The Lipids of Fish

8. THE TRIGLYCERIDES AND CHOLESTEROL ESTERS OF HADDOCK FLESH*

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Triglycerides and cholesterol esters are minor components of the total lipid mixture of haddock flesh, and are almost completely extracted by acetone (Olley & Lovern, 1954). Only two processes for the effective separation of these lipids from each other have been reported, namely selective enzymic hydrolysis of the glycerides (Kelsey, 1939) and chromatography on silicic acid (Borgström, 1952; Fillerup & Mead, 1953; Mead & Fillerup, 1954). Although cholesterol esters can be recovered from a mixture with triglycerides by chromatography on alumina, the triglycerides cannot be recovered quantitatively (Borgström, 1952). Other workers with alumina (Clément, Clément-Champougny & Louedec, 1954) have been predominantly interested in the cholesterol esters and have not attempted to recover the more strongly adsorbed triglycerides. Clément *et al.* (1954) found Kelsey's (1939) procedure unsatisfactory, in that hydrolysis of the glycerides was always far from complete. This agrees with other work on the enzymic hydrolysis of triglycerides *in vitro* (e.g. Desnuelles, Naudet & Rouzier, 1948; Mattson & Beck, 1955).

In the present studies the cholesterol esters and triglycerides contained in an acetone extract of haddock flesh were separated by chromatography on silica gel, and the respective fatty acid compositions determined.

EXPERIMENTAL

The raw material was that portion of the acetone extract (Lovern, 1953) of haddock flesh which would contain all of the cholesterol esters and triglycerides, but virtually no phospholipids. The crude acetone extracts were in each case purified by re-extraction with light petroleum (b.p. 40–60°) and the washing procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951). They were further freed from much phospholipid by acetone precipitation, and from the remaining phospholipid by countercurrent distribution (Lovern & Olley, 1953*a*) between ethanol–water (85:15, v/v) and light petroleum (b.p. 40–60°). The total phospholipid-free material from this countercurrent separation, i.e. all material accumulating in the fractions distributed more towards the light petroleum end of the sequence than the

point at which phosphorus and choline contents became negligible (Lovern & Olley, 1953*a*), was combined and washed in ethereal solution with aqueous Na₂CO₃ to remove free fatty acids and any complex acidic lipids present (Olley & Lovern, 1954). The weight of lipid finally available was 67 g., representing a total of 87 kg. (wet wt.) of haddock flesh. In addition to cholesterol esters and triglycerides, it would contain free cholesterol, waxes, hydrocarbons and possibly still other classes of compound (Lovern & Olley, 1953*a*).

A considerable proportion (15 g.) of the free cholesterol was removed by dissolving the lipids in a minimal amount of warm (approx. 60°) ethyl acetate and cooling to room temperature. Cholesterol is the least soluble of the lipids present. After filtration and washing with ethyl acetate until quite colourless, analysis showed that the crystalline product was entirely free cholesterol. Additional free cholesterol was removed by virtue of its greater solubility than the other major lipid components in cold ethanol. The lipid mixture was dissolved in about 10 vol. of boiling ethanol. Cooling to room temperature gave an emulsion which, on centrifuging, formed two liquid phases. The upper one, composed largely of ethanol, was evaporated to dryness and the residue crystallized from ethyl acetate as before, yielding 9 g. of free cholesterol (analytically verified). The residue from this crystallization was combined with the lower phase obtained from the ethanol emulsion (total lipid 43 g.).

A portion of this material was analysed for combined fatty acids, combined glycerol, free and esterified cholesterol and total unsaponifiable matter. The last was then largely freed from cholesterol, by crystallization from ethyl acetate as above, and the residue acetylated to permit calculation of the approximate proportions of higher aliphatic alcohols and non-alcoholic (hydrocarbon?) compounds respectively, assuming the acetates of the alcohols to have a mean saponification equivalent of 300. The analytical methods used were as described in previous papers (e.g. Lovern & Olley, 1953*a*), except for free and total cholesterol, which were determined by the method of Zlatkis, Zak & Boyle (1953). This had been tested satisfactorily on cholesterol and cholesteryl acetate, and on a range of possible contaminants. However, it was found later, on examination of chromatographic fractions, that this method was unreliable for some of them, where unsaponifiable material could give an apparent cholesterol value considerably in excess of 100%. Tests on such fractions by the method used previously (Lovern & Olley, 1953*a*) gave much lower and more probable values, and the method of Zlatkis *et al.* (1953) was abandoned. The chromogenic contaminants were also at least partially precipitable with digitonin, by the rapid AlCl₃

* Part 7: Olley (1956).

method of Brown, Zlatkis, Zak & Boyle (1954), which was likewise abandoned in favour of the earlier procedure (Lovern & Olley, 1953a).

