309. *Fatty Acids of the Lugworm,* Arenicola marina. *Part I.*

By Wesley Cocker, Thor Dahl, and T. B. H. McMurry.

Hydrolysis of the fats of *Arenicola marina* affords a mixture of saturated acids of the normal, iso-, and anteiso-series containing from 10 to 18 carbon atoms, and mono-, di-, tri-, and poly-ethenoic acids of the three series containing from 11 to 26 carbon atoms.

It is known [1] that the accumulation of fat in *Arenicola marina* (lugworm) diminishes when spawning starts, so that apparently the fat is utilised during reproduction. It is also known that the fats extracted by benzene from mature lugworms when injected into live, sexually mature worms increase spawning, and that the activity lies in the unsaturated fats.[2] It seemed desirable therefore to investigate the composition of these fats and this paper describes initial work.

The fatty acid mixture obtained on saponification of the benzene-extracted fats was esterified with methanol and boron trifluoride [3] and then treated with mercuric acetate in methanol.[4] The complexes so formed were chromatographed by Mangold and Kammereck's method [5] in two systems on a thin layer of silica supported on glass. Well-defined zones of the saturated esters and the complexes of mono-, di-, tri-, and poly-ethenoic esters were obtained. In this way we found the composition of the fatty acid mixture to be saturated 31—33%, mono- 15—22%, di 36—40%, tri- 7%, and poly-ethenoid 3—4%. The acetoxymercurimethoxy-complexes were decomposed with acid, giving the free unsaturated esters, which were submitted to gas–liquid chromatography

[1] Howie, *J. Marine Biol. Assoc.*, 1961, **41**, 127, 771.
[2] Personal communication from Dr. D. I. D. Howie.
[3] Metcalfe and Schmitz, *Analyt. Chem.*, 1961, **33**, 363; Vorbeck, Mattick, Lee, and Pederson, *ibid.*, p. 1512.
[4] Jantzen and Andreas, *Chem. Ber.*, 1959, **92**, 1427; cf. Birks and Wright, *J. Amer. Chem. Soc.*, 1935, **57**, 1993; 1940, **62**, 2412.
[5] Mangold and Kammereck, *Chem. and Ind.*, 1961, 1032; cf. Stahl, *Pharmazie*, 1956, **11**, 633; *Chem.-Ztg.*, 1958, **82**, 323.

on three columns, containing, (*a*) diethylene glycol succinate, (*b*) sucrose diacetate hexa-isobutyrate, S.A.I.B., and (*c*) silicone elastomer, S.E.30, in each case deposited on firebrick. The individual unsaturated esters have not yet been identified and further work is in progress, but hydrogenation and identification of the products by gas–liquid chromatography has, however, given a picture of their carbon skeletons. On the basis of hydrogen uptake the polyunsaturated esters have the same order of unsaturation as the acids of fish oils; fatty acids containing six ethenoid groups are found in fish oils.[6]

The components of the saturated ester fraction were identified by gas–liquid chromatography in the three columns mentioned. Each gave the same pattern (see Table 1). Authentic normal and iso-acids were used for comparison and plots of log (retention time) (log T_R) of their esters against carbon number gave parallel straight lines. Values of log T_R which did not fall on these lines gave a third parallel line corresponding to anteiso-esters.[7]

TABLE 1.

Ester fraction	Carbon number of saturated esters *		
	Normal	iso	anteiso
Saturated	10—18	13—18	15, 17, 18
Mono-ethenoid	11—20	17—20	
Di- ,,	12—20	17, 19	
Tri- ,,	12—23	17—22	
Poly- ,,	14—26	15, 17—24	16, 17

* Throughout this paper, " carbon number " denotes the number of carbon atoms in the corresponding acid.

The approximate composition of each fraction is given in the Experimental section. We may note here that palmitic acid is the major saturated acid, and octadecenoic and octadecadienoic acid are predominant in their respective fractions.

For comparison by gas–liquid chromatography a number of isoalkanoic acids were synthesised. 7-Methyloctanoic and 8-methylnonanoic acids were made by anodic synthesis [8] from, respectively, isovaleric and γ-methylvaleric acid with ethyl hydrogen adipate. 9-Methyldecanoic, 11-methyldodecanoic, and 12-methyltridecanoic acid were similarly made from isovaleric acid with ethyl hydrogen suberate and ethyl hydrogen sebacate, and from γ-methylvaleric acid with ethyl hydrogen sebacate. The yields of chromatographically pure acids never exceeded 10%. Better yields were obtained by the thiophen method of chain extension.[9] In this way 13-methyltetra-, 14-methylpenta-, and 15-methylhexa-decanoic acid were synthesised in ~15% overall yields of chromatographically pure acid.

