SEPARATION OF OCTADECADIENOIC AND OCTADECATRIENOIC ACIDS IN THE OIL OF *CYPRINUS AURATUS*

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The oil from a variety of the fish *Cyprinus auratus* caught in the Lake Biwa was studied by Tsujimoto.[1] The highly unsaturated acids of this oil was found by Tsujimoto to contain docosapentaenoic acid (clupanodonic acid) together with C_{20}- and C_{18}-acids among which eicosatetraenoic acid and possibly also trienoic acid yielding hexabromide appear to be present. The same author[2][3] also pointed out that the ether-insoluble bromide from the fresh water fish oil fatty acids, as compared with that from the marine fish oil fatty acids, has in most cases a lower bromine content. However, he also noted that the fatty acids of an oil from the carp fed on chrysalis yielded an ether-insoluble bromide of Br-content 63.77% approximating the theoretical value for hexabromostearic acid whereas the fatty acids of an oil from the carp fed on sardine yielded an ether-insoluble bromide of Br-content 69.90% approximating that of the ether-insoluble bromides usually obtainable from the fatty acids of marine fish oils. It would appear, therefore, that the fatty acid composition of the oil of bred carps is much influenced by diets on which the fish are fed. Kafuku and Hata[4] indicated the occurrence of linoleic and linolenic acids in the oil of the Formosan fresh water bred fish, *Chanos chanos*, by separating these acids as their bromides from the mixed fatty acids. Takagi and Toyama[5] demonstrated the presence of linoleic acid in the oils from two species of fresh water crustaceans, *Palaemon nipponensis* and *Cambarus clarkii*, by separating a bromide fraction which was found to consist substantially of tetrabromostearic acid obtainable from linoleic acid. On the other hand, the occurrence of linoleic and linolenic acids in some marine fish oils has frequently been stated in the literature, but it appears mostly lacking in confirmation. An octadecatrienoic acid[6][7] is surely present in sardine oil, but this acid is reported to be an isomer of linolenic acid, 6, 10, 14-octadecatrienoic acid.[8][9]

In this study, oils were extracted separately from viscera, roes and the body after removal of viscera and roes of the fish *Cyprinus auratus*, and characteristics of the oils were determined. The fatty acids from the viscera oil and those from the roe oil were fractionated by the urea adduct segregation to obtain fractions relatively rich in diethenoid and triethenoid acids. Two fractions thus obtained (the fraction 3 in Table 2 and the fraction 2 in Table 3) were united and converted to methyl ester, and the latter was fractionally distilled. The methyl ester fractions corresponding to a methyl ester of C_{18}-acid (the fractions 5 and 6 in Table 4) were united, and the united material was fractionated further by elution chromatography on silica gel to give eventually an octadecadienoate fraction and an octadecatrienoate fraction. Ultraviolet absorption curves for these two fractions

after alkali-isomerization were found very close to the corresponding curves for methyl linoleate and methyl linolenate, respectively. The fatty acids of an eluate fraction adjacent to the octadecadienoate fraction yielded on bromination a bromide which was recognized as tetrabromostearic acid obtainable from linoleic acid, while the fatty acids from an eluate fraction adjacent to the octadecatrienoate fraction yielded an bromide which was considered to consist substantially of hexabromostearic acid obtainable from linolenic acid.

