- Exley, D. (1956). *Biochem. J.* **63**, 496.
 Haworth, W. N., Kent, P. W. & Stacey, M. (1948). *J. chem. Soc.* p. 1220.
 Holter, H. (1943). *C.R. Lab. Carlsberg (Sér. chim.)*, **24**, 18, 399.
 Horowitz, H. N., Ikawa, M. & Fling, M. (1950). *Arch. Biochem.* **25**, 226.
 Hughes, D. E. (1951). *Photoelect. Spectr. Gr. Bull.* **5**, 121.
 Johnston, J. P., Ogston, A. G. & Stanier, J. E. (1951). *Analyst*, **76**, 88.
 Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of Amino-sugars*, 1st ed. London: Butterworths.
 Kirk, P. L. (1950). *Quantitative Ultra-micro Analysis*. New York: John Wiley and Sons Inc.
 McClean, D. (1943). *Biochem. J.* **37**, 169.
 McCrea, J. F. (1951). *Biochem. J.* **48**, xlix.
 McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
 Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
 Newth, F. H. (1951). *Advanc. Carbohydr. Chem.* **6**, 83.
 Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
 Schloss, B. (1951). *Analyt. Chem.* **23**, 1321.
 Vasseur, E. & Immers, J. (1949). *Ark. Kemi*, **1**, 253.

South African Pilchard Oil

6. THE ISOLATION AND STRUCTURE OF A DOCOSAHEXAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL*

By J. M. WHITCUTT†

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa

(Received 25 February 1957)

Docosahexaenoic acid has been reported many times in marine oils. Toyama & Tsuchiya (1935) isolated such an acid from sardine oil, and suggested it was either the 4:8:12:15:18:21- or the 4:8:11:14:17:20-hexaene. Farmer & Van den Heuvel (1938) isolated it from cod-liver oil, and gave five possible structures, all with the end double bonds in positions 4 and 20. Matsuda (1942) obtained a docosahexaenoic acid from bonito oil, and proposed a $\Delta^{4:8:12:15:18:21}$ structure for it. Notevarp, Roald, Sletness & Nordli (1953) have reported the isolation of docosahexaenoic acid from herring oil, while Abu-Nasr & Holman (1954) obtained it from cod-liver oil and determined some of the properties of the ethyl ester.

Docosahexaenoic acids have also been obtained from other natural sources. In each the 4:7:10:13:16:19-hexaene structure has been proposed. Klenk & Lindlar (1955) isolated one from the glycerophosphatides of ox brain, and Klenk & Dreike (1955) have reported the presence of the acid in ox-liver phospholipid. A similar acid was isolated by Hammond & Lundberg (1953*b*) from hog-brain lipids. These authors inferred the structure on biogenetic grounds and from alkali-isomerization evidence.

The isolation of a docosahexaenoic acid from South African pilchard oil (*Sardina ocellata* Jenyns) is now reported. The methods of separation and identification were similar to those described in earlier papers of this series (see Silk & Hahn, 1954*b*; Whitcutt & Sutton, 1956). The main product

is considered to be the all-*cis* form of n-docosa-4:7:10:13:16:19-hexaenoic acid. The acid is believed to be between 84 and 90% pure.

EXPERIMENTAL AND RESULTS

Melting points are uncorrected. General methods have been described in a previous paper (Whitcutt & Sutton, 1956). Ultraviolet spectra were determined on a Unicam model SP.500 spectrophotometer and infrared spectra on a Perkin-Elmer model 21 double-beam infrared spectrophotometer.

Isolation of the docosahexaenoic acid

Preparation of an unsaturated acid concentrate. Total pilchard-oil acids (967 g.) were fractionated by means of the lithium salt-acetone and urea-complex procedures to give 254 g. of unsaturated acid concentrate (equiv. wt., 310; iodine value, 395). The procedures were the same as those described by Silk, Sephton & Hahn (1954), except that the precipitation of the acids forming urea complexes was carried out in a single stage.

Esterification of the acid concentrate. The concentrate (232 g.) was dissolved in methanol (1 l.), boiled at 60 mm. pressure for $\frac{1}{2}$ hr. with Norit F.Q.P. decolorizing charcoal (20 g.), filtered and allowed to stand for several hours at 0° in the presence of excess of diazomethane in ether. After removal of diazomethane and some ether, the product was separated between pentane and 10% aq. Na_2CO_3 . Methyl esters (232 g.) were recovered from the pentane layer.

