a qualitatively and quantitatively different picture, also suggest a variety of linkages between the lipids and the protein of the flesh.

The various large-scale extracts revealed considerable differences in appearance, consistency, etc., and it seems probable that they have been separated not only according to the relative strength of their linkages to protein, but also according to their solubility properties. A considerable overlap in their composition would, therefore, be expected. The following paper records the results of investigation of two sub-fractions of the first (acetone) extract.

SUMMARY

1. Haddock flesh contains a total of about 1% lipid, but only about 0.1–0.2% of 'free' lipid, the rest being probably 'bound' to protein.

2. Extraction with a series of solvents suggests the presence of more than one type of linkage between lipid and protein although the picture is complicated by probable differences in solubility of the various lipid constituents.

This work has been carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Lipids of Fish

2. THE ACETONE-SOLUBLE LIPIDS OF THE FLESH OF THE HADDOCK

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In the previous paper (Lovern, 1953) the preparation of a series of lipid extracts from the flesh of the haddock (*Gadus aeglefinus*) was described. The first of these consisted of material extracted from the tissue by acetone and soluble in light petroleum. It is well known that in the presence of such soluble lipids as triglycerides, sterols and sterol esters, acetone will dissolve other lipids, e.g. phospholipins, which alone are insoluble in acetone. This particular extract contained 2.6% phosphorus and 1.6% nitrogen. There has been controversy, particularly in earlier work, about the occurrence of phospholipins genuinely soluble in acetone, i.e. soluble in the absence of such lipids as fats and sterols. MacLean (1914) showed that such phospholipins do occur but can be completely precipitated from acetone in the presence of traces of electrolytes. Removal of impurities, presumably electrolytes, can render an originally acetone-insoluble phospholipin soluble in acetone (MacLean, 1909a). In the purification of phospholipin preparations by repeated precipitation from ether or light petroleum solution by a large excess of acetone, many workers add an electrolyte, commonly magnesium chloride. There are, however, objections

that such a procedure gives a variable yield according to the amount of electrolyte added, and that the nature of the phospholipin is altered in the process, e.g. the proportion of ether-insoluble phospholipin increases with increasing amount of electrolyte (Sinclair & Dolan, 1942). Cahn, Houget & Agid (1949) discussed in detail the relative merits of precipitation with acetone in the presence or absence of added electrolytes and showed that electrolytes also precipitate large proportions of non-lipid contaminants.

We considered it desirable to examine separately the phospholipin fraction genuinely soluble in acetone in the absence of added electrolytes and that present in the total acetone extract but precipitable when accompanying non-phosphatidic lipids had been removed. The technique of counter-current distribution, used in our earlier studies on lipid separation (Lovern, 1952; Olley, 1953), was employed to examine the fractions obtained by acetone precipitation. The non-phosphatidic lipids, together with the acetone-soluble phospholipins, form the subject of the present paper.

Part of this work was presented at the Second International Congress of Biochemistry, Paris, 1952.

EXPERIMENTAL

Analytical methods

Choline and serine plus ethanolamine were estimated as described by Lovern (1952), N, P and plasmal as described

latter being precipitated according to the procedure of Sobel & Mayer (1945) but retaining the acetone/ether wash of the original Schoenheimer & Sperry (1934) method. Glycerol was determined by Whyte's (1946) method scaled down 10 times. Fatty acid analysis by ester distillation was

Fig. 1. Schematic summary of the fractionation of the acetone extract of the flesh of the haddock.

by Olley (1953), glycerophosphate as in Olley & Blewett (1950), total cholesterol by the method of Rose, Schattner & Exton (1941) applied direct to the lipid; free cholesterol was estimated by the same method applied to the digitonide, the

essentially as described by Guha, Hilditch & Lovern (1930), with some later modifications (cf. Lovern, 1934, 1942).

All lipid materials were stored under N_2 at -30° until required for analysis.

Fractionation with acetone

A summary of the fractionation procedure is shown in Fig. 1.

Acetone-extract (about 230 g.) of haddock flesh prepared according to Lovern (1953) was mixed with 300 ml. of ether to a viscous solution and poured slowly with constant stirring into 6.6 l. of acetone at 0°. There was an immediate precipitation of a thick gum and on standing overnight at 0° a further precipitation of a flocculent nature. The total insoluble material (1a) weighed 185 g. and the soluble (1b) 44 g. Fraction 1b was made up to 100 ml. with ether and poured into 2 l. of acetone at 0°, but proved entirely soluble. It contained 1.0% P, no N and gave a strong reaction for cholesterol. Fraction 1a was again taken up in ether and precipitated with acetone as before. In this case the first appearance of the precipitate was flocculent and it later became gummy. Yield of insoluble fraction (2a) 153 g. and of soluble fraction (2b) 32 g. Fraction 2b contained 2.1% P, 0.8% N and much cholesterol. The same reprecipitation procedure applied to 2a gave a flocculent precipitate which settled overnight to a spongy mass. This precipitate, unlike the previous ones, proved to be insoluble in ether, but readily soluble in CHCl_3 . All subsequent precipitates in this acetone-fractionation series were insoluble in ether but soluble in CHCl_3 , which was, therefore, used instead. We have repeatedly observed that when crude phospholipin mixtures are fractionated, either by precipitation methods or by counter-current procedures, certain fractions progressively become less soluble or even abruptly virtually insoluble in ether. We feel that this is probably due to the previously mentioned effects of accompanying electrolytes, or other contaminants, which are being concentrated in certain fractions, and is similar to the effects on solubility in acetone and to the effect on ether solubility noted by Sinclair & Dolan (1942).