The octadecadienoate and octadecatrienoate fractions were subjected to ozonolysis. The volatile aldehydes formed by ozonolysis were separated as their DNPHs (2, 4-dinitrophenylhydrazones). The DNPH fraction of m.p. 103°–104°C from the octadecadienoate fraction was found to consist substantially of hexanal DNPH. The octadecatrienoate fraction gave a DNPH fraction of m.p. 142°–144°C which, in comparison with acetaldehyde DNPH (m.p. 160°–161°C),[10] propionaldehyde DNHP (m.p. 154°–155°C)[10] and butyraldehyde DNPH (m.p. 120°–121°C),[10] was relatively close to propionaldehyde DNPH in its melting point. It agreed with propionaldehyde DNPH in its elementary analysis and was judged as unpurified propionaldehyde DNPH. Carbon dioxide was formed by the ozonolysis of both octadecadienoate and octadecatrienoate fractions, suggesting the occurrence of the group $=CHCH_2CH=$ in both octadecadienoic and octadecatrienoic acids. The non-volatile compounds formed by ozonolysis were oxidized with potassium permanganate, and the oxidation product (monomethyl ester of dibasic acid) was saponified and acidified to give free dibasic acid. Azelaic acid was identified in the oxidation product from both octadecadienoate and octadecatrienoate fractions. From these results, the octadecadienoic acid is found to have the groups $CH_3(CH_2)_4CH=$, $=CHCH_2CH=$ and $=CH(CH_2)_7COOH$, while the octadecatrienoic acid is found to have the groups $CH_3CH_2CH=$, two of $=CHCH_2CH=$, and $=CH(CH_2)_7COOH$. It is thus indicated that the octadecadienoic and octadecatrienoic acids in the oil of *Cyprinus auratus* are identical with linoleic (9, 12-octadecadienoic) and linolenic (9, 12, 15-octadecatrienoic) acids, respectively. It is worthy of a particular note that the octadecatrienoic acid in the oil of *Cyprinus auratus* is identical with linolenic acid whereas the octadecatrienoic acid in sardine oil is reported to be 6, 10, 14-octadecatrienoic acid.

Experimental

1. Properties of the body, viscera and roe oils

The fish used in this study are a variety of the species *Cyprinus auratus* Linne, locally called "nigoro". They were caught in the Lake Biwa in June, 1954. Ninety six fishes of an average body length of 35 cm. weighed 55.6 kg. in total and were all full-roed female. Viscera and roes were separated from the fish, and oils were extracted separately from the remainder of fish body, viscera and roes by the following procedures. The fish body after removal of viscera and roes was cut into small pieces, and an equal amount of hot water was added, and the material was heated at the boiling point of water for 15 min. The liquor was then separated by decantation, and the residue was again boiled with water for 15 min. The liquors obtained by two boiling were united and allowed to settle until a creamy layer separated at the upper part. The oil contained in this creamy layer was separated by extraction with ether followed by removal of ether from the ether solution. The viscera were heated in an open pan so as to remove the bulk of

water by evaporation, and the viscera oil was then extracted by soaking the material in ether. The roes were first heated in an open pan until water was removed to some extent by evaporation and then dried in an oven at 80°C. The roe oil was extracted by soaking the dried material in ether. The oils obtained from the viscera and roes were refluxed with ten times weight of acetone for a while and the mixtures were allowed to cool at room temperature. The acetone-insoluble matter (non-fat lipid) was removed, and the fatty oils were obtained after removal of acetone from the acetone solutions. Yields and properties of the oils obtained from the fish body after removal of viscera and roes, viscera and roes are shown in Table 1.

TABLE 1. Yields and Properties of Body, Viscera and Roe Oils from *Cyprinus auratus*

	Body oil	Viscera oil	Roe oil
Weight of material (kg.)	43.6	1.56	10.2
Ether-extraction oil (g.)	1,412	369	302
Ether-extraction oil (%)	3.2	23.7	3.0
Acetone-soluble oil (g.)	—	359	269
Acetone-soluble oil (%)	—	23.1	2.5
d_4^{20}	0.9242	0.9238	0.9462*
n_D^{20}	1.4774	1.4766	1.4750**
Acid value	0.36	4.14	4.25
Saponification value	189.5	186.6	169.6
Iodine value (Wijs)	161.3	154.2	145.1
Unsaponifiable matter (%)	0.42	1.67	9.25
Ether-insoluble bromide from the fatty acids (%)	41.4	39.5	32.8
M.p. (°C) of ether-insol. bromide	215	227	214

