SEPARATION AND STRUCTURE DETERMINATION OF EICOSATETRAENOIC ACID IN OX LIVER LIPID

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Arachidonic acid, an eicosatetraenoic acid, has been known for a long time to occur in the lipid of internal organs of ox and hog. Relating to its structure, the 6, 10, 14, 18-tetraenoic structure[1] was first reported for the eicosatetraenoic acid separated from ox suprarenal lipid. However, in consequence of later studies by several authors,[2][3][4] the 5, 8, 11, 14-tetraenoic structure is now generally regarded as correct. On the other hand, concerning the structure of eicosatetraenoic acid in fish oil, the 4, 8, 12, 16-tetraenoic structure was assigned to the acid separated from sardine oil.[5] The same structure was given for the acid separated from bonito oil,[6] and this structure has recently been reconfirmed for the acid in sardine oil.[7] As it is known that some unsaturated acids exist in different oils as different isomers, and that in some cases isomers of an unsaturated acid co-exist in the same kind of oil, it is by no means utterly improbable that the eicosatetraenoic acid in the lipid of internal organs of land animal and the eicosatetraenoic acid in fish oil are isomers differing in the locality of their ethylenic linkages. However, since it seemed desirable to the authors to re-investigate the structure of eicosatetraenoic acid in the lipid of internal organs of land animal, the authors carried out the present study.

Eicosatetraenoic acid was separated from ox liver lipid, and its ethyl ester was subjected to oxidative scission with potassium permanganate in acetone. The scission product was separated into oily substances (I), hexane insoluble substances (II) and hexane soluble substances (III). The oily substances (I) consisting chiefly of monoethyl ester of dibasic acid had a neutralization value which is considerably lower than the calculated value for ethyl hydrogen succinate and is rather close to the calculated value for ethyl hydrogen glutarate. This seems to be ascribed to a contamination of the oily substances (I) with some neutral substances. Saponification of the oily substances yielded free dibasic acids from which succinic acid was isolated and identified. Besides succinic acid a substance resembling glutaric acid in its melting point, 94-96° C, was obtained, but it was not a single dibasic acid since its melting point was much raised by a further recrystallization. From the hexane insoluble substances (II), succinic acid was obtained after recrystallization. An examination of the portion recovered from the mother liquor of recrystallization indicated the absence of malonic acid, and re-oxidation of this portion with permanganate yielded succinic acid. The hexane soluble substances (III) had a neutralization value which is close to the calculated value for caproic acid rather than for butyric acid. On removal of neutral substances, however, the presence of butyric acid was indicated.

The results obtained above indicate that the eicosatetraenoic acid in ox liver lipid consists of $=CH(CH_2)_2COOH$ and $CH_3(CH_2)_2CH=$ as two terminal groups and two of $=CH(CH_2)_2CH=$ as intermediate group. Accordingly, this acid has the 4, 8, 12, 16-tetraenoic structure like the eicosatetraenoic acid in fish oil. If the eicosatetraenoic acid in ox liver lipid had the 5, 8, 11, 14-tetraenoic structure, the oxidative scission products should be glutaric acid from the carboxyl side, caproic acid from the methyl group side, and malonic acid or acetic acid produced by secondary decomposition of malonic acid from the intermediate groups, whereas no succinic acid should be formed. However, as mentioned above, succinic acid was isolated and identified, and none of glutaric, caproic and malonic acids was detected in the present experiments. Although it can not be concluded that the eicosatetraenoic acid in ox liver lipid contains no isomer, the main portion of the eicosatetraenoic acid, even if it contains any isomer, must have the 4, 8, 12, 16-tetraenoic structure and not the 5, 8, 11, 14-tetraenoic structure. From the results obtained above, the 5, 8, 11, 14-tetraenoic structure given for the acid in ox suprarenal lipid appears quite doubtful.