EXPERIMENTAL

Extraction of Lugworms.—Killed worms were emulsified and evaporated to dryness at 80°. Dry material (652 g.), containing much sand, was extracted for 20 hr. (Soxhlet) with benzene, and the solution was concentrated to a black viscous residue (25·18 g.).

Fractionation of the Extract.—The residue was dissolved in ether (50 c.c.), acetone (200 c.c.) was added with stirring, and the phospholipids (8 g.) were removed at the centrifuge and washed with acetone (3 × 150 c.c.). The phospholipids were not investigated. The combined acetone solutions were evaporated to dryness and the residue was dissolved in ether (30 c.c.), which was then treated with acetone (100 c.c.), and the precipitate was removed. Evaporation of the ether–acetone solutions gave a phosphorus-free fatty residue (13·7 g.).

Saponification.—The fats were refluxed for 3 hr. with potassium hydroxide (4·4 g.) in 75% ethanol (70 c.c.), and the mixture was cooled, diluted with water (300 c.c.), and extracted with

[6] Sinclair, " Essential Fatty Acids," Butterworths Scientific Publns., London, 1958; Markley, " Fatty Acids," Interscience Publ., Inc., New York, 1960, Part I, p. 163.

[7] James and Martin, *Biochem. J.*, 1956, **63**, 144.

[8] Greaves, Linstead, Shephard, Thomas, and Weedon, *J.*, 1950, 3326; Linstead, Lunt, and Weedon, *J.*, 1951, 1130; Linstead, Lunt, Weedon, and Shephard, *J.*, 1952, 3621; Linstead, Shephard, Weedon, and Lunt, *J.*, 1953, 1538.

[9] Grey, McGhie, and Ross, *J.*, 1960, 1502; McGhie, Ross, Evans, and Tomlin, *J.*, 1962, 350.

ether. The extract was washed with 5% aqueous potassium hydroxide, then with water, and evaporated, giving a yellow oil (3·8 g.) which has not yet been investigated. The combined alkaline solutions were acidified and the oil was collected in ether, which was washed with water and then distilled in steam. The steam-volatile product (0·5 g.) was not investigated. The non-volatile product, collected in ether, gave the fatty acids (7·1 g.) as a light brown semi-solid.

Esterification of the Acids.[3]—Freshly distilled boron trifluoride–ether complex (124 g.) was slowly added to anhydrous methanol (500 c.c.), and the reagent was preserved. The fatty acid fraction (1 g.) was added to the reagent (50 c.c.) and heated at 100° for 3 min., then the whole was poured into water and extracted with ether, which was washed with 5% sodium hydrogen carbonate solution, then with water, and dried ($MgSO_4$) and the solvent was removed.

Mercuration of the Unsaturated Esters.—The reagent was mercuric acetate (14 g.), methanol (250 c.c.), glacial acetic acid (2·5 c.c.), and water (1 c.c.). The mixture of esters (1 g.) was left in the dark for 24 hr. with the reagent (30 c.c.), methanol was removed under reduced pressure at 20°, and the residue was extracted with ether (4 × 20 c.c.), washed with water, and dried.

Fractionation of the Acetoxymercurimethoxy-complexes.—Silica gel (Merck's Kieselgel G) was deposited as a layer 275 μ thick on plate-glass sheets, 20 × 20 cm., and heated at 110° for 0·5 hr. The ether solution of the complexes was applied by a fine spray as a narrow band near the edge of each plate. Five such plates were developed simultaneously in a glass tank with two consecutive solvent systems, namely, (*a*) light petroleum (b. p. 40—60°)–ether (4 : 1 v/v), (*b*) propan-1-ol–glacial acetic acid–pyridine (150 : 1 : 1). In system (*a*) the saturated esters moved rapidly, and the band was scraped from the plates leaving the acetoxymercurimethoxy-complexes of the unsaturated esters near the origin. These were then separated in system (*b*). In order to assist rapid equilibration in the latter system, two strips of filter paper (15 × 3 cm.) were pasted on opposite walls of the tank with their narrow sides dipping into

TABLE 2.