Notes: Body oil and viscera oil were a clear liquid at 20°C. Roe oil deposited a considerable amount of solids at 20°C. * d_4^{30}; ** n_D^{30}

2. Fractionation of the fatty acids from the viscera and roe oils

The fatty acids from the viscera oil (acetone-soluble oil) had d_4^{30} 0.8998, n_D^{30} 1.4695, neutralization value 197.7 and iodine value 162.3. A saturated methyl ester fraction of I.V. 2.4 was separated from the methyl ester of mixed fatty acids in a yield of 22.8% by the permanganate oxidation in acetone. The fatty acids were subjected to urea fractionation. One *l.* of methanol saturated with urea at 60°C was added to 300 g. of fatty acids. The mixture was heated for a while at a temperature above 60°C and allowed to cool at room temperature. The crystalline urea adduct (I) formed was filtered, giving the filtrate (II). The crystalline urea adduct (I) was dissolved in hot methanol and the solution was cooled. The crystalline urea adduct formed thereby was filtered. The filtrate (II) was concentrated. Addition of a further quantity of urea to the concentrated filtrate formed a crystalline urea adduct which was separated by filtration. Such fractionation procedures were repeated nineteen times in total, and some of the fractions obtained were united to give eventually five fractions. Yields and properties of the free fatty acids from these five fractions are given in Table 2.

The fatty acids of the roe oil (acetone-soluble oil) had d_4^{30} 0.8990, n_D^{30} 1.4678, N.V. 196.0 and I.V. 156.1. A saturated methyl ester fraction of I.V. 0.7 was separated in a yield of 20.2% by the permanganate oxidation in acetone. The fatty acids (242

g.) were fractionated by way of urea adduct in a similar way as described for the fatty acids of viscera oil. After repeating the urea fractionation procedures sixteen times in total, four fractions of fatty acids given in Table 3 were eventually obtained.

Among the fractions in Tables 2 and 3, those having an iodine value comparable with the iodine value of dienoic or trienoic acid show a neutralization value which is more or less lower than the neutralization value of C_{18}-acid. The fraction 3 in Table 2 and the fraction 2 in Table 3 were united. The united fraction was converted to methyl ester, and the latter (78 g.) was fractionally distilled with the results shown in Table 4.

TABLE 2. Urea Fractionation of the Viscera Oil Fatty Acids

Fraction	Yield (g.)	Neutralization value	Iodine value
1	95	184.5	288.8
2	37	190.8	226.1
3	44	196.5	188.4
4	70	199.7	92.5
5	84	208.0	26.8

TABLE 3. Urea Fractionation of the Roe Oil Fatty Acids

Fraction	Yield (g.)	Neutralization value	Iodine value
1	90	186.1	265.0
2	30	196.4	175.2
3	74	198.5	108.2
4	48	211.0	7.3

TABLE 4. Fractional Distillation of the Methyl Ester

Fraction	B.p. (°C/4 mm Hg)	Yield (g.)	n_D^{20}	Saponif. value	Iodine value
1	-173	5.5	1.4503	209.0	99.2
2	173–179	6.8	1.4533	206.6	121.5
3	179–183	10.3	1.4542	200.6	137.1
4	183–186	10.7	1.4578	196.8	157.6
5	186–191	7.4	1.4602	192.8	164.5
6	191–195	9.0	1.4613	191.3	169.2
7	195–200	5.1	1.4631	187.6	185.1
8	200–205	6.3	1.4702	183.2	242.5
9	205-	6.9	1.4769	181.4	292.2
Residue	—	5.0	—	168.0	181.0

