RESEARCH REPORTS

SEPARATION AND STRUCTURE DETERMINATION OF EICOSATETRAENOIC ACID IN SWINE LIVER LIPID

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A previous study by the authors[1] established that eicosatetraenoic acid separated from ox liver lipid has the 4, 8, 12, 16-tetraenoic structure and is identical with eicosatetraenoic acid of fish oils. Since this finding by the authors may be worthy of special attention in contrast to the 5, 8, 11, 14-tetraenoic structure[2] assigned to eicosatetraenoic acid separated from ox suprarenal lipid, the authors undertook the present study in which eicosatetraenoic acid was separated from swine liver lipid and its structure determined. Although the eicosatetraenoic acid separated from swine liver lipid was not pure and was found to be contaminated with pentaenoic acid, conjugated acid and others, it was used for structure determination without further purification. It was converted into ethyl ester, and the latter was oxidized with potassium permanganate in acetone. On examining the oxidative scission products, it was found that both the terminal group containing carboxyl and the intermediate groups yielded mainly succinic acid while the terminal group containing methyl yielded chiefly butyric acid. Accordingly the eicosatetraenoic acid under examination was found to have the 4, 8, 12, 16-tetraenoic structure. Although the possibility of the occurrence of isomers in the eicosatetraenoic acid of swine liver lipid is not utterly excluded, its major component, even if it is accompanied with any isomer at all, must have the 4, 8, 12, 16-tetraenoic structure which is the same with that of the eicosatetraenoic acid of ox liver lipid. From the foregoing results, the 5, 8, 11, 14-tetraenoic structure reported for the acid in ox suprarenal lipid appears quite doubtful.

Experimental

1. Separation of Crude Eicosatetraenoic Acid from Swine Liver Lipid

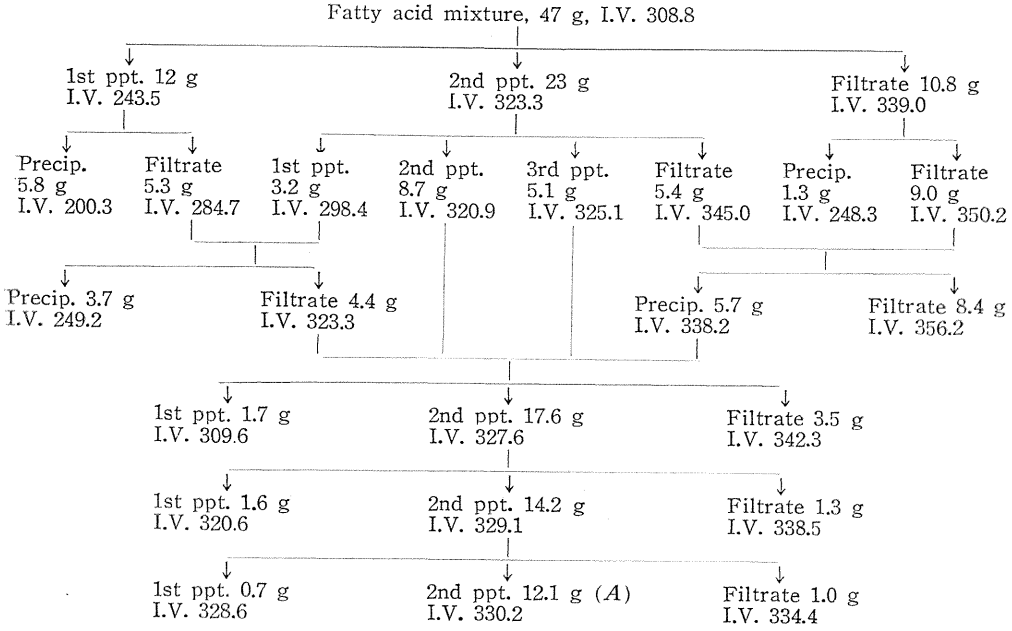
Fresh swine liver (17.6 kg) procured from a slaughter house in Nagoya in January and February, 1954 was minced and boiled in a pan in order to evaporate moisture to some extent. The boiled meat was then dried at 80° C in an oven. The dried material (5.5 kg), after being reduced to powder, was extracted with ether, yielding 780 g of lipid. Saponification of the lipid with alcoholic potash (concentration, 15%) and acidification of the saponification product with dilute hydrochloric acid followed by ether extraction in the usual way gave 510 g of

fatty material. Five liters of acetone was added to the fatty material, and the mixture was refluxed for a short time and then cooled to the room temperature of about 10° C. The portion insoluble in acetone (11. 5 g) was removed by filtration, the filtrate was cooled with ice, and the solid deposit (70 g) formed was filtered off. The filtrate was neutralized with 500 cc of about 13% solution of lithium hydroxide, refluxed for a short time, and cooled to about −5° C.

