84. *Unsaturated Acids of Natural Oils. Part VII. Docosahexaenoic Acid, an Abundant Highly-unsaturated Acid of Cod-liver Oil.*

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The C_{22}-fraction isolated (in ester form) from the mixed di- and poly-olefinic acids of cod-liver oil by systematic evaporative distillation below 110° in a continuous "molecular" still gives every evidence of being composed of a single, structurally distinct acid. This acid, which displays hexaene unsaturation and yields pure behenic acid on complete hydrogenation, must be regarded as docosahexaenoic acid, $C_{22}H_{32}O_2$. Unlike all previously described polyene acids from fish oils, the acid shows no exaltation of the molecular refraction and hence is entirely unconjugated; moreover, unlike most of the previously isolated polyene acids, it has escaped alteration by heat.

The nature and proportions of the fission products obtained from the hexaene acid by ozonolysis and by oxidation agree with an unconjugated constitution and show definitely that the acid must possess one of five cited formulæ.

The highly unsaturated acids which occur together with saturated and mono-olefinic forms as glycerides in Japanese sardine oil, cod-liver oil, halibut-liver oil and other fish oils are highly sensitive to heat and below 200° begin to undergo changes which are in part polymerisations but appear to some extent to be isomerisations. As the result of experience gained several years ago by one of us with Dr. F. C. Webb (unpublished work) it became clear that ordinary fractional distillation of the mixtures of more highly unsaturated acids from Japanese sardine oil (even at the lowest pressures at which equilibrium distillation can satisfactorily be carried out, *i.e.*, 0·1—1 mm.) is hopelessly wasteful and inefficient, and the result is not improved by elaborating the routine of fractionation, by increasing the reflux ratio, or by making use of fractionating columns, since these all serve to increase the duration or intensity of heating. It is remarkable how meagre are the yields of the final fractionation products in those cases where the isolation of pure, or substantially pure, polyene acids has been claimed.*

One of the most unsatisfactory features of *all* the specimens of supposedly homogeneous tri- and poly-olefinic acids which have hitherto been isolated from fish oils is the appearance therein of abnormal refractivity: all the specimens show an exaltation of the molecular refraction approximating to 1 unit (see, for example, the values reported by Toyama and Tsuchiya, *Bull. Chem. Soc. Japan*, 1935, **10**, 232, 241, 433, 441, for acids derived from sardine oil; the refractivities observed by Farmer and Webb for sardine oil acids also showed exactly similar exaltations). Presumably the optical anomaly is due to conjugation, and since the original fish oils and their freshly derived acids appear to be entirely free from conjugated chains, as judged by their absorption spectra (Tsujimoto, *Bull. Chem. Soc. Japan*, 1928, **3**, 299; Edisbury, Morton, and Lovern, *Biochem. J.*, 1933, **27**, 1457; see also Morrell, *J. Soc. Chem. Ind.*, 1936, **55**, 101), the conclusion seems justified that the constitutions of the acids have become changed during heating.

In order to overcome the separative difficulties presented by (*a*) the complexity of the mixture of physically similar unsaturated acids in the fish oils and (*b*) the sensitivity of the acids or their esters to heat, we resorted to fractional evaporative ("molecular") distillation of the mixed di- and poly-olefinic acids, the acids being first separated from the accompanying saturated and mono-olefinic forms by fractional crystallisation of the mixed lithium salts prepared from the saponified oils (Tsujimoto's method) and then converted for ease of distillation into their methyl esters. Although the unsaturated ester fraction of each of several fish oils was examined, the present account is concerned only with that obtained from genuine cod-liver oil. Many practical details of the fractionations have been published elsewhere (*J. Soc. Chem. Ind.*, 1938, **57**, 24) together with comparisons of the products with those isolated by other workers; it is sufficient, however, here to state that sharp separation of the mixed acids (as their esters) from the cod-liver

* The supposedly pure clupanodonic acid ($C_{22}H_{34}O_2$) isolated by Toyama and Tsuchiya (*loc. cit.*, p. 433) from Japanese sardine oil amounted to less than 5 g. from 10 kg. of oil. Other yields of polyene acids reported in the literature are comparable.

oil into fractions consisting respectively of C_{16}-, C_{18}-, C_{20}-, C_{22}- and higher acids was accomplished, and of these only one, the C_{22}-fraction, appeared to be homogeneous as regards the degree of unsaturation of its material. The C_{22}-fraction was the most abundant of the series of fractions, and we regard it on the evidence discussed below as composed of a structurally homogeneous (but not necessarily geometrically homogeneous) compound.

