THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL.

II. THE CONSTITUTION OF HIRAGONIC ACID $C_{16}H_{26}O_2$.*

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In the previous paper,(1) the authors described the isolation of hiragonic acid $C_{16}H_{26}O_2$ from Japanese sardine oil. The present paper records the results of the experiments concerning the constitution of this acid.

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* Translated from the paper published in Japanese in the Report of Tokyo Imperial Industrial Research Laboratory, **27** (1932), No. 2.

(1) Toyama and Tsuchiya, this Bulletin, **4** (1929), 83.

For the determination of the positions of three ethylenic linkings in hiragonic acid, its methyl ester was oxidised by the permanganate method and also by the ozonide method, and the products were examined. Among the oxidation products of methyl hiragonate by the permanganate method, acetic acid $CH_3{\cdot}COOH$, succinic acid $HOOC{\cdot}(CH_2)_2{\cdot}COOH$, and methyl hydrogen adipate $HOOC{\cdot}(CH_2)_4{\cdot}COOCH_3$ were identified. The products obtained by the ozonide method contained, besides the above-mentioned three compounds, acetaldehyde $CH_3{\cdot}COH$ and semi-aldehyde of methyl hydrogen adipate $HOC{\cdot}(CH_2)_4{\cdot}COOCH_3$, and also the presence of semi-aldehyde of succinic acid $HOC{\cdot}(CH_2)_2{\cdot}COOH$ and succinic aldehyde $HOC{\cdot}(CH_2)_2{\cdot}COH$ (or its polymerised products) was indicated. It is seen from these results that the free hiragonic acid has the groups: $CH_3{\cdot}CH=$, $=CH{\cdot}(CH_2)_2{\cdot}CH=$ and $=CH{\cdot}(CH_2)_4{\cdot}COOH$, of which hiragonic acid should contain two of the $=CH{\cdot}(CH_2)_2{\cdot}CH=$ group, since it has three ethylenic linkings. This is also verified by the fact that the actual yield of the oxidation products having four carbon atoms was considerably higher than the value obtainable by assuming only one $=CH{\cdot}(CH_2)_2{\cdot}CH=$ group. The constitution of hiragonic acid was thus established as $\Delta^{6:7,\,10:11,\,14:15}$- hexadecatrienoic acid which is expressed by the following formula:

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

Besides hiragonic acid, two other unsaturated C_{16}-acids have hitherto been found with certainty in natural fatty oils. These are zoomaric acid, which occurs widely in marine animal oils, and lycopodium-oleic acid in lycopodium oil. In comparison of these two acids, it should be mentioned here that the positions of three ethylenic linkings in hiragonic acid differ entirely from those in zoomaric and lycopodium-oleic acids. Whilst the ethylenic linking of zoomaric acid(2) lies in 9:10-position and that of lycopodium-oleic acid(3) in 12:13-position, hiragonic acid has three ethylenic linkings in 6:7-, 10:11- and 14:15-positions, but no ethylenic linking in 9:10- or in 12:13-position.

Experimental.

1. Preparation of Methyl Hiragonate. For the preparation of methyl hiragonate used in these experiments, a methyl ester fraction containing a large amount of highly unsaturated C_{16}- and C_{18}-acids as their methyl esters was prepared first from the sardine oil, and it was then converted into bromides, from which the ether insoluble,

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(2) Toyama, *J. Soc. Chem. Ind. Japan*, **30** (1927), 603.

(3) K. H. Bauer and W. Piners, *Pharm. Zentralhalle*, **71** (1930), 33.

but benzene soluble portion was separated. Debromination of this portion followed by a repeated fractionation gave a fraction corresponding to methyl hiragonate.

