specialized role. The function of vitamin A in preventing xerophthalmia, in maintaining healthy epithelia and in promoting growth, is so little understood that the work of Kaunitz & Slanetz (1950*a*, *b*) on the lard factor may provide a new approach if the active substance can be characterized.

SUMMARY

1. The claim that lard contains a vitamin A-replacing factor has been confirmed.

2. A molecular distillate (210–220°) of lard, administered at the level of 0·6 g./day, cured xerophthalmia and restored normal growth in avitaminotic rats.

3. Neither preformed vitamin A nor carotenoid provitamin A could be detected by spectrophotometric or colorimetric tests on lard or lard unsaponifiable matter before or after chromatography, or on lard distillate.

We wish to thank Dr H. Jasperson, Dr R. E. Stuckey and Mr A. W. Davidson for valuable help. One of us (J. S. L.) has participated in this work as holder of a University Fellowship.

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The Lipids of Fish

4. THE LIPIDS EXTRACTED BY AN ETHANOL:ETHER MIXTURE FROM HADDOCK FLESH PREVIOUSLY EXTRACTED WITH ACETONE

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The material discussed in the present paper was the lipid fraction extracted at room temperature from the flesh of the haddock by ethanol:ether (3:1, v/v) after the tissue had been exhaustively extracted with acetone, also at room temperature, and was the second in the series of extracts described in part 1 of this series of papers (Lovern, 1953). The total crude extract available weighed 94 g., being 0·22 % of the wet weight of the haddock flesh. Although this had been heated on a steam can under vacuum for about an hour it was later found still to contain 16 g. of solvent. The difficulty of removing the last traces of solvent or water from phospholipids is well known, but it was considered inadvisable further to prolong the original heating. A large mass of sticky phospholipid could obviously trap some residual solvent almost indefinitely. The corrected weight of the present crude lipid extract is, therefore, 0·18 % of the wet weight of the tissue.

ANALYTICAL METHODS

The analytical methods used were as described in previous papers (Lovern & Olley, 1953; Olley & Lovern, 1953), with the following exceptions.

Inositol. We have found that the filter-paper chromatographic examination of lipid hydrolysates, using an ammoniacal silver nitrate spray reagent (Hough, 1950), is unreliable for the detection of inositol in very low concentrations. Thus in examining the hydrolysates of various fractions of the acetone extract by the chromatographic method we were unable to detect any inositol (Olley & Lovern, 1953), whereas we have since found that it was present to the extent of 1·1 % in a product corresponding to the combined fractions *B* and *C* (Lovern & Olley, 1953) of the acetone extract. In the present work inositol was determined microbiologically after hydrolysis by refluxing for 6 hr. with 6 N-HCl, using Northam & Norris's (1951) procedure and the organism *Kloeckera brevis* with basal medium I plus a bacteriological yeast extract prepared as by Northam & Norris (1952). There are different views on the

hydrolysis period necessary for complete liberation of inositol (Woolley, 1943; Brante, 1949; Folch, 1949), but we have found that hydrolysis up to 15 hr. gives the same value as 6 hr. with our lipids and *K. brevis*. Each sample was assayed at three levels and with two levels of added inositol. The slope of all curves was the same as that of a standard inositol estimation. Thus no other constituent of these lipid hydrolysates (which were freed from HCl by vacuum evaporation) affected the response of the organism to inositol. It was confirmed that choline at a level of 30 times the inositol present caused no inhibition, such as Taylor & McKibbin (1952) found when using *Saccharomyces carlsbergensis*, and it would appear that *K. brevis* is a particularly suitable organism for inositol assay in lipid hydrolysates.

Choline. The usual assay by precipitation of the reineckate (Lovern, 1952) proved unsuitable with certain fractions, since a brownish precipitate of some other reineckate accompanied the choline reineckate through all manipulations into the final acetone solution. Moreover, it was found that the yield of this other reineckate varied with the amount of choline precipitated simultaneously. Addition of extra choline to a lipid hydrolysate of this kind, followed by assay as the reineckate, gave values greater than the sum of the original assay and the added choline. Thus a direct reineckate assay on the 'lower phase' lipids after treatment with ion-exchange resin (see Fig. 1) gave a choline value of 8·55 % (the true choline content being only 4·8 %), whereas an assay in the presence of approximately the same amount of added choline gave a value of 12·8 %, after allowing for the added choline.

Hence, in addition to colorimetric choline assays by the reineckate method, all fractions examined for choline were assayed by the method of Street, Kenyon & Watson (1946), involving the oxidation of choline to trimethylamine. Because the aqueous phase of the hydrolysates of some fractions contained large ratios of other organic matter to choline, it was found desirable to conduct the permanganate oxidation on the precipitated reineckates. During countercurrent distribution between aqueous ethanol and light petroleum (Fig. 1), the non-choline compound which interfered with the colorimetric reineckate assay was concentrated towards the light petroleum end of the sequence, whereas lecithin samples at the ethanol end gave the same choline value by both procedures. Thus fractions *P*1–3, *P*4–7, *P*8–10 (Fig. 1) had the following choline contents (%) as determined by the colorimetric and oxidative procedures respectively: 11·45, 11·2; 7·53, 7·55; 5·3, 4·47. Interference was thus first noticeable at fractions *P*8–10. Fractions distributed still further towards the light petroleum end of the sequence contained very little choline (see below) but fractions *L*19–20 (Fig. 1) gave a heavy reineckate precipitate with the solubility properties of choline reineckate.

