THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL. IV. THE SEPARATION OF HIGHLY UNSATURATED C_{20}-ACIDS.

By Yoshiyuki TOYAMA and Tomotaro TSUCHIYA.

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In previous reports[1] of this series, the highly unsaturated C_{16}- and C_{18}-acids of sardine oil were studied with the results that two individual components, hiragonic acid $C_{16}H_{26}O_2$ and moroctic acid $C_{18}H_{28}O_2$, were isolated and their constitutions were established as follows:

(1) This Bulletin, **4** (1929), 83; *ibid.*, **10** (1935), 192, 232.

Hiragonic acid : $CH_3{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_4{\cdot}COOH$

Moroctic acid: $CH_3{\cdot}CH_2{\cdot}CH{=}CH{\cdot}CH_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}CH{=}CH{\cdot}(CH_2)_2{\cdot}COOH$

The present paper deals with the highly unsaturated C_{20}-acids in sardine oil. The occurrence of highly unsaturated C_{20}-acids, such as $C_{20}H_{32}O_2$ and $C_{20}H_{30}O_2$, in sardine oil has already been reported by several authors, among whom Majima and Okada,[2] Tsujimoto,[3] and Brown and Beal[4] may be mentioned. Furthermore, such highly unsaturated C_{20}-acids have been found to occur also in various kinds of marine animal oils besides the sardine oil. Thus, we[5] found the acids $C_{20}H_{32}O_2$ and $C_{20}H_{30}O_2$ in several kinds of whale oils (oils obtained from the whales belonging to *Mysticete*), in cod liver oil, in sperm body oil, in several kinds of liver oils from *Elasmobranch* fish, and also in pilot-whale head and body oils. Tsujimoto and his coworkers[6] reported the presence of highly unsaturated C_{20}-acids in giebel oil, herring roe oil, eel oil, the liver oil from *Chionecetes phalangium*, cod liver oil, the oil from *Atheresthes evermanni*, halibut liver oil, and in the liver oil from *Paralithodes camtschatica*. According to Ueno and Iwai,[7] menuke oil and the liver oil from *Scoliodon laticaudes* contain a highly unsaturated acid $C_{20}H_{32}O_2$. Suzuki and his coworkers,[8] examining the glycerides of marine animal oils, stated the occurrence of an acid $C_{20}H_{32}O_2$ as glycerides in whale oil, cod liver oil, herring oil, sardine oil, sand eel oil, cuttle-fish oil, red salmon oil, shark oil and in the liver oil from *Theragra chalcogramma*. A glyceride containing an acid component $C_{20}H_{30}O_2$ was stated by I. Okada[9] to occur in tunny oil, whereas an acid $C_{20}H_{32}O_2$ was considered by Tomiyama[10] to occur in tunny liver oil. Bull[11] stated the occurrence of the acids $C_{20}H_{30}O_2$ and $C_{20}H_{28}O_2$ in cod

(2) *J. Tokyo Chem. Soc.*, **35** (1914), 13.

(3) *J. Soc. Chem. Ind. Japan*, **26** (1923), 1013.

(4) *J. Am. Chem. Soc.*, **45** (1923), 1289.

(5) Toyama, *J. Soc. Chem. Ind. Japan*, **28** (1925), 95, 104; *ibid.*, **29** (1926), 531, 538, 624; *ibid.*, **30** (1927), 519; Toyama and Tsuchiya, *ibid.*, **30** (1927), 63, 116, 207; Toyama, *Report of the Tokyo Imperial Industrial Research Laboratory*, **27** (1932), No. 2.

(6) Tsujimoto, *Report of the Tokyo Imperial Industrial Research Laboratory*, **18** (1923), No. 2; Tsujimoto and Koyanagi, *ibid.*, **25** (1930), No. 4; Tsujimoto, *ibid.*, **26** (1931), No. 10; *ibid.*, **27** (1932), No. 15; Tsujimoto and Kimura, *J. Soc. Chem. Ind. Japan*, **26** (1923), 1162; Tsujimoto, *ibid.*, **30** (1927), 402; *ibid.*, **31** (1928), 136, 1191.

(7) *J. Soc. Chem. Ind. Japan*, **37** (1934), 121, 562.

(8) *Proc. Imp. Acad. Japan*, **5** (1929), 265, 269.