Molecular distillation of the unsaturated esters. The ester concentrate was subjected to three-stage molecular distillation in the still described by Sutton (1953), the temperature and the rate of flow being adjusted so that about one-third of the material was removed as distillate after each cycle of three distillations. The fractions shown in Table 1 were obtained.

* Part 5: Whitcutt & Sutton (1956).

† Marine Oils Research Fellow.

Distillate 4 (Table 1) was mixed with 100 ml. of 8% methanolic KOH at -70° and allowed to stand with occasional shaking for 4 hr. at 30° under vacuum. Water (100 ml.) was then added and the solution extracted twice with pentane to remove unchanged ester. After acidification with HCl and further extraction with pentane, 11.5 g. of a clear yellow oil was obtained. This material (distillate-4 acids) had equiv. wt., 333; iodine value, 424.

Reversed-phase partition chromatography of distillate-4 acids. The conditions were the same as those described by Whitcutt & Sutton (1956). The graph obtained when portions of the column eluate were titrated with methanolic 0.01 N-KOH is shown in Fig. 1. The acids were recovered by diluting each fraction with an equal volume of water and extracting with pentane (see Silk & Hahn, 1954*b*). In all, about 500 mg. of fraction II was recovered from six chromatograms. Corresponding samples were not mixed but were assumed to be identical.

Fraction I was found to consist of the eicosapentaenoic acid described by Whitcutt & Sutton (1956).

Properties and structure of the docosaheptaenoic acid

General properties. The docosaheptaenoic acid recovered from fraction II was a clear, faintly yellow oil of m.p. -44.5 to -44.1° , refractive index n_D^{20} 1.5017 [Found: C, 80.5, H, 9.7%. No. of double bonds/mol., 5.96 (hydrogen

uptake over Pd-BaSO₄); equiv. wt., 324; iodine value, 446. C₂₂H₃₂O₂ requires C, 80.4; H, 9.8%. No. of double bonds/mol., 6.00; equiv. wt., 328; iodine value, 464.]

A Kuhn-Roth determination (Eisenbraun, McElvain & Aycock, 1954) gave the number of C-Me groups/mol. as 1.02.

Hydrogenation product. Chain-length analysis of the above hydrogenation products (Silk & Hahn, 1954*a*) showed the presence of only C₂₂ saturated acid.

The unsaturated acid (60 mg.) was hydrogenated over Pd-BaSO₄ in acetic acid. The product was filtered, extracted with pentane and crystallized twice from acetic acid. This material was used in the melting-point determinations below. The residues were recrystallized from acetone and used in all other determinations.

The saturated acid had equiv. wt. 342, m.p. $79.0-79.3^{\circ}$, compared with equiv. wt. 340, m.p. 79.95° for *n*-docosanoic acid (Francis & Piper, 1939). Mixed with synthetic *n*-docosanoic acid (m.p. $79.3-79.9^{\circ}$), it had m.p. $79.0-79.5^{\circ}$.

The X-ray long spacing of the product was $48.2 \text{ \AA}$ compared with $48.0 \text{ \AA}$ for synthetic *n*-docosanoic acid. Francis & Piper (1939) give a value of $48.3 \text{ \AA}$ for the *C* modification of *n*-docosanoic acid.

The X-ray powder patterns and infrared spectra in carbon disulphide of the hydrogenation product and of our synthetic acid showed no significant differences.

Oxidation products. The conditions of oxidation were similar to those described by Whitcutt & Sutton (1956). The unsaturated acid (30 mg.) was dissolved in dry ethyl acetate (5 ml.) and acetic anhydride (1 ml.) and cooled to -80° . Ozonized oxygen (3%) was passed through the solution at 120 ml./min. until a faint blue colour appeared. A stream of nitrogen was then blown through for 45 sec., after which acetic anhydride (1 ml.), H₂O₂ (1 ml. of '130 vol.') and acetone (3 ml.) were added with shaking. The solution stood overnight at 30° and was then boiled for 30 sec. and most of the solvent removed under vacuum. Potassium hydroxide solution (3 ml. of 25%, w/v) was added, and the product