Glycerol and glycerophosphate. Certain fractions were very deeply coloured and the aqueous phase after alkali hydrolysis was too dark for satisfactory colorimetric determination of glycerol or glycerophosphate. Such hydrolysates were decolorized by cautious addition of phosphotungstic acid. The coloured substances were precipitated before all the other precipitable compounds had been removed, so that a colourless solution without excess phosphotungstic acid was readily obtained and proved perfectly satisfactory for both glycerol and glycerophosphate assay by the methods used previously (Lovern & Olley, 1953; Olley & Lovern, 1953). It was found that a small excess of phosphotungstic acid would not, in any case, interfere with either of these estimations.

EXPERIMENTAL

Removal of non-lipids

(In this section all weights quoted are corrected for analytical and manipulative losses.) The crude extract (77·3 g.) was re-extracted with light petroleum (b.p. 40–60°), giving 3·5 g. insoluble and 73·8 g. soluble material. The insoluble material was also insoluble in ethyl ether, chloroform and water. Successive alkali and acid hydrolyses showed it to contain only 0·7 % fatty acids and 1·5 % unsaponifiable matter, but six amino acids (paper chromatography). It presumably consisted largely of tissue protein, and was discarded.

The light-petroleum-soluble material was next treated by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951), by dissolving it in 1600 ml. of $CHCl_3$:methanol (2:1, v/v) and submerging this solution beneath 21 l. of water at 0° overnight. The process was repeated twice more on the lipids remaining in $CHCl_3$ solution. These three treatments yielded 20·1 g., 16·5 g., and a negligible amount, respectively, of water-soluble material. The combined water-soluble material (about 50 % of the original petroleum-soluble material) yielded on hydrolysis only 1·5 % fatty acids and 3·4 % unsaponifiable matter. It contained 9·7 % N and 0·6 % P. Paper chromatography (without hydrolysis) in butanol:acetic acid:water (40:10:50, v/v) showed the presence of ten substances staining with ninhydrin. The material presumably consisted largely of amino acids, and was discarded.

In the hope of removing further basic or amino acid contaminants, the lipid was next passed through a column of a sulphonated, lightly cross-linked polystyrene (specification of Partridge & Brimley, 1952). The solvent for this purpose should be as aqueous as possible. The lipid (37·2 g.) was not completely soluble in 50 % aqueous ethanol but by gradual addition of other solvents it was found to give a clear solution in a final mixture of 400 ml. water, 450 ml. ethanol, 90 ml. ether and 90 ml. $CHCl_3$. The base-absorbing capacity of the column used was equivalent to 3·6 g. NaOH. After passage through the column, the lipid solution separated abruptly into two phases, some substance being removed which had contributed to the original complete miscibility. The column was washed with a further 2 l. of the solvent mixture, and the combined eluate also settled into two phases. These were separately evaporated, yielding 30·2 g. of lipid from the lower phase and 5·7 g. from the upper phase. The column was then washed with aqueous 0·075 N-NaOH, which removed the remaining 1·3 g. of the original extract. This latter material yielded no fatty acids, but 2·3 % of unsaponifiable material on hydrolysis. It contained 10·6 % N and 0·7 % P. It was thus essentially non-lipid and was discarded. These various manipulations are illustrated in Fig. 1 and some analytical data on various products are given in Table 1. The glycerophosphate P is the true (soluble Ba salt) value (Olley & Lovern, 1953). A trace of 'false' glycerophosphate P was found in the original extract and this was concentrated in the upper phase lipid after the polystyrene treatment, which gave an 'apparent' glycerophosphate P value of 2 %. For the lower phase lipids, true and apparent glycerophosphate values were identical.