Ester zones	Solvent system (*a*) R_F	Solvent system (*b*) R_F
Saturated esters	0·9—0·95	
Complexes of:		
mono-unsaturated esters	0·05—0·1	0·85—0·9
di- ,, ,,	<0·05	0·5, 0·65
tri- ,, ,,		0·25
poly- ,, ,,		0—0·1

the solvent. In both solvent systems the plates were suspended for 10 min. in the tank before being dipped in the solvent. Identification of the saturated ester zone was made by exposure of a narrow band to iodine vapour which gave a yellow colour.[10] The acetoxymercurimethoxy-complexes were identified by spraying a small strip with a 0·1% solution of diphenylcarbazone in 95% ethanol which gave a purple colour with the complexes.[5] Table 2 gives R_F values of the various zones, which are in close agreement with the values obtained previously.[5]

Recovery of Esters from Chromatoplates.—The zone containing the saturated esters was stirred with ether (4 × 15 c.c.), and the solution was filtered through Celite, dried ($MgSO_4$), evaporated to dryness, and submitted to gas–liquid chromatography in chloroform or methylene chloride. The zones of the acetoxymercurimethoxy-complexes were removed from the plates and stirred with 5% methanolic hydrochloric acid (3 × 15 c.c.). The supernatant solutions were diluted with water (100 c.c.) and extracted with ether. The ether extract from each zone was washed with water, dried, and evaporated to dryness. In this way, by using 25 chromatoplates, 212 mg. of esters were fractionated.

Gas–Liquid Chromatography.—The apparatus used was an Aerograph Hy-Fi 600 (Wilkens Instrument and Research, Inc.), with hydrogen flame detector. The columns were stainless-steel coils, 5′ × 1/8″, packed with the stationary phases described above.

Table 3 shows the composition of the mixture of esters obtained from the saturated acid fraction of lugworm extract. Similar results were obtained with all three columns.

Hydrogenation of Unsaturated Esters.—Each fraction was hydrogenated at 17° over palladised

[10] Mangold, Lamp, and Schlenk, *J. Amer. Chem. Soc.*, 1955, **77**, 6070.

TABLE 3.

Column: 20% quadrol S.A.I.B. Temp. 193° ± 1°; chart speed 20 in./hr.; N_2 flow 26 c.c./min.; H_2 flow 24 c.c./min.; T_R of methyl octadecanoate = 1. A = authentic specimen used as standard.

Peak no.	T_R	Carbon number of ester and type of chain: normal	iso	anteiso	Approx. (%)	Peak no.	T_R	Carbon number of ester and type of chain: normal	iso	anteiso	Approx. (%)
1	0·0608	10			0·17	10	0·3082	15			12·5 A
2	0·0797	11			0·13	11	0·3836		16		1·29 A
3	0·1069	12			0·21 A	12	0·4528	16			56·0 A
4	0·1300		13		0·07 A	13	0·5325			17	0·71
5	0·1488	13			0·36 A	14	0·5849		17		6·19 A
6	0·1824		14		0·39 A	15	0·6855	17			4·61 A
7	0·2117	14			7·5 A	16	0·7989			18	0·59
8	0·2369			15	0·35	17	0·8553		18		0·13
9	0·2872		15		2·84 A	18	1·0000	18			5·98 A

The composition of the mixture was unaffected by hydrogenation over palladised charcoal.

charcoal in ethyl acetate. The saturated esters obtained were directly submitted to gas–liquid chromatography on the three columns previously mentioned. Consistent results were obtained with each column. Table 4 shows the composition of the saturated ester fractions obtained on hydrogenation.

TABLE 4.

