Island, Mie Prefecture, in April, 1953, yielded 7.9 g. of lipid by ether extraction, from which acetone-soluble oil was obtained in a yield of 86%; d_4^{20} 0.9356, n_D^{20} 1.4792, acid value 112.5, saponification value 185.6, iodine value 131.2, unsaponifiable matter 11.43%. Crude sterol mixture was separated by recrystallizing the unsaponifiable matter from methanol, and its acetate was repeatedly recrystallized from ethanol. The melting points of recrystallized products were121~123°C., 123.5~125.5°C. and 127~129°C., respectively, after 3, 4 and 6 recrystallizations. The final product showed a positive Tortelli-Jaffé reaction, and reacted very fast with the Liebermann-Burchard reagent. It showed no characteristic absorption maximum in the region of 230~310 mμ. The product after 3 and 4 recrystallizations showed $[\alpha]_D^{26}=+3.9°$ and $+6.5°$, respectively.

Summary

Sterol mixture of the chitons caught around Osaki-Shimojima Island in the Inland Sea of Seto contains predominantly Δ^7-cholestenol, the acetate of which is easily obtainable in a pure state by several recrystallizations of the acetate of sterol mixture. From sterol-free components of unsaponifiable matter, a saturated fraction, possibly consisting of chimyl and batyl alcohols, was separated.

The acetate of sterol mixture of another lot of chitons caught around Sugashima Island, Mie Prefecture, shows a higher melting point than Δ^7-cholestenyl acetate after several recrystallizations, as was the case with the chitons caught in the same locality which were described in the first report of this series.

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* The iodine value by the pyridine sulfate dibromide method showed an enormously high value of 232.4 against the calculated value 59.2.

* Supplied through the courtesy of Dr. E. Nakano, Biology Department, Faculty of Science, Nagoya University.