suggested will result in less-complex fractions amenable to quantitative analysis. The mild procedures employed in fractionation of the acids might be expected not to cause modification of structure during processing. Consideration of the results obtained in this investigation illustrates that the composition of marine oils is of a far greater complexity than hitherto envisaged.

SUMMARY

1. A new approach has been suggested for the analysis of total marine oil acids without isolation of individual components.

2. The techniques of lithium soap-acetone segregation, molecular distillation, reversed-phase partition chromatography and urea complex fractionation have been applied in the successive subdivision of pilchard acid fractions. Relatively simple mixtures of acids have been obtained; the composition of one of which has been calculated without isolation of an individual component.

3. The techniques employed have been such as might be expected to have caused little or no modification of the component acids.

The authors wish to thank Dr D. A. Sutton for his interest and advice throughout the undertaking of this work which was made possible through the endowment of a Fellowship

by Messrs Marine Oil Refiners of Africa Ltd., Simonstown, Cape Province. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

REFERENCES

- Ahrens, E. H. & Craig, L. C. (1952). *J. biol. Chem.* **195**, 299.
 Lovern, J. A. (1942). *Spec. Rep. Fd Invest. Bd, Lond.*, no. 51. London: H.M. Stationery Office.
 Norris, F. A., Rusoff, I. I., Miller, E. S. & Burr, G. O. (1941). *J. biol. Chem.* **139**, 199.
 Norris, F. A., Rusoff, I. I., Miller, E. S. & Burr, G. O. (1943). *J. biol. Chem.* **147**, 273.
 Savary, P. & Desnuelle, P. (1953). *Bull. Soc. chim. Fr.* **10**, 939.
 Silk, M. H. & Hahn, H. H. (1954a). *Biochem. J.* **56**, 406.
 Silk, M. H. & Hahn, H. H. (1954b). *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. (1935a). *Bull. chem. Soc. Japan*, **10**, 411.
 Toyama, Y. & Tsuchiya, T. (1935b). *Bull. chem. Soc. Japan*, **10**, 539.
 Toyama, Y. & Yamamoto, T. (1953). *Mem. Fac. Eng., Nagoya*, **5**, no. 1.
 Tsujimoto, M. (1906). *J. Coll. Engng Tokio*, **4**, no. 1.
 Ueno, S. & Iwai (1934). *J. Soc. chem. Ind. Japan*, **37**, S. b. 251.
 Ueno, S. & Yonese (1936). *J. chem. Soc. Japan*, **57**, 180.

South African Pilchard Oil

4. THE ISOLATION AND STRUCTURE OF A HEXADECATETRAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL

By M. H. SILK* AND H. H. HAHN†

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

(Received 2 February 1954)

In the preceding communication of this series (Silk & Hahn, 1954), the presence of a C_{16} tetraene acid in the body oil of the South African pilchard (*Sardina ocellata* Jenyns) was posulated in order to account for certain observed analytical values. The isolation of such an acid is now reported, together with its characterization by physical and chemical means.

The techniques of lithium soap-acetone segregation, urea complex fractionation and molecular distillation have been applied in successive subdivision of total pilchard acids yielding a concentrate of highly unsaturated material. A C_{16} tetraene

acid has been isolated from this concentrate by reversed-phase partition chromatography on a preparative scale, and has been characterized as *hexadeca-6:9:12:15-tetraenoic acid*. The techniques employed for its isolation have been sufficiently mild in nature to render it unlikely that any modification of structure could have taken place during processing.

EXPERIMENTAL

General methods. See Silk, Sephton & Hahn (1954).

Ultraviolet spectra were determined on a Beckman model DU quartz spectrophotometer. Infrared spectra were determined on a Perkin-Elmer single-beam infrared spectrophotometer.

* Marine Oils Research Fellow.

† Assistant in the Fellowship.

Table 1. *Molecular distillation of the concentrate of unsaturated acids*

Fraction	Distillation temp. (°)	Weight (g.)	Equiv. wt.	Refractive index (n_D^{25})	No. of double bonds/mol.
Concentrate of unsaturated acids	—	54.3	308	—	4.85
A	85	2.60	260	1.4801	3.67
B	90.5	1.78	263	1.4811	3.72
C	96	5.53	268	1.4827	3.93
Residue R (3rd stage)	—	1.18	295	1.4889	4.54
Main bulk residue	—	approx. 41.7	325	—	5.38

Fig. 1. Siphon arrangement for collection and titration of chromatographic column eluate. (Showing siphon having drained, leaving titration sample in side arm.) A, tap of chromatographic column; B, main receiver (21 ml.); C, side arm for collection of sample for titration, 2.75 ml.; D, siphon; E, plunger for altering sample volume by raising or lowering; F, titration vessel with N_2 circulation (cf. Howard & Martin, 1950); G, receiver for collection of main bulk of eluate under N_2 ; H, burette tip.

