by treating with Amberlite IR-120 (H^+). The aqueous acid solution thus obtained was passed through Amberlite IR-4B (OH^-) column (1×26 cm.) and the column was washed with water. The phosphate was eluted from the column with 0.5*N* NaOH solution. From the fractions which showed a positive phosphorus test, Na ion was again removed with Amberlite IR-120 (H^+). $Ba(OH)_2$ was added to the aqueous acid solution up to pH 10.0, and after removal of excess $Ba(OH)_2$ with CO_2, the aqueous solution was lyophilized to give Ba salt of (IV). The product was reprecipitated from MeOH three times with acetone. The sample for analysis was dried to constant weight over P_2O_5 *in vacuo* at 120°. *Anal.* Calcd. for $(C_4H_{10}O_6P)_2Ba$: C, 18.92; H, 3.74; P, 12.22. Found : C, 18.64; H, 3.53; P, 12.23. Rf_1 0.55, Rf_2 0.59.

Bis(2-hydroxypropyl) Phosphate (V)—1.7 g.(1 mole) of 2-hydroxypropyl potassium hydrogen phosphate, 1.2 cc. (2 moles) of propylene oxide, and 30 cc. of water were reacted similarly as in the case of (II). After removal of water and propylene glycol, the reaction mixture was converted into free acid by treatment with Amberlite IR-120 (H^+) and lyophilized. The cation-free product thus obtained was dissolved in 50 cc. of dehyd. *iso*-PrOH and saturated with NH_3. The mixture was centrifuged to remove insoluble ammonium 2-hydroxypropyl phosphate (630 mg.). The residue obtained on evaporation of the supernatant was dissolved in 20 cc. of water and passed through a column of Amberlite IR-120 (H^+) to remove cation. The effluent was added with cyclohexylamine to pH 9~10 and lyophilized. On keeping with trace of dry acetone, the residue solidified into crystals (0.9 g.). Recrystallization was performed with aqueous acetone ($H_2O:Me_2CO=1:9$). The sample for analysis was dried to constant weight over P_2O_5 *in vacuo* at 75°. *Anal.* Calcd. for $C_{12}H_{28}O_6NP$: C, 46.00; H, 9.01; N, 4.47; P, 9.89. Found : C, 45.63; H, 8.90; N, 4.69; P, 10.22. Rf_1 0.74, Rf_2 0.81.

Summary

Three methyl 2-hydroxyalkyl phosphates, methyl 2-hydroxyethyl phosphate (I), methyl 2-hydroxypropyl phosphate (II), and methyl 1-methyl-2-hydroxypropyl phosphate (III), and two bis(2-hydroxyalkyl) phosphates, bis(2-hydroxyethyl) phosphate (IV), and bis-(2-hydroxypropyl) phosphate (V), were synthesized. The Rf values of the products were given.

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38. **Takaaki Aoyagi*** : Studies on the Lipid of *Pelteobagrus nudiceps*. I. The Isolation of a Substance Stimulating the Repair of Tuberculosis Foci.

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Shoyama, *et al.* found that *Pelteobagrus nudiceps*, heat-dried and powdered, could stimulate the repair of tuberculosis foci in various organs of guinea pig as well as in human lung. They[1] identified the substance responsible for this activity as a lipid and also found that lipid of eel (*Anguilla japonica*) had no such activity. This paper is to report which fraction of this lipid is responsible for this activity. Results of pathological examinations carried out concurrently with this separation were reported elsewhere.[2,3] Very few data, so far, are available on the chemical constituents of *Pelteobagrus nudiceps*. Kamijo[4] described the general properties of ether-soluble substances of *Pelteobagrus fulvidraco* and *P. vacheli*.

* This work was carried out while the author maintained the scholarship given by Kamakura Institute of Tuberculosis.