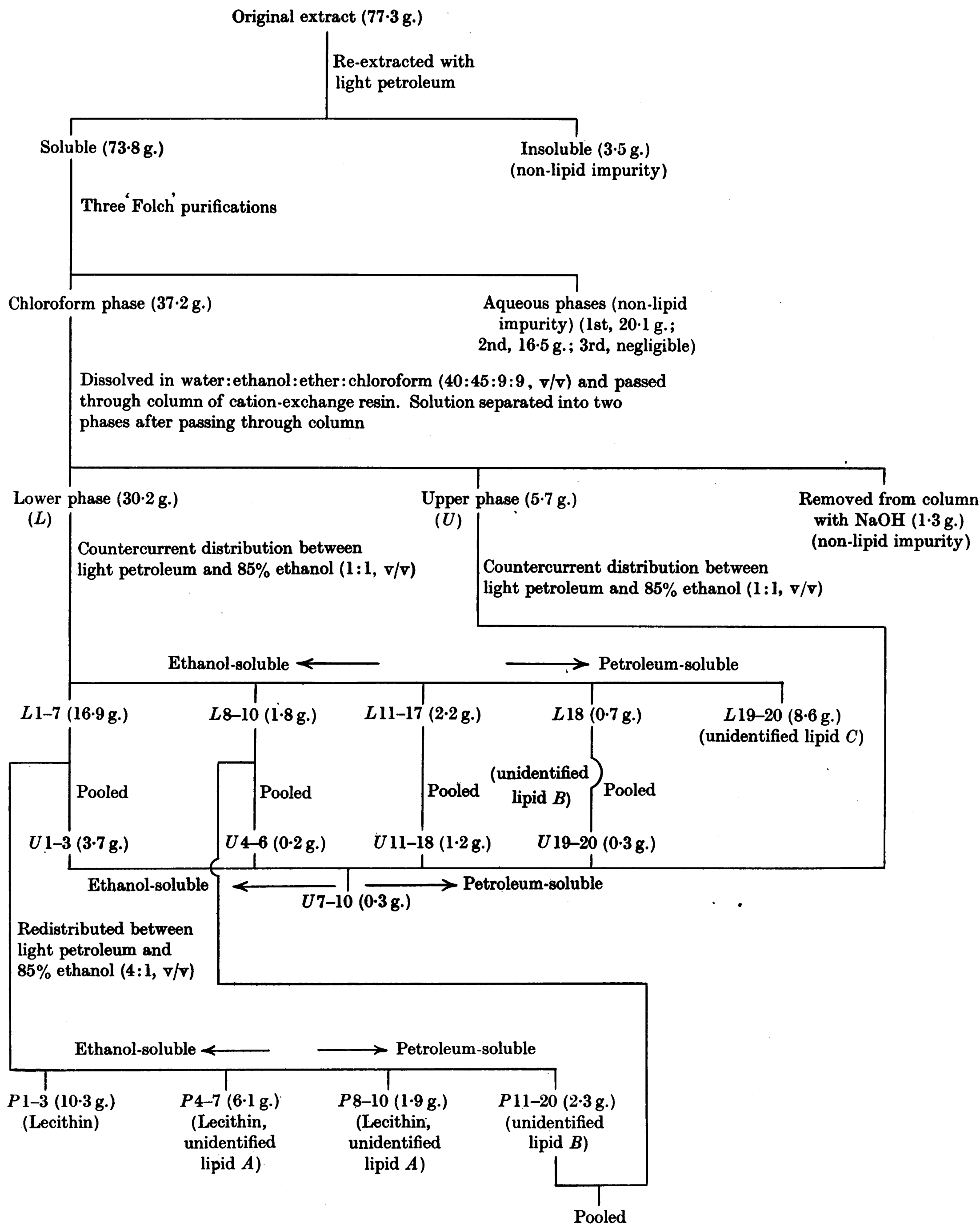

Fig. 1. Fractionation scheme for ethanol:ether extract of haddock flesh. All weights corrected for analytical and manipulative losses. For 'Folch' purification see Folch *et al.* (1951).

Table 1. *Analytical data on various products during lipid purification*

(All values are % constituent in fraction except N:P ratio which is atomic ratio.)

Material	N	P	N:P	Choline	Glycero-phosphate P
Original extract	6·4	1·6	8·9	7·0*	1·0
Lipid after three Folch treatments	3·3	3·1	2·4	—	—
Lower phase after resin treatment	3·8	3·1	2·7	8·6*, 4·8†	2·0
Upper phase after resin treatment	1·2	2·9	0·9	5·0*†	1·8

* Direct reineckate estimation. † True values, Street *et al.* (1946).

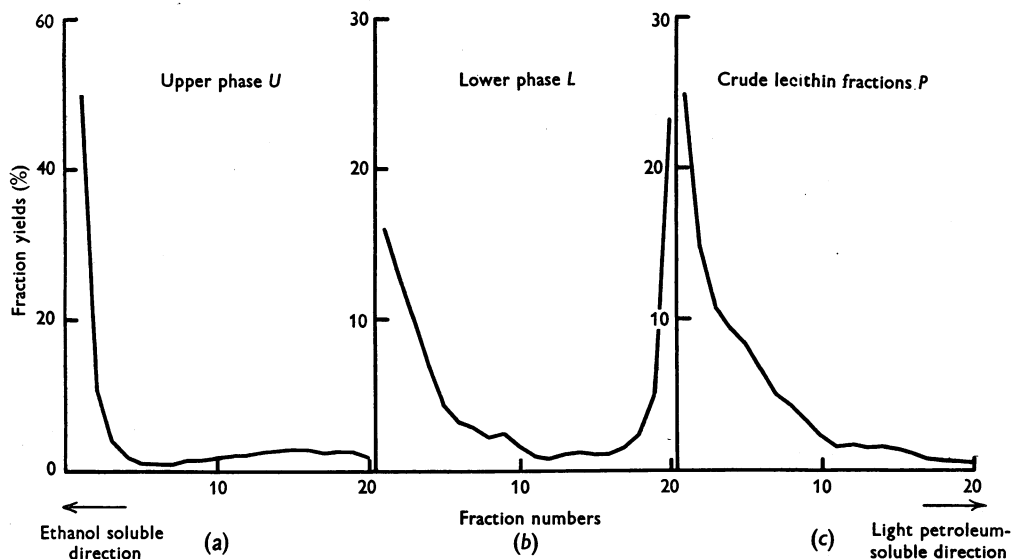

Fig. 2. Countercurrent distribution of (*a*) 'upper phase' lipids (*U*), (*b*) 'lower phase' lipids (*L*), (*c*) combined crude lecithin fractions (*P*) of ethanol:ether extract of haddock flesh. Solvents, light petroleum (b.p. 40–60°) and 85% ethanol (v/v). Volume ratio (*a*) and (*b*) 1:1; (*c*) 4:1. Ordinates: fraction yields (%). Abscissae: fraction numbers. Ethanol-soluble direction to left, petroleum-soluble to right.

Countercurrent distribution

The upper and lower phase lipids were each submitted to countercurrent distribution between light petroleum (b.p. 40–60°) and 85% ethanol (v/v), in the manner described previously (Lovern & Olley, 1953). In both cases a series of ten flasks of light petroleum was used and ten successive batches of ethanol were passed through the system and run off to give fractions 1–10 respectively. The petroleum solutions remaining in flasks 10–1 (in that order) were evaporated to give fractions 11–20, the numbered fractions thus forming a continuous sequence according to changing partition ratio. For the upper phase lipids the volumes of solvent were 100 ml. of each phase in all batches, and for the lower phase lipids 300 ml. The fraction yield curves are shown in Fig. 2 (*a*) and (*b*) for upper and lower phase respectively.