Preliminary stages of isolation

Lithium salt-acetone separation. Total pilchard acids (300 g., average equiv. wt., 282; average number of double bonds per molecule, 2.40) were subjected to lithium soap-acetone segregation twice in 95% aqueous acetone and finally in 97% aqueous acetone (cf. Silk *et al.* 1954). A concentrate (93 g.) of unsaturated acids was obtained which had an average equiv. wt., 307 and an average number of double bonds per molecule 4.55 (H_2 uptake Pd-BaSO₄).

Urea complex fractionation. The above material was fractionated further, using the urea complex method (cf. Silk *et al.* 1954). A non-complex forming concentrate of very highly unsaturated acids was obtained having an average equiv. wt., 308 and an average number of double bonds per molecule, 4.85 (H_2 uptake Pd-BaSO₄).

Molecular distillation of the unsaturated acid concentrate. The molecular still described by Sutton (1953) was used to distil the concentrate of unsaturated acids obtained by urea complex fractionation. All distilling stages were maintained at the same temperature and the distilland was recycled twice before removal of each fraction. Successive fractions were obtained using solvents of increasing boiling point to heat the distilling stages. The lowest boiling fraction (A) which distilled at 85° contained the required C₁₈ tetraene acid in the highest concentration and was reserved for subsequent chromatography. Results are shown in Table 1.

Reversed-phase partition chromatography of unsaturated acid concentrate on a preparative scale

A portion of the above fraction A was chromatographed on a column 80 cm. high × 3 cm. diameter which was maintained at 10° and packed with non-wetting kieselguhr supporting medicinal paraffin as stationary phase (cf. Silk & Hahn, 1954). The mixed acids (413 mg.) were dissolved in aqueous acetone (55%, v/v previously equilibrated with paraffin), and the solution was allowed to run into the top of the column. Last traces of acid were washed in with 20 ml. 55% aqueous acetone. The chromatogram was then developed at a flow rate of 150 ml./hr. with 55% aqueous acetone (pre-cooled to 10°).

The column eluate was collected under N_2 in a siphon constructed such that each sample was divided into two portions; the main bulk and a smaller sample which was titrated for the purpose of following the course of chromatographic resolution (Fig. 1). Methanolic 0.01N-KOH solution with bromothymol blue (0.2% in 70% aqueous acetone) as indicator, was used for titration of samples in a circulating vessel similar to that described by Howard & Martin (1950). The results for fraction A are shown in Fig. 2.

Recovery of hexadecatetraenoic acid from chromatographic column eluate

The column eluate representative of the first peak shown on chromatographic resolution of fraction A (that between the two arrows on Fig. 2) was collected separately. The

Fig. 2. Chromatographic resolution of fraction *A* (preparative scale). Weight of fraction *A*, 413 mg.; column: 80 × 3 cm. Temp. 10°; Flow rate, 150 ml./hr. Solvent system: aqueous acetone (55%, v/v)–medicinal paraffin supported on non-wetting kieselguhr. Eluate between arrows collected separately as representative of first peak.

Fig. 3. Ultraviolet spectra of fatty acids in the region 170–240 mμ. *P*, oleic acid; *Q*, linoleic acid; *R*, linolenic acid; *S*, methyl arachidonate. (These curves are from Rusoff, Platt, Klevens & Burr (1945).) *S* (●) methyl arachidonate in region 220–240 mμ. from Barnes, Rusoff, Miller & Burr (1944). *T*, heptadec-2-enoic acid from Lauer, Gensler & Miller (1941). *U*, hexadecatetraenoic acid isolated from pilchard oil.

Fig. 4. Ultraviolet absorption of alkali-isomerized hexadecatetraenoic acid. (For details see text.)

solution (350 ml.) was evaporated to approximately half its volume at 35° and 15 mm. under N_2 .