** Chojamaru, Kamiohsaki, Shinagawa-ku, Tokyo (青柳高明).

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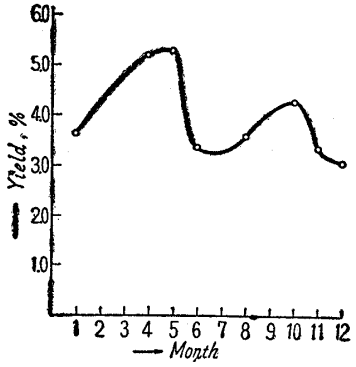

Fig. 1.
Seasonal Variation in the Yield of lipid from *Pelteobagrus nudiceps*

Pelteobagrus nudiceps (6250 g.)
extd. with ether
(B) Soln. (343 g.) N.E.=5.9 I.N.=93.7 acetone
Residue
(C) Sol. (336 g.)(97.6%) N.E.=6.5 I.N.=95.2 saponification ether
Precipitate (D) (6.5 g.) N.E.=0.0 I.N.=0.0
(E) Sol. (16.0 g.)(4.7%)
Cholesterol $C_{27}H_{46}O$
Mother liquor
acidified with HCl, ether
Sol. (296.0 g.)(86.3%) N.E.=175.9 I.N.=97.5
lead salt-EtOH
Aq. layer
concd.
extd. with acetone
Glycerol $C_3H_8O_3$ (30.4 g.)(8.9%)
(F) Solid (102 g.)(29.7%) N.E.=216.5 esterified with CH_2N_2
rectification
Liquid (G) (192 g.)(56.0%) I.N.=138.8
Li salt-acetone
Palmitic acid $C_{16}H_{32}O_2$
Stearic acid $C_{18}H_{36}O_2$
Cerotic acid $C_{26}H_{52}O_2$
Sol. (64.5 g.)(18.8%) I.N.=197.3
Insol. (I) (120.5 g.)(35.1%) I.N.=92.8 esterified with CH_2N_2
Li salt-acetone
rectification
(H) Sol. (60.7 g.)(17.7%) I.N.=214.4 esterified with CH_2N_2
Insol.
Hexadecenoic acid $C_{16}H_{30}O_2$
Octadecenoic acid $C_{18}H_{34}O_2$
rectification
Docosatetraënoic acid $C_{22}H_{36}O_2$

All percentages of yield calculated on the basis of total lipid.
N.E.=Neutralization equivalent
I.N.=Iodine number

Chart I. Separation of Lipid from *Pelteobagrus nudiceps*

The present material was obtained during May from a lake in the Kansai region. The ether-soluble lipid content of this fish varies according to the season as shown in Fig. 1.

The fish was dried in cool, cut finely, extracted with ether, and fractionated as shown in Chart 1. Unless otherwise stated, acids isolated were subjected to identification by chemical analysis of their anilide, amide, and *p*-bromophenacyl ester and, in addition, by examination of their physical characteristics.[5~7]

The fraction (D) in Chart 1, when further treated repeatedly with ether and acetone, contained no nitrogen or phosphorus. In view of the small yield of this fraction and lack of activity in it, no further details of the analysis will be given. The fraction (E), Liebermann-positive substance,[8] recrystallized from ethanol and benzene to white leaflets, which was identified as cholesterol. After the removal of (E) with ether, the aqueous layer was acidified, extracted with ether, and the fatty acid mixture obtained was converted to lead salts. The water-soluble fraction gave glycerol which was identified as its tribenzoate. Lead salt of acids were divided into ethanol-soluble (G) and -insoluble (F) fractions. Ethanol-insoluble lead salt of fatty acids (F) contained $C_{16}H_{32}O_2$, $C_{18}H_{36}O_2$, and $C_{26}H_{52}O_2$. The first two were identified as palmitic and stearic acids, respectively, and from its analytical data, the last one was assumed to be cerotic acid, showing depression in melting point when mixed with C_{20}-, C_{22}-, and C_{24}-acids. The ethanol-soluble lead salt (G) of liquid acids was subjected to lithium salt-acetone method. The fraction (H), acetone-soluble fraction of lithium salt of liquid fatty acids, was esterified with diazomethane and rectified repeatedly until each fraction gave constant iodine number.[9]

The fraction of $b.p_{0.05}$ 157~158° was obtained in the highest yield and gave the highest iodine number. This was brominated[10,11] to give an octabromide and catalytic hydrogenation with PtO_2 gave a saturated acid, m.p. 49~50°, which was saponified to give white crystals, $C_{22}H_{44}O_2$, m.p. 77~78°, and identified as behenic acid. The major composition of the original acid with the highest iodine number was thus proved to be docosatetraënoic acid, $C_{22}H_{36}O_2$. It can not be denied that this acid is not contaminated with a small amount of C_{22}-acid with different number of double bonds, because of its instability and of the method used for purification, but, at least, this tetraënoic acid is presumed to be the major component. This kind of tetraënoic C_{22}-acids were isolated by chromatographic separation from organs of cattle by Klenk[12] and also this acid has been reported to occur in alligator oil, badger fat, ox liver fat, and algae oil, and proposed to be present in sardine oil by Toyama and Tsuchiya,[13] and by Ki.[14]