Experimental

1. Separation of Eicosatetraenoic Acid from Ox Liver Lipid

Fresh ox liver (12 kg) procured from a slaughter house in Nagoya was minced and boiled in a pan in order to evaporate the moisture to some extent. The boiled meat was then dried at 80° C in an oven. The dried material (2.4 kg), after being reduced to powder, was extracted with ether, and 528 g of lipid (4.4% of liver) was obtained. Saponification of the lipid and acidification of the soap solution with dilute hydrochloric acid followed by ether extraction in the usual way gave 296 g of ether-extract. Two liters of acetone was added to the ether-extract, and the portion (29 g) insoluble in acetone at the room temperature was removed. The acetone solution was cooled with ice, and the crystalline solid (17 g) separated was removed. To the acetone filtrate was added 290 cc of a 10% solution of lithium hydroxide. The mixture was cooled with ice, and the precipitate of lithium salts was filtered off. After distilling off acetone from the acetone filtrate, the residue was decomposed with dilute hydrochloric acid, and extracted with ether, yielding 140 g of ether-extract which was an oily liquid contaminated with solid material and had N.V. 76.7 and I.V. 172.5. Saponification of this ether-extract and extraction of the soap solution with ether yielded 41 g of unsaponifiable matter and 56 g of fatty acids which had N.V. 183.7 and I.V. 250.7. Remarkably low yield of unsaponifiable matter and fatty acids (97 g in total) from 140 g of the ether-extract may be explicable by assuming that the ether-extract contained more or less phosphatide constituents which had not been completely saponified in the initial saponification procedure of ox liver lipid but was completely saponified in the saponification procedure of the ether-extract, yielding some saponification products soluble in water. The fatty acids (N.V. 183.7, I,V. 250.7) obtained above were converted into methyl ester and the latter was subjected to fractional distillation. A fraction of B.P. 200-205° C/ca. 1 mm Hg, S.V. 174.5 and I.V. 277.5 was saponified, and 20.4 g of fatty acid mixture (N.V. 183.7, I.V. 297.1) obtained was fractionally precipitated as sodium salt from acetone as shown in Table 1.

TABLE 1

Fatty acid mixture, 20.4 g, N.V. 183.7, I.V. 297.1

1st ppt. 2.0 g
I.V. 261.5

2nd ppt. 9.2 g
I.V. 287.0

3rd ppt. 7.8 g
I.V. 313.6

Filtrate 1.1 g
I.V. 329.8

Precipitate 3.8 g
I.V. 266.0

Filtrate 5.2 g
I.V. 302.1

1st ppt. 3.3 g
I.V. 305.0

2nd ppt. 1.4 g
I.V. 316.2

Filtrate 2.9 g
I.V. 321.0

Precipitate 6.0 g
I.V. 298.5

Filtrate 3.5 g
I.V. 322.5

Precipitate 4.2 g
I.V. 285.9

Filtrate 1.6 g
I.V. 324.6

1st ppt. 2.0 g
I.V. 313.3

2nd ppt. 4.9 g
I.V. 328.7

Filtrate 0.4 g
I.V. 327.6

1st ppt. (*A*)
0.6 g
N.V. 184.5
I.V. 320.6

2nd ppt. (*B*)
5.2 g
N.V. 184.1
I.V. 328.8

Filtrate (*C*)
0.4 g
N.V. 184.0
I.V. 330.8

The neutralization values of the final fractions *A*, *B* and *C* in Table 1 agree with the calulated value for eicosatetraenoic acid (184.3). The iodine value of *A* is a little lower, while the iodine values of *B* and *C* are close to the calculated value for eicosatetraenoic acid (333.5). The fraction *B* had d_4^{20} 0.9261, n_D^{20} 1.4911 and Mol. Refr. 95.23 (calculated for $C_{20}H_{32}O_2F_4$, 94.23). The ether insoluble bromide from the fraction *C* (yield 102%) had Br content 66.83% (calculated for $C_{20}H_{32}O_2Br_8$, 67.74%) and decomposed at 240° C with darkening. Hydrogenation of the fraction *A* yielded arachidic acid which had M.P. 75° C and N.V. 179.9 (calculated, 179.5) after recrystallization from ethanol, and showed no depression of melting point when mixed with a pure specimen of arachidic acid.

2. Oxidation of Ethyl Eicosatetraenoate with Potassium Permanganate in Acetone

The ethyl ester (5.0 g) prepared from the eicosatetraenoic acid (the fraction *B* in Table 1) was dissolved in 200 cc of acetone, and 70 g of powdered potassium permanganate was added in small portions. The mixture was refluxed for 20 hours. Acetone was distilled off, and sodium bisulfite solution was added to the residue in order to reduce the excess of potassium permanganate. The mixture was then filtered through a wet filter paper, and manganese oxide together with oily substances (I) remaining on the filter paper was washed with hot water. The filtrate and washing were combined, neutralized with sodium carbonate, and evaporated to dryness. The residue was acidified with sulfuric acid, and extracted twice with 500 cc of ether. The ether solution was washed with a saturated solution of sodium chloride, dehydrated with anhydrous sodium sulfate, and distilled, leaving a residue which separated some solid at ordinary temperature. The residue was washed twice with 30 cc of hexane, giving hexane insoluble substances (II) and hexane soluble substances (III).