The resulting aqueous suspension of fatty acid was then saturated with Na_2SO_4 and exhaustively extracted with distilled *n*-pentane under N_2 . The pentane extract was washed with water and evaporated to yield the fatty acid (144 mg.) as a pale yellow (almost colourless) oil of refractive index n_D^{20} 1.4870. (Found: C, 77.1, H, 9.9%. No. of double bonds/mol., 3.96 (H_2 uptake over Adams's catalyst); equiv. wt., 250. $C_{16}H_{24}O_2$ (hexadecatetraenoic acid) requires C, 77.3; H, 9.7%. No. of double bonds/mol., 4.0, equiv. wt., 248.5.)

Hydrogenation product. Recovery of the hydrogenation product, followed by crystallization from 96% ethanol gave a fatty acid of equiv. wt., 258 and m.p., 62.0–62.5°. (Palmitic acid has equiv. wt. = 256.4 and m.p. 62.85°.) Mixed m.p. with an authentic sample of palmitic acid (m.p. 62.3–62.6°) showed a m.p. of 62.1–62.6°, thus confirming that the hydrogenation product was palmitic acid.

Fig. 5. Infrared absorption of hexadecatetraenoic acid (smooth curve 3.04 %, w/w, CS_2) and undec-10-enoic acids (broken curve), 2.47 %, w/w, CS_2 .

Fig. 6. Infrared absorption 1325–1425 cm^{-1} in CCl_4 of hexadecatetraenoic acid (4.97 %, w/w, U) undec-10-enoic acid (5.00 %, w/w, V) and palmitic acid (4.84 %, w/w, W).

Ultraviolet absorption. The ultraviolet absorption curve of the hexadecatetraenoic acid showed no peaks in the region 220–320 $\text{m}\mu$. A faint inflexion at 231 $\text{m}\mu$ indicated that a negligible amount of oxidation had taken place. Comparison of the $E_{1\%}^{1\text{cm}}$ observed at 231 $\text{m}\mu$ (1.95) with that of pure methyl linoleate hydroperoxide (780) at the same wavelength (Sephton & Sutton, 1953) showed that the maximum amount of oxidation that could have taken place was 0.33 %.

The ultraviolet absorption of the hexadecatetraenoic acid in the region 206–240 $\text{m}\mu$ is shown in Fig. 3, together with that of several other acids in the far ultraviolet region (170–240 $\text{m}\mu$).

Alkali isomerization. This was carried out according to the method of Herb & Riemenschneider (1952) by heating a 13 mg. sample for 15 min. at 180° in 21 % KOH–glycol reagent. The ultraviolet absorption of the product is shown in Fig. 4.

Infrared absorption. The infrared absorption curve of the hexadecatetraenoic acid in CS_2 is shown together with that of undec-10-enoic acid in Fig. 5. The infrared absorption of

hexadecatetraenoic, undec-10-enoic and palmitic acids in CCl_4 in the region 1325–1425 cm^{-1} is illustrated on a larger scale in Fig. 6.

Permanganate oxidation of hexadecatetraenoic acid and paper chromatography of the oxidation products

Oxidation of the hexadecatetraenoic acid with cold alkaline KMnO_4 yielded adipic acid and a little glutaric acid.

A sample of the hexadecatetraenoic acid was oxidized at 0° with cold alkaline KMnO_4 , according to the method of Dolby, Nunn & Smedley-Maclean (1940). Removal of the monobasic acid products by steam distillation followed by continuous ether extraction of the (HCl) acidified residue, furnished the dibasic oxidation products as a crystalline mass after evaporation of the ether. The dibasic products were spotted on to sheets of Whatman no. 1 paper, together with reference C_2 – C_8 dicarboxylic acids, and the chromatograms developed with NH_3 (sp.gr. 0.88)–ethanol (20:80, v/v), according to Long, Quayle & Stedman (1951). Development was carried out in a descending direction for 14 hr. at 30° and the solvent allowed to run off the bottom of the paper. The papers were dried for 45 min. at 80° and sprayed with British Drug Houses Universal Indicator to contrast the acid spots. All spots were separated by at least 1.5 cm., and the major product of oxidation was shown to be adipic acid by its exact correspondence with a spot of authentic adipic acid. Some glutaric acid was also evidenced by a spot clearly resolved from the adipic acid. Several chromatograms were carried out with the same result.

