

FATTY OILS OF AQUATIC INVERTEBRATES
XXII. UNSAPONIFIABLE COMPONENTS OTHER THAN STEROL IN
THE FATTY OIL OF THE SEA-HARE *APLYSIA KURODAI*

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In the preceding report¹⁾ of this series, oils (*A*, *B*, *C* and *D*) were extracted from four lots of the sea-hare *Aplysia kurodai*, and their characteristics were determined. The sterol mixture from the oil *C* was found to contain cholesterol and a $\Delta^{5,7}$ -sterol, the acetate of which had a relatively low melting point. In the present work, unsaponifiable components other than sterol of the oils *C* and *D* were examined with the results described below.

The non-sterol fraction separated from the unsaponifiable matter of the oil *C* was acetylated and the acetate was fractionally distilled (Tables 2 and 3). The distillate fractions had a considerably lower boiling point than the acetates of batyl alcohol and kindred alcohols which have frequently been found in oils of molluscs. They had also a high refractive index, and the free unsaponifiable substances obtained by saponification of the distillate fractions showed an exceedingly higher iodine value than the original acetate fractions. From these facts it was supposed that some unusual components are likely to be present in the unsaponifiable matter of the oil of *A. kurodai*. At the outset of this study, an acetate fraction (the fraction 2 in Table 3) and free unsaponifiable matter prepared from this acetate fraction were examined to find a clue to the structure of the substance in question. Thereby it was found that this substance has an aromatic ring structure and a phenolic hydroxyl group and contains bromine as an unexpected component element. In this connection the unsaponifiable matter of the oil from *Philine japonica*²⁾ which belongs in the same order *Pleurocoela* as *A. kurodai* was tested for halogen, but the result was negative. The crystalline solid contained in higher distillate fractions (the fractions 6 and 7 in Table 2) was separated and purified to give a substance which crystallized in plates of a constant melting point of 93.5°–94.5°C. Its absorption spectra (Figs. 1 and 2) like those of the above mentioned distillate fraction 2 in Table 3 had strong and sharp absorption bands at 1761 cm^{-1} and 1214 cm^{-1} in the infrared region and a weak absorption maximum near 280 $\text{m}\mu$ in the ultraviolet region. It had an iodine value of nearly zero, and upon saponification gave a free alcohol (*P*), which crystallized in fine needles of m.p. 69°–71°C, Mol. Wt. 309 and I.V. 89.7 and accorded with the molecular formula $\text{C}_{16}\text{H}_{21}\text{OBr}$. Like the free unsaponifiable matter from the above distillate fraction 2 in Table 3, this free alcohol had a single absorption maximum at 283 $\text{m}\mu$, in the ultraviolet region, and a hydroxyl absorption at 3300–3400 cm^{-1} , a group of absorption band at 3054, 1612, 1565 and 1497

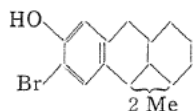
cm^{-1} attributable to the aromatic ring structure, and the bands at 1391 and 1205 cm^{-1} corresponding to the phenolic hydroxyl group, in the infrared region (Figs. 1 and 2).

Since a remarkable proportion of the acetate mixture remained undistilled by the fractional distillation (Tables 2 and 3), chromatographic fractionation was attempted in the next place. Thus the unsaponifiable matter of the oil *D*, after removal of hexane-insoluble matter and sterol, was chromatographically fractionated into fourteen eluate fractions (Table 4). The earlier eluate fractions were liquid while the later eluate fractions were solid. The united material of the eluate fractions 3-6 in Table 4, 52% of the total eluate, showed the characteristic absorption at 283 $\text{m}\mu$. Further purification of this material by chromatography (Tables 5 and 6) brought about a rise of the bromine content to an approximately constant value (25%) giving eventually a substance (*Q*) which crystallized from hexane in the form of fine needles, m.p. 68°C. Another portion of the unsaponifiable matter of the oil *D*, after removing hexane-insoluble matter but without removing sterol, was chromatographed. A combined eluate fraction obtained thereby was freed from sterol using digitonin, treated with the Claisen solution, and purified further by chromatography to give eventually a substance (*R*) of m.p. 69°-71°C and Br-content 25.47%. Another combined eluate fraction which was a crystalline solid and obtained in a relatively small amount, yielded on recrystallization a crystalline substance of m.p. 146.5°-147.5°C and Br-content 20.60%.