Table 1. *Molecular distillation of the unsaturated ester concentrate at 96°*

Distillate	Wt. (g.)
1	135
2	30
3	17
4	13
Residue	30

Fig. 1. Chromatogram of acids from distillate 4 (Table 1). Weight of sample, 500 mg. Column, heptane supported on non-wetting kieselguhr, 280 cm. $\times$ 1.5 cm. Temperature, 10° . Flow rate, 100 ml./hr. Solvent system: aq. methanol 70% (v/v) to 415 ml. of eluate, 70% (v/v) to 1200 ml., 75% (v/v) to 1265 ml., 80% (v/v) to 2600 ml. Solvents were equilibrated with *n*-heptane. Acetic acid (6 ml./l.) was added to the first solvent. Eluates between the arrows were collected separately as fractions I and II.

heated on a water bath for a few minutes, cooled, and acidified with a slight excess of conc. HCl. The product was evaporated to dryness under vacuum, and the residue extracted with boiling acetone (about 40 ml. in all). The dicarboxylic acids obtained by evaporation of the acetone were chromatographed on paper with chloroform-formic acid-water-acetone solvent as described by Whitcutt & Sutton (1956). Only malonic and succinic acids were identified.

Fig. 2. Ultraviolet spectra of fatty acids in the region 200–240 $m\mu$. HH_1 , Heptadeca-2-enoic acid from Lauer, Gensler & Miller (1941); EE_1 , eicosapentaenoic acid from Whitcutt & Sutton (1956); DD_1 , docosahexaenoic acid isolated from pilchard oil.

A further sample of the unsaturated acid (17 mg.) was oxidized with CrO_3 under the conditions described by Eisenbraun *et al.* (1954), and the steam distillate concentrated to a small volume. Paper chromatography (Lindquist & Störgards, 1953) showed that the only volatile oxidation products were acetic and propionic acids.

Ultraviolet absorption. Apart from a slight inflexion at 232 $m\mu$ corresponding to 0.2% autoxidation (Silk & Hahn, 1954b), the docosahexaenoic acid showed no absorption in the 220–300 $m\mu$ region. The spectrum is given in Fig. 2.

Alkali isomerization. The docosahexaenoic acid (11.2 mg.) was heated at 180° under nitrogen in a 21% (w/w) solution of KOH in ethylene glycol for 8 min. (see Herb & Riemen-schneider, 1952; Hammond & Lundberg, 1953a). The absorption spectrum is shown in Fig. 3.

Infrared absorption. The spectrum shown in Fig. 4 was taken in carbon tetrachloride solution (1.30%, w/w) except from 850 to 670 cm^{-1} , where carbon disulphide solution (1.26%, w/w) was used.

Synthesis and degradation of methyl tricosahexaenoate. The docosahexaenoic acid (41 mg.) was used to prepare the methyl ester of its higher homologue (see Whitcutt & Sutton, 1956). The product was ozonized, worked up as described above and the dicarboxylic acid fragments were chromatographed on paper. Malonic and glutaric acids were detected together with a smaller amount of succinic acid. The ozonolysis was repeated with the following modifications.

Fig. 3. Ultraviolet absorption of docosahexaenoic acid after alkali isomerization. For details see text.

Fig. 4. Infrared absorption of docosahexaenoic acid. For details see text. The band at 759 cm^{-1} may be due to traces of chloroform.

After blowing nitrogen through the ozonized solution, H_2O_2 (0.4 ml. of '130 vol.') was added, together with enough acetone (about 3 ml.) to give one phase. This solution was allowed to stand for 20 min. at room temperature before being boiled for a few seconds. Most of the solvent was then removed under vacuum. Water (3 ml.) was added, and excess of solid Na_2SO_3 to reduce all the H_2O_2 present. After warming and then standing for a few minutes, KOH solution (4 ml. of 25%, w/v) was added, and the procedure continued as described above. Only malonic and glutaric acids were detected when the final product was chromatographed on paper.

DISCUSSION

The unsaturated acid melted over a range of 0.4° . Its elementary analysis, equivalent weight and hydrogen uptake correspond to the values which would be expected for a docosahexaenoic acid.

Chromatographic examination of the hydrogenated material showed that only C_{22} acids were present. The hydrogenated counterpart, recrystallized from acetic acid, began to melt 0.9° below the upper limit of melting of our authentic specimen of *n*-docosanoic acid, and this latter point coincided with the literature value. A mixed melting point showed no depression. The X-ray long spacing of $48.2\text{\AA}$ is in agreement with the result ($48.0\text{\AA}$) on our authentic acid and with the value ($48.3\text{\AA}$) reported by Francis & Piper (1939). Neither the X-ray-powder pattern nor the infrared spectrum differed significantly from those of *n*-docosanoic acid.