Carbon number of ester and type of chain: normal	iso	anteiso	Type of unsaturated ester fraction and % of the saturated acids found after hydrogenation: Mono-	Di-	Tri-	Poly-ethenoid
11			0·6			
12			0·8	0·15	3·1	
13			0·45	0·1	6·6	
14			0·3	0·3	4·2	6·0
	15					3·6
15			0·25	0·95	7·25	5·1
		16				2·4
16			11·7	29·9	8·4	19·4
		17				3·6
	17		2·1	1·4	2·5	4·6
17			4·9	6·65	2·3	5·2
	18		0·5		1·5	2·0
18			56·9	51·45	10·1	16·8
	19		2·7	0·65	1·6	3·0
19			12·6	3·25	5·2	4·2
	20		1·8		2·1	4·7
20			4·3	5·35	21·2	9·0
	21				2·6	1·5
21					6·6	3·6
	22				0·1	2·0
22					9·8	5·2
	23					0·5
23					2·6	1·8
	24					0·9
24						1·1
25						0·7
26						0·5

Preparation of Isoalkanoic Acids.—The following new compounds were prepared as intermediates in the synthesis of isoalkanoic acids by the thiophen chain-extension method.[9]

2-*Isovalerylthiophen.* Anhydrous stannic chloride (196 g.) was added during 0·5 hr. to a stirred mixture of thiophen (59 g.) and isovaleryl chloride (86 g.) in dry benzene (250 c.c.) at 0°. After a further 1 hr. the mixture was decomposed with ice-cold 10% hydrochloric acid, and the benzene layer was separated, washed with 10% aqueous sodium carbonate, then with water, dried, and fractionated, giving the required *ketone* (90·3 g.), b. p. 145·5—146°/30 mm. (Found:

C, 64·6; H, 7·0. $C_9H_{12}OS$ requires C, 64·3; H, 7·2%). Its 2,4-*dinitrophenylhydrazone* consisted of red needles (from ethanol), m. p. 159—160° (Found: C, 52·0; H, 4·9. $C_{15}H_{16}N_4O_4S$ requires C, 51·7; H, 4·6%).

2-*Isopentylthiophen.* 2-Isovalerylthiophen (56 g.), 99% hydrazine hydrate (25 g.), and ethylene glycol were refluxed for 2 hr. Sodium hydroxide (30 g.) was added, and the mixture was again refluxed for 2 hr. and then distilled until no more oil was collected. This was separated in ether (3 × 50 c.c.) which was washed with water, dried, and distilled, giving 2-*isopentylthiophen* (22·8 g.), b. p. 75—77°/10 mm. (Found: C, 70·2; H, 9·3. $C_9H_{14}S$ requires C, 70·1; H, 9·15%).

2-γ-*Methylvalerylthiophen.* Thiophen (13·5 g.), γ-methylvaleryl chloride (20 g.), benzene (150 c.c.), and stannic chloride (42 g.) gave the required ketone (20·3 g.), b. p. 124—125°/9 mm. Its 2,4-*dinitrophenylhydrazone* consisted of red needles (from ethanol), m. p. 171—172° (Found: C, 53·1; H, 4·9. $C_{16}H_{18}N_4O_4S$ requires C, 53·0; H, 5·0%).

2-*Isohexylthiophen.* The preceding ketone (3·6 g.), 85% hydrazine hydrate (23 g.), and ethylene glycol (50 c.c.), when refluxed for 3 hr., treated as above with potassium hydroxide (25 g.), and distilled, gave 2-*isohexylthiophen* (2·1 g.), b. p. 95—96°/10 mm. (Found: C, 71·6; H, 9·0. $C_{10}H_{16}S$ requires C, 71·4; H, 9·6%).

Methyl δ-(5-*isopentyl*-2-*thenoyl*)*valerate.* 2-Isopentylthiophen (10·1 g.) and δ-methoxycarbonylvaleryl chloride (12·1 g.) in dry benzene (50 c.c.) were stirred at 0° and stannic chloride (18·2 g.) was added during 30 min. The required *ester* was first distilled (b. p. 170—175°/0·5 mm.) and then crystallised as needles (ligroin) (15·5 g.), m. p. 36—37° (Found: C, 64·5; H, 8·4. $C_{16}H_{24}O_3S$ requires C, 64·8; H, 8·2%). Its 2,4-*dinitrophenylhydrazone* consisted of red needles (from ethanol), m. p. 103—105° (Found: C, 55·3; H, 6·1. $C_{22}H_{28}N_4O_6S$ requires C, 55·4; H, 5·9%).

δ-(5-*Isopentyl*-2-*thenoyl*)*valeric acid.* Its methyl ester (14 g.) was refluxed for 2 hr. in ethanol (30 c.c.) and water (100 c.c.) with potassium hydroxide (15 g.). The required *acid* consisted of plates (ligroin) (11·5 g.), m. p. 60—61° (Found: C, 63·8; H, 7·75. $C_{15}H_{22}O_3S$ requires C, 63·8; H, 7·85%).