There were marked changes in the appearance of the various fractions obtained in both cases. Of the upper phase lipids, fractions *U* 1–3 had a typical 'lecithin' appearance, fraction *U* 4 was a gum, *U* 5–6 were liquid, *U* 7 was partly liquid and partly crystalline, *U* 8–10 were mainly crystalline, *U* 11–17 resembled soft fats, *U* 18 was mixed and rather wax-like, *U* 19 was wax-like and *U* 20 was again a liquid. Clearly the original material was a very complex mixture, but about 65% of the total was concentrated in the lecithin-like fractions 1–3. Mere traces of cholesterol could be detected in *U* 16, where cholesterol might be expected to be concentrated (Lovern & Olley, 1953).

Of the lower phase lipids, fractions *L* 1–6 had a typical lecithin appearance, *L* 7 was clearly a mixture, *L* 8–10 were gums, *L* 11–17 were waxy, *L* 18 was a liquid and *L* 19–20 were again phosphatidic in appearance but exceptionally dark in colour. Mere traces of cholesterol were detected in *L* 14 and 18. The lower phase lipids were thus also a complex mixture, showing many features in common with the upper phase lipids, but differing mainly in that some 28% was found as a phosphatide-like substance in *L* 19 and 20. This material is quite different from lecithin or any other known phosphatide (including sphingomyelin, see below) in its distribution behaviour.

Fractions were pooled in both series, on the basis of similarity in appearance, partition ratio, etc., and then certain of these were pooled between one series and the other. Thus fractions *U* 4–6 and *L* 8–10 were combined as a predominantly gummy fraction (2·0 g.), *U* 7–10 as a crystalline

fraction (0·3 g.), *U* 11–18 and *L* 11–17 as a soft fat-like or waxy material (3·4 g.), *U* 19–20 and *L* 18 as a liquid fraction (1·0 g.) and *L* 19–20 as a phosphatide-like substance (8·6 g.).

The main lecithin-containing fractions (*U* 1–3 and *L* 1–7) were not pooled into one fraction, since it was considered desirable to effect some further separation by countercurrent distribution with a higher petroleum : ethanol ratio. Fractions *U* 1 and *L* 1 were combined and added to 400 ml. of light petroleum in the first of a series of ten flasks. Fractions *U* 2 and *L* 2 were similarly added to 400 ml. of light petroleum in flask 2, fractions *U* 3 and *L* 3 in flask 3, and fractions *L* 4–7 in flasks 4 to 7. Flasks 8–10 each contained 400 ml. of light petroleum only. Ten batches of 100 ml. of 85% ethanol were passed through the series, giving fractions *P* (pooled material) 1–10 respectively and the petroleum solutions in flasks 10–1 gave fractions *P* 11–20. The yield curve of this distribution is shown in Fig. 2 (*c*). Fractions *P* 11–20 (2·3 g.) were all viscous liquids, generally similar to fractions *U* 4–6 and *L* 8–10, with which they were, therefore, combined to give 4·3 g. material. Fractions *P* 1–10 all had a typical phosphatidic appearance and, on the basis of the distribution curve in Fig. 2 (*c*), they were pooled into three groups: *P* 1–3 (10·3 g.), *P* 4–7 (6·1 g.) and *P* 8–10 (1·9 g.).

All these manipulations are illustrated in Fig. 1.

Examination of pooled fractions

Pooled fractions were analysed for the following constituents: N, P, glycerophosphate (true and apparent), choline, ethanolamine (serine was absent), glycerol, inositol, fatty acids, unsaponifiable matter, plasmal. The results are given in Table 2. Detailed analyses by ester distillation were made of the fatty acids of fractions *P* 1–3 and *L* 19–20. The fatty acids of fractions *P* 4–7 were combined with those of *P* 8–10, and those of *U* 4–6 etc. with those of *U* 11–18 etc., giving in each case about the minimum amount of material on which it is possible to carry out such an analysis. The results of these four fatty acid analyses are given in Table 3.

Table 2. *Analytical data on groups of pooled fractions from countercurrent distributions*

(All values except those in parentheses are % constituent in fraction.)

Constituent	Fraction numbers							
	P1–3	P4–7	P8–10	U4–6, L8–10, P11–20	U7–10	U11–18, L11–17	U19–20, L18	L19–20
Wt. of fraction (g.)	10·3	6·1	1·9	4·3	0·3	3·4	1·0	8·6
N	2·9	1·9	1·8	1·2	0·7	0·6	2·2	5·9
P	4·7	3·7	3·4	2·3	0·3	0·7	0·8	1·4
(N:P)	(1·3)	(1·1)	(1·2)	(1·1)	(5·2)	(1·9)	(6·1)	(9·1)
Glycerophosphate P (apparent)	3·5	2·6	2·4	1·5	0·3	0·7	0·6	1·3
Glycerophosphate P (true)	3·5	2·5	2·5	1·1	0·3	0·3	0·1	0·9
Choline	11·5	7·5	5·3	0·5	Nil	Nil	0·1	0·3
Ethanolamine	0·5	0·3	0·5	0·9	0·1	0·1	0·2	0·5
Glycerol	1·3	1·2	1·0	0·5	—	1·0	2·2	1·5
Inositol	1·5	1·2	0·7	1·3	0·1	0·2	0·4	1·3
Fatty acids	50·0	59·5	58·0	53·9	18·7	60·8	20·5	44·3
(Iodine value of fatty acids)	—	—	—	(228)	(141)	(167)	(135)	(204)
Unsaponifiable matter	2·4	2·4	3·4	7·7	32·4	9·8	20·8	7·0
(Iodine value of unsaponifiable)	(172)	(160)	(162)	(154)	(170)	(120)	(121)	(102)
(Sap. equiv. of acetylated unsaponifiable)	—	—	—	(381)	—	(435)	(442)	(458)
Plasmal	—	—	11·4	5·1	11·7	6·2	7·0	1·5
(Iodine value of plasmal)	—	—	(212)	(242)	(154)	(184)	(115)	(229)

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

(All weights as % of total acids. Figures in parentheses indicate average unsaturation in hydrogen atoms.)

