THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL. V. THE CONSTITUTION OF EICOSATETRAENOIC ACID $C_{20}H_{32}O_2$.

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Received May 7th, 1935. Published July 28th, 1935.

As described in the 4th report of this series,⁽¹⁾ the highly unsaturated C_{20}-acids were separated from sardine oil. On further fractionation of these acids into individual components, 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 confirmed by separating a concentrated fraction of this acid. The present paper deals with the constitution of the eicosatetraenoic acid thus obtained. For the determination of its constitution, its amyl ester was subjected to ozonolysis. Among the products of ozonolysis, butyric acid $CH_3\cdot(CH_2)_2\cdot COOH$, butyric aldehyde $CH_3\cdot(CH_2)_2\cdot COH$, succinic acid $HOOC\cdot(CH_2)_2\cdot COOH$, and amyl hydrogen succinate $HOOC\cdot(CH_2)_2\cdot COOC_5H_{11}$ were identified, and also the presence of succinic semi-aldehyde, succinic aldehyde and amyl ester of succinic semi-aldehyde was indicated. Besides these compounds, minor amounts of carbon dioxide and acetaldehyde were found; these compounds, however, are

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(1) This Bulletin, **10** (1935), 241.

believed to be derived from a minor ingredient (probably amyl eicosapentenoate) which contaminates the amyl ester used for this experiment. It is seen from these results that amyl eicosatetraenoate has the following groups: $CH_3\cdot(CH_2)_2\cdot CH=$, $=CH\cdot(CH_2)_2\cdot CH=$ and $=CH\cdot(CH_2)_2\cdot COOC_5H_{11}$, of which it must contain three of the group $=CH\cdot(CH_2)_2\cdot CH=$, since it has four ethylenic linkings. Although in the case of moroctic acid described in a previous paper,⁽²⁾ the positions of three groups which lied between CH_3- and -COOH were determined from the experimental results found by examining the products of ozonolysis of methyl ester of tetrathiocyano-moroctate prepared by the selective addition of thiocyanogen to methyl moroctate, the positions of the respective groups in amyl eicosatetraenoate were confirmed without further proof, since all the products of ozonolysis consisted of C_4-compounds. The constitution of eicosatetraenoic acid is thus established as $\Delta^{4:5,\ 8:9,\ 12:13,\ 16:17}$-acid which is expressed by the following formula:

$$CH_3\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot COOH.$$

In comparing the constitution of eicosatetraenoic acid with that of gadoleic acid ($\Delta^{9:10}$-eicosenoic acid)⁽³⁾ which is a constituent distributed widely in marine animal oils, it should be noted here that eicosatetraenoic acid has no ethylenic linking in 9:10-position. As stated in previous papers,⁽⁴⁾ similar relations were found to exist between the polyethylenic and monoethylenic acids having sixteen and eighteen carbon atoms respectively. Thus hiragonic acid ($\Delta^{6:7,\ 10:11,\ 14:15}$) has no ethylenic linking in the same position as zoomaric acid ($\Delta^{9:10}$), likewise moroctic acid ($\Delta^{4:5,\ 8:9,\ 12:13,\ 15:16}$) has no ethylenic linking in the same position as oleic acid ($\Delta^{9:10}$).

Experimental.

The methods of isolation of eicosatetraenoic acid from sardine oil have already been described in the previous paper.⁽⁵⁾ The same acid as described in the previous paper was used for this experiment. It showed d_4^{15} 0.9300, n_D^{15} 1.4935, neutralisation value 184.3 (calc. for $C_{20}H_{32}O_2$: 184.4), iodine value by the Wijs method 334.0 (calc. 333.7). It was heated with an equal amount of amyl alcohol containing 2.5% of hydrogen chloride on the water-bath under a reflux condenser for one hour, and the resulting amyl ester was separated from the excess of amyl alcohol and some unchanged free acid. The amyl ester (5 g.) was dissolved in 50 c.c. of chloroform

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(2) This Bulletin, **10** (1935), 232.

(3) Toyama and Tsuchiya, *J. Soc. Chem. Ind. Japan,* **37** (1934), 31, 34.