The above three fractions *P*, *Q* and *R* were recognized to consist of an identical, homogeneous substance by spectrophotometric analysis and other means. In regard to the structure of this substance, the following considerations are taken into account: (1) From the results of the present study, the presence of one phenolic hydroxyl group in this substance is evident. Furthermore, the fact that this substance exhibits a single absorption maximum at 283 $\text{m}\mu$, ϵ_{283} = about 2700, in the ultraviolet region appears to indicate that this substance is a monophenol with a single aromatic ring. (2) Analytical data show the presence of one atom of bromine in the molecule. The difficulty of debromination suggests that the bromine atom is attached to the aromatic ring. (3) While this substance shows an iodine value of about 90, determined by the pyridine sulphate dibromide method, its acetate shows scarcely any iodine value. Hence it seems probable that the iodine value of this substance is caused by the reaction of the halogen reagent with the aromatic ring under influence of a free phenolic hydroxyl group (and a substituted bromine atom originally present in the molecule). This postulation is supported by the fact that on bromination this substance absorbs two atoms of bromine to give a product which shows no iodine value. Accordingly this substance appears to have no unsaturated linkage other than that in the aromatic ring. This substance is less easily soluble in an aqueous solution of potassium hydroxide and is faintly positive for a qualitative test for phenol. These behaviors of this substance suggest the presence of a long alkyl side chain or cycloparaffin structure. (4) Although an detailed interpretation of the infrared spectra of this substance is difficult because of their complexity, it is inferred that the 895 cm^{-1} band of this substance is due to a 1:2:4:5-substitution of aromatic ring while the 1761 cm^{-1} band of its acetate is due to an ester with an α -electronegative substituent (bromine). Furthermore, it is inferred that the bands

at 1451, 1380 and 1373 cm^{-1} are caused by the methyl group and the bands at 1022 and 977 cm^{-1} by the cyclohexane ring.*

From these considerations the following structure, for example, appears to be probable for this substance of the formula $\text{C}_{16}\text{H}_{21}\text{OBr}$.



It is seen from the results of this study that the unsaponifiable matter of the oil from *A. kurodai* contains a remarkable amount of peculiar substance containing bromine. Such a fact has never been found in natural oils hitherto studied and is very interesting. The pigment of the shellfish *Murex brandaris*³⁾ and the pigment of *M. brandaris* and *M. trunculus*⁴⁾ were studied by previous authors, and the former was identified with 6, 6'-dibromoindigo. It is supposed that the substance, $\text{C}_{16}\text{H}_{21}\text{OBr}$, in *A. kurodai* is likely to have some relation to the violet pigment contained in the secretion of this animal.

Experimental

The oil samples *C* and *D* used in this study are the same as described in the preceding report.¹⁾ Characteristics of their unsaponifiable matter are shown in Table 1.

TABLE 1. Unsaponifiable Matter of the Oils
from *A. kurodai*

Oil	<i>C</i>	<i>D</i>
Unsaponifiable matter (%).....	61.7	59.8
Iodine value*	157.8	152.0
Sterol (%).....	4.73	2.66
$\Delta^{5,7}$ -Sterol (% in the total sterol)	33.0	27.5

* Iodine values recorded in this paper were determined by the pyridine sulphate dibromide method.

1. Fractional distillation of the non-sterol fraction of the unsaponifiable matter from the oil *C*

The non-sterol fraction (I.V. 151.4) was recovered from the mother liquor of digitonide which was obtained in the separation of sterol from the unsaponifiable matter of the oil *C* using digitonin (see the preceding report¹⁾). This was acetylated to give an acetate of S.V. 174.8 and I.V. 82.3. The acetate (33.6 g) was fractionally distilled with the results shown in Table 2. During the distillation there was an indication of decomposition with an evolution of white smoke, and there remained a remarkable amount of residue.