Material	Saturated acids				Unsaturated acids		
	C_{16}	C_{18}	C_{20}	C_{22}	C_{18}	C_{20}	C_{22}
P 1–3 (Lecithin)	15	6	2	2	10 (−4·3)	37 (−6·8)	28 (−9·9)
P 4–7 + *P* 8–10 (Lecithin plus unidentified *A*)	9	10	2	6	22* (−5·0)	47 (−7·0)	4 (−10·7)
U 4–6 etc. + *U* 11–18 etc. (Unidentified *B*)	16	5	6	—	28 (−4·0)	45 (−8·0)	—
L 19–20 (Unidentified *C*)	8	7	2	4	16 (−4·0)	45 (−8·0)	18 (−10·0)

* Also contained a trace of C_{16} unsaturated acids.

DISCUSSION

Calculation of compositions

It is clear from Table 2 that all pooled fractions are complex mixtures. Thus, although fractions *P* 1–3 are clearly largely lecithin, they contain glycerol, inositol and unsaponifiable matter, none of which is produced on alkaline hydrolysis of lecithin. Most of the other fractions are even more heterogeneous. It seemed best to calculate the composition of each group of fractions along the lines followed in previous papers (Lovern & Olley, 1953; Olley & Lovern, 1953). In attempting to do this, however, it proved impossible in some cases to achieve a balance among the various constituents.

Plasmals. A particular difficulty concerns the plasmals. These were determined gravimetrically (Olley & Lovern, 1953), after it had been ascertained that all fractions except *P* 1–3 and *P* 4–7 gave weak positive spot tests with the fuchsin reagent. The quantities of aldehyde actually recovered were sometimes larger than anticipated from the spot test (e.g. in fractions *P* 8–10 and *U* 7–10) and were far larger than would be possible from classical plasmalogens based on ethanolamine. Unfortunately, the small amounts of plasmals left over from the iodine value determinations were not tested for an aldehyde reaction, such as was satisfactorily demonstrated in earlier work (Olley & Lovern, 1953), until some 4 or 5 days after isolation. At this stage they failed to show any reaction with the fuchsin reagent. Since their iodine values were not only extremely high for plasmals, but roughly paralleled those of the fatty acids isolated from the same fractions, suspicion was aroused that they might represent soaps which had remained in the ether layer when the fatty acids were removed by washing with aqueous alkali. That this was most unlikely, however, was shown by carrying out exactly similar processes on a sample of cod-liver oil, which yielded the expected amounts of fatty acids and unsaponifiable matter, but no plasmal fraction. Thus the material described in Table 2 as plasmal represents a neutral substance, present originally in a larger molecule which on alkali hydrolysis gives a water-soluble potassium salt, and which is liberated virtually immediately on acidification of this salt. Of all the known lipids, only the plasmalogens meet these requirements.

Plasmalogens have been reported analogous to all the established glycerophosphatides. In addition, there is evidence of the existence of other plasmalogens of unknown structure. Thus, Lovern (1952) found in ox-brain phosphatides certain fractions containing plasmal in excess of that attributable to all known plasmalogens based on choline, ethanolamine or serine. The same was noted for the plasmalogens of the haddock lipids described in the preceding paper (Olley & Lovern, 1953). In the present work, as in that reported in the previous papers, evidence was found of the occurrence of new classes of fatty acid derivatives (see below), and we suggest that they may be accompanied by corresponding classes of plasmalogens.

In calculating the distribution of hydrolytic fragments such as choline, ethanolamine and glycerophosphate, it has been assumed that they are uniformly distributed between ester phosphatides and acetal phosphatides, with one aldehyde radical equivalent to two fatty acid radicals. A similar distribution has been assumed, of necessity, for 'unidentified lipid', but waxes and inositol lipid have been calculated as if exclusively fatty acid esters. Plasmalogen contents, without subdivision into different types, have been calculated directly from the plasmal values, as if all plasmalogen was the classical ethanolamine-containing material.

Unsaponifiable matter. As in previous papers, the unsaponifiable matter has been assumed to consist essentially of higher aliphatic alcohols, and saponification equivalents of the acetates in excess of 300 have been attributed to the presence of non-alcoholic matter, e.g. hydrocarbons. In the present series, a steady increase in saponification equivalents in the more petroleum-soluble fractions is in line with previous experience. For calculations, the unsaponifiable matter of fractions *P* 1–3, *P* 4–7 and *P* 8–10 has been assumed to be entirely alcoholic, by analogy with previous findings (Olley & Lovern, 1953), and the saponification equivalent of the acetylated material from fraction *U* 7–10, which was insufficient for analysis, has been taken as 400.

The occurrence of nitrogen in the unsaponifiable matter has been mentioned previously (Olley & Lovern, 1953) and, largely on the basis of paper chromatography, it was concluded that this nitrogen could not be attributed to sphingosine. However, our hydrolytic procedure, involving 2 hr. refluxing with 0·5 N ethanolic potassium hydroxide, has been chosen primarily as giving the fatty acids of glycerophosphatides in relatively undamaged condition (hydrolysis by refluxing with 6 N aqueous hydrochloric acid, for instance, producing much darkening and fall in iodine value of the fatty acids) and permitting separation of plasmals from other unsaponifiable matter.