The analytical data obtained were: fatty acids 53.3, glycerol 4.1, free cholesterol 16.8, esterified cholesterol 19.2, total unsaponifiable matter 42.6%. The partly purified unsaponifiable matter contained 55.4% cholesterol and, after acetylation, had a saponification equivalent of 498. This requires an equivalent of 588 for the acetate of the non-cholesterol components. From these values the approximate percentage composition of the starting material was calculated to be: triglycerides 40, cholesterol esters 33, free cholesterol 17, waxes and higher aliphatic alcohols 5, hydrocarbons 3, non-lipids (by difference) 2.

Chromatography

The column, measuring 40 cm. $\times$ 6 cm., was composed of approx. 600 g. of a mixture of 2 parts of silica gel (B.D.H. 'For chromatographic adsorption') and one part of Hyflo Super-Cel (Johns Manville, London, S.W. 1). Before use this mixture had been washed with 0.5N-HCl, followed by water until neutral, and was then activated for 24 hr. at 120°. An atmosphere of N₂ was maintained permanently in the entire chromatographic assembly (cf. Riemenschneider, Herb & Nichols, 1949). The adsorbed oxygen film on the column was removed with a solution of 13 g. of linoleic acid in 500 ml. of light petroleum (b.p. 40–60°), a procedure based on a suggestion of Professor T. P. Hilditch. Elution with 10 l. of ether and 5 l. of ether-methanol (95:5, v/v) removed both unchanged linoleic acid and its oxidation products. The column was used repeatedly in all the following separations, being washed with light petroleum after each run until all traces of other solvents had been removed. It was kept permanently covered with solvent. All solvents were dry and de-aerated.

The lipid (41 g.) was chromatographed in three equal batches, in successive runs [cf. Borgström (1952) who used 1 g. of adsorbent/30 mg. of lipid]. Each batch was added to the column dissolved in 500 ml. of light petroleum (b.p. 40–60°). The eluting solvents in the first run were light petroleum, light petroleum-benzene (1:1, v/v), benzene and benzene-ether (1:1, v/v). In the second and third runs, the benzene-ether mixture contained only 5% ether (v/v) and was followed by pure ether to obtain complete elution of all lipid. Each eluant was used until it virtually ceased to bring any material off the column, and was collected in fractions of 1 l. In each case a final fraction, representing some 10 l. of solvent, was collected during the washing of the column with light petroleum ready for its re-use. The results of the three runs are shown in Fig. 1.

The significance of certain features of Fig. 1 will be discussed later. It is, however, immediately obvious that in spite of sharp separation of four main groups of product in each run, these cannot represent essentially pure substances, since material contributing to one peak in one curve must be contributing to a different peak in another curve. However, corresponding peaks in the three runs showed considerable resemblance. Thus the first peak (light petroleum) in each case ranged from a soft white wax to a pale-yellow liquid as elution continued, and gave only a trace reaction for cholesterol. The second peak (light petroleum-benzene) was in each case a pale-yellow, mobile liquid, which gave a strong reaction for cholesterol. The third peak (benzene) was similar to the second in appearance, but gave

only a faint reaction for cholesterol. The fourth peak (benzene-ether) was predominantly solid, containing crystals of free cholesterol. The small final peak of the second and third runs (ether), together with the last two fractions of the first run, was a brown viscous oil (total, 2.7 g.), giving only a weak reaction for cholesterol.

From Borgström's (1952) work it was considered that these peaks must represent concentrates of hydrocarbons, cholesterol esters, triglycerides and triglycerides plus free cholesterol, respectively, the final ether peak possibly representing partially oxidized triglycerides. The material from the three runs was accordingly combined into five main fractions, the first four of which were rechromatographed.