Oily substances (*I*). Manganese oxide with oily substances on the filter paper was dried under vacuum and extracted first with acetone and then with ether. The acetone-extract, after removing the acetone, was added to the ether solution, and it was washed with a solution of sodium carbonate in order to separate the acidic substances from the ether solution as their sodium salts. The aqueous solution was acidified with dilute hydrochloric acid, and the oily liquid separated was collected by using ether. The oily liquid (0.8 g) had N.V. 358.5 (calculated for ethyl hydrogen succinate 383.9 and for ethyl hydrogen glutarate 350.3). Saponification of this oily liquid with alcoholic potash followed by acidification with dilute hydrochloric acid gave a solution with a small amount of insoluble oil. The solution was filtered through a wet filter paper, and the clear filtrate free from oily mater was concentrated and finally evaporated to almost dryness under vacuum. The residue thus obtained was refluxed with ethanol, the inorganic salts insoluble in ethanol were removed, and the ethanol solution was concentrated and cooled. And then a small amount of crystalline solid separated. It had M.P. 94–96° C, but the melting point was raised to 145–146° C after one recrystallization from water. The ethanol mother liquor separated from crystalline solid was evaporated, and hot water was added to the residue, when a small amount of oily matter separated. The aqueous solution freed from oily matter was concentrated and cooled, and the crystalline solid was separated. It had M.P. 179–180° C and N.V. 928.5 after two recrystallizations from water, and showed no depression of melting point when mixed with a specimen of succinic acid (M.P. 182–183° C, N.V., calculated, 950.3) in various proportions.

Hexane insoluble substances (*II*). This portion (1.0 g) gave, after two recrystallizations from water, a crystalline solid which had M.P. 178–179° C and N.V. 938.3, and showed no depression of melting point when mixed with a specimen of succinic acid. From the mother liquor of recrystallization, 0.7 g of crystalline solid was recovered, and a portion of it was heated to 150° C in a test tube. There was no sign of decomposition. The air expelled from the test tube was introduced into a solution of calcium hydroxide, but it caused no turbidity in the solution. Another portion of the crystalline solid recovered from the mother liquor was subjected to a further oxidation with permanganate in acetone, and the ethanol soluble portion, freed from inorganic salts, was separated from the oxidation products and recrystallized from water, yielding a crystalline substance of M.P. 179–180° C which was identified with succinic acid.

Hexane soluble substances (*III*). On treating this portion with a little cold hexane, a small amount of insoluble portion was removed, and an oily liquid (0.7 g) was obtained as hexane soluble portion. It had n_D^{20} 1.4250 and N.V. 511.1 (n_D^{20} for butyric and caproic acids are 1.3391 and 1.4164, respectively. Neutralization values, calculated for these acids, are 636.8 and 483.0, respectively). Since this portion appeared to be contaminated more or less with neutral substances, the alcoholic solution of soap resulting from the determination of neutralization value was evaporated to dryness, the residue was dissolved in water, and the neutral substances were extracted with ether. After these procedures, the aqueous solution of soap was evaporated to dryness, the residue was acidified with dilute sulfuric acid, and the acidified solution was extracted with a large amount of ether. The ether solution was washed with a saturated solution of sodium chloride, the ether

was distilled off, and a liquid acid having N.V. 625.2 and a butyric acid like odor was obtained. Hydroxamic acid prepared from this liquid acid was analyzed by paper chromatography iu ethanol with the following results.

	R_f (ascending method)	Coloration
Hydroxamic acid from the liquid acid	0.55	Purple
Butyrohydroxamic acid	0.57	Purple

Notes. Filter paper: "Toyo Filter Paper" No. 2; developer: ethyl acetate; temperature: 12-13° C; chromogenic reagent: 10% solution of ferric chloride in ethanol.

Summary

Eicosatetraenoic acid was separated from ox liver lipid and its ethyl ester was subjected to permanganate oxidation in acetone. Among the oxidative scission products, succinic acid resulting from both carboxyl side and intermediate groups and butyric acid resulting from the methyl group side were identified. Accordingly it is shown that the eicosatetraenoic acid in ox liver lipid has the 4, 8, 12, 16-tetraenoic structure and is identical with the eicosatetraenoic acid in fish oil.

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