The iodine value (446) is the only analytical result appreciably different from the theoretical value (464) for docosahexaenoic acid, but the difference is too small to be accounted for by the presence of acetylenic or allenic unsaturation. To settle the question of the exact degree of unsaturation of the acid our microhydrogenation procedure was tested with pure samples of unsaturated acids. Results were found to be reproducibly 1–2% high, and on this basis the correct unsaturation of the acid is 5.84–5.90 double bonds/molecule. Assuming, as is most probable from the known chromatographic behaviour of these compounds, that docosapentaenoic acid is the main contaminant, the docosahexaenoic acid is between 84 and 90% pure.

Spectral evidence shows that the double bonds are not conjugated with each other nor with the carboxyl group, since there are no bands in the ultraviolet, and absorption below $240\text{ m}\mu$ is much less than that shown by *n*-heptadec-2-enoic acid. The absence of infrared bands at 968, 990 and 3300 cm^{-1} rules out the possibility of *trans* or terminal unsaturation (see Bellamy, 1954). A broad absorption band between 1370 and 1500 cm^{-1} may have obscured the characteristic 1379 cm^{-1} band of the methyl group. A distinct shoulder possibly due to the latter appears at 1395 cm^{-1} .

The only oxidation products detected by paper

chromatography were acetic, propionic, malonic and succinic acids. Acetic acid may have arisen from further oxidation of propionic acid; both acids were present to approximately the same extent. Malonic acid was by far the most abundant product.

The advantages of using an Arndt-Eistert synthesis to determine the position of the double bond nearest to the carboxyl group have been previously discussed (Whitcutt & Sutton, 1956). Ozonolysis of the C_{22} homologue of the docosahexaenoic acid gave only malonic and glutaric acids.

On the basis of the evidence outlined above the main component of the material isolated can only be $\text{H}_3\text{C}\cdot\text{CH}_2\cdot[\text{CH}:\text{CH}\cdot\text{CH}_2]_6\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$ (all *cis-n*-docosa-4:7:10:13:16:19-hexaenoic acid). However, the possibility that other docosahexaenoic acids with different structures are also present in smaller amounts cannot be excluded.

The alkali-isomerization spectrum of the acid shows bands corresponding to conjugated diene, triene, tetraene, pentaene and hexaene, and is supporting evidence for the group

since de Surville, Sutton & Rivett (1957) have shown that the doubly methylene-interrupted double bonds of trideca-5:9-dienoic acid do not isomerize into conjugation under the usual conditions for alkali rearrangement. The low proportion of conjugated pentaene and hexaene obtained in the isomerization of the docosahexaenoic acid contrasts with the results reported by other authors (see Hammond & Lundberg, 1953*a*; Abu-Nasr & Holman, 1954).

Since the structure proposed above is in disagreement with all the earlier structures for docosahexaenoic acids of marine origin, it is desirable to review the evidence upon which these structures were based. Although the acids were obtained from various sources, the fatty acid compositions of different fish oils are often similar with regard to the distribution of chain length and unsaturation, and it is reasonable to assume that the same acids are present.

Toyama & Tsuchiya (1935) proposed a $\Delta^{4:8:12:15:18:21}$, or alternatively a $\Delta^{4:8:11:14:17:20}$, structure, on the basis of a quantitative examination of oxidation products, but it is difficult to assess the value of their results as only minor amounts of pure products appear to have been isolated.

Matsuda (1942) proposed a $\Delta^{4:8:12:15:18:21}$ structure based in part on identification of the ozonolysis products after partial hydrogenation of the acid in the presence of a nickel catalyst. Boelhouwer, Gerkens, Ong Tian Lie & Waterman (1953), however, have shown that double bonds migrate readily under such conditions.

Farmer & Van den Heuvel (1938) based their alternative structures, all with double bonds in positions 4 and 20 and with a

group somewhere in the chain, upon (i) identification of acetaldehyde, acetic acid and carbon dioxide as the only volatile oxidation products; (ii) identification of methyl hydrogen succinate after oxidation of the ester; (iii) production of 4.8 mol.prop. of acetic and 1.3 mol.prop. of succinic acid on complete oxidation of the free acid. It would be desirable to confirm these results by more modern techniques.