The same column was used, without any re-activation. The combined first peaks (fractions 1–7, 1–5 and 1–9 from the three respective runs, giving fraction A, 7.5 g.) were added to the column dissolved in 250 ml. of light petroleum. Elution, as before, was continued with each successive solvent until further elution appeared to be unprofitable. Fifty-nine fractions of 250 ml. (first fraction 500 ml.) were collected and the eluants employed were light petroleum (b.p. 40–60°), petroleum-benzene mixtures (95:5, 90:10, 80:20, 60:40 by vol. successively), benzene and ether. The

Fig. 1. Chromatography of crude lipids on silica gel. Curves 1–3 from successive batches of lipids. Elution data: batches 1–3 respectively; light petroleum (b.p. 40–60°), fractions 1–7, 1–7, 1–8; light petroleum-benzene (1:1, v/v), fractions 8–13, 8–11, 9–15; benzene, fractions 14–19, 12–16, 16–20. Batch 1 only; benzene-ether (1:1, v/v), 20–22. Batches 2 and 3; benzene-ether (95:5, v/v), 17–22, 21–27; ether, 23–25, 28–30. Final fraction in each run from column washings.

final fraction, as usual, consisted of column washings with a large volume of light petroleum. The results are illustrated in Fig. 2. On the basis of adsorption, appearance and cholesterol reaction the fractions were pooled into *A* 1-13 (3.3 g.), *A* 14-19 (2.0 g.), *A* 20-31 (1.6 g.), *A* 32-48 (0.2 g.), *A* 49-59 (0.4 g.).

Fraction *B* (fractions 8-13, 6-10, 10-14 from the three successive runs, total 9.4 g.) was rechromatographed similarly to fraction *A* except that one light petroleum-benzene mixture (95:5, v/v) was omitted. Elution proved to be very slow and seventy-two fractions were collected, as shown in Fig. 3. These were pooled into *B* 1-4 (0.1 g.), *B* 5-8 (0.7 g.), *B* 9-21 (2.1 g.), *B* 22-39 (1.5 g.), *B* 40-50 (1.8 g.), *B* 51-58 (0.1 g.), *B* 59-67 (2.6 g.), *B* 68-72 (0.5 g.).

Fig. 2. Rechromatography of fraction *A*. Eluant (see text) changed at fractions 10, 22, 31, 37, 45, 50. Final fraction from column washings.

Fig. 3. Rechromatography of fraction *B*. Eluant (see text) changed at fractions 5, 40, 50, 57, 64. Final fraction from column washings.

Fraction *C* (fractions 14-19, 11-17, 15-21 from the three runs, total 8.2 g.) was rechromatographed, eluting successively with light petroleum (b.p. 40-60°), light petroleum-ether (99:1, v/v) and light petroleum-ether (80:20, v/v). Twelve fractions of 500 ml. were collected, plus the usual final column-washing fraction, the details being shown in Fig. 4. The fractions were pooled into *C* 1 (0.7 g.), *C* 2-5 (0.5 g.), *C* 6-8 (6.8 g.), *C* 9-12 (0.2 g.) and *C* 13 (86 mg.).

Fraction *D* (20, 18-22, 22-27 from the three runs, total 9.0 g.; fraction 21 of the first run was unfortunately lost) was rechromatographed, eluting successively with light petroleum (b.p. 40-60°), light petroleum-ether mixtures (1, 2, 4, 8, 20, 50% ether, by vol.) and ether, with a final

Fig. 4. Rechromatography of fraction *C*. Eluant (see text) changed at fractions 5 and 11. Final fraction from column washings.

Fig. 5. Rechromatography of fraction *D*. Eluant (see text) changed at fractions 4, 7, 10, 15, 24, 42 and 48. Final fraction from column washings.

fraction from column washings. The separation into fifty-two fractions is shown in Fig. 5. Fractions were pooled into *D* 1-3 (0.4 g.), *D* 4-15 (0.1 g.), *D* 16-26 (1.8 g.), *D* 27-40 (5.0 g.), *D* 41-46 (1.0 g.), *D* 47-52 (0.7 g.).