Fraction 2a yielded 142.5 g. of insoluble (3a) and 10.5 g. of soluble material (3b). The latter gave a definite reaction for cholesterol. Further reprecipitations of the acetone-insoluble portion under the same conditions of dilution and temperature yielded a series of acetone-soluble fractions as illustrated in Fig. 2. (Insoluble and soluble fractions are given the suffixes *a* and *b*.) Fraction 4b no longer gave a reaction for cholesterol, and it and all subsequent ones had a typically phosphatidic appearance, in contrast to fractions 1b and 2b which were oily liquids, 3b being intermediate.

Fig. 2 illustrates the marked effect of electrolytes or other effective contaminants on solubility in acetone. Only on the basis of selective removal or concentration of such agents is it possible to explain the rise from the minimum at 3b to the maximum at 5b. The levelling out of the weight curve for the last three fractions suggests that this value of about 5 g. represents the solubility of the 'insoluble' fraction in 6.6 l.

of acetone (containing 200 ml. of CHCl_3) at 0°. Fraction 10b was accordingly combined with the final acetone-insoluble fraction 10a, giving 74 g. of material (fraction C), fractions 1b-3b were combined to give 80 g. (fraction A) of material containing, presumably, all the non-phosphatic lipid (e.g. all the cholesterol), together with some phospholipin, and fractions 4b-9b were combined to give 70 g. of a fraction (B) consisting mainly of acetone-soluble phospholipins.

Fig. 2. Reprecipitation from acetone of the lipids extracted by acetone from haddock flesh. Total initial wt. 224 g. Curve shows weight of lipid remaining in solution after each precipitation.

A general comparison of the phospholipins contained in these three fractions is given in Table 1. It should be noted that for fraction A the data relate only to the phospholipin portion separated by counter-current distribution from the non-phosphatidic lipids as described below, whereas it was assumed that fractions B and C consist mainly of phospholipins.

Table 1. *Analytical data for fractions B and C and the crude phospholipin portion of fraction A*

(Values are percentages of constituents in the fractions.)

Fraction	N (%)	P (%)	N:P ratio (atomic)	Choline (%)	Serine + ethanolamine		Plasmal (%)
					N (%)		
A (1-12)*	2.32	3.25	1.58	11.2	Nil		Trace
B	1.80	3.05	1.31	10.1	0.17		4.7
C	2.21	3.34	1.47	6.2	0.13		2.0

* Phospholipin portion, see p. 131.

Table 1 shows that all preparations contained too high an N:P ratio for monoaminophospholipins and that neither repeated precipitation from acetone nor counter-current distribution in the case of fraction *A* had removed the excess N. The fractions also contain considerable uncharacterized lipid, the recognized phospholipin bases and P contents being less than required for a mixture of the classical phospholipins. The greatest discrepancy in respect of nitrogenous bases is shown by fraction *C*.

In view of the unexpected course of the acetone-precipitation curve in Fig. 2, and as we have been unable to find any comparable published experiments, it may be mentioned that prior to carrying out the present series of studies we had conducted exploratory work with an exactly comparable series of haddock flesh lipid extracts, prepared some 2 years earlier. This preliminary work gave the same form of acetone-precipitation curve.

Since the solubility of fraction *C* in acetone at 0° was apparently about 5 g./6.6 l., it seemed that fractions 1–9 might each be contaminated with about 5 g. of *C* and hence fraction *B* might contain about 30 g. of material really belonging to fraction *C*. Fraction *B* was accordingly dissolved in 250 ml. ether and precipitated in 6 l. of acetone at 0°, yielding 24 g. soluble and 46 g. insoluble lipid. The latter was a thick gum probably occluding some soluble lipid, and hence was reprecipitated with 100 ml. of ether and 4 l. of acetone, yielding an additional 8 g. soluble and 38 g. insoluble lipid. A total of 10 l. of acetone could dissolve about 7.5 g. of fraction *C*, so that the predicted yield of insoluble matter was about 23 g. instead of the 38 g. actually obtained, again illustrating the complexity of the factors affecting the solubility of phospholipins in acetone. As shown later, fraction *A* also contained much phospholipin really belonging to fraction *C*. For the present examination, the 38 g. of insoluble lipid obtained from *B* were added to *C*, leaving 32 g. of the final fraction *B'*. *B'* contained 2.1% N before hydrolysis, 1.8% water-soluble N after hydrolysis, 9.2% choline, 2.0% plasmal and 3.3% P.