The fraction (I), the acetone-insoluble fraction of lithium salts, was esterified with diazomethane and rectified similarly as (H). Two acids were isolated. The acid with low boiling point, $b.p_{0.04}$ 151~155°, obtained in a small amount was identified as hexadecenoic acid, $C_{16}H_{30}O_2$. The second acid, $b.p_{0.01}$ 151~154°, was identified as octadecenoic acid, $C_{18}H_{34}O_2$.

In view of the results obtained above, it was found that the lipid of *Pelteobagrus nudiceps* contained cholesterol as a nonsaponifiable substance, palmitic, stearic, and

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cerotic acid as saturated acids, and hexadecenoic, octadecenoic, and docosatetraënoic acids as unsaturated acids. Most of these acids are found as a glyceryl ester. While the distribution of docosatetraënoic acid in nature has been reported in marine fish,[13,14] this acid was isolated for the first time from the component of fresh water fish. Pathological examinations carried out concurrently with the present fractionation showed that the active substance responsible for the repair of tuberculous foci was found in (B), (C), (G), and (H). Antituberculous activity against $H_{37}Rv$ of the (H) fration was 5 γ/cc. by the conventional technique.

Therefore, at the present stage, docosatetraënoic acid is assumed to be the main component which is responsible for biological activity. Results of detailed examinations on the structure of the docosatetraënoic acid will be reported in the following paper.

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Experimental

Fractionation of the Lipid from *Pelteobagrus nudiceps*—*Pelteobagrus nudiceps* collected in May from a lake in Kansai region was dried thoroughly in cool, cut finely, and repeatedly extracted with ether for 200 hrs., yielding 343 g.(neutral. equivalent; 5.9, iodine number, 93.7) of yellow oil. 3500 cc. of acetone was added to this oil, allowed to stand at room temperature over night, by which insoluble white yellow solid separated. This solid was washed with cold acetone, yielding 6.5 g.(neutral. equivalent, 0.0; iodine No. 0.0,) of acetone-insoluble substance (D) N (Kjeldahl) $<0.05\%$, P (Allen) $<0.1\%$.

Acetone-soluble fraction (C) (neutral. equivalent, 6.5; iodine No. 95.2) was saponified with 1.5 *N* ethanolic KOH (3000 cc.) and the non-saponifiable material was removed by ether, yielding 16.0 g. (E) of white crystals. The saponified solution was acidified with HCl and extracted with ether, yielding 296 g.(neutral. equivalent, 175.9, iodine No. 97.5) of soft brown solid. Mother liquor was concentrated on a water bath and extracted with acetone, yielding 30.4 g. of brown syrup, which showed positive acrolein test. This syrup was converted into a benzoate to give a crystalline product which was recrystallized from EtOH and ligroine to needles, m.p. 72~73°. No depression of the melting point was observed when mixed with the authentic sample of glyceryl tribenzoat. *Anal.* Calcd. for $C_{24}H_{20}O_6$: C, 71.29; H, 4.95. Found: C, 71.22; H, 4.72.

Non-saponifiable fraction (E) which showed positive Liebermann reaction, was recrystallized from EtOH and benzene to white leaflets, m.p. 147°; $[\alpha]_D^{18}$ −37.14. Acetate: Needle crystals, m.p. 112.5~113.5°. *Anal.* Calcd. for $C_{29}H_{48}O_2$ (Cholesteryl acetate): C, 81.25; H, 11.29. Found: C, 81.40; H, 11.21. Cholesteryl acetate dibromide, m.p. 116~117°. *Anal.* Calcd for $C_{29}H_{47}O_2Br_2$: Br, 27.26. Found: Br, 27.19.

Oppenauer oxidation gave cholestenone as white needles, m.p. 79~79.5°. *Anal.* Calcd. for $C_{27}H_{44}O$: C, 84.31; H, 11.53. Found: C, 84.17; H, 11.32.