(9) *Journal of the Imperial Fisheries Institute*, **28** (1932), 105.

(10) *Bulletin of the Japanese Society of Scientific Fisheries*, **2** (1933), No. 1.

(11) *Tidskrift Kemi Farm. Terapi*, **14** (1917), 11.

liver oil. Brown and Beal[12] indicated the presence of two highly unsaturated C_{20}-acids, $C_{20}H_{32}O_2$ and $C_{20}H_{30}O_2$, in menhaden, cod liver, herring and salmon oils besides sardine oil. It is seen from the foregoing notes that the highly unsaturated C_{20}-acids, such as $C_{20}H_{32}O_2$ and $C_{20}H_{30}O_2$, occur widely in various kinds of marine animal oils, including sardine oil, but no individual acid has hitherto been isolated with certainty, and little has been known regarding the properties of these acids.

The above is a review of the most important works in literature on highly unsaturated C_{20}-acids in sardine and other marine animal oils. A survey of the literature shows that the highly unsaturated C_{20}-acids have also been found to occur in terrestrial animal oils. Thus Hartley[13] found an acid $C_{20}H_{32}O_2$ in the liver fats of pig and ox, which was termed afterward arachidonic acid by Lewkowitsch.[14] The presence of arachidonic acid in the fats from the organs of domestic animals has frequently been reported since then, but it appears not to have been isolated in a state of purity.

In the experiments described below, the highly unsaturated C_{20}-acids have been separated from sardine oil, and on a further separation into individual components, an eicosatetraenoic acid $C_{20}H_{32}O_2$ has been isolated in fairly pure state, and also a concentrated fraction of eicosapentenoic acid $C_{20}H_{30}O_2$ has been separated. Although the eicosatetraenoic acid in marine animal oils is described as arachidonic acid by some authors, it should not be assumed without experimental evidence that both acids are identical. In order to decide whether the eicosatetraenoic acid isolated from sardine oil, the constitution of which will be reported in a succeeding paper, is identical with arachidonic acid or not, it is necessary to gain a fuller information concerning arachidonic acid.

Experimental.

1. **Separation of Highly Unsaturated C_{20}-Acids.** As described in the second report,[15] 60 kg. of the ethyl esters, prepared by the ethanolysis of sardine oil, were subjected to distillation, and there was obtained 27 kg. of a fraction boiling over 215°/10 mm. Fifteen kg. of this fraction was used for this experiment. It was first

(12) *Loc. cit.*, (4).

(13) *J. Physiol.*, **38** (1909), 353.

(14) "Chemical Technology and Analysis of Oils, Fats and Waxes", 6th Edition, Vol. I, p. 215.

(15) This Bulletin, **10** (1935), 192.

treated by sodium-soap-acetone method,[16] and crude highly unsaturated acids having neutralisation value 179.3 and iodine value[17] 327.6 were obtained from the acetone-soluble sodium soaps. These were converted into methyl esters, and 8 kg. of the latter were subjected to fractional distillation with the results shown in Table 1.

During the distillation of the fraction (7), the temperature of bath was raised to about 260°, the pressure was reduced to 5 mm., and the distillation was continued till the rate of distillation became very slow. The large yield of the residue is probably caused by a partial polymerisation of methyl esters having high boiling point.

Table 1.

Fraction	B.p./10 mm.	Yield (g.)	n_D^{20}	Saponif. value	Iodine value
(1)	Below 210°	750	1.4700	189.0	245.7
(2)	210–215°	650	1.4766	181.4	275.8
(3)	215–220°	1020	1.4800	177.8	300.4
(4)	220–222°	900	1.4852	175.3	315.2
(5)	222–225°	850	1.4872	173.0	328.7
(6)	225–230°	600	1.4891	170.9	334.3
(7)	Over 230°	2050	1.4930	163.0	353.2
Residue and loss	—	1180	—	—	—