Analysis of fractions

Fractions *A* and *B'* were submitted to counter-current distribution between 85% (v/v) ethanol and light petroleum (b.p. 40–60°), using conical flasks as units, as described by Lovern (1952). When conducted manually this operation is particularly flexible, and the sequence of transfers can be modified at any stage according to the appearance of both phases at various positions in the chain of units, or the progress of elution from the end of the chain. Periodical emulsification, usually late in the sequence, was again observed (cf. Lovern, 1952). Emulsified material was separated centrifugally, and a marked tendency was noted for such material to re-emulsify on each transfer right through the series.

Fraction A

Fraction *A* (80 g.) was added to the first of thirty flasks containing 400 ml. of light petroleum and successive batches of 400 ml. of 85% ethanol were passed down the chain and run off as a series of fractions. By the fifteenth such fraction the yield had become very low and the appearance of the petroleum in flasks 19–30 suggested that these flasks contained almost no lipid, hence they were withdrawn from the system and the contents of each separately evaporated. The fifteen ethanol fractions run off are

numbered 1–15 in Fig. 3 and flasks 30–19 of light petroleum yielded fractions 16–27 in Fig. 3. Seventeen further batches of ethanol were then run through the remaining eighteen flasks of light petroleum (1–18), and these yielded fractions 28–44 in Fig. 3. Finally the light petroleum in flasks 18–1 was evaporated to yield fractions 45–62 in Fig. 3. The sequence of counter-current events is thus arranged in Fig. 3 to run continuously according to the tendency towards one or the other phase, vertical dotted lines indicating where the curves are really discontinuous. Certain fractions were analysed for N, P, choline, free and esterified cholesterol, the results being shown in Fig. 3.

Fractions were pooled according to analyses, appearance and weight yield. Fractions 1–12 were combined to include virtually all of the phospholipin, fractions 13–33 to give a fraction relatively sterol free, fractions 34–44 a mixed fraction rich in free sterol, fractions 45–52 a main free sterol fraction, and fractions 53–62 to include all esterified cholesterol. (From other experiments we know that in counter-current distribution with these solvents, triglycerides largely accompany esterified cholesterol and should, therefore, also be almost exclusively contained in fractions 53–62. As will be shown below, such was the case.)

Fractions A 1–12. The general analysis of fractions *A* 1–12 is given in Table 1. In an attempt to remove the excess N, this fraction (about 33 g.) was submitted to counter-current separation in a series of four flasks between light petroleum and 20% (v/v) ethanol (400 ml. each phase). Three ethanol fractions were eluted and contained only 0.253 g. in the first eluate and 0.185 g. in the second and third combined. These fractions contained 6.9 and 6.2% N and 0.4 and 0.7% P respectively. They were only partially soluble in ether but readily soluble in water. The removal of larger amounts of N-rich material was attempted in seven flasks between light petroleum and 50% (v/v) ethanol, but abandoned because of severe emulsification (not found with 20% ethanol). The small yield of ethanol phase recovered contained 0.13 g. of material with 5.7% N and 1.9% P. The experience of Olley (1953) suggested that a very high lipid concentration might prove effective and hence the material (approx. 32 g.) was dissolved in 100 ml. of light petroleum and shaken with 100 ml. of 85% (v/v) ethanol. The two phases proved completely miscible, although in previous work with phospholipins we have used such concentrations. The volumes of both phases were doubled, when ready separation occurred. A series of seven ethanol fractions was run through nine batches of light petroleum, but examination of the fractions showed no helpful removal of excess N; thus fractions 1, 2 and 5 of the petroleum phase contained only 1.4, 1.9 and 2.0% N respectively, the excess N accompanying the main weight of material into the ethanol. The combined petroleum fractions (5.8% of the total) were taken up in ether and washed with dilute aqueous Na₂CO₃, since free fatty acids were found in many other fractions (see below). This material yielded 0.6 g. free fatty acid and 1.1 g. neutral substance.

The ethanol-phase crude phospholipin fraction contained 2.3% N, 3.2% P (N:P ratio, 1.6), 11.2% choline, 0.07% α -amino-acid N and 0.3% N liberated as NH₃ by addition of alkali to an acid hydrolysate.

Since non-phosphatidic lipids had now been largely removed, a test was made of the acetone solubility properties of this fraction. About 30 g. was dissolved in 100 ml. of ether and poured into 1500 ml. of acetone at 0°, giving 35% soluble (fraction *a*) and 65% insoluble lipid (fraction *b*). This again illustrates the effect of the presence of other lipids

or of a particular concentration of electrolytes. Fraction (a) proved devoid of free fatty acids (fraction (b) would naturally be so), and the 1.1 g. of neutral product from the petroleum phase was combined with it. Fractions (a) and (b) contained 2.4 and 2.6% N and 3.3 and 3.8% P respectively. They were separately hydrolysed by refluxing for 2 hr. with 0.5N-ethanolic KOH. After addition of water the soap solutions were extracted with ether giving 8.8 and 3.6% unsaponifi-

able matter respectively. This had I_2 values of 159 and 222 and, after acetylation, saponification equivalents of 484 and 300. Fractions (a) and (b) further yielded 9.5 and 12.6% choline and 56.5 and 60.4% fatty acids respectively, the latter having I_2 values of 276 and 243. The two lots of fatty acids were combined, giving enough material for a quantitative analysis by ester distillation, the results of which are given in Table 2.