The fractions 5 and 6 in Table 4 have saponification values which are in the neighbourhood of the saponification values for methyl esters of C_{18}-acids. These fractions were united, and the united material (16.0 g.) was chromatographed on silica gel using hexane in the early stage of elution and hexane containing 0.5–1% ether in the later stage as eluants. Twenty two eluate fractions were obtained. Refractive indices of each eluate fraction and also iodine values and ultraviolet absorption values after alkali-isomerization for some eluate fractions were determined. The 1st eluate fraction had n_D^{20} 1.4465 and I.V. 94.1 while the 22nd fraction had n_D^{20} 1.4737 and I.V. 295.0. The refractive indices and iodine values of the eluate

fraction were lower than the corresponding values of the next succeeding fraction. In the following, a united fraction (*A*) from the 4th to 8th fractions (n_D^{20} 1.4546–1.4573) and a united fraction (*B*) from the 11th to 18th fractions (n_D^{20} 1.4640–1.4723) were used for the separation of methyl octadecadienoate and methyl octadecatrienoate, respectively.

3. Separation of methyl octadecadienoate

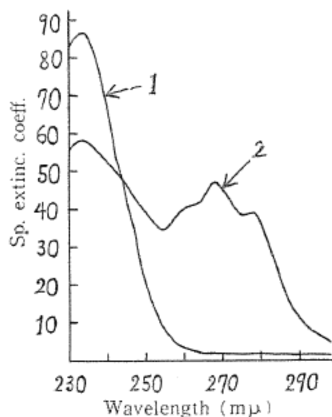
The eluate fraction (*A*), 3.8 g., was again chromatographed on silica gel, and fractionated into seventeen eluate fractions of n_D^{20} 1.4472–1.4685.

The fatty acids from the 7th and 8th fractions and the fatty acids from 15th and 16th fractions were hydrogenated. Both hydrogenation products melted at 68°–69°C and showed no depression of melting point when mixed with a pure specimen (m.p. 70°C) of stearic acid.

The fatty acids (0.6 g.) from the 9th to 11th fractions were brominated in hexane, and the crystalline bromide insoluble in cold hexane was separated. The bromide had m.p. 112°–113°C and showed no depression of melting point on admixture with a pure specimen (m.p. 114°C) of tetrabromostearic acid prepared from linoleic acid.

The 12th to 14th fractions all had n_D^{20} 1.4610. The united material of these fractions had S.V. 190.1 and I.V. 170.8 (Calcd. for $C_{18}H_{31}O_2CH_3$, S.V. 190.5 and I.V. 172.4). The ultraviolet absorption curve (Fig. 1) of this material after the isomerization under the condition of 6.5% KOH-ethylene glycol, 180°C and 45 min. exhibited the characteristic maximum, $k_{233}=86.9$ based on the methyl ester or $k_{233}=91.2$ based on the free acid, which is very close to the reported value,[11] $k_{233}=93.5$, for alkali-isomerized linoleic acid.

Ozonolysis. The united material (0.15 g.) of the 12th to 14th fractions was dissolved in 20 cc. of chloroform, and a current of ozonized oxygen was passed into the solution under cooling with ice. After the completion of ozonization, chloroform was distilled off under vacuum. The ozonide was decomposed with 40 cc. of water by heating for 30 min. on a water bath while a current of hydrogen was passed into the mixture. The volatile compounds formed during the decomposition of ozonide were passed along with the hydrogen current successively through a solution of 2, 4-dinitrophenylhydrazine in 2 N-hydrochloric acid and a barium hydroxide solution. The latter became turbid indicating the formation of carbon dioxide by the ozonolysis. Yellowish orange precipitate formed in the 2, 4-dinitrophenylhydrazine solution was separated by filtration and dried under vacuum. Yield 105 mg. Recrystallization of this precipitate from



Curve 1. Methyl octadecadienoate fraction

Curve 2. Methyl octadecatrienoate fraction

FIG. 1. Ultraviolet absorption curves of the methyl octadecadienoate and methyl octadecatrienoate fractions after alkali-isomerization.

hexane gave yellowish orange needles of m.p. 103°–104°C. The ultraviolet absorption spectrum in ethanol exhibited an absorption maximum at 360 mμ and an absorption minimum at 293 mμ.

Anal. Found: C, 51.15%; H, 5.80%.

Calcd. for $C_{12}H_{16}N_4O_4$: C, 51.42%; H, 5.78%.

