RESEARCH REPORTS

FATTY OILS OF AQUATIC INVERTEBRATES

XVIII. FATTY OIL OF *TONNA LUTEOSTOMA* AND ITS $\Delta^{5,7}$-STEROL COMPONENT

TATSUO TANAKA and YOSHIYUKI TOYAMA

Department of Applied Chemistry

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It was noted in the 9th report[1] of this series that sterols in the oil of the shellfish *Tonna luteostoma* (Küster) appeared to contain a component differing from other shellfish sterols since the sterol mixture from this shellfish had a relatively large content of 5,7-diene (provitamin D) and both the sterol mixture and its acetate had a low melting point. In the present study, fatty oil was extracted from a larger lot of this shellfish (samples *A* and *B*), the characteristics of oil were determined, and its sterol components were examined.

The meat from the shellfish sample *A* was separated into the flesh and viscera portions, and fatty oil was extracted separately from each portion. Comparing the flesh portion and the viscera portion, the yield of lipid (ether-extract) and the proportion of fatty oil (acetone-soluble oil) in lipid were larger for the viscera portion than for the flesh portion. The content of unsaponifiable matter in oil and the sterol content in unsaponifiable matter were smaller for the viscera portion than for the flesh portion while the 5,7-diene content in sterol was smaller for the flesh portion than for the viscera portion. These features are all the same as previously noted in the case of several kinds of shellfish.[1),2),3)] From the shellfish sample *B*, the viscera oil alone was extracted. Comparing the viscera oils from both samples, the content of unsaponifiable matter in oil and the 5,7-diene content in sterol were alike for both oils, but the sterol content in unsaponifiable matter was remarkably smaller for the oil from the sample *B* than for the oil from the sample *A* (see Tables 1 and 2). The composition of polyethenoid acids in the viscera oil from the sample *B* was estimated by ultraviolet absorption measurements of the alkali-isomerized fatty acids, and it was found as a characteristic feature of this oil that unlike most aquatic animal oils, this oil contains triethenoid acids in the largest proportion among polyethenoid acids (Table 3).

The crude sterol mixture from the viscera oil of the sample *A* was acetylated, and the steryl acetate mixture was subjected to repeated recrystallizations from ethanol, by which its melting point was raised and its 5,7-diene content was increased by first several recrystallizations but rather decreased by a further repetition of recrystallization. Also fractional crystallization of the steryl acetate mixture from ethanol was found unsuccessful to separate a pure $\Delta^{5,7}$-component since

it gave steryl acetate fractions in which the largest content of 5,7-diene was 60% (see Table 4). Chromatographic separation of the steryl acetate mixture from the sample *B* on alumina column gave eventually a $\Delta^{5,7}$-steryl acetate of the highest purity; m.p. 115°–116°C. Saponification of this acetate gave a free sterol of m.p. 118°–119°C. This sterol is characterized by a low melting point of both free sterol and acetate. It is recognized as a C_{27}-sterol on the basis of the saponification value of its acetate. The infrared absorption spectrum of this sterol (Fig. 2) exhibited absorption bands at 962, 891 and 1,643 cm^{-1}. Hence, this sterol is found to have 22 : 23-double bond and terminal methylene group, since *trans* 22 : 23-double bond in a steroid is known to exhibit an absorption band[4] at about 970 cm^{-1} and terminal methylene group $CH_2=CR_1R_2$ exhibits absorption bands[5] at about 890 and 1,640 cm^{-1}. The possible locations of the terminal methylene group in a C_{27}-sterol are 20 : 21, 25 : 26 or 25 : 27. If the terminal methylene group in this sterol is assumed to be located at 20 : 21, the double bonds at 22 : 23 and 20 : 21 are conjugated and this sterol should exhibit an absorption maximum in the region of 220–250 mμ.[6] However, the ultraviolet absorption spectrum of this sterol (Fig. 1) shows no such absorption maximum. Accordingly this sterol is presumably concluded to be $\Delta^{5,7,22,25}$-cholestatetraenol. Although 14-dehydroergosterol[7] and 24(28)-dehydroergosterol[8] are known as tetra-unsaturated sterols occurring in fungi, the occurrence of tetra-unsaturated sterol in animal kingdom appears not to have been known before the present study.