No depressions of the melting points were observed when these pure samples were mixed with the corresponding authentic samples.

Separation of Solid and Liquid Fatty Acids by Lead Salt-Alcohol Method—Crude fatty acids obtained by the above saponification were treated by lead salt-alcohol method in the following manner. A mixture of crude fatty acids (296 g.) was dissolved in 1500 cc. of hot EtOH. The solution was added with ethanolic solution of 185 g. of $Pb(AcO)_2$, allowed to stand at room temperature over night, and the precipitate was filtered off. The insoluble lead salts and the lead salts remaining in the filtrate were decomposed with dil. HNO_3 to give the corresponding free fatty acids which mainly

Table I.

Fraction number	b.$p_{0.5}$(°C)	Yield (g.)	Neutral. enquiv.	m.p. of free acid (°C)
a	~100	0.6	217.1	59~61
b	101~130	3.0	220.7	61~61.5
c	131~137	57.5	222.3	61~61.5
d	138~148	2.7	212.5	55~58
e	149~156	22.5	198.5	67.5~68.5
f	157~167	1.0	200.4	65~67
g	168~	1.1	145.9	74.5~76
h	residue	12.5		

consisted of saturated fatty acids (F) (102 g., neutral. equivalent, 216.5) and mainly unsaturated fatty acids (G) (192 g., iodine No., 138.8), respectively.

The solid acid (F) was esterified with CH_2N_2, subjected to rectification, and divided into 8 fractions as shown in Table I.

Fractions (a), (b), and (c) showed the same neutralization equivalent and melting point, and saponification of these fractions yielded white scales which were recrystallized from a mixture of MeOH and acetone (3:1), m.p. 61~61.5. *Anal.* Calcd. for $C_{16}H_{32}O_2$ (Palmitic acid): C, 74.94; H, 12.58; COOH, 17.56. Found: C, 75.17; H, 12.57; COOH, 17.49. *p*-Bromophenacyl ester, m.p. 84.5~85.5°. *Anal.* Calcd. for $C_{24}H_{37}O_3Br$: C, 63.58; H, 8.16; Br, 17.64. Found: C, 63.72; H, 8.02; Br, 17.83.

No depression of the melting point was observed on admixture of the acid and its derivatives with palmitic acid and its corresponding derivatives.

Fraction (e) after saponification gave white leaflets, m.p. 68~69°. *Anal.* Calcd. for $C_{18}H_{36}O_2$ (Stearic acid): C, 75.99; H, 12.76; COOH, 15.84. Found: C, 75.74; H, 12.85; COOH, 16.14. Anilide, m.p. 93~94°. *Anal.* Calcd. for $C_{24}H_{41}ON$: C, 80.15; H, 11.49; N, 3.90. Found: C, 80.24; H, 11.20; N, 3.83. No depression of the melting point was observed on admixture of the acid and its derivative with stearic acid and its corresponding derivative.

Fraction (g) after saponification gave white crystalline powder, m.p. 74.5~76°. *Anal.* Calcd. for $C_{26}H_{52}O_2$: C, 78.72; H, 13.21; COOH, 11.36. Found: C, 78.68; H, 13.10; COOH, 11.44.

This acid showed depression of the melting point on admixture with authentic samples of C_{20} arachidic acid, C_{22} behenic acid, and C_{24} lignoceric acid. Cerotic acid is the most probable from its analytical data.

Separation of Liquid Fatty Acids by Lithium Salt-Acetone Method—The mixture of liquid fatty acids (G) isolated as above was dissolved in 5000 cc. of acetone and neutralised with LiOH. After the complete precipitation of the lithium salts, it was dissolved by warming the vessel on a water bath and the solution was allowed to cool to reprecipitate the sparingly soluble lithium salts. The insoluble lithium salts were collected and acidified with HCl to give the free fatty acids (I) (120.5 g.). The fatty acids remaining in the filtrate as lithium salts were once recovered and dissolved again in acetone, partially neutralized with LiOH as before, and divided into soluble fraction and precipitate. The precipitate was added to (I). Yellow oil (H) obtained from the filtrate weighed 60.7 g.

