FATTY OILS OF AQUATIC INVERTEBRATES

XXI. FATTY OIL OF THE SEA-HARE *APLYSIA KURODAI* AND ITS STEROL COMPONENTS

TATSUO TANAKA and YOSHIYUKI TOYAMA

Department of Applied Chemistry

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As to the fatty oils from the opisthobranches, no work has been published except the 14th report[1] of this series on the fatty oils of *Actinocyclus japonicus* of the order *Acoela* and *Philine japonica* of the order *Pleurocoela*. In the present study, oils were extracted from four lots of the sea-hare *Aplysia kurodai* which belongs in the order *Pleurocoela* like *P. japonica*, and their properties and sterol components were examined.

Among various kinds of opisthobranches, the sea-hare *A. kurodai* has a larger size and is one of the most common members found in Japanese waters. As is seen from Table 1, there is a striking difference in the yield of ether-extract from the dried material. This appears to be attributable chiefly to a different catching locality and season for each lot of the animals used in this study, as frequently experienced in the case of other aquatic invertebrates used in our previous studies. However, the proportion of acetone-soluble oil (fatty oil) in ether- extract is found to be alike for each oil. Oils extracted from each lot were a dark brownish orange, viscous liquid and had a large content of unsaponifiable matter (Table 2). The fatty acids and unsaponifiable matter were separated in the usual way, and the composition of polyethenoid acids in the total fatty acids was estimated by the alkali-isomerization method for the oils *A* and *C*. The unsaponifiable matter from each oil was a viscous semi-solid and had a characteristic odor. The content of sterol in the unsaponifiable matter was very small except in the case of the oil *A*. However, the content of $\Delta^{5,7}$-sterol in the total sterol was found to be about 30% for each oil.

The crude sterol mixture obtained by recrystallization of the unsaponifiable matter of the oil *A* from methanol was subjected to a fractional crystallization to give three fractions, but no further fractionation was possible because of the scarcity of the material. The unsaponifiable matter from the oil *C* was treated with digitonin to separate a sterol mixture. The acetate of this sterol mixture was fractionated by chromatography. The more easily eluted fractions obtained thereby showed no characteristic absorption peak in the ultraviolet region and, on recrystallization, yielded cholesteryl acetate. Consequently, the non-conjugated sterol components of this oil were found to consist mainly of cholesterol. The $\Delta^{5,7}$-conjugated sterol components were concentrated in the more difficultly eluted fractions, from which a fraction composed mainly of $\Delta^{5,7}$-steryl acetate could be separated by further purification. Although this acetate fraction eventually ob-

tained was not yet pure, its comparatively low melting point appeared to suggest that the $\Delta^{5,7}$-sterol in the oil of *A. kurodai* is closely related to, if not identical with, the sterol previously separated from *Pinna pectinata japonica*[2] and *Tonna luteostoma*[3].

Experimental

1. Sea-hares used for extraction of oil

Among the four lots of sea-hares used in this study (Table 1), the lot *A* consists of raw animals, the lot *B* is a sun-dried material of raw animals and the lots *C* and *D** are a salted material of raw animals. Prior to extraction of oil, the lot *A* was heated on a water bath for the removal of water to some extent and then dried at about 80°C in an electric oven. The lot *B* was dried in an electric oven. The lots *C* and *D* were first rinsed with water for the removal of salt and then dried in the same manner as the lot *A*. The dried material was reduced to powder and then extracted with ether. The ether-extract thus obtained, except that from the lot *D*, was refluxed with about ten times its weight of acetone for a while and then cooled to the ordinary temperature, the acetone-insoluble matter (phosphatide) was removed by filtration, and the acetone-soluble oil was recovered from the acetone-filtrate. The catching locality, weight and number of animals and the yields of ether-extract and acetone-soluble oil are given in Table 1.