Experimental

1. Shellfish samples used for extraction of oil

Two samples (*A* and *B*) of the whole shellfish, *Tonna luteostoma* (Küster), were used in this study. The sample *A* was procured from the Aichi-ken Toyohama Fisheries Co-operation in middle August, 1955 and the sample *B* from the Aichi-ken Mikawa Isshiki Fisheries Co-operation in early May, 1956. These samples were shelled in this laboratory, and the meat was separated into the flesh and

TABLE 1. Shellfish Samples

	Sample *A*	Sample *B*
Number of shellfish	142	170
Wt. of whole shellfish (kg.)	—	25.90
Wt. of meat (kg.)	12.96	14.80
Wt. of flesh portion (kg.)	7.25	5.90
Dried material of flesh portion (g.)	1,210	980
Ether-extract from flesh portion (g.)	28	—
Ether-extract from flesh portion (%)	2.3	—
Acetone-soluble oil from flesh portion (g.)	18	—
Acetone-soluble oil from flesh portion (%)	64	—
Wt. of viscera portion (kg.)	5.71	8.20
Dried material of viscera portion (g.)	1,905	2,700
Ether-extract from viscera portion (g.)	319	472
Ether-extract from viscera portion (%)	17	17
Acetone-soluble oil from viscera portion (g.)	252	396
Acetone-soluble oil from viscera portion (%)	79	84

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.

viscera portions. The flesh portion was dried at about 80°C in an electric oven. The viscera portion was first heated on a water bath for the removal of water to some extent and then dried in an electric oven. The dried material was reduced to powder and then extracted with ether. The ether-extract thus obtained 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 proportions of flesh and viscera portions and the yields of oil are given in Table 1. From the sample *B*, oil was extracted from the viscera portion alone.

2. Fatty oils and their fatty acids

Fatty oils (acetone-soluble oils) extracted from both samples are a dark reddish orange, viscous liquid with some solid at the ordinary temperature. Properties of the fatty oils and their fatty acids and unsaponifiable matters separated in the usual way are given in Table 2. The fatty acids of viscera oil from the sample *B* were isomerized under the condition of 21% KOH-ethylene glycol, 180°C and 15 min. with a current of nitrogen, the ultraviolet absorptions of the isomerized fatty acids were measured, and the polyethenoid acids were estimated by applying the formula given by Hammond and Lundberg.[9] The results are shown in Table 3.

TABLE 2. Fatty Oils

	Flesh oil from the sample *A*	Viscera oil from the sample *A*	Viscera oil from the sample *B*
d_4^{40}	—	0.9129	0.9128
n_D^{40}	—	1.4707	1.4721
Acid value	57.7	19.0	84.0
Saponification value	124.4	163.1	163.8
Iodine value	135.9	133.1	141.0
Unsaponifiable matter (%)	50.21	15.30	14.72
Fatty acids			
n_D^{40}	1.4665	1.4616	1.4653
Neutralization value	190.2	189.6	188.5
Iodine value	139.9	138.6	143.3
Saturated acids (%)	—	28.0	29.2
Neutr. value of saturated acids	—	215.0	213.9
Iodine value of saturated acids	—	3.4	1.3
Unsaponifiable matter			
Iodine value	93.5	122.4	90.4
Sterol (%)	64.7	37.1	13.5
5,7-Diene (%, on the basis of total sterol)	5	31	32

Notes: Iodine values recorded in this paper were determined by the Wijs method for fatty oils and fatty acids and by the pyridine sulfate dibromide method for 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 determination of 5,7-diene ($\Delta^{5,7}$-sterol) in the total sterol, the digitonide obtained in the determination of the total sterol in unsaponifiable matter was used for ultraviolet absorption measurements,[10] and the percentage of 5,7-diene in the total sterol was calculated by assuming the mean molecular weight of sterols to be 385 (mol. wt. of C_{27}-diunsaturated sterol).

TABLE 3. Polyethenoid Acids in the Fatty Acids of Viscera Oil from the Sample *B*

	Wave length (mμ)	Specific extinction coefficient	% Assuming pentaethenoid acids to be C_{22}	% Assuming pentaethenoid acids to be C_{20}
Hexaethenoid ·	376	0.89	3.0	3.0
Pentaethenoid·	348	5.53	9.3	5.4
Tetraethenoid·	316	11.15	8.2	9.7
Triethenoid ···	270	18.48	9.6	11.0
Diethenoid ····	235	17.88	5.2	5.9

Notes: Since absorption maxima were observed at 376, 348. 316, 270 and 235 mμ in our experiments, these wave lengths were adopted in place of the wave lengths, 374, 346. 315, 268 and 233 mμ in the formula given by Hammond and Lundberg. Monoethenoid acids (%) can be obtained by subtracting saturated acids (%) and polyethenoid acids (%) from 100. Assuming the monoethenoid acids to have an iodine value of 89.9 (iodine value of oleic acid), the iodine value of total fatty acids can be calculated from the observed composition as 145.1, if pentaethenoid acids are C_{22}, and 142.2, if pentaethenoid acids are C_{20}. These values are close to the observed value, 143.3, given in Table 2.