Work now in progress in our laboratory on the properties of sphingomyelin has confirmed the findings of others (Thannhauser & Fränkel, 1931; Freytag & Smith, 1933; Rouser, Berry, Marinetti & Stotz, 1953) that alkaline hydrolysis in ethanolic solution does not yield free sphingosine, at least as a major product. With our procedure much of the sphingomyelin was converted into an acidic product, presumably by loss of choline only, with a nitrogen: phosphorus ratio of nearly 1. The unsaponifiable

matter obtained had a nitrogen content of 2·5 %, with only traces of phosphorus, and was presumably fatty acid amides of sphingosine (ceramides) as found by Thannhauser & Fränkel (1931) and Freytag & Smith (1933).

It is, therefore, probable that part of the nitrogen in our unsaponifiable matter represents ceramides. However, ceramides do not make up the major part of this unsaponifiable matter. A countercurrent distribution between light petroleum and 85 % ethanol of a sterol-free unsaponifiable preparation from the entire acetone extract of haddock flesh (corresponding to the combined fractions *A*, *B* and *C* of our previous papers), which contained 0·8 % nitrogen, showed that most of it was concentrated at the petroleum end with a nitrogen content of about 0·4 %, and at the ethanol end was about 30 % of the original with a nitrogen content of 2·0 %, suggesting that of a ceramide (about 2·2 %).

We have found that sphingomyelin gives a countercurrent distribution pattern very like that of lecithin in our system. The solubility properties of ceramides (Thannhauser & Fränkel, 1931) suggest that if they occurred free in our lipid extracts (cf. Fränkel & Bielschowsky, 1932), they also would accompany the lecithin on countercurrent distribution. This, together with our finding with the unsaponifiable matter from the total acetone extract, suggests that ceramides should not occur in the unsaponifiable matter of fractions well towards the petroleum end of the countercurrent sequence.

The presence of nitrogen (and phosphorus) in fatty acids (Olley & Lovern, 1953) might now, apparently, also be attributed to partially split sphingomyelin but, as with the unsaponifiable matter, not all of it can be accounted for in this way. Although, unfortunately, we have not routinely determined the nitrogen and phosphorus contents of fatty acids from various fractions, we have observed an atomic nitrogen:phosphorus ratio of nearly unity only with fractions obtained near the ethanol end of the sequence. With fractions nearer the petroleum end the fatty acid nitrogen is far in excess of the fatty acid phosphorus, e.g. the fatty acids from fractions *L* 12, 15, 18 and 20 in the present series contained 0·13, 0·17, 0·27 and 0·18 %(weight) nitrogen respectively but only 0·05, 0·06, 0·09 and 0·16 % (weight) phosphorus.

The high degree of unsaturation of the unsaponifiable matter, particularly in the more ethanol-soluble fractions where ceramides should be concentrated, may be contrasted with the typical sphingomyelin product with predominantly saturated fatty acids.

Since routine nitrogen analyses were not made on the various unsaponifiable fractions it is impossible to calculate the possible ceramide content. However, in view of the items raised above, it would appear rather unsafe to do, and would certainly be so with the more petroleum-soluble fractions, which are rich in unsaponifiable matter.

The unsaponifiable matter gave no colour with antimony trichloride and hence does not contain vitamin A.

Inositol. The chemistry of inositol-containing lipids is still little known, but it is clear that more than one type of compound is involved. The countercurrent distribution of the present extract shows the existence of two inositol lipids concentrated respectively at the extremities of the system, and possibly another at fraction *U* 4–6, etc. Countercurrent analysis of soybean and maize phosphatides has likewise demonstrated the existence of two types of inositol lipids concentrated respectively at opposite ends of the distribution sequence (Scholfield, Dutton, Tanner & Cowan, 1948; Scholfield, McGuire & Dutton, 1950). In the absence of any information as to structure, the diphosphoinositide of brain (Folch, 1949) containing 21 % of inositol has been taken as a basis for calculating the amount of inositol lipid in the various fractions. A unimolecular fatty acid : inositol ratio has been assumed in calculating fatty acid distribution.

Glycerol. All fractions yielded some free glycerol on hydrolysis by refluxing 2 hr. with 0·5 N-ethanolic potassium hydroxide (there was insufficient of fractions *U* 7–10 for glycerol determination). It is improbable that this was derived from triglycerides. The original tissue contained only very small proportions of triglycerides and these should have gone entirely into the acetone extract (Lovern & Olley, 1953). The possibility of 'bound' triglycerides, liberated by ethanol but not by acetone, is remote, since triglycerides are usually lacking in bound lipids, although they have been reported in the thromboplastic lipoprotein of ox lung (Chargaff, Bendich & Cohen, 1944). However, even if triglycerides were present in the ethanol:ether extract, they would have been sharply concentrated at the extreme petroleum end of the countercurrent distribution (Lovern & Olley, 1953).

It is known that some inositol lipids give free glycerol on hydrolysis (De Sütö-Nagy & Anderson, 1947; Folch, 1949); the former workers employed alkaline hydrolysis. It has already been mentioned that an acetone extract corresponding to the earlier fractions *B* + *C* (which must have been free from triglycerides, after repeated acetone precipitation) contained 1·1 % inositol. This same material yielded 0·5 % glycerol on alkaline hydrolysis.

The ratio of glycerol to inositol varies considerably in the different fractions, and there is more glycerol than corresponds to the 1:1 ratio of brain diphosphoinositide. Nevertheless, it seems likely that much of the glycerol has been derived from inositol lipids. There is the same general tendency to con-

centration at the ends of the countercurrent distribution. No direct use of the glycerol values has been made in calculating the composition of the various fractions.