Fig. 3. Haddock flesh lipids. Counter-current distribution of acetone-soluble fraction A between light petroleum and 85% (v/v) ethanol. Fifteen successive batches of ethanol (fractions 1-15) passed through a chain of thirty flasks containing light petroleum. Flasks 30-19 withdrawn, contents giving fractions 16-27. A further seventeen batches of ethanol (fractions 28-44) passed through the remaining eighteen flasks, the contents of flasks 18-1 finally becoming fractions 45-62. Curves for yield, free and esterified cholesterol pass through experimental values for every fraction. Experimental values for fractions 1-11, 15, 22, 30, 36, 40, 57 and 62 on nitrogen curve and for fractions 1-12 and 15 on phosphorus curve. Zero phosphorus values found on fractions 22, 30, 33, 36 and 40. Free cholesterol content of pooled fractions 16-27 = 8%.

Table 2. Fatty acid composition of certain lipids of the haddock

(Weights as % of total fraction.)

Material	Saturated acids				Unsaturated acids					
	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄
Lecithin 'A' (flesh)	—	13.5	5.7	2.9	—	—	12.6 (5.4)*	33.7 (8.0)	27.3 (12.0)	4.3
Lecithin 'B' (flesh)	—	16.4	4.0	2.1	—	—	5.4 (5.5)	42.2 (8.0)	29.9 (11.0)	—
Free fatty acids (flesh)	—	12.1	7.3	3.7	—	—	20.6 (4.0)	37.4 (7.5)	18.9 (10.0)	Trace
Depot fat† (liver)	4.3	14.1	0.3	—	0.5	12.4 (2.0)	30.5 (2.6)	29.3 (5.9)	8.6 (7.3)	—

* Figures in parentheses show average unsaturation as lack of hydrogen atoms.

† Lovern (1932).

In view of the presence of uncharacterized N, it may be noted that after alkaline hydrolysis only 1.3 and 1.4% N could be found in the aqueous phase of (a) and (b), in contrast to the 2.4 and 2.6% before hydrolysis. Part of the loss may correspond to the 0.3% N released as NH_3 on adding alkali to an acid hydrolysate, but a further source of loss is N present in the fatty acids. Other workers (MacLean, 1909a, b; Trier, 1913a, b, c; Bülow & Page, 1932) have reported that fatty acids liberated by either alkali or acid hydrolysis of phospholipins contain about 0.1–0.5% N which cannot be removed by thorough washing or prolonged hydrolysis. Our fatty acids contained 0.2 and 0.5% for fractions (a) and (b) respectively. Trier (1913a) further found that fatty acids recovered after hydrolysis with $\text{Ba}(\text{OH})_2$ contained P equal to 0.12% of the original lipid. We did not examine either fatty acids or unsaponifiable matter for P, but we did note that the aqueous solution after hydrolysis of (a) and (b) contained P equivalent to only 2.3 and 3.2% of the original lipids respectively, compared with 3.3 and 3.8% before hydrolysis. The glycerophosphate P in the solution was even less, being 2.0 and 2.4% respectively of the original lipid.

Fractions A 13–33. Fractions 13–33 (total weight 9.35 g.) gave 95% of ether-soluble lipid, which was a mobile liquid at room temperature, and 5% of ether-insoluble, water-soluble substance, which was discarded. The purified lipid (7.45 g.) was washed in ether solution with Na_2CO_3 solution, yielding 4.70 g. free fatty acids and 2.75 g. neutral material. The latter, an oily liquid at room temperature, was saponified with ethanolic KOH to yield 64.4% unsaponifiable matter and 38.6% fatty acids (I_2 val., 236.2). The unsaponifiable matter contained 48.7% cholesterol, hence the fraction contained 33.0% non-sterol unsaponifiable matter. It further contained 0.3% glycerol. Allowing for fatty acids combined with this, the percentage of fatty acid apparently combined with the 33% of non-sterol unsaponifiable matter is 35.6%. (All the cholesterol in this fraction is in the free state.) Such a close ratio strongly suggests a wax ester, with the unsaponifiable portion consisting of higher aliphatic alcohols. The amounts available were too small for satisfactory further examination.

Fractions A 34–44. Fractions 34–44 (total weight 9.77 g.) yielded 0.1 g. ether-insoluble impurity, which was soluble in water or CHCl_3 . It was a light brown powder containing 0.8% N and 0.5% P. Hydrolysed with 0.5 N-ethanolic KOH it yielded 36.5% fatty acids (I_2 val., 99.0) and no unsaponifiable matter. The main portion of fractions 33–44 (8.93 g.) was crystallized from ethyl acetate to yield 2.5 g. cholesterol and 6.34 g. concentrate. The latter (6.06 g.) was worked up in a similar manner to fractions 13–33, giving 4.27 g. free fatty acids and 1.79 g. neutral product, which on saponification yielded 13.4% fatty acids (I_2 val., 174.6) and 55.9% unsaponifiable. The latter contained 76.3% cholesterol, hence the content of non-sterol unsaponifiable matter in the fraction was 13.2%. The glycerol content was 0.2%, hence fatty acids not present as triglycerides amount to 11.7%, again in close agreement for a wax ester with the amount of non-sterol unsaponifiable matter.