TABLE 1. Sea-hares

Lot No.	*A*	*B*	*C*	*D*
Catching locality	Sakushima, Aichi-ken	Owase, Mie-ken	Hakodate, Hokkaido	Hakodate, Hokkaido
Date of receipt	Middle July, 1956	Late Aug., 1956	Late Sept., 1956	Middle Nov., 1957
Number of animals	9	33	76	204
Wt. of raw animals (g)	2,300	—	—	26,820
Dried material (g)	251	(2,200)	1,090	2,200
Dried material (%)	11	—	—	8
Ether-extract (g)	4.3	16.0	111.5	551
Ether-extract (%)	1.7	—	10.2	25.0
Acetone-soluble oil (g)	3.7	14.8	104	—
Acetone-soluble oil (%)	86	93	93	—

Notes: Percentage of ether-extract is expressed on the basis of dried material. Percentage of acetone-soluble oil is expressed on the basis of ether-extract. The lot *B* (sun-dried material) is contaminated with a noticeable amount of difficultly removable foreign matter such as sands.

2. Fatty oils and their fatty acids

The acetone-soluble oils from the lots *A*, *B* and *C* and the ether-extract from the lot *D* were a dark brownish orange, viscous liquid. The color of the oil *A* had a dash of green. Each oil was saponified with alcoholic potassium hydroxide,

* Received by courtesy of Dr. M. Yamada, Faculty of Fisheries, Hokkaido University.

and the diluted soap solution was extracted with ether in the usual way to separate the fatty acids and unsaponifiable matter. A complete saponification of the oil *B* appeared to be somewhat difficult. In the case of the oils *B*, *C* and *D*, the fatty acids thus obtained were refluxed with ten times their weight of hexane for a while and then allowed to stand overnight at the ordinary temperature, the hexane-insoluble matter was removed by filtration, and the hexane-soluble fatty acids were recovered from the filtrate and analyzed. The fatty acids from the oils *B* and *D* gave especially large amounts of hexane-insoluble matter. The fatty acids had a dark reddish orange or dark brownish orange color and solidified at the ordinary temperature. The color of the fatty acids from the oil *A* had a dash of green. As for the unsaponifiable matter, that of the oil *D* was treated with hexane for the removal of hexane-insoluble matter in the same way as in the case of the fatty acids.

Properties of the oils and their fatty acids and unsaponifiable matter are given in Table 2.

TABLE 2. Properties of Oils

Oil No.	*A*	*B*	*C*	*D*
n_D^{40}	1.4953	—	1.4833	—
Acid value	42.0	42 9	22.2	—
Saponification value	127.3	—	137.6	—
Iodine value	157.1	138.1	—	—
Unsaponifiable matter (%)	41.9	39.4	61.7	59.8
Fatty acids				
n_D^{40}	1.4747	—	—	1.4798
Neutralization value	180.7	—	—	179.5
Iodine value	138.9	144.7	173.8	157.6
Saturated acids (%)	—	—	22.9	23.0
Neutr. V. of saturated acids	—	—	203.3	204.0
Iodine V. of saturated acids	—	—	1.9	3.3
Unsaponifiable matter				
Iodine value	166.8	135.6	157.8	152.0
Sterol (%)	26.10	4.94	4.73	2.66
$\Delta^{5,7}$-Sterol (%, on the basis of total sterol)	28.3	28.7	33.0	27.5

Notes: The fatty acids of the oils *B*, *C* and *D* and the unsaponifiable matter of the oil *D* were freed from hexane-insoluble matter. Iodine values recorded in this paper were determined by the Wijs method for the fatty oils and fatty acids and by the pyridine sulphate dibromide method for the unsaponifiable matter and sterol. For the determination of saturated acids (%) in the total fatty acids, the methyl esters of total fatty acids were subjected to the permanganate-acetone oxidation method. For the spectrophotometric determination of $\Delta^{5,7}$-sterol in the total sterol, the digitonide obtained in the determination of the total sterol in the unsaponifiable matter was used[4], and the percentage of $\Delta^{5,7}$-sterol in the total sterol was calculated by assuming the mean molecular weight of sterol to be 399 (Mol. Wt. calculated for diunsaturated C_{28}-sterol).