(i) *Preparation of methyl ester fraction in which highly unsaturated* C_{16}- *and* C_{18}-*acid are concentrated.* The Japanese sardine oil used in these experiments had d_4^{20} 0.9265, n_D^{20} 1.4795, saponification value 190.0, iodine value by the Rosenmund-Kuhnhenn method 164.0, and the mixed fatty acids yielded 54.14% of ether insoluble bromides. It was converted into ethyl esters(4) by ethanolysis, and the latter were subjected to the distillation, by which the fraction boiling below 215°/10 mm. and the fraction boiling over 215°/10 mm. were collected separately. Whilst the fraction boiling below 215°/10 mm. was used in these experiments, the higher fraction was used for the examination of highly unsaturated C_{20} and C_{22}-acids which will be reported later. Sixty kg. of the ethyl esters gave 28.5 kg. of the fraction boiling below 215°/10 mm. This was converted into free fatty acids by saponification followed by acidification in the usual way, the latter were dissolved in 5 times of their weight of 85% methanol, allowed to cool down to about 10°, and the separated crystals consisting largely of saturated fatty acids were filtered. The filtrate was neutralised with aqueous lithium hydroxide solution (2.5%), the insoluble lithium soaps formed were removed by filtration, and on acidification of the filtrate there were obtained 7.5 kg. of 85% methanol, allowed to cool down to about 10°, and the separated crystals consist- jected to fractional distillation, which yielded 5.9 kg. of a fraction boiling below 215°/15 mm. It had saponif. value 199.6 and iodine value by the Rosenmund-Kuhnhenn method 182.8, and was considered to contain a large proportion of highly unsaturated C_{16}- and C_{18}- acids as their methyl esters.

(ii) *Preparation of ether insoluble bromides and separation of the bromides by means of benzene.* From the methyl ester fraction boiling below 215°/15 mm., the ether insoluble bromides were prepared in the following manner: 100 g. of the methyl ester fraction was dissolved in 1 l. of ether and cooled to –10°. Bromine, dissolved in an equal part of glacial acetic acid, was added with constant stirring until the brown colouration remained permanent. After standing for 15 hours at 2–3°, the supernatant ethereal solution was removed by decantation, the residual precipitate of insoluble bromides was washed 2 to 3 times with cold ether by decantation as before, and then transferred on the filter where it was washed thoroughly until the washings became colourless. In total, about 2 l. of ether was used for washings. The precipitate appeared to be soluble in ether to a certain extent. For the recovery of the solid bromides dissolved in the washings, the united washings were treated with sodium thiosulphate to remove the excess of bromine, concentrated by distilling off a portion of ether, and on cooling the solid bromides separated from the solution. In our previous experiment,(5) the precipitate of solid bromides to be recovered was filtered and washed with ether, but a large amount of ether was required in order to remove oily bromides which adhere tenaciously to the precipitate, and the washing of precipitate was not successful as the precipitate itself dissolved in ether before it was completely washed. In this experiment, the precipitate was filtered and it was boiled with five volumes of alcohol, then cooled down to room temperature and filtered with suction. By repeating the washing in this way with the use of alcohol four times, the filtrate became

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(4) Excepting this case, the methyl esters were used instead of the ethyl esters.

(5) This Bulletin, **4** (1929), 83.

colourless and a white powder of bromide was obtained. The latter was added to the main portion of ether insoluble bromides obtained before. The ether insoluble bromides thus obtained were then treated with benzene in the following manner: 20 g. of ether insoluble bromides were refluxed with 1.5 l. of benzene for about 40 minutes, and filtered while hot. From the filtrate, the dissolved bromides were recovered by distilling off benzene. By these treatments, 5.9 kg. of the methyl ester fraction yielded 1.5 kg. of benzene soluble, but ether insoluble bromides and 900 g. of benzene insoluble bromides.