The methyl ester of the C_{22}-acid consistently gave hydrogenation values corresponding exactly with hexaene unsaturation, and the reduced acid therefrom agreed immediately (*i.e.*, without recrystallisation) in melting point and *X*-ray diagram with behenic acid of a degree of purity greater than 99%. The oxidation results also are entirely consistent with the possession by the acid of a high degree of structural homogeneity. Thus the evidence leaves little doubt but that one of the chief highly unsaturated acids of cod-liver oil is docosahexaenoic acid, $C_{22}H_{32}O_2$, and it is to be noted that no *pentaene* C_{22}-acid identifiable with Tsujimoto's clupanodonic acid from Japanese sardine oil * is present as an original constituent in cod-liver oil.

On ozonolysis the methyl ester gave immediately acetaldehyde (uncontaminated with formic acid) and carbon dioxide, and the residues on further oxidation with hydrogen peroxide gave succinic acid, crystalline methyl hydrogen succinate, and some acetic acid; no oxalic acid, or dibasic acid other than succinic acid, was formed. The total absence of formaldehyde or formic acid, and of fatty aldehydes or acids higher than acetaldehyde or acetic acid, showed that one of the end groups of the polyene acid must be $CH_3{\cdot}CH{:}$; and the production of methyl hydrogen succinate showed the other end group to be ${:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$. Estimation of the proportion of acetic acid and succinic acid formed by complete oxidation of the *free* polyene acid gave the values 4·8 mols. and 1·3 mols. respectively; the proportion of carbon dioxide formed was through oversight not accurately measured, but it was observed to be in excess of 2·7 mols. (see p. 430).

In view of the large proportions of acetic acid and carbon dioxide thus formed and the absence of oxalic acid, it follows that a large proportion of the polyene acid was built up by repetition of the same group, $:CH{\cdot}CH_2{\cdot}CH:$, which characterises linoleic and linolenic acid: this conclusion, moreover, agrees with the fact that docosahexaenoic acid as now obtained by us, unlike the previously isolated polyene acids from fish oils referred to above, showed *no* exaltation of the molecular refraction. The molecule of *free* succinic acid formed (as distinct from the half ester) must of course be derived from a non-terminal $:CH{\cdot}[CH_2]_2{\cdot}CH:$ group.

The formula for docosahexaenoic acid must then contain four $:CH{\cdot}CH_2{\cdot}CH:$ groups, and these can be arranged between the above-mentioned terminal groups and in relation to the single group $:CH{\cdot}[CH_2]_2{\cdot}$ in five ways, shown in the formulæ (I)—(V):

(I) $CHMe[:CH{\cdot}CH_2{\cdot}CH]_4{:}CH{\cdot}[CH_2]_2{\cdot}CH{:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$

(II) $CHMe[:CH{\cdot}CH_2{\cdot}CH]_3{:}CH{\cdot}[CH_2]_2{\cdot}CH{:}CH{\cdot}CH_2{\cdot}CH{:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$

(III) $CHMe[:CH{\cdot}CH_2{\cdot}CH]_2{:}CH{\cdot}[CH_2]_2{\cdot}CH[:CH{\cdot}CH_2{\cdot}CH]_2{:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$

(IV) $CHMe{:}CH{\cdot}CH_2{\cdot}CH{:}CH{\cdot}[CH_2]_2{\cdot}CH[:CH{\cdot}CH_2{\cdot}CH]_3{:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$

(V) $CHMe{:}CH{\cdot}[CH_2]_2{\cdot}CH[:CH{\cdot}CH_2{\cdot}CH]_4{:}CH{\cdot}[CH_2]_2{\cdot}CO_2H$

Docosahexaenoic acid possesses one of these formulæ, but a decision between the five must await detailed examination of the constitution of the partial addition products of the hexaene acid.

As mentioned above, docosahexaenoic acid was observed by Toyama and Tsuchiya (*loc. cit.*, pp. 433, 539) and by Farmer and Webb to accompany clupanodonic acid in sardine oil, but neither group of workers isolated it in pure condition. The hexaene acid is with little doubt an original constituent of sardine oil and is probably identical with that which we have found to be an important constituent of cod-liver oil. The clupanodonic acid, on the other hand, we believe to be a secondarily formed product, probably derived from docosahexaenoic acid by the action of heat; for in fractional distillations which we have carried out on Japanese sardine oil esters (" molecular " still), no less than in those we have conducted on cod-liver oil esters, we have failed to recognise a pentaene C_{22}-fraction.

* Clupanodonic acid was first isolated from Japanese sardine oil by Tsujimoto. Later, Toyama and Tsuchiya (*loc. cit.*) and simultaneously Farmer and Webb observed that this pentaene C_{22}-acid was accompanied by a hexaene C_{22}-acid.

Experimental.