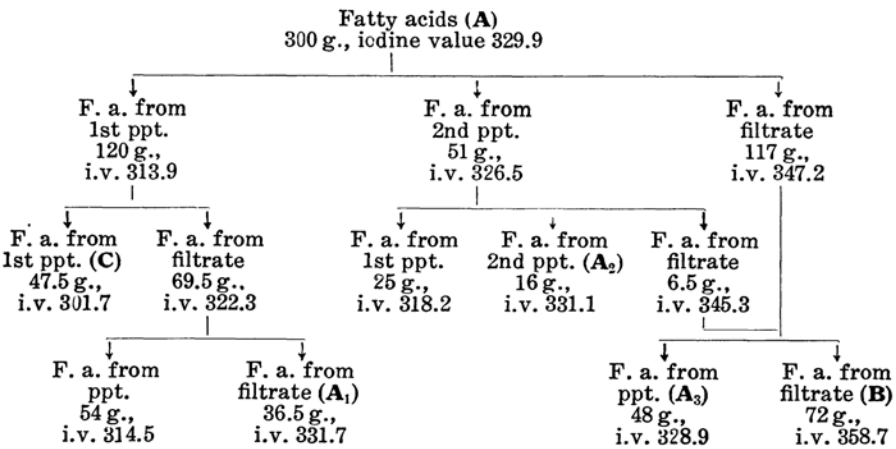
The fractions (3) and (4) were subjected to a further fractionation, and there was obtained a fraction boiling at 217–221°/10 mm.; saponif. value 177.0 and iodine value 303.0. Since this fraction was considered to contain a little of less unsaturated methyl esters, it was again treated by sodium-soap-acetone method, by which a small amount of acetone-insoluble sodium soaps was formed. The fatty acids liberated from acetone-soluble sodium soaps were then reconverted into methyl esters, and the latter were repeatedly fractionated to give 580 g. of a fraction boiling at 217–221°/10 mm.; saponif. value 177.0 and iodine value 316.0. The free fatty acids (**A**) obtained from this methyl ester fraction showed neutralisation value 185.0 and iodine value 329.9, and yielded 101% of ether insoluble bromides which had Br-content 69.41%. Hydrogenation yielded arachidic acid which had neutr. value 178.9 (calc. for $C_{20}H_{40}O_2$: 179.6) and m.p. 74–74.5° after recrystallisation from 95% alcohol. The melting point was not lowered when the substance was admixed with an authentic specimen of arachidic acid which was prepared from erucic acid by fusion with caustic potash and had m.p. 74.5–75°. Accordingly, the fatty acids (**A**) are proved to consist essentially of highly unsaturated C_{20}-acids. It is true that their iodine value is close to the calculated value (337.7) for the acid $C_{20}H_{32}O_2$, but they are found by the separative methods described below to contain other acids of different degrees of unsaturation in addition to the acid $C_{20}H_{32}O_2$.

(16) Toyama and Tsuchiya, *Chemische Umschau Fette, Oele, Wachse und Harze*, **32** (1925), 204; *ibid.*, **34** (1927), 51; also *J. Soc. Chem. Ind. Japan*, **28** (1925), 653, 962.

(17) Unless otherwise stated, the iodine values recorded in this paper were determined by the Wijs method.

2. **Fractional Precipitation of Sodium Soaps in Acetone.** The fatty acids (**A**) obtained above were fractionally precipitated from an acetone solution as their sodium soaps, and were separated into the portions of different degrees of unsaturation. The separative operation was carried out in the following manner: 50 g. of the fatty acids (A) were dissolved in 1 l. of acetone, and a portion of the sodium hydroxide solution prepared by dissolving 50 g. of sodium hydroxide in 50 c.c. of water and 100 c.c. of 95% alcohol was added to effect a partial neutralisation of the fatty acids, by which there was formed a precipitate of sodium soaps. After the precipitated sodium soaps were once brought into solution by a gentle boiling of the solution under a reflux condenser, the solution was allowed to cool to reprecipitate the sodium soaps which were then filtered. The filtrate was again neutralised partially by adding a portion of the sodium hydroxide solution, and after being treated as before, the precipitate of sodium soaps was filtered. Each precipitate of sodium soaps (1st and 2nd precipitates) was decomposed with hydrochloric acid to liberate the fatty acids. The remaining fatty acids in the filtrate were recovered by removing the solvent followed by acidification. The fatty acids (**A**) were thus separated into three portions, and each portion was subjected to a further separation by a similar operation as before. In these experiments, 300 g. of the fatty acids (**A**) were used instead of all the quantity of the fatty acids (**A**) obtained by the foregoing procedure. The results are given in Table 2.

Table 2.