The fatty acids of the oils *A* and *C* were isomerized under the condition of 21% KOH-ethylene glycol, 180°C and 15 minutes with a current of nitrogen, the ultraviolet absorptions of the isomerized fatty acids were measured, and the

TABLE 3. Polyethenoid Acids in the Fatty Acids of the Oils *A* and *C*

Polyethenoid acid	Fatty acids from the oil *A*				Fatty acids from the oil *C*			
	Wave length (mμ)	Specific extinc. coeff.	%, taking pentaethenoid acid as		Wave length (mμ)	Specific extinc. coeff.	%, taking pentaethenoid acid as	
			C_{22}	C_{20}			C_{22}	C_{20}
Hexaethenoid	376	0.77	2.63	2.63	374	0.70	2.4	2.4
Pentaethenoid	348	3.44	5.40	3.11	346	10.53	19.6	11.3
Tetraethenoid	316	8.79	8.18	9.03	316	19.03	11.7	15.0
Triethenoid	270	17.66	10.95	11.72	270.5	16.65	0.74	3.9
Diethenoid	235	21.23	10.24	10.65	233	21.50	7.6	9.0

Notes: Since characteristic absorption maxima were observed at the wave lengths shown in Table 3, the specific extinction coefficients at these wave lengths were adopted in place of those at 374, 346, 315, 268 and 233 mμ in the formula given by Hammond and Lundberg. In the case of the fatty acids of the oil *C*, monoethenoid acid (%) can be calculated by subtracting saturated acids (%) and polyethenoid acids (%) from 100. Assuming the monoethenoid acid to have an iodine value of 89.9 (iodine value Calcd. for oleic acid), the iodine value of the total fatty acids can be calculated from the percentage composition as 173.2, if pentaethenoid acid is taken as C_{22}, and 167.9, if pentaethenoid acid is taken as C_{20}. The former value is close to the observed value, 173.8, given in Table 2.

polyethenoid acids were estimated by applying the formula given by Hammond and Lundberg[5]. The results are shown in Table 3.

3. Unsaponifiable matter

The unsaponifiable matter from each oil was a viscous semi-solid with a characteristic odor. The unsaponifiable matter from the oil *A* had a reddish orange color while that from the remaining three oils was of a dark brownish orange color.

i. Sterol from the oil *A*. The unsaponifiable matter (0.9 g) separated from the oil *A* was recrystallized from methanol to give 0.1 g of a crude sterol mixture; fine laminae, m.p. 135°–138°C and $\Delta^{5,7}$-sterol content* of 30%. Concentration of the mother liquor gave 0.02 g of the 2nd crop of m.p. 134°–137°C, $[\alpha]_D^{24} = -46.0°$** and $\Delta^{5,7}$-sterol 29% and 0.01 g of the 3rd crop of m.p. 130°–133°C and $\Delta^{5,7}$-sterol 20%. The lst crop of crude sterol mixture was refluxed with acetic anhydride to give an acetate which had m.p. 102°–105°C after recrystallization from methanol.

ii. Sterol from the oil *C*. About 41 g of the unsaponifiable matter from the oil *C* was dissolved in 2.1 *l* of hot 90% ethanol, and 10 g of digitonin dissolved in 1 *l* of hot 90% ethanol was added. The solution was allowed to stand overnight at the ordinary temperature, and the digitonide formed was separated by filtration.

* $\Delta^{5,7}$-sterol contents (%) recorded in this paper, except those recorded in Table 2, were determined spectrophotometrically by applying the formula given in the 5th report[6] of this series.