Analysis

All pooled fractions were analysed for cholesterol, total unsaponifiable matter, fatty acids and glycerol. Hydrolysis involved refluxing for 8 hr. with 0.5*N* ethanolic KOH. The methods used for cholesterol and glycerol assay were as given by Lovern & Olley (1953*a*). When enough was available the unsaponifiable matter was acetylated, the saponification equivalent determined and, allowing for the cholesterol present, the equivalent of the non-sterol unsaponifiable matter calculated. On the basis of an average value of 300 for the acetates of higher aliphatic alcohols, the relative proportions of alcoholic and non-alcoholic (hydrocarbon?) material were calculated. An average mol.wt. of 300 was assumed for the fatty acids and the approximate composition of each pooled fraction calculated by dividing the available fatty acids among the other constituents. Some fractions yielded water-soluble products of hydrolysis other than glycerol; this was assumed to represent non-lipid impurities. Non-sterol alcoholic compounds were calculated as free or esterified according to the availability of fatty acids after the requirements of cholesterol esters and triglycerides had been met. Cholesterol was assumed to be esterified in fractions *A*, *B* and *C*, and to be free in fractions *D* and *E*. This is in agreement with chromatographic behaviour for *A*, *B*, *D* and *E* (Borgström, 1952; Garcia, Lovern & Olley, 1956) and with the appearance of the

fractions. For fraction *C* the assumption is more doubtful, but fraction *C* is poor in cholesterol.

The analytical results are given in Table 1, and the calculated compositions in Table 2. The latter were considered adequate as a basis for pooling fractions representing concentrates of the cholesterol esters and triglycerides, respectively, but in view of the various assumptions enumerated above can only be regarded as approximations. The analytical results on fraction *C* 13 (86 mg.) were inadequate for even a rough calculation of composition.

The percentage composition of the starting material, calculated from the results in Table 2 and the weights of the various fractions is: triglycerides 36, cholesterol esters 24, free cholesterol 10, waxes and higher aliphatic alcohols 12, hydrocarbons 15 and non-lipids 3.

The fatty acids of fractions *A* 14-19, *A* 20-31, *A* 32-48, *B* 5-8, *B* 9-21, *B* 22-39, *B* 40-50 and *B* 51-58 were combined, representing some 92% of the total cholesterol esters. Other fractions containing cholesterol esters were of too mixed a nature to be included. The fatty acids of fractions *C* 6-8, *D* 16-26, *D* 27-40, *D* 41-46, *D* 47-52 and *E* were likewise combined (Table 3, triglycerides 2), representing 85% of the total triglycerides. The fatty acids of fraction *B* 59-67, representing 14% of the total triglycerides, were examined separately (Table 3, triglycerides 1), in view of their sharp chromatographic separation. Each group of acids was analysed by fractional distillation of the methyl esters (semi-micro procedure of Lovern, 1934), but the small quantities available (3.5, 10.5 and 1.8 g. respectively) mean that the results can only be regarded as rough approximations. In the last sample no attempt was made to separate

Table 1. *Analytical data on pooled fractions*

All values except saponification equivalents are g./100 g. of lipid. A dash indicates no determination made.

Fraction	Fatty acids	Cholesterol	Total unsaponifiable	Saponification equiv. of acetylated unsaponifiables	Glycerol
<i>A</i> 1-13	Trace	Nil	100	9000	—
<i>A</i> 14-19	43.8	53.9	59.2	—	—
<i>A</i> 20-31	43.1	59.4	59.4	—	—
<i>A</i> 32-48	30.5	41.6	62.3	—	Trace*
<i>A</i> 49-59	19.3	13.2	68.0	877	Trace*
<i>B</i> 1-4	5.8	2.7	78.0	1248	—
<i>B</i> 5-8	38.6	40.4	55.4	466	—
<i>B</i> 9-21	44.4	43.3	50.9	414	Nil*
<i>B</i> 22-39	38.9	39.6	48.6	412	Nil*
<i>B</i> 40-50	39.1	44.4	52.3	416	Nil*
<i>B</i> 51-58	26.4	24.7	49.8	—	Nil*
<i>B</i> 59-67	76.5	5.0	16.2	302	7.0
<i>B</i> 68-72	22.2	6.6	60.1	610	Present*
<i>C</i> 1	13.0	8.5	78.8	2644	Trace*
<i>C</i> 2-5	15.8	10.6	53.9	629	Trace*
<i>C</i> 6-8	82.5	1.7	12.1	303	7.7
<i>C</i> 9-12	52.0	8.3	37.1	—	2.6
<i>C</i> 13	30.8	13.3	64.8	—	Present*
<i>D</i> 1-3	1.8	Nil	92.2	3721	Trace*
<i>D</i> 4-15	66.3	1.2	14.8	—	6.0†
<i>D</i> 16-26	71.9	5.9	20.6	341	5.0†
<i>D</i> 27-40	33.1	60.9	62.1	—	4.8
<i>D</i> 41-46	61.6	9.0	25.9	376	6.3
<i>D</i> 47-52	50.9	5.0	28.6	424	8.7
<i>E</i>	43.2	8.0	45.4	805	5.4