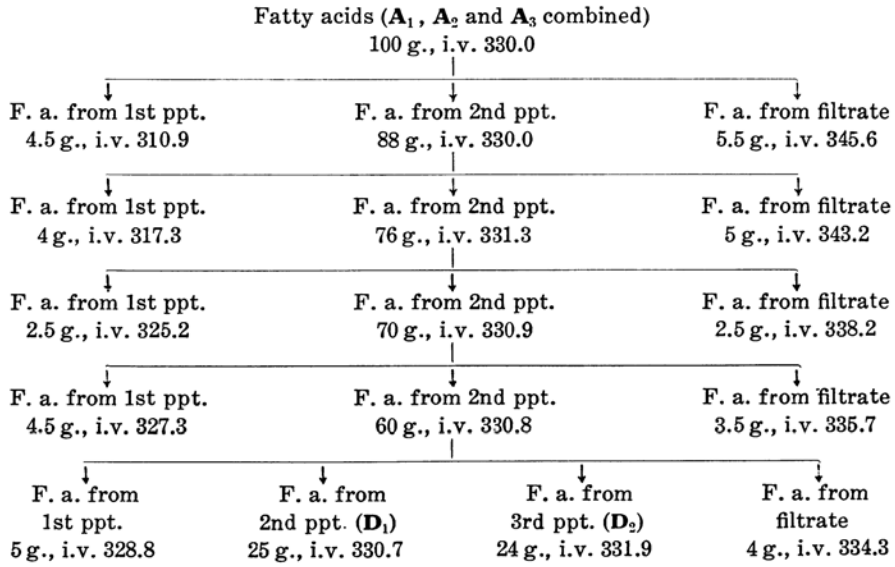


From their iodine values, the fatty acids fractions ($\mathbf{A_1}$), ($\mathbf{A_2}$) and ($\mathbf{A_3}$) are considered to consist essentially of an acid $C_{20}H_{32}O_2$; the fraction (**B**) contains a more highly unsaturated acid $C_{20}H_{30}O_2$ in addition to the acid $C_{20}H_{32}O_2$, while the fraction (**C**) contains less unsaturated acids besides the acid $C_{20}H_{32}O_2$.

3. **Isolation of Eicosatetraenoic Acid $C_{20}H_{32}O_2$.** In order to isolate the eicosatetraenoic acid $C_{20}H_{32}O_2$ from the fatty acids fractions ($\mathbf{A_1}$), ($\mathbf{A_2}$) and ($\mathbf{A_3}$), 100 g. of the combined fractions (iodine value 330.0) were dissolved in 2.5 l. of acetone, and a small portion of the sodium hydroxide solution prepared by dissolving 50 g.

of sodium hydroxide in 50 c.c. of water and 100 c.c. of 95% alcohol was added, by which a small amount of the precipitate (1st precipitate) of sodium soaps was formed. This was filtered, and the filtrate was then neutralised completely with the same solution of sodium hydroxide. The precipitated sodium soaps (2nd precipitate) were filtered, and the fatty acids liberated on acidification were again subjected to fractional precipitation of sodium soaps in acetone as before. These separative operations were repeated till the results shown in Table 3 were obtained. In the final fractionation, the fatty acids were separated into four portions.

Table 3.



The fatty acids fractions obtained by the final fractionation in Table 3 showed the iodine values which were close to each other. The fractions ($\mathbf{D}_1$) and ($\mathbf{D}_2$) were converted into methyl esters, and the latter were subjected to a repeated fractional distillation which yielded a fraction boiling at 217–220°/10 mm. as methyl eicosatetraenoate. It had the following constants: d_4^{15} 0.9140, d_4^{20} 0.9106, n_D^{15} 1.4854, n_D^{20} 1.4834, mol. refr. 99.87 (calc. for $C_{21}H_{34}O_2$: 98.96), saponif. value 176.0 (calc. 176.3), iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 321.2 and 309.5 respectively (calc. 319.0), thiocyanogen value 160.5 (calc. for the formation of tetrathiocyanate: 159.5). Bromination in ethereal solution yielded 103% of ether insoluble bromide (Found: Br, 67.02. Calc. for $C_{21}H_{34}O_2Br_8$: Br, 66.76%).