Unidentified lipids. The acetone extract of haddock flesh contained considerable proportions of a lipid, probably a phospholipid, with an unidentified base (Olley & Lovern, 1953). The same base was detected in the ethanol:ether extract up to fractions U11–18 etc., but not further towards the petroleum end. In the acetone extract, the major portion of unidentified lipid overlapped and immediately followed the lecithin on countercurrent distribution, while there was a trail of very small quantities right through to the petroleum end of the sequence. The major portion had a phosphatidic appearance whereas the later material was gummy or waxy and was probably a different substance. In the ethanol:ether extract, a significant part of the unidentified lipid also overlapped with and closely followed the lecithin on countercurrent distribution. This material occurred in fractions P4–7 and P8–10, which had an entirely phosphatidic appearance. However, important proportions of unidentified lipid occurred in fractions U4–6 etc. and U11–18 etc. which were gummy or waxy in consistency. This material was probably analogous to the unidentified lipid of the more petroleum-soluble fractions of the acetone extract. Since this gummy lipid forms an appreciable proportion of the total unidentified lipid in the ethanol:ether extract, it seems inappropriate to combine it, as was done with the acetone extract, into one group with the other phosphatide-like unidentified lipid. The latter has accordingly been given the temporary designation 'unidentified lipid A' and the former 'unidentified lipid B'. Calculations of the amounts of these have been made from the quantity of fatty acid available, assuming the same fatty acid content as in lecithin (cf. Olley & Lovern, 1953).

Fractions L19–20 (and presumably to a much less extent fractions U19–20 etc.) contained an unidentified lipid of an entirely different type. It again had a phosphatidic appearance and its countercurrent distribution behaviour is distinctive. Apart from traces of choline and ethanolamine, the high nitrogen content represents unidentified material. The bases present in the aqueous phase after alkaline hydrolysis were precipitated with phosphotungstic acid and from the precipitate a base fraction equivalent to only 3·4% of the original lipid was recovered by extraction with aqueous barium hydroxide. Most of the nitrogenous substances remained firmly linked to the phosphotungstic acid. The recovered material was a pale viscous liquid, readily soluble in water and ethanol, containing 8·3% nitrogen and 2·5% amino nitrogen. The latter required 1·5 hr. for complete liberation by nitrous acid. Spectroscopic examination showed only a progressive increase in absorption with decreasing wavelength in the ultraviolet, the absorption curve becoming very steep below about 260 mμ. Paper chromatography in butanol:acetic acid:water (40:10:50, v/v) showed one spot reacting with ninhydrin ($R_F = 0·18$), but no spots reacting with phosphomolybdic acid or ammoniacal silver nitrate. Fraction L19–20 was rich in the base interfering in the reineckate assay of choline.

The unidentified lipid in fractions U19–20 etc. and L19–20 has been calculated from the available fatty acids, as with the other unidentified lipids. To distinguish it from these it has been temporarily named 'unidentified lipid C'.

Non-lipids. These have been estimated by difference, as in previous papers. In two cases (P8–10 and U11–18), the sum of the calculated lipid constituents amounted to over 100% (105 and 102% respectively). In these fractions it was, therefore, necessary to reduce the amounts of all hypothetical lipids slightly and to ignore the possible presence of non-lipid.

Unused data. The presence of small but increasing amounts of choline and ethanolamine in fractions above U11–18, etc., may be noted. The choline cannot be attributed to lecithin or sphingomyelin; possibly both bases are present in plasmalogens. They have not been included in calculations with these fractions. In fractions P1–3 only there is a considerable excess of choline and a small excess of glycerophosphate over the fatty acids available for lecithin. A similar finding was made with the acetone extract of haddock flesh (Olley & Lovern, 1953). In fractions P1–3, therefore, the lecithin content has been calculated from the available fatty acids after allowing for inositol- and ethanolamine-containing lipids and wax esters, whereas in later fractions lecithin has been calculated from the choline content.

Composition of ethanol:ether extract

The results of the above calculations are given in Table 4, which also shows the composition of the total extract. The latter may be compared to the acetone extract (Olley & Lovern, 1953). The ethanol:ether extract differs from the acetone extract not only in items which might be anticipated, such as cholesterol, triglycerides and free fatty acids, but in its content of unidentified lipids B and C (there must be some overlap between the various unidentified lipids, which has had to be ignored in the calculations). Lecithin, however, is again the most plentiful component of the total lipid mixture. The two extracts are very similar in their content of phosphatidylethanolamine, waxes, and hydrocarbons, but the ethanol:ether extract contains more plasmalogen, which is of doubtful composition,

Table 4. *Composition of fractions from countercurrent distribution*

(Calculated as shown in text, pp. 691–3. All weights in g.)

Fractions	Lecithin	Phosphatidyl ethanolamine	Unidentified lipid *A*	Unidentified lipid *B*	Unidentified lipid *C*	Plasmalogens	Alcohols and waxes	Inositol lipid	Hydro-carbons	Non-lipids
P 1–3	5·9	0·6	—	—	—	—	0·5	0·7	—	2·6
P 4–7	3·2	0·2	1·3	—	—	—	0·3	0·3	—	0·8
P 8–10	0·5	0·1	0·7	—	—	0·4	0·1	0·1	—	—
U 4–6, etc.	0·1	0·4	—	2·2	—	0·4	0·5	0·3	0·1	0·3
U 7–10	—	—	—	—	—	0·1	0·1	Trace	Trace	0·1
U 11–18, etc.	—	—	—	2·4	—	0·4	0·5	Trace	0·1	—
U 19–20, etc.	—	—	—	—	0·1	0·1	0·3	Trace	0·1	0·4
L 19–20	—	—	—	—	4·6	0·2	0·8	0·5	0·2	2·3
Total	9·7	1·3	2·0	4·6	4·7	1·6	3·1	1·9	0·5	6·5
Total (as % of original material)	(27·1)	(3·6)	(5·6)	(12·8)	(13·1)	(4·4)	(8·6)*	(5·3)	(1·4)*	(18·1)

* Only about 62 % of the total, due to selective losses in removal of non-lipids.

however. In spite of the large proportion of non-lipids removed in the preliminary treatments, the purified ethanol:ether extract appeared still to contain about as much non-lipid as the total light-petroleum-soluble fraction of the acetone extract. However, the assumptions made in calculating the large proportions of unidentified lipids in the present extract probably result in inaccurate estimates of non-lipid matter.