* By paper chromatography

† Too low, because of volatilization during concentration.

Table 2. *Calculated approximate composition of pooled fractions*

The composition is given as g. of component/100 g. fractions. A dash indicates not determined, but probably negligible.

Fraction	Cholesterol esters	Free cholesterol	Triglycerides	Waxes and higher alcohols	Hydrocarbons etc.	Non-lipids
A 1-13	Nil	Nil	—	3	97	Nil
A 14-19	90	—	—	10	—	Nil
A 20-31	100	—	—	Nil	Nil	Nil
A 32-48	70	—	—	21	—	9
A 49-59	22	—	—	28	37	13
B 1-4	5	—	—	23	56	16
B 5-8	70	—	—	14	8	8
B 9-21	75	—	—	18	—	7
B 22-39	69	—	—	17	—	14
B 40-50	77	—	—	12	—	11
B 51-58	43	—	—	32	—	25
B 59-67	9	—	70	21	—	Nil
B 68-72	11	—	Present	45	25	19
C 1	15	—	5	4	67	9
C 2-5	18	—	5	6	40	31
C 6-8	3	—	77	20	—	Nil
C 9-12	14	—	26	44	7	9
D 1-3	Nil	Nil	2	7	85	6
D 4-15	—	1	74	14	—	11
D 16-26	—	6	80	14	—	Nil
D 27-40	—	61	37	1	—	1
D 41-46	—	9	68	17	—	6
D 47-52	—	5	57	18	5	15
E	—	8	48	12	25	7

Table 3. *Composition of the fatty acids of certain lipids of haddock flesh*

Values are g./100 g. of total acids. Average unsaturation shown in parentheses, as lack of hydrogen atoms.

Lipid	Saturated acids			Unsaturated acids			
	C ₁₄	C ₁₆	C ₁₈	C ₁₆	C ₁₈	C ₂₀	C ₂₂
Cholesterol esters	—	8	6	—	20 (3.4)	29 (6.1)	37 (7.8)
Triglycerides (1)	3	9	4	25 (> 2)	51 (4.0)	8 (?)	—
Triglycerides (2)	Trace	15	7	12 (> 2)	22 (4.0)	29 (7.0)	15 (10.8)
Triglycerides (total)	Trace	14	7	14 (> 2)	26 (4.0)	26 (7.0)	13 (10.8)
Lecithins*	—	18	7	—	14 (4.2)	35 (7.6)	24 (10.8)

* Also contains 2% of C₂₀ saturated acids.

the acids into saturated and unsaturated fractions before methylation and distillation. Instead, the total acids were methylated and distilled, giving five fractions, in which the proportions and equivalents of saturated esters were subsequently determined by oxidative removal of the unsaturated components. With the acids of the cholesterol esters, the saturated fraction (0.4 g.) was too small for fractional distillation, and its composition had to be estimated directly from its analytical characteristics, involving assumptions that could not be checked, e.g. that palmitic and stearic were the only saturated acids present.

The compositions of the three groups of fatty acids are given in Table 3, together with the values for the total triglyceride mixture.