Isolation of Di- and Poly-ene Acids from Cod-liver Oil.—The freshly derived oil (6 kg.) from selected cod livers was diluted with methylated spirit (4 l.) and heated to 60°. To this solution an excess of 50% aqueous sodium hydroxide solution (1000 g. of sodium hydroxide) at 60° was added little by little with stirring. The mixture was simmered and stirred for 45 minutes, then cooled, and strongly acidified with 15% hydrochloric acid. The lower aqueous layer was syphoned off, the oily layer, consisting of free acids, washed successively with 15% hydrochloric acid (3 times) and with hot water (4 times), and then taken up in acetone (4 l.), and the solution dried overnight with calcium chloride (1 lb.).

The filtered acetone solution of free acids was further diluted with acetone (15 l.) and then treated at 50° very gradually (with stirring) with a slight excess of saturated aqueous lithium hydroxide solution (440 g. of lithium hydroxide in 3·2 l. of water) warmed to 50°. The alkaline acetone solution of lithium salts was simmered for 20 minutes and then diluted with acetone (about 45 l. necessary) until the total of the solvents added represented a mixture containing 95% of acetone and 5% of water. On standing overnight, the solution deposited a precipitate consisting of the bulk of the salts of the saturated and mono-olefinic acids. The precipitate was removed by centrifuging, and the filtrate freed from acetone by distillation: a solution of the lithium salts of the di- and poly-ene acids remained, and this was first freed from the unsaponifiable matter of the oil by thorough extraction with ether and then acidified with 15% hydrochloric acid. The mixture of free unsaturated acids was extracted with ether, and the ethereal solution washed with water, dried over sodium sulphate, and evaporated.

The di- and poly-ene acids were esterified by refluxing them for 3 hours with 8 times their volume of 4% methyl-alcoholic hydrogen chloride. A part (60%) of the alcohol was distilled from the product, and the residual material treated with ice. Two layers separated, of which the upper was separated, washed well with water and with sodium carbonate, and then taken up in ether. The well-dried (sodium sulphate) ethereal solution gave the mixed unsaturated acids as a rather thick oil. Yield, 1200 g.

The Unsaponifiable Matter.—The oily neutral material extracted from the lithium salts with ether deposited (i) a solid, crystallising in prisms, m. p. 148°, and forming with acetic anhydride an acetate, m. p. 113°, (ii) a very small amount of an amorphous solid, insoluble in most organic solvents, and (iii) a highly unsaturated, orange-yellow oil, b. p. 160—173°/0·5 mm. The crystalline product (i) gave the reactions of a sterol and agreed in m. p. and mixed m. p. with cholesterol * (cholesterol acetate melts at 115°); the other products have not been investigated.

The C_{22}-fraction of Unsaturated Acids.—Evaporative distillation of the mixed unsaturated acids in the form of their methyl esters at about 10^{-4} mm. pressure was conducted in a continuous "molecular" still by a technique which will be described elsewhere. The highest temperature employed was 110° and the total time of contact with the heated surface was about 60 secs. The fraction containing only the ester(s) of C_{22}-acid(s) amounted to 225 g., but a further small quantity was present in the lower and the higher connecting fractions; this fraction had $n_D^{20°}$ 1·49300, $[R_L]_D$ 107·25, hydrogen value 356 (Calc. for $C_{23}H_{34}O_2$ $|\bar{=}_6$: $[R_L]_D$ 107·2; hydrogen value 353·5).

No departure from hexaene unsaturation was observed when this fraction was afterwards divided into two portions by a further evaporative distillation; moreover, the ester gave *at once* on hydrogenation and evaporation of the solvent a saturated ester, m. p. 53°. This ester was in every way identical with pure methyl behenate (m. p. 54°) and the acid derived therefrom by saponification (m. p. 82°) was found to be behenic acid (m. p. 82°, mixed m. p. 82°). We are indebted to Dr. S. H. Piper for examining the *X*-ray spacing of this acid, and to Professor A. C. Chibnall for comparing the acid directly with synthetic behenic acid: from the results of these observations the acid is estimated to have a degree of purity exceeding 99%.

Oxidation of Methyl Docosahexaenoate.—The ester (5 g.) was dissolved in acetone (300 c.c.), the solution placed in a flask surmounted by a percolator containing an excess of powdered potassium permanganate, and the percolator fitted with a reflux condenser. The solvent was refluxed until the colour of permanganate in the reaction flask remained just permanent, the residual colour being then discharged by addition of a drop of perhydrol. The manganese mud and admixed salts were filtered off, dried, and thoroughly extracted with boiling water; the filtrate was distilled until only 20 c.c. of liquid remained in the flask. To this liquid residue

* A beautifully crystalline hydrocarbon (fine needles, m. p. 76°) appeared in the unsaponifiable matter isolated by Dr. Webb from Japanese sardine oil. This has now been definitely identified as cholesterilene (Found: C, 88·0; H, 12·05. Calc. for $C_{27}H_{44}$: C, 87·95; H, 12·05%), doubtless formed from cholesterol by the action of alcoholic hydrogen chloride, followed by distillation.