The acetone-insoluble fraction of Li salts (I) (120.5 g.) was esterified with CH_2N_2, subjected to rectification, and divided into 6 fractions as shown in Table II. The free acids liberated from the methyl ester were hydrogenated with PtO_2 in glacial AcOH and the products, saturated acids, were recrystallized.

Table II.

Fraction No.	b.$p_{0.05}$ (°C)	Yield (g.)	Iodine No. of unsatd. ester	H_2 (moles calcd. from unsatd. ester)	m.p. of satd. acid (°C)
II-1	~110	10.5	78.3	1.07 (methyl hexadecenoate)	57~60
II-2	111~116	6.0	73.4	1.1 (〃)	60~64
II-3	117~125	77.0	69.5	1.01 (methyl octadeconoate)	66~68
II-4	126~135	8.0	118.5	1.4 (〃)	65~70
II-5	136~	3.5	135.7	1.9 (〃)	67~72
II-6	residue	15.5			

The free acid liberated from the methyl ester fraction (II-1) in Table II was again subjected to redistillation and divided into 4 fractions as shown in Table III.

Table III.

b.$p_{0.04}$ (°C)	Yield (g.)	Iodine No. of unsatd. acid	H_2 (moles calcd. from hexadecenoic acid)	m.p. of satd. acid (°C)
~150	2.5	83.5	0.97	55~60
151~155	2.5	92.7	1.01	60~61
156~	1.5	79.8	0.91	63~65
Residue	3.5			

The second fraction was hydrogenated and recrystallized from a mixture of MeOH and acetone to white scales, m.p. 60~61°. *Anal.* Calcd. for $C_{16}H_{32}O_2$ (palmitic acid): C, 74.94; H, 12.58; COOH, 17.56. Found: C, 75.37; H, 12.31; COOH, 17.49. *p*-Bromophenacyl ester, m.p. 84~85°. *Anal.* Calcd. for $C_{24}H_{37}O_3Br$: C, 63.57; H, 8.16. Found: C, 63.99; H, 8.20.

No depression of the melting point was observed on admixture of pure samples with the corresponding authentic samples.

The free acid (m.p. 12~14°) liberated from the methyl ester fraction (II-3) was subjected to redistillation and divided into 5 fractions as shown in Table IV.

Table IV.

b.$p_{0.01}$ (°C)	Yield (g.)	Iodine No. of unsatd. acid	H_2 (moles calcd. from octadecenoic acid)	m.p. of satd. acid (°C)
~145	2.5	97.7	1.06	62~64
146~150	7.5	71.3	1.04	66~68
151~154	55.0	83.8	1.03	68~69
155~	3.5	75.6	1.03	67~68
Residue	8.5			

The 2nd and 3rd fractions which showed the same iodine number and melting points after hydrogenation were recrystallized to white leaflets, m.p. 68~69. *Anal.* Calcd. for $C_{18}H_{36}O_2$ (Stearic acid): C, 75.99; H, 12.76; COOH, 15.84. Found: C, 75.99; H, 12.65; COOH, 15.50. *p*-Bromophenacyl ester, m.p. 88~89°, and anilide, m.p. 93~93.5°. No depression of the melting point was observed when these pure samples were mixed with the corresponding authentic samples.

The free acids (H) (60.7 g.) recovered from acetone-soluble fractions of Li salts were esterified with CH_2N_2, subjected repeatedly to rectification in N_2 gas, and divided into 5 fractions as shown in Table V.

Table V.

Fraction number	b.$p_{0.2}$ (°C)	Yield (g.)	Iodine No. of unsatd. ester	H_2 (moles calcd. from methyl docosatetraënoate)
V-1	~150	2.5	128.4	2.7
V-2	151~156	7.7	237.4	3.85
V-3	157~170	40.5	247.4	3.95
V-4	171~	3.5	220.7	3.72
V-5	residue	7.5		

Fraction V-3 obtained in the highest yield and showing the activity responsible for the repair of tuberculosis foci was subjected to redistillation and divided into 6 fractions as shown in Table VI.