Fractions A 45–52. Fractions 45–52 (11.41 g. total weight) were entirely soluble in ether; 10.07 g. crystallized from ethyl acetate yielded 4.01 g. cholesterol and 6.06 g. concentrate. Treated in the same way as the preceding fractions 5.54 g. of this concentrate gave 2.17 g. free fatty acids and 3.37 g. neutral product which, on saponification, gave 12.2% fatty acids (I_2 val., 166.3) and 62.6% unsaponifiable,

which itself contained 44.9% cholesterol. The glycerol content was 0.4%, hence fatty acids not present as triglycerides amount to 8.3%, to be compared with 34.5% non-sterol unsaponifiable matter. Here there is a considerable discrepancy compared with the requirements for wax esters, and it seems likely that types of unsaponifiable matter other than higher aliphatic alcohols, e.g. hydrocarbons, are also present.

Fractions A 53–62. Fractions 53–62 (total weight 14.94 g.) proved to contain 0.23 g. free fatty acids. The neutral material contained free and esterified cholesterol and presumably also triglycerides. An attempt was made to separate these by counter-current distribution between light petroleum and tetrahydrofurfuryl alcohol. Ethanol is not a sufficiently good solvent for cholesterol esters or triglycerides to move them at any useful rate through light petroleum. Unfortunately, although the new solvent pair gave very efficient separation of free and esterified cholesterol, it did not effectively separate the latter from triglycerides. Many other solvent pairs were tried without success, part of the difficulty, no doubt, arising from the large range of fatty acids present in both types of compound, giving very broad and overlapping bands. Of the twenty subfractions obtained by the use of tetrahydrofurfuryl alcohol, nos. 16–20 (those most preferentially soluble in the lower phase), amounting to 7% of the total, were the only ones containing no esterified cholesterol. They were pooled and found to contain 2.5% glycerol, 30% unsaponifiable matter other than cholesterol and yielded 39.8% fatty acids on hydrolysis. The excess of fatty acids (assuming average chain length C_{19}) over the requirements of triglycerides is 15.8%, hence the unsaponifiable matter was assumed to be about half higher aliphatic alcohols occurring as wax esters, and half hydrocarbon or similar substance.

An attempt to examine separately the fatty acid mixture of the cholesterol esters and of the triglycerides in the combined first fifteen subfractions by selective enzymic hydrolysis of the latter (Kelsey & Longenecker, 1941) was spoiled by accidental loss of most of the material. The combined material contained 3.3% glycerol, 33% esterified cholesterol and 6% free cholesterol. Again assuming an average fatty acid chain length of C_{19} , the content of cholesterol esters would be 58.3% and of triglycerides 33.6%, thus accounting with the 6% free cholesterol for 98% of the total.

The free fatty acids removed from the various fractions were combined and analysed by fractional distillation of the methyl esters, the results being given in Table 2. The occurrence of free fatty acids in a tissue extract is always suspect, since they may be a product of rapid post-mortem autolysis. Fairbairn (1945) has shown how rapid this may be in the case of liver phospholipins. The fish used in preparing these extracts had been preserved with ice for up to 18 hr. before extraction. To test this point, a sample of 10 kg. of haddock flesh was obtained from freshly killed fish at sea and minced directly into a large excess of acetone. Subsequent extraction was as before. The product was fractionated by acetone precipitation, separated by counter-current extraction between light petroleum and ethanol, and the phospholipin-free portion extracted with Na_2CO_3 solution. The yields of various lipids at all stages were comparable to those obtained in the main experiment, and a total of 3.4 g. of free fatty acids was obtained from the 10 kg. of fish. Bloor (1926) found free fatty acids amounting to about 2% of the total fatty acids in the lipids of ox heart.

The total composition of this first subfraction *A* of haddock flesh lipids has been derived by summation of the foregoing data and the results are given in Table 3. It has been assumed that combined fatty acid in excess of that directly accountable as triglyceride, esterified cholesterol or lecithin was present as wax esters. In all cases where there was evidence of such 'excess' fatty acid there was enough unsaponifiable matter other than cholesterol to support this assumption. The content of lecithin was based on the choline contents. 'Non-lipid impurity' was obtained by difference, 84% of it being concentrated in the lecithin fractions. It could be calculated that the 'impurity' in lecithin fractions (*a*) and (*b*) respectively contained 5.9 and 17.1% N and 4.5 and 10.9% P. An average chain length of 19 carbon atoms has been assumed for the fatty acids and unsaturation has been disregarded in making certain calculations, e.g. weight of cholesterol esters from weight of esterified cholesterol.