The fractions 4 and 5 in Table 2 were united and the united material was saponified to give a free unsaponifiable matter of I.V. 147.6. The acetate regener-

* Interpretation of the infrared spectra was made mostly by reference to L. J. Bellamy, "The Infra-red Spectra of Complex Molecules", Methuen and Co. Ltd., London (1954),

TABLE 2. Fractional Distillation of the Acetate of Non-sterol Fraction from the Oil C

Fraction	b.p. (°C/4 mm Hg)	Yield		n_D^{40}	S.V.	I.V.
		(g)	(%)			
1	-145	0.9	2.7	1.5178	65.8	165.6
2	145-160	1.7	5.0	1.5201	92.5	131.1
3	160-170	1.9	5.7	1.5253	141.0	91.1
4	170-180	5.1	15.2	1.5272	171.7	63.7
5	180-184	5.2	15.5	1.5313	174.8	49.6
6	Gradual fall of temp.	4.0	11.9	1.5355	163.3	46.9
7		3.4	10.1	1.5364	157.4	56.1
Residue and loss	—	11.4	33.9	—	—	128.7

ated from this free unsaponifiable matter by refluxing with acetic anhydride showed S.V. 150.5. Since the fractions 6 and 7 deposited some crystalline solid at the ordinary temperature, they were recrystallized from methanol, and the liquid acetate was recovered from the filtrate and jointed with the regenerated acetate from the fractions 4 and 5. The united material was fractionated further with the results shown in Table 3. Thereby the distillate fractions showed a considerably lower saponification value as compared with the material before distillation.

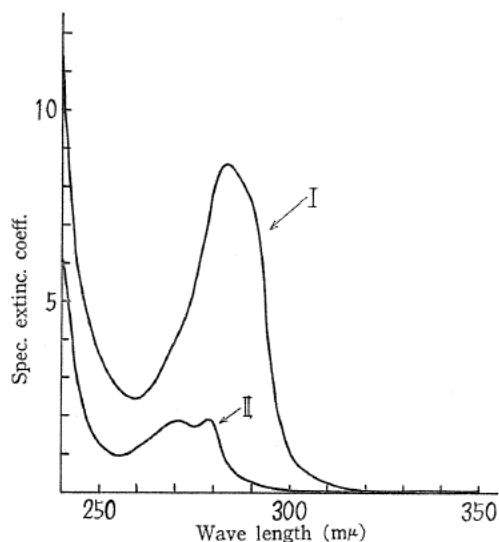
TABLE 3. Redistillation of the Acetate Fractions 4-7 in Table 2

Fraction	b.p. (°C/6 mm Hg)	Yield		n_D^{40}	S.V.	I.V.
		(g)	(%)			
1	-182	1.80	24.3	1.5331	135.2	67.2
2	182-185	4.10	55.4	1.5361	139.1	62.5
Residue	—	1.50	20.3	—	120.9	80.0

i. The fraction 2 in Table 3. Both the acetate fraction and the free unsaponifiable matter prepared by saponifying the acetate had a brownish yellow color and a characteristic odor and were easily soluble in organic solvents such as ether, hexane, methanol, carbon tetrachloride and others while difficultly soluble in a dilute aqueous solution of potassium hydroxide. The acetate fraction exhibited some absorption near $280\text{ m}\mu$ in the ultraviolet region* and strong and sharp absorptions at 1761 and 1214 cm^{-1} in the infrared region. On the other hand, the free unsaponifiable matter from this acetate fraction exhibited absorption maxima at $283\text{ m}\mu$ in the ultraviolet region, at about 3400 cm^{-1} and at 3054 , 1612 , 1565 and 1497 cm^{-1} in the infrared region. Qualitative test for the component element revealed the absence of nitrogen, sulphur and phosphorus and the presence of bromine. In this connection, it may be mentioned here that while the crude unsaponifiable matter from the oils C and D in the present study and A in the previous study¹⁾ was positive for the flame reaction for halogen, an unsaponifiable fraction of the oil of *P. japonica* obtained by removing a greater