Non-volatile substances formed by ozonolysis were extracted with ether, and the ether-extract was oxidized with an alkaline solution of potassium permanganate. The oxidation product (monomethyl ester of dibasic acid) obtained by acidification of the solution followed by extraction with ether was converted to free dibasic acid by saponification followed by acidification. The dibasic acid fraction thus obtained was treated with hexane for the removal of hexane-soluble matter. The hexane-insoluble crystalline fraction (40 mg.) was separated and recrystallized from ether-hexane, giving crystals of m.p. 103°–104°C and N.V. 599.4 (Calcd. for $C_9H_{16}O_4$, 596.2). The melting point was not lowered when mixed with a specimen (m.p. 106°C) of azelaic acid.

4. Separation of octadecatrienoic acid

The eluate fraction (*B*), 2.4 g., was fractionated further by chromatography on silica gel, giving twenty eluate fractions of n_D^{20} 1.4518–1.4720.

A portion of the fatty acids (0.5 g.) from the 10th to 14th fractions was brominated in ether, and an ether-insoluble bromide of m.p. 175°–176°C was obtained. The melting point of this bromide was not lowered on admixture with various proportions of a pure specimen (m.p. 180°C) of hexabromostearic acid prepared from linolenic acid. Another portion of the same fatty acids was hydrogenated. The product after recrystallization from ethanol melted at 67°–68°C and showed no depression of melting point on admixture of a pure specimen of stearic acid.

The 15th to 19th eluate fractions had n_D^{20} 1.4705-1.4715, suggesting that these fractions consist substantially of the same component. The united material (0.25 g.) of these fractions had S.V. 191.0 and I.V. 261.6 (Calcd. for $C_{18}H_{29}O_2CH_3$, S.V. 191.9 and I.V. 260.4). The ultraviolet absorption curve (Fig. 1) of this material after alkali-isomerization exhibited the characteristic maxima, $k_{233}=57.0$ and $k_{268}=46.3$ on the basis of methyl ester or $k_{233}=59.9$ and $k_{268}=48.6$ on the basis of free acid, which are very close to the reported values, $k_{233}=59.8$ and $k_{268}=49.3$,[11] for alkali-isomerized linolenic acid.

Ozonolysis. The united material (0.12 g.) of the 15th to 19th fractions was subjected to ozonolysis in a similar way as described for the octadecadienoate fraction. Carbon dioxide was formed by ozonolysis. 2,4-Dinitrophenylhydrazones obtained from volatile carbonyl compounds melted at 142°–144°C after recryatallization from hexane. The absorption spectrum of the recrystallized material in methanol exhibited an absorption maximum at 359 mμ and an absorption minimum at 293 mμ.

Anal. Found: C, 44.68%; H, 4.22%.

Calcd. for $C_9H_{10}N_4O_4$: C, 45.38%; H, 4.23%.

The non-volatile product of ozonolysis gave, after similar treatments as described in the case of octadecadienoate fraction, 52 mg. of crystalline dibasic acid which had m.p. 102°–103°C and N.V. 589.6 after recrystallization from ether-hexane. The melting point was not lowered on admixture of azelaic acid.

Summary

Properties of the oils extracted separately from viscera, roes and the remainder of the body of the fresh water fish, *Cyprinus auratus*, were examined. The fatty acids of viscera and roe oils were fractionated by way of urea adduct, giving fractions relatively rich in dienoic and trienoic acids. The fractions from the respective oils were united, and the united fraction was fractionated further by fractional distillation of the methyl ester followed by elution chromatography of the fraction consisting substantially of methyl esters of C_{18}-acids on silica gel. Two methyl ester fractions were eventually separated, the fatty acid of which were found to consist substantially of octadecadienoic and octadecatrienoic acids, respectively. The structures of these two acids were identified with those of linoleic (9,12-octadecadienoic) and linolenic (9,12,15-octadecatrienoic) acids, respectively, by ozonolysis.

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