3. Separation of $\Delta^{5,7}$-sterol from unsaponifiable matter

The unsaponifiable matter separated from viscera oil was recrystallized from methanol to give crystals of crude sterol mixture. The crude sterol mixture from the viscera oil of the sample *A* had m.p. 115°–119°C, and its acetate mixture (I) had m.p. 107°–109°C, iodine value 161.7 and 5,7-diene content 33%. The crude sterol mixture from the viscera oil of the sample *B* had m.p. 116°–118°C, and its acetate mixture (II) had m.p. 109°–111°C and 5,7-diene content 33%.*

(i) Fractional crystallization of crude steryl acetate mixture. Eight g. of crude steryl acetate mixture I was subjected to repeated recrystallizations from

TABLE 4. Fractional Crystallization of Crude Steryl Acetate I

Fraction	Yield (g.)	m.p. (°C)	5,7-Diene content (%)
1	0.35	129.5–130.5	37
2	0.11	123 –124	44
3	0.29	121 –122	44
4	0.25	117.5–119	48
5	0.51	113.5–114.5	60
6	0.29	116 –118	45
7	0.67	112 –114	32
8	0.25	106 –108	32
9	1.45	108 –110	37
10	0.33	99 –101	32
11	1.10	99 –101	30
12	0.50	99 –101	28
13	0.53	—	18
Mother liquor	—	—	—

Notes: The fraction 1 was obtained after seven recrystallizations of the crude steryl acetate mixture I from ethanol. The fractions 2–13 were obtained by fractional crystallization of the steryl acetate mixture recovered from the united mother liquor of seven recrystallizations.

* 5,7-Diene contents (%) for the samples recorded in this paper, except those recorded in Table 2, were determined by ultraviolet absorption measurements of the samples by applying the formula given in the 5th report[2] of this series.

ethanol, by which the melting point of steryl acetate was steadily raised to 129.5°–130.5°C after seven recrystallizations. 5,7-Diene content of steryl acetate was increased by first three recrystallizations to 50%, but it was rather decreased by further recrystallizations and became 37% after seven recrystallizations. The steryl acetate mixture remaining in the united mother liquor of recrystallizations was fractionally crystallized from ethanol. Among the steryl acetate fractions eventually obtained, the largest 5,7-diene content was 60% as shown in Table 4.

(ii) Chromatography of crude steryl acetate mixture. Recrystallization of the crude steryl acetate mixture II from methanol gave an steryl acetate mixture of m.p. 115°–117°C and 5,7-diene content 33%. This steryl acetate mixture (3.3 g.) was chromatographically fractionated using an adsorption column of alumina, 57 mm in diameter and 415 mm in height, hexane as solvent and hexane-ethanol (200 : 1) as developer and eluant. The results are shown in Table 5. The eluate fractions 3, 4, 5, 9 and 10 in Table 5 were united, and the united material was again chromatographically fractionated to give 0.08 g. of an eluate fraction of 5,7-diene content 98%.

TABLE 5. Chromatography of Crude Steryl Acetate Mixture II

Eluate fraction	Yield (g.)	m.p. (°C)	5,7-Diene content (%)
1	0.29	118–120	0
2	1.43	113–115	4
3	0.83	109–111	30
4	0.20	107–109	58
5	0.10	108–110	76
6	0.09	110–112	98
7	0.07	110–112	100
8	0.05	110–112	95
9	0.06	114–116	69
10	0.04	—	16
11	0.04	—	2
12	0.02	—	0

Notes: For the elution of the fractions 11 and 12, hexane-ethanol (10 : 1) and hexane-methanol (4 : 1), respectively, were used. The recovery of 5,7-diene, calculated from the 5,7-diene content of each fraction, is found to be about 70%.

Several experiments on chromatography were preliminary made with the crude steryl acetate mixture I. First, the steryl acetate fractions, which were obtained from I by fractional crystallization and had 5,7-diene content about 30%, were united, and the united material was chromatographically fractionated to give a fraction of 5,7-diene content 53%. In this experiment, the recovery of 5,7-diene was more than 90%. Next, the fraction of 5,7-diene content 53% was chromatographically fractionated to give fractions of 5,7-diene content 70–80% and a minor amount of a fraction of 5,7-diene content 100%. In this case the recovery of 5,7-diene was 74%. Finally, the united material of the fractions of 5,7-diene content 70–80% was subjected to a further fractionation by chromatography, by which a very minor amount of a fraction of 5,7-diene content 100% together with fractions of 5,7-diene content 26–90% and viscous liquid fractions free from 5,7-diene were obtained. The recovery of 5,7-diene in this case was only 29%. From these

results, it appears that the $\Delta^{5,7}$-sterol is relatively unstable and undergoes some changes, possibly oxidation and polymerization, during chromatography, and the rate of these changes rapidly increases after the lapse of some period.