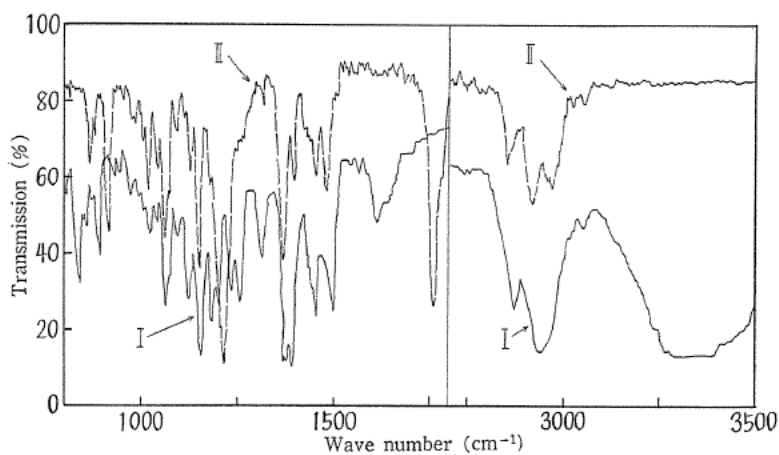
* Ultraviolet absorption spectra recorded in this paper were taken in ethanol.

part of sterol by recrystallization of the total unsaponifiable matter from methanol gave a negative test. The fraction 2 in Table 3 had a bromine content of 15.31 %,* which even after refluxing with zinc dust and glacial acetic acid for 15 hours showed an approximately same value, 15.23%. The free unsaponifiable matter from this fraction developed only a very faint brown color on the ferric chloride test in methanol and a reddish pink color on the Guareschi's alkali test.



I. Free alcohol *P* II. Its acetate

FIG. 1. Ultraviolet absorption curves of the free alcohol *P* and its acetate



I. Free alcohol *P* II. Its acetate

FIG. 2. Infrared absorption spectra of the free alcohol *P* and its acetate (KBr-tablet method)

* Bromine contents recorded in this paper were determined by the Carius method.

ii. Crystalline solid from the fractions 6 and 7 in Table 2. These fractions were recrystallized from methanol to give a crystalline solid of m.p. 90°–92°C. Further recrystallizations of this crystalline solid from aqueous methanol yielded a substance (0.6 g) which crystallized in the form of plates and had a constant melting point of 93.5°–94.5°C, S.V. 163.0 (Calcd. for $C_{18}H_{22}O_2Br$, 159.7) and I.V. 0.8. Its ultraviolet and infrared spectra are shown in Figs. 1 and 2, respectively. On saponification it yielded a free alcohol (*P*) which crystallized in fine needles of m.p. 69°–71°C, I.V. 89.7** and Mol. Wt. 309*** (Calcd. for $C_{16}H_{21}OBr$, 309) and did not show the Liebermann-Burchard reaction. This free alcohol had an absorption maximum at 283 $m\mu$ (Fig. 1). Its infrared spectra are given in Fig. 2. It was faintly positive for the Guareschi's alkali test.

Anal. Found: C 61.49%, H 6.83%, Br 25.52%

Calcd. for $C_{16}H_{21}OBr$: C 62.14%, H 6.84%, Br 25.84%

2. Chromatography of the non-sterol fraction of the unsaponifiable matter from the oil *D*

Fifty g of the unsaponifiable matter from the oil *D* was refluxed with ten times its weight of hexane for a while and then cooled to the ordinary temperature, the hexane-insoluble matter was removed by filtration, and the hexane-soluble portion (45.6 g) was recovered from the hexane filtrate. The hexane-soluble portion was treated with digitonin in 90% ethanol to remove sterol. The non-sterol fraction thus obtained was a dark reddish orange, viscous liquid and had n_D^{20} 1.5425, I.V. 147.3 and Br 13.57%. Thirty g of this non-sterol fraction was chromatographically fractionated, using 150 g of silica gel as adsorbent and ether-hexane (1:10-5), ethanol-hexane (1:20-10) and methanol-hexane (10:1) as develop-