Precipitates of lithium soap (161 g as fatty acid) were filtered off. After distilling off acetone from the filtrate, the residue was decomposed with dilute hydrochloric acid, and the oily liquid contaminated with crystalline solid was collected by using ether. This was refluxed with alcoholic potash, and the extraction of the soap solution with ether in the usual way yielded 45 g of unsaponifiable substances and 204 g of fatty acids rich in highly unsaturated acids which had N.V. 194.3 and I.V. 236.6. The fatty acids were converted into methyl ester, and the latter was subjected to fractional distillation by which a fraction of B.P. 200°-205° C /*ca.* 1 mm Hg was separated. The fatty acid mixture (N.V. 181.7 and I.V. 308.8) from this fraction was fractionally precipitated as sodium salt from acetone, as shown in Table 1.

The fatty acid fraction (*A*) in Table 1 had N.V. 183.8 and I.V. 330.2 which agree with the calculated values (N.V. 184.3 and I.V. 333.5) for eicosatetraenoic acid. It had d_4^{20} 0.9264, n_D^{20} 1.4915 and Mol. Refr. 95.27 (calculated for $C_{20}H_{32}O_2F_4$ 94.23). Bromination of the fraction (*A*) in ether yielded 103% of insoluble bromide which had Br content 67.05% (calculated for $C_{20}H_{32}O_2Br_8$ 67.74%) and decomposed at about 240° C with darkening. Hydrogenation product of the fraction (*A*) gave arachidic acid which had M.P. 75° C and N.V. 178.3 (calculated, 179.5) after recrystallization from ethanol. Ultraviolet absorption curves of the fraction (*A*) and its alkali-

TABLE 1



isomerization product obtained under the isomerization condition of 21% KOH-glycol, 180° C and 15 minutes are shown in Figs. 1 and 2, respectively. Table 2 shows the specific extinction coefficients of the fraction (*A*) and its alkali-isomerization product at the characteristic wave lengths.

TABLE 2

Wave length (mμ)	Specific extinction coefficient	
	Fatty acid fraction (*A*)	After alkali-isomerization
235	6.61	42.0
270	1.20	46.1
316	0.14	60.9
348	—	12.8

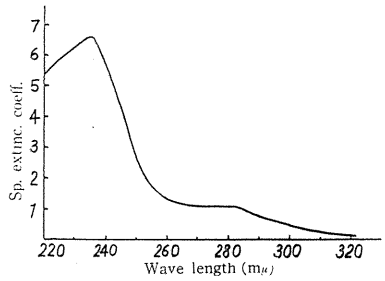


FIG. 1. Ultraviolet absorption curve of the fraction (*A*).

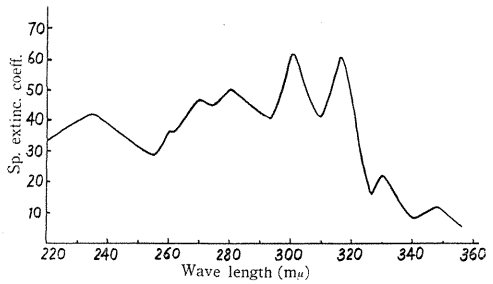


FIG. 2. Ultraviolet absorption curve after alkali-isomerization.

As is seen from Fig. 1, the absorption curve for the fraction (*A*) has a peak at 235 mμ. Accordingly the presence of conjugated diene in the fraction (*A*) is indicated. The absorption curve for the alkali-isomerization product shown in Fig. 2 has distinctly a peak at 348 mμ, indicating the presence of pentaenoic acid in the original fraction (*A*). Assuming that the specific extinction coefficient of the conjugated diene in the fraction (*A*) at 235 mμ be 119 which is the specific extinction coefficient of C_{18}-conjugated dienoic acid at 233 mμ, the diene content of the fraction (*A*) is calculated to be $100\times6.61/119=5.6\%$. But the actual content is smaller than 5.6% since the fraction (*A*) possibly contains some other compounds which have also some absorption at 235 mμ. Taking the specific extinction coefficient of the alkali-isomerization product of eicosatetraenoic acid at 348 mμ as 87.8,[3] the content of pentaenoic acid in the fraction (*A*) is calculated to be $100\times12.8/87.8=14.6\%$. The actual content, however, is deemed to be lower than 14.6% because of the possible occurrence of some other compounds having absorption at 348 mμ in the fraction (*A*). At any rate the results of the spectrophotometric examination indicate that the fraction (*A*) is not a pure eicosatetraenoic acid, but contaminated with less than 15% of pentaenoic acid and a small amount of conjugated compounds consisting mainly of conjugated diene.