4. Properties of $\Delta^{5,7}$-sterol and its derivatives

The eluate fractions of 5,7-diene content of more than 95%, obtained from the steryl acetate mixture II by chromatography, were united, and the united material was purified by recrystallization from methanol to give 0.2 g. of an steryl acetate of m.p. 115°–116°C (on melting, it became colored with dark orange-red); $[\alpha]_D^{23} = -74.1°$ (in chloroform); saponification value 132.2 (calcd. for $C_{29}H_{42}O_2$, 132.8); $k_{272} = 27.423$, $k_{282} = 29.017$ and $k_{294} = 16.246$.

The free sterol obtained by saponification of the acetate had m.p. 118°–119°C (with darkening as observed for the acetate) after recrystallization from methanol; $[\alpha]_D^{28} = -109.9°$; $k_{272} = 29.224$, $k_{282} = 30.929$ and $k_{294} = 17.287$. With digitonin, this sterol formed quantitatively digitonide. Ultraviolet and infrared absorption spectra of this sterol are shown in Figs. 1 and 2, respectively. The benzoate prepared from this sterol had m.p. 133°–138°C after recrystallization from methanol (it began to become turbid at 133°C and completely melted to a dark orange-red liquid at 138°C); $k_{230} = 28.104$, $k_{272} = 25.726$, $k_{282} = 26.036$ and $k_{294} = 14.330$.

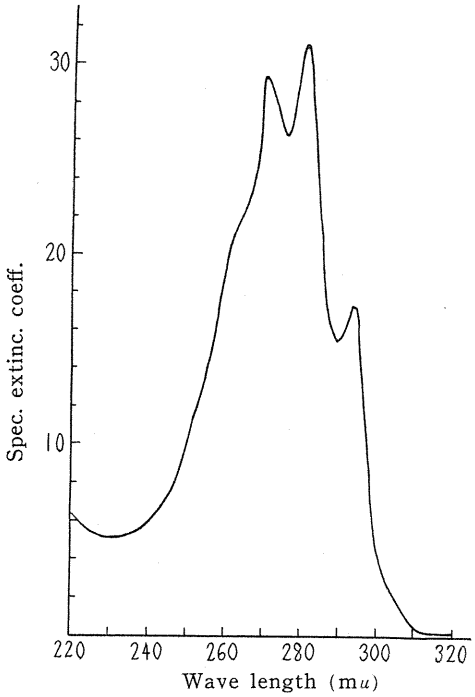

FIG. 1. Ultraviolet absorption curve of $\Delta^{5,7}$-sterol.

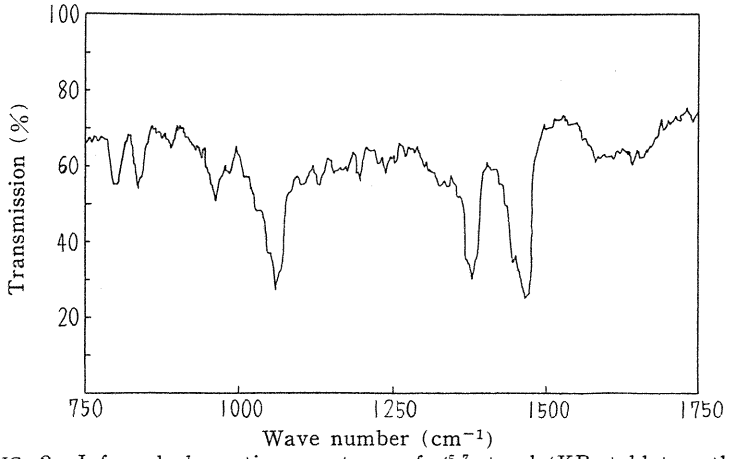

FIG. 2. Infrared absorption spectrum of $\Delta^{5,7}$-sterol (KBr-tablet method).

Summary

1. Fatty oils were extracted separately from the flesh and viscera portions of the shellfish, *Tonna luteostoma*. Properties of fatty oils and their fatty acids were determined.

2. The sterol mixture from viscera oil was acetylated, and the acetate mixture was chromatographically fractionated to give eluate fractions rich in 5,7-diene. These fractions were united, and the united material was purified by recrystallization, by which a pure $\Delta^{5,7}$-steryl acetate of m.p. 115°–116°C (with darkening) and $[\alpha]_D^{28} = -74.1°$ was eventually obtained. The free sterol from this acetate had m.p. 118°–119°C (with darkening) and $[\alpha]_D^{28} = -109.9°$. This sterol was recognized as a C_{27}-sterol on the basis of the saponification value of its acetate. Ultraviolet and infrared spectra of this sterol indicated the presence of *trans* isolated double bond and terminal methylene group besides 5,7-diene in this sterol. Hence, this sterol is presumably concluded to be a $\Delta^{5,7,22,25}$-cholestatetraenol.

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