TABLE 4. Chromatography of the Non-sterol Fraction of the Unsaponifiable Matter from the Oil *D*

Eluate fraction	Yield		n_D^{20}	I.V.	Bromine (%)
	(g)	(%)			
1	0.50	1.7	1.5086	234.9	1.83
2	0.52	1.7	1.5203	204.9	—
3	1.08	3.6	1.5455	126.3	—
4	0.39	1.3	1.5456	133.7	—
5	13.09	43.6	1.5514	152.3	15.76
6	1.07	3.6	1.5476	154.7	—
7	1.01	3.4	1.5287	168.7	—
8	0.76	2.5	—	158.0	—
9	0.51	1.7	1.5336	150.2	—
10	0.68	2.3	1.5475	191.8	—
11	0.52	1.7	1.5376	163.5	—
12	5.02	16.7	—	133.4	—
13	3.90	13.0	—	117.3	15.82
14	0.95	3.2	—	136.6	—

** Assuming two atoms of bromine to be absorbed in the determination of iodine value, the iodine value calculated for $C_{16}H_{21}OBr$ is 82.1.

*** Molecular weights recorded in this paper were determined in benzene by the cryoscopic method.

per and eluant. The results are shown in Table 4.

The eluate fractions 1 and 2 in Table 4 were a light yellow liquid. The fractions 3-6 were a reddish orange yellow, viscous liquid and showed an absorption maximum at 283 $m\mu$ in the ultraviolet region, while the other eluate fractions had no characteristic absorption in this region. The fraction 8 was an orange yellow crystalline solid. The fractions 12 and 13 were a dark reddish orange, resinous solid. The fraction 13 had m.p. 30°-40°C and Mol. Wt. 740.

The fractions 3-6 in Table 4 were united and 12.57 g of the united material was fractionated by chromatography, using silica gel as adsorbent, to give the results shown in Table 5.

TABLE 5. Chromatography of the Eluate Fractions 3-6 in Table 4

Eluate fraction	Yield		n_D^{40}	I.V.	Bromine (%)
	(g)	(%)			
1	0.07	0.6	1.5411	—	—
2	0.62	4.9	1.5456	129.4	—
3	1.54	12.3	1.5522	127.6	18.68
4	1.81	14.4	1.5568	114.5	21.09
5	4.20	33.4	1.5530	148.5	16.32
6	2.28	18.1	1.5425	192.6	6.83
7	1.44	11.5	1.5477	186.1	11.59
8	0.43	3.4	—	115.6	—
9	0.18	1.4	—	113.7	—

The fraction 4 in Table 5 had an absorption maximum at 283 $m\mu$ and Mol. Wt. 300. A portion (0.83 g) of this fraction was dissolved in 17 cc of ether, an excess of bromine in glacial acetic acid (1:4) was added dropwise to the solution under cooling at about -10°C. No precipitate was formed after standing overnight at the same temperature. The excess bromine was removed from the solution, and 1.44 g of a bromination product was recovered, which was a light yellow solid of a melting point of about 70°C and had a single absorption maximum at 297 $m\mu$; I.V. 0, Br 51.75% and Mol. Wt. 470 (Calcd. for $C_{16}H_{19}OBr_3$, Br 51.33% and Mol. Wt. 467).

The united material of 1.10 g of the fraction 3 and 0.70 g of the fraction 4 in Table 5 was fractionated by chromatography, using alumina as adsorbent, with the results shown in Table 6.

The eluate fractions 3 and 4 in Table 6 were united and the united material was recrystallized from hexane at about -5°C to give a substance which crystal-

TABLE 6. Chromatography of the Eluate Fractions 3 and 4 in Table 5

Eluate fraction	Yield		n_D^{40}	I.V.	Bromine (%)
	(g)	(%)			
1	0.18	10.0	1.5335	161.2	—
2	0.92	51.1	1.5536	131.9	18.46
3	0.45	25.0	1.5616	92.9	} 25.20
4	0.17	9.4	1.5619	88.0	
5	0.08	4.5	—	117.8	—

lized in white needles (*Q*) of m.p. 66°–68°C and showed a single absorption at 283 $m\mu$. Its bromine content was found to be 25.27%. The residue remaining after evaporation of the solvent from the mother liquor had I.V. 92.2 and Br 25.21%.