(4) This Bulletin, **10** (1935), 192, 232.

(5) This Bulletin, **10** (1935), 241.

ozonised oxygen was passed into the solution under cooling with ice-salt until it became saturated, and on distilling off chloroform under reduced pressure, the ozonide remained as a light yellow viscous oil. Fifteen g. of the amyl ester yielded 24 g. (or 160%) of ozonide which was not thoroughly freed from chloroform. The calculated yields for normal ozonide $C_{25}H_{42}O_{14}$ and ozonide peroxide $C_{25}H_{42}O_{15}$ are 151.3 and 155.6% respectively. Water (150 c.c.) was added to the ozonide, and the liquid was heated in a flask on the water-bath for about 30 minutes. During the heating, a current of hydrogen was passed into the flask, and the volatile decomposition products (**A**) carried over with hydrogen were collected in other three flasks (**a**, **b** and **c**) which were connected in succession to the initial flask by a delivery tube; the first (**a**) was filled with 200 c.c. of water under cooling with ice, the second (**b**) and the third (**c**) with 400 c.c. of approximately 1/3N barium hydroxide solution. The decomposition products remaining in the initial flask partly dissolved in water, and partly separated as an oily substance (**C**) under the aqueous solution (**B**), which was separated by means of filtration. By these treatments the decomposition products of ozonide were separated into three portions (**A**, **B** and **C**).

1. **Volatile Decomposition Products (A).** The aqueous solution in the flask (**a**) produced a pink colouration with Schiff's reagent. On adding *p*-nitrophenylhydrazine and hydrochloric acid, there was formed a precipitate of *p*-nitrophenylhydrazone which on recrystallisation from 50% alcohol yielded *p*-nitrophenylhydrazone of butyric aldehyde in yellow needles of m.p. 86.5–87° (Found: N, 20.29. Calc. for $C_{10}H_{13}O_2N_3$: N, 20.29%). Harries(6) gives m.p. 87° for the same compound. The solution in the flask (**a**) showed also the presence of acetaldehyde by a deep blue colouration produced with diethylamine and sodium nitroprusside. The quantity of acetaldehyde must, however, be small, since *p*-nitrophenylhydrazone prepared from this solution, as stated above, yielded *p*-nitrophenylhydrazone of butyric aldehyde by a single recrystallisation. The solution in the flask (**a**) contained also acidic substances which were not further examined. The barium hydroxide solution in the flask (**b**) was found to contain a small amount of precipitate of barium carbonate, from which the amount of carbon dioxide formed by ozonolysis was calculated as being 1.02% of the amyl ester used for ozonolysis. Should amyl eicosatetraenoate contain the group $=CH{\cdot}CH_2{\cdot}CH=$, there would be a formation of carbon dioxide by the secondary decomposition of the products of ozonolysis, such as malonic acid and the corresponding aldehyde derived from that group, and the yield of carbon dioxide would be 11.75% of amyl eicosatetraenoate, provided that the secondary decomposition proceeds to a quantitative extent. As is seen from the results obtained with methyl moroctate in the third report and also with amyl esters of highly unsaturated C_{22}-acids which will be reported elsewhere, the secondary decomposition due to the presence of the group $=CH{\cdot}CH_2{\cdot}CH=$ takes place to a considerable extent, though not to a quantitative extent, and it never gives such a minor yield of carbon dioxide as in the present experiment. The formation of carbon dioxide in the present experiment is most likely to be due to contamination of the amyl ester used for ozonolysis with a minor ingredient having the group $=CH{\cdot}CH_2{\cdot}CH=$, such as amyl eicosapentenoate. Also the possibility is not excluded that a small portion of the ozonide of amyl eicosatetraenoate undergoes abnormal

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(6) "Untersuchungen über das Ozon und seine Einwirkung auf organische Verbindungen", p. 185.

decomposition with the formation of carbon dioxide. The presence of a minor amount of acetaldehyde in the solution of the flask **(a)** as stated above is also explainable by the same reason.