(iii) *Debromination of benzene soluble, but ether insoluble bromides and fractionation of debrominated products.* For the debromination of benzene soluble bromides, 100 g. of the bromides was mixed with 50 g. of zinc powder, and the mixture was dropped gradually into a flask containing 150 c.c. of alcohol. The flask was immersed in cold water to avoid a violent heat evolution. When no further evolution of heat occurred, the flask was placed on the water-bath, and 150 c.c. of 5N alcoholic solution of hydrogen chloride was added in the course of 1.5 hours; during the reaction further 50 g. of zinc powder was added in several portions. After all the alcoholic solution of hydrogen chloride has been added, the solution was refluxed for another 1 hour, and the filtered. The residue, which seemed to contain some unchanged bromides, was heated with 100 c.c. of alcohol and 25 g. of zinc powder on the water-bath with an addition of 50 c.c. of alcoholic solution of hydrogen chloride during 30 minutes. After heating for one hour more, the alcoholic solution was filtered. The alcoholic solutions and the washings obtained by these treatments were combined and the debrominated product which was separated by an addition of a large quantity of water was taken up with ether. Since it was suspected that the debrominated product might contain a small portion of unsaponifiable substance, it was saponified, the unsaponifiable substance was removed by extraction with ether, and the fatty acids liberated from the soap solution on acidification were reconverted into methyl esters. These were subjected to a repeated fractionation, by which a fraction boiling at 186–188°/15 mm. was finally obtained as methyl hiragonate; yield 58 g. It had the following constants: d_4^{15} 0.9155, d_4^{20} 0.9122, n_D^{15} 1.4783, n_D^{20} 1.4764, molecular refraction 81.74 (calc. for $C_{17}H_{28}O_2|\!\overline{=}_3$: 80.96), saponif. value 212.8 (calc. 212.4), iodine value by the Rosenmund-Kuhnhenn method 288.7 (calc. 288.2). On brominating in ethereal solution, it gave an ether-insoluble bromide (yield 57%) which melted at about 180° to a light yellow liquid (Found: Br, 64.61. Calc. for $C_{17}H_{28}O_2Br_6$: Br, 64.47%).

Hiragonic acid was obtained on saponification of its methyl ester followed by acidification (Found: C, 76.69; H, 10.32. Calc. for $C_{16}H_{26}O_2$: C, 76.74; H, 10.47%). It had the following constants: d_4^{15} 0.9330, d_4^{20} 0.9296, n_D^{15} 1.4870, n_D^{20} 1.4850, molecular refraction 77.13 (calc. for $C_{16}H_{26}O_2|\!\overline{=}_3$: 76.22), neutralisation value 223.1 (calc. 224.2), iodine value by the Rosenmund-Kuhnhenn method 303.1 (calc. 304.4). Bromination of hiragonic acid in ethereal solution gave an ether insoluble bromide (yield 57%) which melted at about 190° (Found: Br, 65.84. Calc. for $C_{16}H_{26}O_2Br_6$: Br, 65.71%). Hydrogenation of hiragonic acid yielded palmitic acid which after recrystallisation from 90% alcohol showed neutralisation value 218.5 (calc. 219.0), m.p. and mixed m.p. 62.5°. It should be noted here that hiragonic acid and its methyl ester showed a slight exaltation of molecular refraction; the cause of this exaltation is unknown to us.

2. Oxidation of Methyl Hiragonate by the Permanganate Method. Methyl hiragonate (20 g.) was dissolved in 200 g. of acetone, the solution was gently boiled.

and 200 g. of powdered potassium permanganate was added in the course of 5 hours. When all the permanganate has been added, the solution was continued to reflux for 5 hours more, and then the acetone was removed by distillation. The residue was mixed with 400 c.c. of water, and a current of sulphur dioxide was passed through the mixture until the excess of permanganate and the insoluble oxides of manganese have disappeared completely. An oily portion (**A**), which separated from the aqueous solution, was collected on a filter, and the filtered solution was shaken with 2 l. of ether and allowed to separate into two layers. For a further recovery of the oxidation products dissolved in the aqueous layer, alcohol was added until the greater portion of inorganic salts separated out from the solution, and the filtered solution was then neutralised with alkali, evaporated to dryness, acidified with hydrochloric acid, and extracted with 300 c.c. of ether. The ethereal solutions were united and the ether distilled off. The residue was extracted three times with petroleum ether, using 150 c.c. for each extraction, and separated into petroleum ether soluble portion (**B**) and petroleum ether insoluble portion (**C**). The three portions thus obtained were examined separately.