3. Alkali treatment and chromatography of the non-sterol fraction of the unsaponifiable matter from the oil *D*

Seventy eight g of the hexane-soluble fraction of the unsaponifiable matter from the oil *D* was chromatographically fractionated using silica gel as adsorbent, and two fractions, the one (47.9 g) having n_D^{40} 1.5405–1.5492 and the other (4.2 g) being a crystalline solid of I.V. 120–130, were separated. Recrystallization of the latter fraction from ethanol-hexane gave 1.2 g of a substance which crystallized in the form of needles of m.p. 146.5°–147.5°C and Br 20.60% and did not show the Liebermann-Burchard reaction. The former fraction which corresponded to the fractions 3–6 in Table 4 was again subjected to a chromatographic fractionation. Several eluate fractions, n_D^{40} 1.5456–1.5488, obtained thereby were united and the united material was treated with digitonin to remove sterol, and a non-sterol fraction of n_D^{40} 1.5472 and I.V. 154.4 was separated and then subjected to an alkali-treatment in the following way: The non-sterol fraction (34.8 g) was dissolved in 320 cc of hexane and the solution was shaken, in a separatory funnel, successively with 150 cc-, 120 cc- and 100 cc-portions of Claisen solution (prepared by dissolving 140 g of potassium hydroxide in 100 cc of water and then diluting the solution with methanol to a volume of 400 cc). The united aqueous methanol layer was then extracted four times with each 150 cc portion of hexane. After these procedures, all the hexane layers were united, washed with water, and dehydrated with anhydrous sodium sulphate, and the hexane was removed from the solution leaving a reddish orange-yellow viscous liquid of n_D^{40} 1.5374, I.V. 170.2 and Br 12.25%. It had no characteristic absorption at 283 $m\mu$. The combined aqueous methanol layer was acidified with a dilute hydrochloric acid under cooling with water, and the substance separated from the aqueous medium was collected using hexane. The substance (20.5 g) thus obtained was a reddish orange, viscous liquid of n_D^{40} 1.5529, I.V. 145.3 and Br 17.98%. This substance was chromatographed to give a fraction (5.0 g) of Br 19.44%. This fraction was again treated with Claisen solution to separate an alkali-soluble fraction of n_D^{40} 1.5607, I.V. 108.8 and Br 22.24%, which was purified further by chromatography to give eventually 0.40 g of an eluate fraction (*R*). It crystallized in white fine needles of m.p. 69°–71°C, I.V. 92.5 and Mol. Wt. 310 (Calcd. for $C_{16}H_{21}OBr$, 309). It may be noted that when the fraction *R* is once melted, it takes a long time to bring out solidification on cooling at the ordinary temperature.

Anal. Found: C 62.21%, H 6.97%, Br 25.47%

Calcd. for $C_{16}H_{21}OBr$: C 62.14%, H 6.84%, Br 25.84%

The fraction *R* showed absorption spectra similar to those of the previously described fraction *P*. Its acetate had S.V. 163.8 (Calcd. for $C_{18}H_{23}O_2Br$, 159.7) and I.V. 0.7. The absorption spectra for this acetate accorded with those for the acetate of the fraction *P*.

Summary

The unsaponifiable components other than sterol in the oil from the sea-hare *Aplysia kurodai* were examined. The non-sterol fraction constituted a major part of the total unsaponifiable matter and was found to contain a remarkable amount of bromine-containing substances, from which a bromophenol derivative, $C_{16}H_{21}OBr$, of m.p. 69° – $71^{\circ}C$ and a crystalline solid of m.p. 146.5° – $147.5^{\circ}C$ were separated.

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