Table 3. *Component lipids of subfraction A of the acetone extract of haddock flesh (%)*

Lecithin	32.1	Wax esters	8.4
Free cholesterol	17.8	Triglycerides	6.8
Free fatty acids	17.7	Hydrocarbons, etc.	2.0
Cholesterol esters	9.8	Non-lipid impurities	5.4

Fraction B'

The counter-current analysis of fraction *B'* was made in a series of twenty flasks, using 150 ml. of each phase (light petroleum and 85% (v/v) ethanol) for about 32 g. lipid. An attempt to use only 100 ml. of each phase, in the light of Olley's (1953) experience, resulted in complete miscibility of the phases. Ethanol fractions were not run off in this case,

Fig. 4. Haddock flesh lipids. Counter-current distribution of acetone-soluble fraction *B'* between light petroleum and 85% (v/v) ethanol. Curve passes through experimental value for every fraction.

the total contents of each flask forming one fraction. The weight distribution is shown in Fig. 4. Fractions 1-6 formed a uniform material and were combined (30.1 g.). An attempt to resolve it further by distribution between light petroleum and tetrahydrofurfuryl alcohol was unsuccessful. Fractions 7-20 ranged in appearance from waxy solids to viscous gums, and were combined to give 2.4 g. of mainly non-phosphatidic material. The latter was too small for

adequate examination, but on saponification it yielded 28% unsaponifiable matter and 36% fatty acids. The former appeared to be a mixture of aliphatic alcohols and other (possibly hydrocarbon) material; acetates: I_2 val., 91, sap. equiv., 660. This fraction also contained 1.0% choline, 0.04% serine + ethanolamine N, 0.26% glycerophosphate P, and 0.48% free phosphate P after alkaline hydrolysis. The fatty acids on lead salt/alcohol separation gave 42.5% solid acids (I_2 val., 39.4) and 57.5% liquid acids (I_2 val., 231.6).

The main phospholipin fraction (1-6), in spite of its relative impurity (choline, 9.4%; glycerophosphate P, 2.2%; acid hydrolysable P, 0.1%; other P, 0.6%) was treated as one substance. Saponification yielded 2% unsaponifiable matter and 58.5% fatty acids. The former was apparently higher aliphatic alcohols; acetates: I_2 val., 147, sap. equiv., 334. The fatty acids were analysed by distillation of the methyl esters, the results being given in Table 2.

The summation of constituent lipids in fraction *B'* would appear to be lecithin (from choline data) 60.6%, wax esters 5.8%, hydrocarbons, etc. 1.0%, unidentified 32.6%. The proportion of unidentified material is far higher than in fraction *A*. Most of it (28.9%) occurs in the main phospholipin fraction (1-6). It is not all non-lipid in nature since fractions 1-6 yielded 58.5% fatty acids, whereas lecithin and wax together would only yield 48.6% fatty acids. Since the glycerophosphate content is in close agreement with the choline content, this excess fatty acid is not present in phospholipins based on glycerophosphoric acid. In the absence of more information, it is impossible to say how much of the 32.6% unidentified material is non-lipid in nature. The small content of plasmalogens, about 3.6% if based on the classical glycerylphosphorylethanolamine, would form part of it.

DISCUSSION

The relative importance of the various lipids described here can be better appreciated by reference to the extraction data in the preceding paper (Lovern, 1953). The acetone-extracted lipids amounted to 0.57% of the fresh weight of the tissue and to almost half of the total tissue lipids. The material in the other extracts, although not yet examined in detail, is clearly more allied to the phospholipins and cerebrosides than to such substances as triglycerides, sterols, waxes, etc. Of the total acetone extract, all the triglyceride and cholesterol was concentrated into fraction *A* (35.7% of the total), of which in turn they comprised only the small percentages shown in Table 3. Assuming that all lipid fractions still to be examined are free from triglyceride and cholesterol, the total content of these in the haddock flesh is triglyceride 0.014%, cholesterol esters 0.020% and free cholesterol 0.036%. The position as regards wax esters is less clear, since it is a fairly common finding that unsaponifiable matter, presumably alcoholic, occurs in the hydrolytic products of crude phospholipin preparations, e.g. in those of ox brain (Lovern, 1952), ox, pig and sheep liver (Hilditch & Shorland, 1937) and soya bean and rapeseed

(Hilditch & Pedelty, 1937). Most workers with phospholipins have not looked for it. This unsaponifiable matter may not arise, as assumed in Table 3, from waxes, but from still more complex compounds akin to phospholipins. The material listed as 'hydrocarbons, etc.' is the least well characterized of the lipids itemized; it is merely unsaponifiable matter in excess of available fatty acid or deduced from unexpectedly high saponification equivalents of acetylated unsaponifiable matter. Certain fractions of unsaponifiable matter were pooled and freed from cholesterol and after acetylation had a saponification equivalent of 1150, far above the expected value of about 330. This product contained 85.5% C and 11.0% H, confirming, therefore, that it almost certainly contained some hydrocarbon. The occurrence of more free fatty acid than triglyceride in the tissue is remarkable, although once again the total concentration in the tissue is very low, being 0.036%. The test on absolutely fresh fish at sea gave a value of 0.034%.