(i) *Oily portion* (**A**). Yield 10.5 g. This portion contained neutral substance which escaped oxidation, besides acidic substance. Sodium carbonate solution was added to this portion, and the resulting soap solution was treated with ether, by which 2.5 g. of neutral substance was obtained from the ethereal extract. The soap solution yielded on acidification an oily liquid having neutr. value 338.5 and saponif. value 691.6 (calc. for methyl hydrogen adipate $C_7H_{12}O_4$: neutr. value 350.5 and saponif. value 700.9). The free acid obtained on saponification followed by acidification consisted of a crystalline solid which crystallised from ethyl acetate in needles; neutr. value 767.1 (calc. for adipic acid $C_6H_{10}O_4$: 768.2), m.p. 152.5–153°, which was unaltered when admixed with an authentic specimen of adipic acid (Found: C, 49.09; H, 6.95. Calc. for $C_6H_{10}O_4$: C, 49.29; H, 6.90%).

(ii) *Petroleum ether soluble portion* (**B**). The petroleum ether solution was heated on the water-bath until the petroleum ether was driven off, and then the residue (2.8 g.) heated in an oil-bath. About 1 g. of a colourless liquid distilled before the temperature of the bath reached 150°, beyond which nearly nothing distilled out; and when the temperature of the bath reached about 210°, the residue showed an indication of decomposition. The petroleum ether which distilled first showed acid reaction. On neutralising it with barium hydroxide solution, it precipitated barium acetate (Found: Ba, 53.66. Calc. for $C_4H_6O_4Ba$: Ba, 53.79%). The distillate boiling below 150° (temperature of bath) was found to consist of acetic acid, since silver acetate was obtained by neutralising with sodium hydroxide and then adding silver nitrate (Found: Ag, 64.59. Calc. for $C_2H_3O_2Ag$: Ag, 64.64%).

(iii) *Petroleum ether insoluble portion* (**C**). Yield 12.8 g. This portion consisted of a crystalline solid, and on recrystallisation from ethyl acetate it gave succinic acid; neutr. value 948.6 (calc. 950.6), m.p. and mixed m.p. 182.5–183° (Found: C, 40.60; H, 5.22. Calc. for $C_4H_6O_4$: C, 40.66; H, 5.12%). It is considered that this portion consists almost exclusively of succinic acid, and its yield is about 64% of the methyl hiragonate used for the oxidation. This is considerably higher than the theoretical yield of succinic acid, 44.68%, with the assumption that methyl hiragonate has only one $=CH\cdot(CH_2)_2\cdot CH=$ group.

3. Ozonolysis of Methyl Hiragonate. Methyl hiragonate (5 g.) was dissolved in chloroform (50 c.c.), and on cooling with ice-salt, a current of ozonised oxygen was passed through the solution until it became saturated. On distilling off the solvent under reduced pressure, the ozonide remained as a light yellow sirup. Twenty g. of methyl hiragonate yielded 32.2 g. of ozonide (161.0%). The yield calculated for normal ozonide $C_{17}H_{28}O_{11}$ and ozonide peroxide $C_{17}H_{28}O_{12}$ are 154.5 and 160.6%, respectively. The ozonide obtained here is probably ozonide peroxide. Water (200 c.c.) was added to the ozonide, and the liquid was heated in a flask on the water-bath for 30 minutes, a gentle current of hydrogen being passed into the flask. In order to recover the volatile decomposition products (**A**) which were carried over with hydrogen, the flask was attached by a delivery tube to other three flasks which were connected in succession, the first (**a**) being filled with 200 c.c. of water and cooled with ice, the second (**b**) and the third (**c**) being filled with 400 c.c. of approximately 1/3 N barium hydroxide solution. The liquid remained in the initial flask consisted of aqueous solution and reddish orange oil ($\mathbf{B}_1$) which separated on the bottom of the flask. These were separated, and the clear aqueous solution was extracted with 2 l. of ether. After distilling off ether from the ethereal solution, 50 c.c. of water was added to the residue, of which a portion remained undissolved as an orange yellow oil ($\mathbf{B}_2$), and was separated from the aqueous solution (**C**). By these treatments, the decomposition products of ozonide were separated into (i) volatile substance (**A**), (ii) oily substance ($\mathbf{B}_1$ and $\mathbf{B}_2$) and (iii) the portion dissolved in water (**C**).