13-*Methyl*-6-*oxotetradecanoic acid.* The previous compound (2·35 g.) was heated at 100° for 3 hr. in N-sodium carbonate (200 c.c.) with Raney nickel (23 g.). The mixture was cooled and filtered, and the nickel was washed with N-sodium carbonate (5 × 100 c.c.). The combined filtrates were acidified, and then extracted with benzene (3 × 100 c.c.), the extract being washed with water. The benzene solution was stirred with a saturated solution of chromium trioxide in 95% acetic acid (100 c.c.) at 0° for 1 hr. and then for 1 hr. at room temperature. The excess of chromium trioxide was destroyed with sulphur dioxide, and the benzene layer was washed till free from acid, dried, and evaporated. The required *acid* was obtained as plates (from light petroleum) (1·35 g.), m. p. 62—63° (Found: C, 70·3; H, 10·9. $C_{15}H_{28}O_3$ requires C, 70·3; H, 11·0%).

Methyl δ-(5-*isohexyl*-2-*thenoyl*)*valerate.* 2-Isohexylthiophen (6·3 g.) condensed at 0° with δ-methoxycarbonylvaleryl chloride (6·8 g.) in dry benzene (50 c.c.) containing stannic chloride (10·5 g.) and gave the required *ester* (9·1 g.) as a colourless solid, b. p. 135—140°/1 mm., m. p. 33° (Found: C, 66·05, 65·9; H, 8·6, 8·7. $C_{17}H_{26}O_3S$ requires C, 65·8; H, 8·4%). Its 2,4-*dinitrophenylhydrazone* consisted of red needles (from ethanol), m. p. 90° (Found: C, 55·7; H, 6·15. $C_{23}H_{30}N_4O_6S$ requires C, 56·3; H, 6·2%).

δ-(5-*Isohexyl*-2-*thenoyl*)*valeric acid.* The methyl ester, hydrolysed with aqueous-ethanolic alkali as above, gave the *acid* as needles (from ligroin), m. p. 61—61·5° (Found: C, 65·0, 65·1; H, 8·05, 8·0. $C_{16}H_{24}O_3S$ requires C, 64·8; H, 8·2%).

14-*Methyl*-6-*oxopentadecanoic acid.* The previous compound (6 g.) was desulphurised in N-sodium carbonate (200 c.c.) with Raney nickel (35 g.), and the product oxidised as above; this gave the required *acid* (2·5 g.) as needles (from ligroin), m. p. 63—64° (Found: C, 71·4; H, 11·0. $C_{16}H_{30}O_3$ requires C, 71·1; H, 11·2%).

Ethyl 7-(5-*isopentyl*-2-*thenoyl*)*heptanoate.* 2-Isopentylthiophen (10·1 g.) with 7-ethoxycarbonylheptanoyl chloride (15·4 g.) gave the required ester (20·5 g.), b. p. 200—210°/1·5 mm. Its 2,4-*dinitrophenylhydrazone* consisted of red needles (from ethanol), m. p. 98—99° (Found: C, 58·4; H, 6·8. $C_{25}H_{34}N_4O_6S$ requires C, 57·9; H, 6·6%).

7-(5-*Isopentyl*-2-*thenoyl*)*heptanoic acid.* Its ethyl ester, hydrolysed as above, gave the *acid* as needles (from benzene–ligroin), m. p. 50—50·5° (Found: C, 65·5, 65·4; H, 8·8, 8·7. $C_{17}H_{26}OS_3$ requires C, 65·8; H, 8·4%),

15-*Methyl*-8-*oxohexadecanoic acid.*—The previous compound (0·8 g.) was desulphurised, as described above, and gave the required *acid* as flakes (from ligroin) (0·25 g.), m. p. 64—65° (Found: C, 71·7; H, 11·35. $C_{17}H_{32}O_3$ requires C, 71·8; H, 11·3%).

The authors thank Dr. D. I. D. Howie of the Department of Zoology for bringing this problem to their notice.

UNIVERSITY CHEMICAL LABORATORY, TRINITY COLLEGE,
DUBLIN, REPUBLIC OF IRELAND. [*Received, October* 19*th*, 1962.]