** Optical rotations recorded in this paper were measured in chloroform.

TABLE 4. Chromatography of Crude Steryl Acetate Mixture from the Oil *C*

Eluate fraction	Yield (g)	m.p. (°C)	$\Delta^{5,7}$-Sterol content (%)
1	0.30	99–102	0
2	0.05	112–113	0
3	0.13	112–113	0
4	0.20	109–111	0
5	0.18	105–107	6
6	0.48	95–100	55
7	0.15	—	17
8	0.01	—	—

The digitonide was decomposed by refluxing with pyridine[7] and 1.5 g of a free sterol mixture was recovered by ether-extraction. The free sterol mixture was a light yellow crystalline solid and had m.p. 130°–134°C and $\Delta^{5,7}$-sterol 30%. Its acetate had m.p. 95°–98°C, $\Delta^{5,7}$-sterol 27% and I.V. 108.9. The acetate (1.5 g) was chromatographically fractionated using an adsorption column of alumina, 20 mm in diameter and 300 mm in height, hexane as solvent and ethanol-hexane (1: 200–50) as developer and eluant. The results are shown in Table 4. In this chromatography, the recovery of $\Delta^{5,7}$-sterol, calculated from the yield and the $\Delta^{5,7}$-sterol content of each eluate fraction, was about 70%.

The eluate fractions 1–4 in Table 4 were united, and the united material was recrystallized from methanol to give 0.32 g of a steryl acetate which had a constant melting point of 112°–113°C, $[\alpha]_D^{10} = -43.7°$ and S.V. 130.9 (Calcd. for $C_{29}H_{48}O_2$, 130.9). The free sterol obtained by saponification of this acetate had m.p. 147.5°–148.5°C, $[\alpha]_D^{10} = -39.4°$ and I.V. 65.3 (Calcd. for $C_{27}H_{46}O$, 65.6) and showed no depression of melting point on admixture with cholesterol. The eluate fraction 6 in Table 4 was subjected to a further chromatography to give three eluate fractions. The 2nd eluate fraction (0.12 g) had the largest $\Delta^{5,7}$-sterol content, 72%. Recrystallization of this fraction from methanol gave 0.03 g of a steryl acetate which had m.p. 114°–116°C and $[\alpha]_D^{7} = -54.3°$. Its $\Delta^{5,7}$-sterol content is estimated at 94% from the absorption data; $k_{272} = 22.02$, $k_{282} = 23.38$ and $k_{294} = 13.09$. Its ultraviolet absorption curve is shown in Fig. 1.

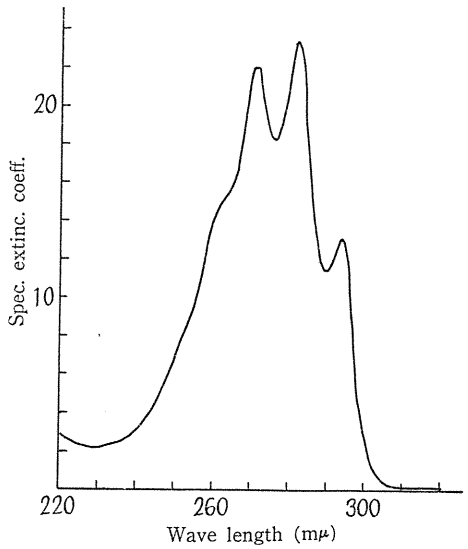

FIG. 1. Ultraviolet absorption curve for $\Delta^{5,7}$-steryl acetate from the oil *C*.

Summary

Oils were extracted from four lots of the sea-hare *Aplysia kurodai*. Characteristics of each oil and its fatty acids were determined, and the sterol components

were examined. The total sterol contained about 30% of $\Delta^{5,7}$-sterol. The non-conjugated sterol components were found to consist mainly of cholesterol. The acetate of the $\Delta^{5,7}$-sterol in *Aplysia kurodai* had a comparatively low melting point. In this respect it is similar to the acetate of the $\Delta^{5,7}$-sterol previously separated from oils of *Pinna pectinata japonica* and *Tonna luteostoma*.

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