(i) *Volatile substance* (**A**). The aqueous solution of volatile substance collected in the flask (**a**) produced a pink colouration with Schiff's reagent, and a deep blue colouration with diethylamine and sodium nitroprusside, indicating that acetaldehyde is present in the aqueous solution. On adding *p*-nitrophenylhydrazine in hydrochloric acid, *p*-nitrophenylhydrazone of acetaldehyde separated, which crystallised from 50% alcohol in yellow needles; m.p. 126.5–127°, which was not lowered when admixed with a pure specimen, m.p. 127.5–128° (Found: N, 23.23. Calc. for $C_8H_9O_2N_3$: N, 23.46%). The aqueous solution showed also acid reaction, and gave a deep red colouration on adding ferric chloride after neutralisation. The barium salt, obtained from the solution by neutralisation with barium hydroxide, was found to be barium acetate (Found: Ba, 53.50. Calc. for $C_4H_6O_4Ba$: Ba, 53.79%).

The barium hydroxide solution in the flask (**b**) was found to contain a small quantity of precipitate of barium carbonate (0.1528 g.), which indicated the formation of carbon dioxide by the decomposition of ozonide. But the yield of carbon dioxide calculated from the quantity of precipitated barium carbonate is only 0.17% of the methyl hiragonate used for ozonolysis. If a highly unsaturated acid having the $=CH{\cdot}CH_2{\cdot}CH=$ group is subjected to ozonolysis, carbon dioxide is formed by secondary decomposition of the oxidation products derived from that group thus: $HOOC{\cdot}CH_2{\cdot}COOH \rightarrow CH_3{\cdot}COOH+CO_2$ and $HOC{\cdot}CH_2{\cdot}COOH \rightarrow CH_3{\cdot}COH+CO_2$. It is, however, found from the results of our experiments which will be reported later that the secondary decomposition takes place to a considerable extent, though not to a quantitative extent, and it never gives such a small yield of carbon dioxide. Consequently, the formation of carbon dioxide in such a minute amount as in the case of above experiment gives an indication of the absence of the $=CH{\cdot}CH_2{\cdot}CH=$ group in methyl hiragonate; it is probably due either to the contamination of highly unsaturated acids having the $=CH{\cdot}CH_2{\cdot}CH=$ group or to an abnormal decomposition of a small portion of the ozonide of methyl hiragonate.

(ii) *Oily substance* (**B**$_1$ and **B**$_2$). Yield 10.9 g. It was separated into neutral and acidic portions by adding sodium hydroxide solution and extracting the resulting soap solution with ether. The neutral portion (2.4 g.) obtained from the ethereal solution had saponif. value 379.0 (calc. for semi-aldehyde of methyl hydrogen adipate $C_7H_{12}O_3$: 389.4). This was saponified and then oxidised with potassium permanganate in alkaline solution. The crystalline products obtained on acidification yielded pure adipic acid after recrystallising from ethyl acetate; neutr. value 766.8 (calc. for $C_6H_{10}O_4$: 768.2), m.p. and mixed m.p. 152–153°.

The acidic portion (8.5 g.) which was obtained from the soap solution on acidification and was collected by using ether, had neutr. value 340.0 and saponif. value 690.2 which are close to the corresponding values for methyl hydrogen adipate (neutr. value 350.5 and saponif. value 700.9). Adipic acid liberated from the ester in the usual way showed neutr. value 767.0 and m.p. 152–153° after recrystallising from ethyl acetate (Found: C, 49.16; H, 6.91. Calc. for $C_6H_{10}O_4$: C, 49.29; H, 6.90%).