DISCUSSION

Ultraviolet evidence shows that the hexadecatetraenoic acid isolated from pilchard oil does not contain conjugated double bonds, since no diene, triene or tetraene peaks were apparent in the region 220–320 $\text{m}\mu$. Comparison of the ultraviolet absorption with that of known acids in the region 170–240 $\text{m}\mu$ (Fig. 3) shows that the curve lies in the expected position close to that of methyl arachidonate (C_{20} tetraene ester). The molar absorption observed at 210 $\text{m}\mu$ is about one-twentieth of that shown by the peak of heptadec-2-enoic acid which

has unsaturation conjugated with the carboxyl group. The hexadecatetraenoic acid cannot therefore be $\alpha\beta$ -unsaturated.

The ultraviolet absorption of the alkali-isomerized hexadecatetraenoic acid (Fig. 4), shows clearly the three peaks of a conjugated tetraene. No other peaks are apparent. The acid is thus probably a tetraene with all the double bonds separated by a single methylene group. If this were not so, then tetraene absorption would not have been evidenced after alkali isomerization because double bonds separated by more than a single methylene group are unlikely to isomerize into conjugation (cf. Farmer, 1942).

Only five structures are possible for a C_{16} tetraene acid in which the double bonds are separated by a single methylene group. These are as follows:

Of these structures (I) is $\alpha\beta$ -unsaturated and is excluded by the ultraviolet evidence. Structures I-IV all contain a $C\text{-CH}_3$ group which exhibits a well defined peak at approximately 1375 cm^{-1} in the infrared. No such peak was apparent in the infrared curve of the hexadecatetraenoic acid (cf. Fig. 6, which shows that the $C\text{-CH}_3$ absorption of palmitic acid is completely absent from the hexadecatetraenoic acid).

Structures I-IV are thus clearly impossible for the isolated acid, which can therefore most probably be represented by structure V containing a terminal vinyl group. This grouping exhibits intense maxima at 910 and 990 cm^{-1} in the infrared, and both these peaks were strongly evidenced in the absorption curve of the hexadecatetraenoic acid (Fig. 5). The spectrum of undec-10-enoic acid, which contains the —CH:CH_2 grouping, was used as a reference curve.

trans-trans Conjugated double bonds such as might arise from autoxidation of an unsaturated acid, also exhibit an infrared maximum at 990 cm^{-1} , but since no peak due to oxidation was shown by the ultraviolet absorption curve of the isolated acid, there is no likelihood that the observed peak at 990 cm^{-1} can have been due to oxidation. The measured intensity of this peak, if due to *trans-trans* conjugated bonds and not to a terminal vinyl group,

would correspond to some 50% oxidation having taken place, which is clearly impossible.

The validity of structure V is also confirmed by the identification of adipic acid among the permanganate oxidation products of the isolated acid. The presence of some glutaric acid in addition is probably the result of further degradation of the adipic acid (cf. Begemann, Keppler & Boekenoogen, 1950).

The related structure VI which includes a terminal vinyl group and which would also give adipic acid on oxidation, cannot be that of the isolated acid, because the conjugated triene-carboxylic acid which might arise on alkali isomerization (VII) would only show one peak at approx. $300\text{ m}\mu$ in the ultraviolet (cf. Hauser, Kuhn, Smakula & Hoffer, 1935, and Weedon & Woods, 1951). Since the three

peaks of a conjugated tetraene were observed for the alkali-isomerized acid (Fig. 4), structure VI cannot obtain.

Thus structure (V), hexadeca-6:9:12:15-tetraenoic acid, is clearly established as that of the acid isolated from pilchard oil. This compound, which has not previously been reported, occurs to an extent of approximately 1.3% of the total pilchard acids.

During the course of preparation of this paper it has come to our notice that a C_{16} tetraene acid has recently been isolated by Toyama & Yamamoto (1953) from Japanese sardine oil. The acid was insufficiently characterized, but one of the two possible structures suggested contained a terminal vinyl group. A second structure in which the double bonds occupy the positions 4, 8, 11, 14 was preferred by these authors, on the grounds that succinic acid was isolated after oxidative fission.

SUMMARY

1. The techniques of lithium soap-acetone segregation, molecular distillation, urea complex fractionation and reversed-phase partition chromatography have been applied in successive subdivision of the total fatty acids of South African pilchard oil.

2. Application of these techniques has resulted in the isolation of a new acid characterized by physical and chemical means as hexadeca-6:9:12:15-tetra-enoic acid.