a few drops of 10% sodium carbonate solution were added, the product combined with the aqueous extract of the manganese mud, and the whole evaporated to dryness. The solid residue (A) was dissolved in excess of 25% sulphuric acid, and the solution distilled into a solution of sodium carbonate (with addition of more water to the flask at intervals) until no more acid passed over. The alkaline solution of the distillate was evaporated to dryness, and the residue acidified and thoroughly extracted with ether. The extract was dried, and the ether cautiously distilled under an efficient column. The acidic residue was distilled under a short column to free it from ether, and only a minute tarry residue remained in the flask. An aliquot portion of the distillate was neutralised with sodium carbonate, and the salt so formed converted into its *p*-bromophenacyl ester. This melted at once at 84° and on recrystallisation at 85°; it did not depress the m. p. (85°) of authentic *p*-bromophenacyl acetate. Formic acid was thus absent and any higher-boiling fatty acid contained in the small tarry distillate was entirely negligible in amount. Fractional distillation of the remainder of the (aqueous) acid distillate gave as the last fraction nearly anhydrous acetic acid, b. p. 114—116° (Found: *M*, monobasic, 59·7. Calc., 60·0). The unused fractions of acetic acid were united and diluted to a known volume, and aliquot parts of the solution titrated. The total yield of acetic acid estimated in this way was 3·13 g., *i.e.*, 1 mol. of ester yielded at least 4 mols. of acetic acid.

The aqueous liquid remaining after the volatile acids had been distilled off yielded on exhaustive continuous extraction with ether a crystalline acid, m. p. 178—184°. This was succinic acid and, when freed from a trace of oily matter, melted at 184° (mixed m. p. 184°).

To determine more accurately the proportions of acetic acid and succinic acid produced by oxidation, a further 3·42 g. (0·01 g.-mol.) of ester were oxidised with potassium permanganate in acetone as above. The solid residue (A) (see above) was dissolved in water and made up to 100 c.c.; then the acetic acid liberated by sulphuric acid from an aliquot part of this solution was estimated by a standard steam distillation–titration method. The succinic acid from the remainder of the solution of potassium salts was isolated as above, recrystallised, and weighed. Total yield of acetic acid, 3·42 g. (4·87 mols.); total yield of succinic acid, 1·75 g. (1·30 mols.).

Ozonolysis of Methyl Docosahexaenoate.—The ester (4 g.) was ozonised in chloroform (20 c.c.) at 0—5°. The ozonide, when freed from solvent, was a colourless thick syrup. This was decomposed by gradual warming with water to 90°, the volatile decomposition products being swept by a stream of hydrogen through a train of five wash-bottles containing dimedon reagent (2 bottles) and freshly prepared 5N-baryta (3 bottles). The combined contents of the first two bottles, when treated with a drop of acetic acid and warmed slightly, yielded a copious precipitate which melted *at once* at 138—139°. This was the dimedon derivative of acetaldehyde (mixed m. p. with authentic specimen, 138—139°). Thus the volatile aldehydic product was free from formaldehyde.

The production of carbon dioxide from the ozonide began in the cold and continued briskly when the reaction flask was warmed. From the amount of barium carbonate formed in the last three wash-bottles, the amount of carbon dioxide passing through the latter was computed to be 2·7 mols. A proportion, however, of the carbon dioxide had been absorbed in the dimedon reagent, which contained sodium carbonate solution.*

The aqueous liquid remaining in the decomposition flask contained a yellowish oil, which was separated by decantation of the aqueous layer. This oil was taken up in ether, and the ethereal solution dried and evaporated. Yield of oil, 1 g. The aqueous liquid, when extracted thoroughly with ether and the solution dried and cautiously distilled (column), yielded a liquid residue. This was repeatedly extracted with light petroleum, the undissolved yellow oil combined with the preceding oily product, and the petroleum extract evaporated. From the petroleum extract acetic acid (*p*-bromophenacyl ester, m. p. 84°) was obtained, which after distillation was estimated by titration. Yield, 3·1 mols. The yellow oil was treated overnight with dilute perhydrol, then taken up in ether, and the solution (after drying) allowed to evaporate slowly. A portion of the oil solidified, yielding crystals of methyl hydrogen succinate, m. p. 57°, which on hydrolysis gave succinic acid; the remainder of the oil was directly hydrolysed, yielding a further quantity of succinic acid.

Continuous extraction of the aqueous portion of the decomposition product with ether gave succinic acid in an amount corresponding to 0·46 mol.

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* Through an oversight the complete yield of carbon dioxide was not determined.