The only comparable data known to the authors are those of Kaucher, Galbraith, Button & Williams (1943) for cod muscle, which they found to contain (calculated to wet weight) about 2.4% lipid, made up of 0.55% triglyceride, 0.03% cholesterol esters, 0.05% free cholesterol, 1.07% phospholipins and 0.66% cerebroside. This tissue differs from haddock flesh, therefore, in containing far more triglyceride and phospholipin plus cerebroside, the amounts of free and esterified cholesterol being of the same order. Neither in the present work nor in that of Kaucher *et al.* was the tissue purely skeletal muscle, but included connective tissue, nerves, blood vessels, blood and lymph. While the preponderance of true muscle tissue may be overwhelming for the bulk of the lipid constituents, these other admixed tissues may contribute significantly to trace constituents such as sterols and triglycerides.

It is common practice in preparing phospholipin extracts of tissues first to dehydrate the tissue with acetone, which also removes unwanted lipids such as fat and cholesterol. Such acetone extracts, especially the first, aqueous, one, are commonly discarded. The present results show that, at least in certain cases, such a practice will result in loss of phospholipin, and probably selective loss. We observed that even the first, largely aqueous, acetone extract contained, in addition to much non-lipid matter, lipids amounting to about 0.06% of the tissue weight (i.e. 10% of the total acetone-extractable lipids), and having roughly the same composition (sterols, free fatty acids, phospholipins, etc.) as the main acetone-extracted lipids.

Counter-current distribution

Certain general features of the counter-current distribution require comment. Thus, Fig. 3 shows

that the free cholesterol is distributed in a broad band, irregular on its advancing (ethanol-soluble) side. A single compound should give a symmetrical and narrower band and the observed effect is probably due to the marked effect of phospholipins on the solubility properties of other admixed lipids and, in the high concentrations used, on the properties of the solvent pair. Thus, the cholesterol is probably 'carried along' by the preceding main phospholipin fraction, 1-12.

Counter-current distribution cannot be effective for the separation of compounds which have partition ratios so high or low that the substances are sharply concentrated at one end of the system, as is true for lecithin in Figs. 3 and 4. In fact, such lecithin concentrates contained up to 20% of other lipids (choline, unsaponifiable matter and total fatty acid data), together with non-lipid impurities.

Both in the present and in other experiments we have noted that lipids are generally distributed so that more unsaturated derivatives are carried further by the polar solvent. This is true for free fatty acids, triglycerides and wax esters. The distribution of these over a great part of the system in the case of fraction A may be partly a genuine distribution and partly a trailing effect of the preceding phospholipins. It should be noted that uncharacterized nitrogen, presumably non-lipid, was similarly found all through the system.

'Excess' nitrogen

The nature of the 'excess' nitrogen is not clear. Part of it is liberated as ammonia during alkaline hydrolysis or addition of alkali to acid hydrolysates. This may well arise from urea, a known contaminant of some phospholipins, e.g. from blood (Christensen, 1939; Folch & Van Slyke, 1939). The balance of excess nitrogen is not present as an amino-acid, since a test with ninhydrin on a paper chromatogram showed only the expected traces of serine and ethanolamine. Glutamic acid, reported as a constituent of phospholipins (Hecht & Mink, 1952) was not present. Paper chromatography of the water-soluble products of alkaline hydrolysis of fraction 1-6 of B' showed the presence of a base reacting with phosphomolybdic acid but not with ninhydrin, also staining with iodine vapour, having an R_f value in butanol/acetic acid/water about half that of choline. It may correspond to a substance noted by Levine & Chargaff (1951). The lipid itself gave a strong Jaffé test for creatinine (equivalent, however, only to 0.04% creatinine) and this effect could not be removed by passing the lipid through a column of base-adsorbing ion-exchange resin. By contrast, the aqueous solution of an alkaline hydrolysate also gave a strong Jaffé reaction, the chromogen being readily removed on a column. While this test is not specific for creatinine, the latter is a known con-

stituent of fish flesh. If, in fact, creatinine occurs in these crude lipids, the evidence from adsorption experiments suggests that it is bound in some way to a larger molecule, possibly a lipid. Creatinine has been reported in a lipid extract of brain (Brante, 1949) and in MacLean & MacLean's (1927) 'carnithin'.

The nitrogen bound to the fatty acids has never been characterized. It would not appear to be present in such well-known derivatives as ureides since it cannot be removed by drastic hydrolysis. An association of nitrogen with partly oxidized fat is a well-known phenomenon in rancidity studies (Lea, 1938), but there is no evidence that similar effects are responsible in the case of phospholipins. This small amount of nitrogen was ignored in carrying out the fatty acid analyses.