DISCUSSION

Chromatographic separation

The variability of silicic acid as an adsorbent has often been noted, e.g. by Borgström (1952). In the author's laboratory extreme variation has been observed between a silicic acid of American origin and silica gel of British manufacture: e.g. the American product adsorbed lecithin more strongly than cholesterol or triglycerides, whereas silica gel ('for chromatographic adsorption') from two British firms, although a strong adsorbent for

cholesterol and triglycerides, failed to adsorb lecithin from light petroleum solution. It is therefore desirable to specify the precise grade and source of silicic acid or silica gel.

The three runs with the same raw material indicate a certain amount of variability in the adsorbent after use, but no loss of adsorptive strength. Indeed, the progressive lowering of the first peak, and other evidence in Fig. 1 of somewhat more difficult elution in the later runs, suggest that the adsorptive power increases with use.

The fact that none of the peaks, in spite of apparently good separation from each other, represents a pure class of lipid was suggested by this variation between runs in Fig. 1, and proved by rechromatography of the primary peaks. Fraction *C* is the most homogeneous, but all primary fractions contain, for instance, small proportions of easily eluted material, apparently largely hydrocarbons, which have previously escaped ready elution. Apparently some lipids can influence the adsorptive behaviour of other lipids, just as they can influence their solubility.

Silica gel is capable of fractionating both cholesterol esters and triglycerides according to fatty acid composition. Thus the main cholesterol-ester fractions of Figs. 2 and 3 (see Table 2) were respectively: *A* 14–19, wax; *A* 20–31, softer wax; *A* 32–48, liquid; *B* 5–8, wax; *B* 9–21, *B* 22–39, *B* 40–50, all liquids at room temperature. Similar progressive changes were evident in the fatty acids of these fractions, and of the principal triglyceride fractions. The more unsaturated compounds were the more strongly adsorbed. This is illustrated in more detail by the fatty acids of the triglycerides of fractions *B* 59–67 compared with those of the main triglyceride fractions eluted later (see below). The hydrocarbon fractions show a similar gradation of properties, e.g. fractions *A* 1–13 range progressively from a fairly hard white solid at room temperature through softer solids to liquids. This is another possible reason for the overlapping of lipid types from one chromatographic peak to another. It is known that silicic acid fractionates free fatty acids or their methyl esters according to unsaturation (Claesson, 1946; Riemen-schneider *et al.* 1949; Lemon, 1949; Kurtz, 1952).

There was no difficulty in obtaining quantitative elution from the column, and no evidence of oxidation occurring on it. Fraction *E* probably represented material already slightly oxidized before chromatography. Although its fatty acids were dark in colour, they behaved normally during methylation and distillation. Apart from fraction *E* all fractions were either colourless or very pale yellow.

Composition

The composition of the total material, estimated by analysis before chromatography, shows certain differences from the composition determined from analyses on the various chromatographic fractions. While triglycerides and non-lipids show satisfactory agreement, both free and total cholesterol are higher before chromatography, at the expense of other unsaponifiable constituents (higher alcohols and hydrocarbons). This is probably due to falsely high values for cholesterol in the first analysis, the other unsaponifiable matter being determined by difference.

It is, of course, not certain that everything calculated as higher aliphatic alcohols really is such material. Some of it may be steroidal alcoholic material other than cholesterol, and interfering in one method of cholesterol assay, but not in the other. The same may be true of some of the so-called hydrocarbon, which is really non-acetylable and, therefore, presumably non-alcoholic unsaponifiable matter. The main peak of such material (fractions *A* 1–13) was distilled under vacuum, and analyses of the fractions confirmed that it was, indeed, almost entirely a range of hydrocarbons corresponding closely to those reported by Garcia *et al.* (1956). However, it can be seen from Table 2 that small proportions of 'hydrocarbons' appeared in the last-eluted fractions of each run, and quite large amounts in fraction *E*. It is improbable that such material consists of normal aliphatic hydrocarbons.

Garcia *et al.* (1956) reported that the hydrocarbons were probably largely contaminants derived from rubber. A chromatographic analysis of the total unsaponifiable matter from cod-flesh lipids, prepared with rigid exclusion of all contact with rubber, has since revealed that it does contain a very small proportion of hydrocarbons. The most readily eluted material was a soft wax at room temperature. It had an iodine value of 59; mean mol.wt. 312; C 85.96; H 12.69%. (C, H and mol.wt. determinations by Weiler and Strauss, Oxford.) The amount was equivalent to about 0.2% of the total lipids, compared to the 3.2% of Garcia *et al.* (1956). The position with haddock is presumably similar.