The authors wish to thank Dr D. A. Sutton for his interest and valuable advice throughout the undertaking of this work. 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

- Barnes, R. H., Rusoff, I. I., Miller, E. S. & Burr, G. O. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 385.
 Begemann, P. H., Keppler, J. G. & Boekennoogen, H. A. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 439.
 Dolby, D. E., Nunn, L. C. A. & Smedley-Maclean, I. (1940). *Biochem. J.* **34**, 1423.
 Farmer, E. H. (1942). *Trans. Faraday Soc.* **38**, 358.
 Hauser, K. W., Kuhn, R., Smakula, A. & Hoffer, M. (1935). *Z. phys. Chem. B* **29**, 371.
 Herb, S. F. & Riemenschneider, R. W. (1952). *J. Amer. Oil Chem. Soc.* **29**, 505.
 Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
 Lauer, W. M., Gensler, W. J. & Miller, E. (1941). *J. Amer. Chem. Soc.* **63**, 1153.
 Long, A. G., Quayle, J. R. & Stedman, R. J. (1951). *J. chem. Soc.* p. 2197.
 Rusoff, I. I., Platt, J. R., Klevens, H. B. & Burr, G. O. (1945). *J. Amer. chem. Soc.* **67**, 675.
 Sephton, H. H. & Sutton, D. A. (1953). *Chem. & Ind.* p. 667.
 Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **57**, 577.
 Silk, M. H., Sephton, H. H. & Hahn, H. H. (1954). *Biochem. J.* **57**, 574.
 Sutton, D. A. (1953). *Chem. & Ind.* p. 1383.
 Toyama, Y. & Yamamoto, T. (1953). *Mem. Fac. Engng Nagoya*, **5**, no. 1. (Abstracted in *J. Amer. Oil Chem. Soc.* 1954, **31**, 34).
 Weedon, B. C. L. & Woods, R. J. (1951). *J. chem. Soc.* p. 2687.

Studies in the Biochemistry of Micro-organisms

93. CYCLOPENIN, A NITROGEN-CONTAINING METABOLIC PRODUCT OF *PENICILLIUM CYCLOPIUM* WESTLING

By A. BRACKEN, ANNA POCKER AND H. RAISTRICK

Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(Received 15 February 1954)

The *Penicillium cyclopium* series of species of *Penicillium* contains four accepted species and one variety, i.e. *P. cyclopium* Westling, *P. cyclopium* Westling var. *echinulatum* Raper & Thom, *P. puberulum* Bainier, *P. martensii* Biourge and *P. aurantio-virens* Biourge. The series has proved to be a fruitful source of mould metabolic products, the following having been described: penicillic acid from *P. puberulum* (Alsberg & Black, 1913) and *P. cyclopium* (Birkinshaw, Oxford & Raistrick, 1936); ergosterol in the mycelium of *P. puberulum* (Birkinshaw, Callow & Fischmann, 1931); the tropolones, puberulic and puberulonic acids, from *P. puberulum* and *P. aurantio-virens* (Birkinshaw & Raistrick, 1932) and *P. johannioli* Zaleski = *P. martensii* Biourge (Oxford, Raistrick & Smith, 1942); *i*-erythritol from *P. cyclopium* (Oxford & Raistrick, 1935); the anthraquinones, ω -hydroxy-emodin and emodic acid, from *P. cyclopium* (Anslow, Breen & Raistrick, 1940); cyclopolic and cyclopaldic acids, two fully substituted benzoic acids, from *P. cyclopium* (Birkinshaw, Raistrick, Ross & Stickings, 1952) and an unnamed photo-

sensitive nitrogenous compound, $C_{17}H_{12}O_2N_2$, m.p. 220° (decomp.), from *P. puberulum*, which gave phenol on heating and *p*-hydroxybenzoic acid on oxidation (Campbell, Foss, Hirst & Jones, 1945).

We now wish to report the isolation from culture filtrates of an authentic strain of *P. cyclopium* of a hitherto undescribed metabolic product for which we propose the name cyclopenin.

CYCLOPENIN

Cyclopenin was isolated, in roughly equal amounts, from culture filtrates of *P. cyclopium*, L.S.H.T.M. no. 72, grown on either Czapek-Dox or Raulin-Thom solutions, by adsorption on charcoal and elution with methanol. Cyclopenin, $C_{17}H_{14}O_3N_2$, crystallizes from methanol in colourless needles containing two molecules of water of crystallization. On heating, these crystals melt at 110–120° with frothing and loss of water, reset on cooling and remelt at 207°. Cyclopenin separates from a variety of other solvents, especially ethyl acetate, in crystals containing half a molecule of water of crystallization which melt sharply at 207°. It