Fatty acids of lecithins

The fatty acid compositions of the two lecithins (Table 2) exhibit several novel features, especially when compared with those of the depot fat. First, the absence of hexadecenoic acid, which is a common constituent of phospholipins from diverse species (Hilditch & Shorland, 1937; Shorland, 1951; Klenk & Böhm, 1951; Klenk, 1935) including phosphatides from fish liver and roe (Shorland & Hilditch, 1938; Shorland, 1939), and which is a characteristic universal constituent of fish depot fats. Secondly, the very high proportions of highly unsaturated C_{20} and C_{22} derivatives (60–70 % of the total acids) and the extremely high average unsaturation of these groups. Thirdly the low content of C_{18} unsaturated acids, normally a major constituent of phospholipins, coupled once again with unusually high average unsaturation. In fact, such a concentration of highly unsaturated derivatives has never previously been reported in phospholipin analysis. The fish liver and roe phosphatides mentioned above had fatty acid compositions closely similar to those of most fish depot fats. Anno (1949) reported that the lecithin fraction of salmon egg lipids contained oleic acid as the principal unsaturated acid, with only small amounts of highly unsaturated C_{20} and C_{22} acids, and Klenk (1933) found considerable proportions of highly unsaturated C_{20} and C_{22} acids in the liver phosphatides of a shark, although they were absent from the liver glycerides of this species.

Until we have examined the other phospholipins in our series of extracts it is impossible to say whether the exceptional fatty acid compositions reported here are a result of fractionation by acetone-solubility or are a general feature of fish-muscle phospholipins. The only previous comparison of the acetone-soluble and acetone-insoluble phosphatides of a tissue appears to be that of Hilditch & Shorland (1937) with sheep liver. The differences were not

striking, but there was a tendency for higher unsaturation in the acetone-soluble fraction, due to increased proportions of highly unsaturated C_{22} acids, largely at the expense of less-highly unsaturated C_{18} acids.

In comparing these fatty acid analyses with those of other workers it should be remembered that the present fractions represented impure lecithins (up to 20 % other lipids) and that both, especially *A*, contained a certain proportion of acetone-insoluble lecithin. It may be that if we had succeeded in obtaining a pure preparation of the acetone-soluble lecithins of haddock flesh we should have found an even higher concentration of C_{20} and C_{22} highly unsaturated acids. It is, however, notable that these products still contained about 20 % of saturated acids, the minimum reported by various workers for phosphatides of diverse origin. Snider (1936) concluded that the fatty acids of muscle phospholipins of many species contain about 27 % saturated acids, in contrast to 40 % in liver phospholipin fatty acids. Sinclair's (1935) work on the effect of dietary fat emphasized the significance of the ratio of saturated to unsaturated acids in phosphatides. Most determinations of detailed fatty acid composition have been concerned with the total phosphatide fraction of a tissue. Consideration of the iodine values of some of the extracts described in the preceding paper (Lovern, 1953) suggests that the fatty acids of the total phospholipin fraction of haddock flesh would be less rich in highly unsaturated derivatives than the sub-fractions described here.

Free fatty acids

Finally, attention may be drawn to the composition of the free fatty acid fraction, which in its saturated acids resembles the two lecithins rather than the depot fat, but in its unsaturated acids occupies an intermediate position, for both proportions and average unsaturation of homologous groups. The significance of this fraction cannot be assessed until data are available on all the other lipids of the tissue.

SUMMARY

1. An acetone extract of haddock flesh was separated into three fractions comprising (*A*) acetone-soluble non-phosphatidic lipids plus some lecithin, (*B'*) mainly lecithin soluble in acetone in the absence of added electrolytes, and (*C*) acetone-insoluble lipids. The lecithin in (*A*) was partly genuinely soluble in acetone and partly soluble in the presence of other lipids. The significance of acetone-insolubility in the preparation of phospholipins is discussed.

2. Fractions *A* and *B'* were studied by counter-current distribution between light petroleum and 85 % (v/v) ethanol, followed by pooling of certain fractions on the basis of similar chemical compositions and analysis of the pooled material for various lipid constituents.

3. Fraction *A* consisted of about 32 % lecithin, 18 % free cholesterol, 18 % free fatty acids, 10 % cholesterol esters, 8 % waxes, 7 % triglycerides, 2 % hydrocarbons, etc. and 5 % non-lipid matter. The free fatty acids were a genuine constituent of haddock flesh and not a post-mortem artifact. Fraction *B'* consisted of about 61 % lecithin, 6 % waxes, 1 % hydrocarbons, etc., and 32 % unidentified material, including probably 4 % plasmalogens.

4. Detailed fatty acid analyses of the lecithins in fractions *A* and *B'* revealed exceptionally high

proportions of highly unsaturated C₂₀ and C₂₂ acids, with a curious total lack of hexadecenoic acid and unusually small amounts of C₁₈ unsaturated acids. The free fatty acids were in some respects intermediate in type between those of the lecithins and those of haddock depot fat. The significance of these fatty acid compositions cannot be assessed until all the lipid fractions of the tissue have been similarly examined.

5. Certain fractions were quite rich in uncharacterized nitrogen, and early observations of the presence of firmly bound nitrogen in the fatty acids liberated from phospholipins were confirmed.

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