The supposed higher aliphatic alcohols were probably esterified in the more readily eluted fractions (*A*, *B* and *C*), since in most cases there was about an equivalent amount of fatty acid available after allowing for cholesterol esters and triglycerides. In fractions *D* and *E*, however, there was little or no available fatty acid and the alcohols were presumably free. By analogy with cholesterol, the alcohols would be expected to be less strongly adsorbed when esterified.

From Table 1 it can be seen that the most strongly adsorbed of the main glyceride fractions (*D* 47–52) had a glycerol:fatty acid ratio considerably higher than that for triglycerides (about 1:10 for fatty acids of mean equivalent 300). In earlier work (Olley & Lovern, 1954; Garcia *et al.* 1956) evidence was found of the possible occurrence of traces of monoglycerides in haddock- and cod-flesh lipids. Monoglycerides, but not diglycerides, would have been removed by the countercurrent procedure from the lipids used in the present work (cf. Olley & Lovern, 1954). Diglycerides would be more strongly adsorbed than triglycerides (Kaufmann, 1940; Borgström, 1954). There is, therefore, a suggestion that fractions *D* 47–52 contain a proportion of diglycerides.

The fact that triglycerides exceeded cholesterol esters in the starting material was unexpected, in view of previous findings with both haddock (Lovern & Olley, 1953*a*) and cod (Garcia *et al.* 1956). It suggests that the triglyceride content of the flesh of these species, although almost negligible, may vary seasonally just as it does in the flesh of fatty species, such as herring, or in the main fat depot (liver) of haddock and cod.

Fatty acids

In Table 3 are given the fatty acid compositions of the cholesterol esters, triglycerides and total lecithins of haddock flesh. The latter value has been derived by appropriately weighted summation of the values for the various lecithin fractions (*A*, *B*, *C*1, *C*2 and *P*1–3) examined previously (Lovern & Olley, 1953*a, b*; Olley & Lovern, 1953).

The cholesterol esters are unusual in that the proportions of homologous unsaturated-acid groups increase with chain length, instead of passing through a maximum. Since the longer-chain acids are also the more highly unsaturated, the effect is to increase the mean degree of unsaturation of the cholesterol-ester fatty acids over that which would result from a more normal group disposition. The effect is heightened by the unusually low proportion of saturated acids. Thus, although the individual groups (*C*₁₈, *C*₂₀ and *C*₂₂) are less highly unsaturated in the cholesterol esters than in the triglycerides, the respective iodine values of the total fatty acids, calculated from Table 3, would be 205 for the cholesterol esters and 183 for the triglycerides. Clément *et al.* (1954), who limited their examination to determination of the mean iodine value (i.v.), found that the fatty acids of their chromatographically separated cholesterol esters of blood serum were more unsaturated (i.v. 130) than those of the accompanying triglycerides (i.v. 72), or even of the total phosphatides (i.v. 110). The lecithin of haddock flesh contains fatty acids of mean iodine value 227, calculated from Table 3. Kelsey &

Longenecker (1941) gave what appears to be the only previous detailed analysis of the fatty acids of cholesterol esters. In ox plasma they were far more unsaturated than those of the accompanying triglycerides. Although the selective enzymic hydrolysis used by Kelsey & Longenecker is open to criticism (see above), their cholesterol-ester fraction would have represented a crude concentrate of these esters and the difference in unsaturation is very great. They found the fatty acid of the cholesterol esters to belong mainly to the *C*₁₈ series, and reported the phospholipid fatty acids to have a mean iodine value similar to that of the triglycerides.

The triglyceride fatty acids differ from those of all the other haddock-flesh lipids studied (Olley & Lovern, 1953; Lovern & Olley, 1953*b*) in containing appreciable proportions of hexadecenoic acid. This is a characteristic component of the depot fat of fish and its virtual absence from the other lipids of haddock muscle was surprising. Haddock-flesh glycerides, however, have a fatty acid composition fairly similar to that of haddock-depot fat (Lovern, 1932), the differences being a somewhat greater mean unsaturation of the homologous groups and more stearic acid in the flesh glycerides.