2. **Aqueous Solution (B).** This was neutralised with sodium carbonate and then extracted with ether in order to separate the neutral substances. The aqueous solution separated from ethereal extract was acidified with hydrochloric acid, and the acidic substances liberated were extracted with a large quantity of ether. By these treatments the substances dissolved in the aqueous solution **(B)** were separated into (i) neutral substances and (ii) acidic substances.

(i) *Neutral Substances.* Yield 0.6 g. These substances consisted of an orange yellow liquid and showed aldehyde reaction. They were oxidised with an alkaline solution of potassium permanganate, the products obtained after acidification were taken up with ether, and on removal of ether, there remained a mixture consisting of solid and liquid, of which the greater part of liquid was removed by absorption when the mixture was poured on a sheet of filter paper. The remaining solid showed m.p. 175–176° after being washed with a little cold ether and was considered to be an impure succinic acid. From these results, the presence of succinic aldehyde in the initial neutral substances is postulated.

(ii) *Acidic Substances.* Yield 11.2 g. These substances consisted of a reddish orange liquid. They were extracted three times with petroleum ether by using 150 c.c. each time, and the petroleum ether solution was separated from the residual insoluble portion.

The petroleum ether solution was heated on the water-bath until the solvent has distilled off, the residue was then heated in an oil-bath, and there was obtained about 1 g. of colourless distillate between 180–200° (temperature of bath). The silver salt prepared from this distillate was found to be silver butyrate (Found: Ag, 55.41. Calc. for $C_4H_7O_2Ag$: Ag, 55.34%). The final residue of distillation showed aldehyde reaction. Oxidation with alkaline permanganate followed by acidification gave a mixture of liquid and solid, from which the liquid portion was removed by absorption when the mixture was poured on a sheet of filter paper. The remaining crystalline solid showed m.p. 175–176° after being washed with a little cold ether; this was considered to be an impure succinic acid.

The petroleum ether insoluble portion (7.4 g.) consisted of a liquid which deposited crystalline solid on keeping at room temperature. The product obtained by oxidation with alkaline permanganate followed by acidification had neutralisation value 946.5 (calc. for $C_4H_6O_4$: 950.6) and m.p. 182–183°, after being washed with a little cold ether. The melting point was unaltered when the substance was admixed with a pure specimen of succinic acid. When the liquid portion was separated from the initial petroleum ether insoluble portion and kept at room temperature, there was formed a deposit of crystalline solid which was separated from the liquid portion. Further standing of the liquid portion gave another quantity of crystalline solid. Both deposits of crystalline solid thus obtained were considered to be succinic acid as they melted at about 180°. Hence the liquid portion is believed to contain semi-aldehyde of succinic acid which gives crystalline succinic acid by the oxidation in air.

3. **Oily Substances (C).** These were neutralised with sodium carbonate solution, and the neutral substances were removed by extraction with ether. The acidic substances liberated from their sodium salts on acidification were taken up with ether.

The neutral substances (3.1 g.), when oxidised with alkaline permanganate and then acidified, yielded an oily product which proved to be amyl hydrogen succinate having neutralisation value 296.3 and saponification value 592.1 (calc. for $C_9H_{16}O_4$: neutr. value 298.2 and saponif. value 596.5). On recrystallising from ethyl acetate, the free acid liberated from this ester in the usual way yielded succinic acid; neutr. value 945.2, m.p. 182–183°.

The acidic substances (3.4 g.) showed neutr. value 286.5 and saponif. value 590.1, and seemed to consist principally of amyl hydrogen succinate. The free acid liberated from this ester yielded succinic acid which had neutr. value 947.2 and m.p. 180–181° after recrystallisation from ethyl acetate.

Summary.

The eicosatetraenoic acid separated from sardine oil was converted into the amyl ester, and the latter was subjected to ozonolysis. Among the products of ozonolysis were found: butyric acid, butyric aldehyde, succinic acid and amyl hydrogen succinate. The presence of succinic semi-aldehyde, succinic aldehyde and amyl ester of succinic semi-aldehyde was also indicated. Accordingly the constitution of the eicosatetraenoic acid is shown to be $\Delta^{4:5,\ 8:9,\ 12:13,\ 16:17}$-eicosatetraenoic acid as expressed by the following formula:

$$CH_3\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot COOH.$$

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