(iii) *The portion dissolved in water* (**C**). Sodium carbonate solution was added to the aqueous solution of this portion, and the resulting soap solution was shaken with 2 l. of ether and allowed to separate into two layers. After removal of the ether from the ethereal solution, there remained 1.2 g. of neutral substance (**C**$_1$). The soap solution was decomposed with hydrochloric acid, extracted with 2 l. of ether, and on distilling off the ether from ethereal extract, 17 g. of acidic substance (**C**$_2$) was obtained.

The neutral substance (**C**$_1$) showed aldehyde reaction. It was subjected to a further oxidation with alkaline permanganate solution, and the product, which after acidification was collected by using ether, was treated with a little water in which a portion remained undissolved. The insoluble portion had neutr. value 342.6 and saponif. value 691.2 (calc. for methyl hydrogen adipate: neutr. value 350.5 and saponif. value 700.9), and the free acid obtained on acidification of the saponified product melted at 149–150° and was considered to be an impure adipic acid. The aqueous extract on evaporation left behind a crystalline solid which melted at 175°, and it was likely to be an impure succinic acid. From these experiments, it is seen that the neutral substance (**C**$_1$) contains semi-aldehyde of methyl hydrogen adipate and succinic aldehyde (or its polymerised products).

The acidic substance (**C**$_2$) was extracted three times with petroleum ether, using 150 c.c. for each extraction. The united petroleum ether solution was heated on the water-bath to remove petroleum ether. The distilled petroleum ether showed an acid reaction and had an odour of acetic acid· The barium salt, obtained on neutralising the petroleum ether with barium hydroxide, was found to consist of barium acetate (Found: Ba, 53.60. Calc. for $C_4H_6O_4Ba$: Ba, 53.79%). After distilling off petroleum ether, the residue (2 g.) was heated in an oil-bath, and a small quantity of distillate was obtained before the temperature of oil-bath reached 150°. Beyond 150°, practically nothing distilled over, and when the temperature of bath reached about 200°, the distillation was stopped since there were the indications of decomposition. The silver salt, prepared from the distillate by neutralising with sodium hydroxide and adding silver nitrate, was found to consist of silver acetate (Found: Ag, 64.50. Calc. for $C_2H_3O_2Ag$: Ag, 64.64%). The final residue from distillation was oxidised with alkaline permanganate solution, and the product obtained after acidification was found to be an impure succinic acid, m.p. 177–177.5°.

The petroleum ether insoluble portion (14.5 g.) consisted of a mixture of crystalline solid and liquid. It was oxidised with alkaline permanganate solution, and the product obtained after acidification was recrystallised from ethyl acetate to yield succinic acid; neutr. value 948·7, m.p. 182.5–183° (Found: C, 40·61; H, 5·10. Calc. for $C_4H_6O_4$: C, 40.66; H, 5.12%). Twenty g. of methyl hiragonate gave 14.5 g. or 72.5% yield of petroleum ether insoluble portion. Since it is considered that the petroleum ether insoluble portion consists almost entirely of succinic acid and its semi-aldehyde, methyl hiragonate should have two $=CH\cdot(CH_2)_2\cdot CH=$ group. (The maximum yield of succinic acid will be 44.68% if it has only one of such group.)

Summary.

Methyl hiragonate has been prepared from Japanese sardine oil, and it was subjected to the oxidation by the permanganate method and by the ozonide method. Among the oxidation products obtained by the permanganate method, succinic acid, acetic acid and methyl hydrogen adipate were identified. The products obtained by the ozonide method contained, besides the above-mentioned three acidic compounds, acetaldehyde and semi-aldehyde of methyl hydrogen adipate, and also the presence of semi-aldehyde of succinic acid and succinic aldehyde (or its polymerised products) was indicated. Accordingly, the constitution of hiragonic acid is $\Delta^{6:7,\ 10:11,\ 14:15}$-hexadecatrienoic acid as expressed by the following formula:

$$CH_3\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_2\cdot CH=CH\cdot(CH_2)_4\cdot COOH$$

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