2. Oxidation of Crude Ethyl Eicosatetraenoate with Potassium Permanganate in Acetone

The fatty acid fraction (*A*) was converted into ethyl ester. Ten g of ethyl ester was dissolved in 400 cc of acetone, and 140 g of powdered potassium permanganate was added in small portions. The mixture was refluxed for 20 hours. Acetone was then 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 oxides together with oily liquid (I) remaining on the filter paper were washed with hot water. The filtrate and washings were combined, neutralized with sodium carbonate, and evaporated to dryness. The residue was acidified with dilute sulfuric acid and extracted twice with 500 cc of ether. The ether solution was washed with a saturated solution of sodium chloride, and the ether was distilled off, leaving a residue consisting of liquid and solid substances. This residue was washed with 30 cc of hexane, giving hexane-insoluble portion (II) and hexane-soluble portion (III).

Oily liquid (*I*). Manganese oxides with oily liquid (I) on the filter paper were dried under vacuum in order to remove moisture to some extent, and extracted first with acetone and then with ether. The acetone-extract obtained after removing acetone was added to the ether solution, and the latter was washed with a solution of sodium carbonate. By this treatment, the acidic substances in the ether solution entered into the carbonate solution as their sodium salts. The carbonate solution was acidified with dilute hydrochloric acid, and the oily liquid (1.8 g) separated was collected by using ether. It had N.V. 368.4 (calculated, 383.9 for ethyl hydrogen succinate and 350.3 for ethyl hydrogen glutarate). Saponification of this material followed by acidification with dilute hydrochloric acid gave a solution contaminated with a small amount of insoluble oil. The solution was filtered through a wet filter paper, and the clear filtrate was concentrated and then extracted with large amounts of ether. The ether solution obtained was washed with a saturated solution of sodium chloride, and after dehydration the ether was distilled off. The residue was dissolved in water. Since there was still a small amount of insoluble oily liquid in the aqueous solution, it was filtered through a wet filter paper for the removal of insoluble oily liquid. The clear filtrate was concentrated and cooled, and a crystalline substance was separated. This had M.P. 180°–181° C and N.V. 935.7, and showed no depression of melting point when mixed with a specimen of succinic acid (M.P. 182°–183° C and N.V., calculated, 950.3) in various proportions.

Hexane-insoluble portion (*II*). This portion (2.8 g), after being washed with a little hexane, was recrystallized from water, yielding crystals which had M.P. 180°–181° C and N.V. 929.2 and showed no depression of melting point when mixed with succinic acid. The portion (1.5 g) recovered from the mother liquor of recrystallization was subjected to a further oxidation with potassium permanganate in acetone, and the ethanol-soluble portion freed from inorganic salts was separated and recrystallized from water, yielding succinic acid of M.P. 180°–181° C.

Hexane-soluble portion (*III*). This portion still contained a small amount of crystalline solid insoluble in hexane. The liquid portion (2.0 g) freed from the solid by a repeated treatment with cold hexane had N.V. 513.5 (calculated for

butyric acid, 636.8) and appeared to be contaminated more or less with non-acidic substances and others. It was neutralized with a dilute aqueous solution of potassium hydroxide, and the aqueous solution was extracted with ether. The aqueous solution was then acidified with dilute sulfuric acid and subjected to steam distillation. The distillate was neutralized and evaporated, and the residue, after acidification with dilute sulfuric acid, was extracted with large amounts of ether. The ether solution was washed with a saturated solution of sodium chloride and then the ether was distilled off, leaving a liquid of N.V. 617.2 having a butyric acid like odor. Hydroxamic acid prepared from this liquid was analyzed by paper chromatography in ethanol with the results shown in Table 3.

TABLE 3

Hydroxamic acid	R_f (ascending method)	Coloration
Under examination	0.56	Purple
Butyrohydroxamic acid	0.57	〃
Caprohydroxamic acid	0.86	〃

Notes. Filter paper: "Toyo Filter Paper" No. 50; developer: ethyl acetate; temperature: 23-24° C; chromogenic reagent: 10% solution of ferric chloride in ethanol.

Summary

Crude eicosatetraenoic acid fraction was separated from swine liver lipid. Although this fraction had neutralization and iodine values which are close to the calculated values for eicosatetraenoic acid, the ultraviolet absorption measurements of this fraction and its alkali-isomerization product revealed that this fraction is contaminated with less than 15% of pentaenoic acid and a small amount of conjugated acids consisting mainly of conjugated diene. Ethyl ester of this fraction was subjected to the permanganate oxidation in acetone. Among the oxidative scission products, succinic acid resulting from both the terminal group containing carboxyl and the intermediate groups and butyric acid resulting from the terminal group containing methyl were identified. Accordingly the eicosatetraenoic acid of swine liver lipid, like the eicosatetraenoic acid of ox liver lipid, has the 4, 8, 12, 16-tetraenoic structure and is identical with the eicosatetraenoic acid of fish oils.

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