The eicosatetraenoic acid liberated from the methyl ester in the usual way showed the following constants: d_4^{15} 0.9300, d_4^{20} 0.9263, n_D^{15} 1.4935, n_D^{20} 1.4915, mol. refr. 95.16 (calc. for $C_{20}H_{32}O_2$: 94.23), neutralisation value 184.3 (calc. 184.4), iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 334.0 and 323.1 respectively (calc. 333.7), thiocyanogen value 167.1 (calc. for the formation of tetrathiocyanate: 166.9). Bromination in ethereal solution yielded 103% of ether insoluble

bromide which turned black at about 240° without melting (Found: Br, 68.06. Calc. for $C_{20}H_{32}O_2$: Br, 67.76%).

4. **Separation of a Concentrated Fraction of Eicosapentenoic Acid $C_{20}H_{30}O_2$.** Seventy g. of the fatty acid fraction (**B**) recorded in Table 2 was dissolved in 1.8 l. of acetone, and a small quantity of the same solution of sodium hydroxide as used in the preceding separative operation was added in order to precipitate the less unsaturated fatty acids as their sodium soaps, which were filtered. The more highly unsaturated fatty acids recovered from the filtrate were again treated with the sodium hydroxide solution as before, the less unsaturated portion was removed as insoluble sodium soaps, and after several repetitions of these separative operations, there was obtained 8 g. of a fatty acid fraction from the final filtrate which had the following constants: d_4^{15} 0.9399, n_D^{15} 1.5019, neutr. value 185.1, iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 392.7 and 378.4 respectively, thiocyanogen value 167.9 (calc. for $C_{20}H_{30}O_2$: neutr. value 185.6, iodine value 419.9, thiocyanogen value calc. for the formation of tetrathiocyanate 168.0). Bromination in ethereal solution yielded 104% of ether insoluble bromide which turned black at about 240° without melting (Found: Br, 71.28. Calc. for $C_{20}H_{30}O_2Br_{10}$: Br, 72.56%). As the iodine value of this fraction is still considerably lower than the value calculated for $C_{20}H_{30}O_2$, it must contain an appreciable amount of $C_{20}H_{32}O_2$ in addition to $C_{20}H_{30}O_2$. Although the acid $C_{20}H_{30}O_2$ could not be isolated in pure state, its existence in sardine oil is thus proved by the present experiments.

5. **Examination of the Less Unsaturated Portion.** Forty-seven g. of the fatty acid fraction (**C**) recorded in Table 2 was dissolved in 1 l. of acetone and was partially neutralised by adding a portion of the sodium hydroxide solution prepared by dissolving 50 g. of sodium hydroxide in 100 c.c. of water and 200 c.c. of 95% alcohol. The solution was refluxed until all the insoluble sodium soaps formed once disappeared, and then it was allowed to cool to reprecipitate the insoluble sodium soaps. By these treatments, the following two portions were separated.

	Yield (g.)	Neutr. value	Iodine value
(i) Fatty acids from insoluble sodium soaps	17	186.0	276.6
(ii) Fatty acids from the filtrate	29	185.0	315.9

The iodine value of the fatty acids (i) was more close to the calculated value for $C_{20}H_{34}O_2$ (248.6) than that for $C_{20}H_{32}O_2$ (333.7), but they yielded 71% of an ether insoluble bromide having Br-content 67.06% which was considerably higher than the Br-content of $C_{20}H_{34}O_2Br_6$ (61.02%) and was close to the Br-content of $C_{20}H_{32}O_2Br_8$ (67.76%). On the other hand, when the lithium soaps prepared from the fatty acids (i) were treated with 50% alcohol, there was obtained a small amount of the lithium soaps insoluble in 50% alcohol, and the latter yielded on acidification fatty acids with an iodine value 91.3 which indicated that the fatty acids (i) contained also a small amount of less unsaturated fatty acids such as $C_{20}H_{38}O_2$ (iodine value 81.8). Accordingly, the fatty acids (i) were found to be a mixture of the fatty acids of different degrees of unsaturation, and though the presence of $C_{20}H_{34}O_2$ was not proved with certainty, it was most probable.

Summary.

Highly unsaturated C_{20}-acids have been separated from sardine oil and they were further separated into the portions of different degrees of unsaturation with the results that an eicosatetraenoic acid $C_{20}H_{32}O_2$ was isolated and also the presence of an eicosapentenoic acid $C_{20}H_{30}O_2$ was proved by separating a fraction, in which this acid is concentrated.

Tokyo Imperial Industrial Research Laboratory,
Shibuya-ku, Tokyo.