Table VI.

b.$p_{0.05}$ (°C)	Yield (°C)	Iodine No. of unsatd. ester	H_2 (moles calcd. from methyl docosatetraënoate)	m.p. of methyl ester	m.p. of free acid
~150	5.3	218.2	3.63	46~48	73~76
151~156	9.5	241.0	3.87	48~50	76~78
156~157	8.0	264.6	4.15	49~50	77.5~78.5
157~158	5.5	278.1	4.20	49~50	77.5~78.5
159~	4.2	260.0	4.08	49~50	77~78
Residue	8.0				

The first and the second fractions gave, after hydrogenation and saponification, a substance showing no depressions of the melting point when mixed with the authentic behenic acid. The third, fourth, and fifth fractions which showed the same iodine number and melting point after hydrogenation were recrystallized from benzene to white crystals, m.p. 77.5~78.5°. *Anal.* Calcd. for $C_{22}H_{44}O_2$ (Behenic acid): C, 77.58; H, 13.02; COOH, 13.24. Found: C, 77.50; H, 13.22; COOH, 13.28. *p*-Bromophenacyl ester, m.p. 95~96°. *Anal.* Calcd. for $C_{30}H_{49}O_3Br$: C, 67.04; H, 9.10; Br, 14.90. Found: C, 67.12; H, 8.84; Br, 14.93. Anilide, m.p. 97.5~98.5°. *Anal.* Calcd. for $C_{28}H_{49}ON$: C, 80.90; H, 11.88; N, 3.37. Found: C, 80.90; H, 11.34; N, 3.49. No depression of the melting point was observed on admixture of the acid and its derivatives with behenic acid and its corresponding derivatives.

The fraction of b.$p_{0.05}$ 157° was dissolved in ether and added with a bromine solution. The precipitate of bromide was allowed to stand in the cold, filtered, and recrystallized from EtOH and benzene to white crystals, m.p. 230°(decomp.). *Anal.* Calcd. for $C_{23}H_{38}O_2Br_8$: C, 27.99; H, 3.85; Br, 64.91. Found: C, 27.66; H, 3.55; Br, 63.98.

Summary

1) The lipid content of *Pelteobagrus nudiceps* varies depending on the season and the peak was observed in May and October.

2) The non-saponifiable fraction (E) of the lipid was found to be cholesterol.

3) The solid fatty acids were found to be composed of palmitic, stearic acid and

probably cerotic acid.

4) Acetone-soluble fraction obtained by lithium-acetone method was found to contain C_{22} tetraënoic acid (docosatetraënoic acid) which is responsible for the stimulation of the repair of tuberculous foci. The acetone-insoluble fraction of lithium salts contained hexadecenoic and octadecenoic acids.

5) Glycerol was isolated in the theoretical amount calculated on the basis of triglyceride of C_{20} fatty acid.

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39. Takaaki Aoyagi*: Studies on the Lipid of *Pelteobagrus nudiceps*. II.[1] The Structure of Octadecenoic Acid and Docosatetraënoic acid isolated from *Pelteobagrus nudiceps*.

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In the preceding paper[1] the active substance that stimulates the repair of tuberculosis foci in guinea pig was shown to be docosatetraënoic acid, $C_{22}H_{36}O_2$. The present paper deals with the chemical structure of this acid. The structure of octadecenoic acid, which occupied the major part of the fatty acids of *Pelteobagrus nudiceps*, was also determined.

Ozonolysis[2~5] was carried out in the determination of both acids. In order to avoid polymerization or the production of peroxide, ozone was saturated at a low temperature (at $-10°$) and to avoid polymerization of aldehyde in the oxidation products, oxidation with hydrogen peroxide after the decomposition of ozonide was also carried out at a low temperature.

Following the above-mentioned procedures, acids separated and isolated after the oxidation were identified as their anilide, amide, *p*-bromophenacyl ester, and by other physical properties.[6~8]

After ozonolysis of methyl octadecenoate and steam distillation of the products, pelargonic acid, $C_9H_{18}O_2$, was identified in the distillate. After saponification of the residue of steam distillation, azelaic acid, $C_9H_{16}O_4$, was isolated and identified. Therefore, for the octadecenoic acid obtained from the lipid of this fish a structure of 9-octadecenoic acid is proposed. This acid is assumed to be oleic acid rather than elaidic acid from its physical constants.

During the ozonolysis of methyl docosatetraënoate, carbon dioxide and acetaldehyde were found in the volatile fraction and after subsequent oxidation with peroxide, acetic acid and caproic acid were isolated from the distillate. The residue from the distilla-

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