The least strongly adsorbed triglycerides (fraction *B* 59–67; Table 3, triglycerides 1) show a remarkable reduction in the highly unsaturated acids (*C*₂₀ and *C*₂₂) compared with the rest of the triglycerides. Indeed, considering the mixed nature of triglyceride fatty acid distribution, it is surprising that such a concentrate of *C*₁₆ and *C*₁₈ acids could be obtained.

Each class of lipid appears to have its characteristic fatty acid composition. While the lecithins show little more mean unsaturation of any particular homologous group than do the triglycerides, they possess far more *C*₂₀ and *C*₂₂ acids, at the expense of *C*₁₆ and *C*₁₈ acids. The analyses in Table 3, in fact, show how uninformative the comparison of mean iodine values alone can be. Of the other lipids of haddock flesh, 'unidentified lipid A' (cf. Garcia *et al.* 1956) appears to be outstanding in its high proportion of stearic acid (Olley & Lovern, 1953), although only crude concentrates of this lipid were available for analysis. The analysis of the free fatty acids of haddock flesh (Lovern & Olley, 1953*a*) has since been found to be unreliable, due to the presence of unsaponifiable matter, some of which may have distilled with the esters rather than accumulated in the residue (Brenner, unpublished work).

SUMMARY

1. An extract of haddock flesh was prepared which contained essentially all of the triglycerides and cholesterol esters, but none of the acidic lipids

or phospholipids. Other components were free cholesterol, waxes, aliphatic alcohols and hydrocarbons.

2. This mixture was separated chromatographically on silica gel, the primary fractions being rechromatographed. Hydrocarbons, cholesterol esters and triglycerides, which were fairly well separated from each other, all showed subfractionation according to unsaturation. Esterified higher aliphatic alcohols were separated from free alcohols.

3. The fatty acids of the cholesterol esters were of an unusually high average unsaturation, owing mainly to a high content of C_{20} and C_{22} unsaturated acids and, to a less extent, to a rather low content of saturated acids.

4. The fatty acids of the triglycerides showed considerable resemblance to those of haddock-depot fat and, alone among the flesh lipids, contained a major proportion of hexadecenoic acid.

5. A triglyceride fraction, less strongly adsorbed on silica gel than the bulk of the triglycerides, contained unsaturated fatty acids much enriched in C_{16} and C_{18} derivatives at the expense of more highly unsaturated C_{20} and C_{22} acids, and hence its mean unsaturation was much lower.

6. Each class of lipid appears to have a characteristic fatty acid composition.

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Amino Acids, Fruit Acids and Polyols of Myrobalans

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Myrobalans are the dried fruit of an Indian tree, *Terminalia chebula* Retz, and they are used extensively for tanning. The ripe fruit is a hard yellow drupe, 3–5 cm. long, ovoid in shape, and containing a single seed, usually 2 cm. long and 1 cm. in diameter. When dry the fruit becomes five-ribbed.

There have recently been isolated from these dried fruit two crystalline ellagitannins, chebulagic acid (Schmidt & Nieswandt, 1950) and corilagin (Schmidt & Schmidt, 1952b), the constitutions of which (Schmidt & Nieswandt, 1950; Schmidt, Schmidt & Herok, 1954; Schmidt, Blinn & Lademann, 1952; Schmidt & Schmidt, 1952a),

together with that of a third tannin, chebulinic acid, have now been elucidated. Reference to two-dimensional chromatograms, Fig. 1 (cf. White, Kirby & Knowles, 1952), of the polyphenols and tannins in methanolic extracts of myrobalans (43–53% tannin, hide-powder test), specially collected in Ceylon and in the Bhimlipatam, Coimbatore, Jubbulpore and Kolhapur districts of India, showed that chebulagic and chebulinic acids and corilagin were major constituents, accompanied by varying proportions of the following products of their complete and incomplete hydrolysis: chebulic acid, 3:6-digalloylglucose, ellagic acid, gallic acid and β -glucogallin. Small quanti-