In no single case has the purity of a highly unsaturated C_{22} fatty acid of natural origin been properly established. Possible contaminants are closely related structures having similar chemical, physical and analytical properties which may counterbalance the effects produced by each other. Some polyene acids described in the literature, and with analytical values close to the theoretical ones, must to-day be considered of doubtful purity because of the relatively inefficient methods used in their isolation.

When closely related compounds are found together in Nature, biogenetic relationships may be of value in deciding the structure to be assigned in uncertain cases. The structure which has been proposed for the docosahexaenoic acid of South African pilchard oil is not only identical with that for the mammalian docosahexaenoic acids, but is also closely similar to the structure of the eicosapentaenoic acid described in Part 5 (Whitcutt & Sutton, 1956).

SUMMARY

1. Docosahexaenoic acid has been isolated from South African pilchard oil. Some of its properties have been determined and its degree of purity is discussed. Evidence is presented that the main component is the 4:7:10:13:16:19-hexaene.

2. The structures previously assigned to docosahexaenoic acids of marine origin are discussed.

The author wishes to thank Dr W. S. Rapsom and Dr D. A. Sutton for their interest and advice throughout this work. Microanalyses were done by Miss P. M. Hughes,

infrared spectra by Miss W. Albrecht and the X-ray spacings by Dr F. H. Herstein. The melting point of the *n*-docosahexaenoic acid was determined by Mr A. E. Carte. The investigation was made possible through the endowment of a Fellowship by Messrs Marine Oil Refiners of Africa Ltd., Simonstown, Cape Province, which is gratefully acknowledged. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

REFERENCES

- Abu-Nasr, A. M. & Holman, R. T. (1954). *J. Amer. Oil Chem. Soc.* **31**, 41.
 Bellamy, L. J. (1954). *The Infrared Spectra of Complex Molecules*. London: Methuen.
 Boelhouwer, C., Gerkens, J., Ong Tian Lie & Waterman, H. I. (1953). *J. Amer. Oil Chem. Soc.* **30**, 59.
 de Surville, B., Sutton, D. A. & Rivett, D. E. A. (1957). *J. chem. Soc.* (in the Press).
 Eisenbraun, E. J., McElvain, S. M. & Aycock, B. J. (1954). *J. Amer. chem. Soc.* **76**, 607.
 Farmer, E. H. & Van den Heuvel, F. A. (1938). *J. chem. Soc.* p. 427.
 Francis, F. & Piper, S. H. (1939). *J. Amer. chem. Soc.* **61**, 577.
 Hammond, E. G. & Lundberg, W. O. (1953*a*). *J. Amer. Oil Chem. Soc.* **30**, 433.
 Hammond, E. G. & Lundberg, W. O. (1953*b*). *J. Amer. Oil Chem. Soc.* **30**, 438.
 Herb, S. F. & Riemenschneider, R. W. (1952). *J. Amer. Oil Chem. Soc.* **29**, 456.
 Klenk, E. & Dreike, A. (1955). *Hoppe-Seyl. Z.* **300**, 113.
 Klenk, E. & Lindlar, F. (1955). *Hoppe-Seyl. Z.* **299**, 74.
 Lauer, W. M., Gensler, W. J. & Miller, E. (1941). *J. Amer. chem. Soc.* **63**, 1153.
 Lindquist, B. & Störgards, T. (1953). *Acta chem. scand.* **7**, 87.
 Matsuda, S. (1942). *J. Soc. chem. Ind. Japan*, suppl. 45, pp. 158–61. [*Chem. Abstr.* (1951). **45**, 546d.]
 Notevarp, O., Roald, A., Sletness, P. & Nordli, S. (1953). *Proc. Symposium on Cured and Frozen Fish Technol., Swedish Inst. Food Preservation Research*, publ. no. 100, X. [*Chem. Abstr.* (1956). **50**, 9644i.]
 Silk, M. H. & Hahn, H. H. (1954*a*). *Biochem. J.* **56**, 406.
 Silk, M. H. & Hahn, H. H. (1954*b*). *Biochem. J.* **57**, 582.
 Silk, M. H., Sephton, H. H. & Hahn, H. H. (1954). *Biochem. J.* **57**, 574.
 Sutton, D. A. (1953). *Chem. & Ind.* p. 1383.
 Toyama, Y. & Tsuchiya, T. (1935). *Bull. chem. Soc. Japan*, **10**, 539. [*Chem. Abstr.* (1936). **30**, 2029³.]
 Whitcutt, J. M. & Sutton, D. A. (1956). *Biochem. J.* **63**, 469.