Fatty acids

The fatty acid compositions given in Table 3 should be compared with those of other fractions of haddock-flesh lipids (Olley & Lovern, 1953). The main lecithin fraction of the ethanol:ether extract (*P* 1–3) resembles the lecithins extracted by acetone. The predominant saturated acid is palmitic and the unsaturated acids are mainly highly unsaturated C_{20} and C_{22} derivatives. A new feature is the presence of a little C_{22} saturated acid. The trend towards higher average unsaturation of the fatty acids in lecithins soluble in acetone compared to lecithins insoluble in acetone has been shown (Olley & Lovern, 1953). Possibly a lecithin containing a saturated C_{22} acid would be so insoluble in acetone as not to occur in the total acetone extract of the tissue. The general unsaturation of the lecithins of the ethanol:ether extract resembles that of the acetone-insoluble lecithins of the total acetone extract.

Fractions *P* 4–7 and *P* 8–10 are largely a mixture of lecithin and an unidentified lipid (*A*) similar to that found in the acetone extract, but containing a higher proportion of lecithin than the previous concentrates of this unidentified lipid. The fatty acids of the latter (Olley & Lovern, 1953) differed from those of the lecithin fractions principally in that stearic acid replaced palmitic acid as the main saturated acid. The same trend, but to a reduced extent, may be noted in the less rich concentrates of unidentified lipid *A* of fractions *P* 4–7 and *P* 8–10. The occurrence of saturated C_{22} acids may again be noted. The unsaturated acids of fractions *P* 4–7 and *P* 8–10 differ widely from those of the unidentified lipid of the acetone extract, much more resembling those of fractions *U* 4–6, etc., which represent largely unidentified lipid *B*. The probability of considerable overlap of lipids *A*, *B* and *C* has been mentioned, and in addition the compositions given in Table 3 can only be approximate in view of the marginal amounts of material available for analysis. Nevertheless, the unsaturated acids of unidentified lipid *A* in the ethanol:ether extract appear to be different from those of the similar fraction of the acetone extract. A lecithin fraction (1*C*) with a somewhat similar trend was encountered in the acetone extract (Olley & Lovern, 1953).

Fractions U4–6 etc. plus U11–18 etc. are a mixture in which the postulated unidentified lipid B is the main component. In spite of the large range of primary fractions included, the pooled groups U4–6 etc. and U11–18 etc. show a general similarity of composition (Tables 2 and 4). It should be noted that the very small intermediate group U7–10 represents upper phase lipids only and was peculiar in being largely crystalline. Its composition was entirely different from that of the two adjacent groups in Tables 2 and 4. Since the lower phase lipids were quantitatively far the more important, fractions U4–6 etc. and U11–18 etc. may be regarded as largely representing a continuous sequence from L8–17.

The fatty acid composition of this group of fractions is quite different from that of all the other haddock flesh lipids yet encountered. The saturated acids resemble those of the various lecithin fractions, but the unsaturated acids are remarkable for the complete absence of C_{22} derivatives, the compensating increase being in C_{18} acids. The lack of C_{22} saturated acids may also be noted.

If it were not for the variations in the saturated acids, it would be tempting to explain the fatty acid composition of fractions P4–7 plus P8–10 as that of a mixture of lecithin and unidentified lipid B, with no evidence of appreciable proportions of unidentified lipid A in the ethanol:ether extract. This is the more plausible because the small amounts of saturated esters available for distillation made the saturated acid percentages particularly liable to error. With the information at present available, the matter must remain unsettled.

Fractions L19–20 include unidentified lipid C as the main component. The fatty acids of this lipid follow the general lecithin pattern in their unsaturated members, but the saturated mixture differs in having equal proportions of palmitic and stearic acids. Saturated C_{22} acids are again in evidence. The remarks above on the greater risk of error in determining the saturated acid than the unsaturated acid percentages apply here also.

All these fractions show general similarity to the various phosphatides of the acetone extract, in containing 20–30 % of saturated acids and in the high content of highly unsaturated acids. In all of them the C_{20} acid, arachidonic acid (or an isomer) is the most plentiful constituent.

Non-lipid impurities

The gross contamination of this ethanol:ether extract with non-lipid material is notable. The haddock flesh had been previously exhausted with acetone, which removed relatively enormous amounts of non-lipid matter, e.g. about 2 % of the tissue weight as material insoluble in light petroleum in the first (wet) acetone extract (Lovern, 1953). The light-petroleum-soluble portion of the combined acetone extracts (0·57 % of the tissue weight) contained a further 16 % of non-lipids (Olley & Lovern, 1953). In spite of this previous extraction, the ethanol: ether extract contained 53·5 % of non-lipid removed prior to countercurrent distribution. Most of this was soluble in light petroleum in the presence of phospholipids. Muscle tissue is, of course, a rich source of 'extractives', but these results emphasize the futility of recording nitrogen and phosphorus contents of crude lipid extracts as indicative of phospholipid content.

The effectiveness of the purification procedure of Folch *et al.* (1951) has also been tested on an acetone extract of haddock flesh corresponding to the combined earlier fractions $B + C$, which contained 22·4 % of non-lipids (Olley & Lovern, 1953). Three treatments removed 17 % of non-lipids. In the present work, the impurities removed by this method contained 72 % of the nitrogen and 16 % of the phosphorus of the original crude extract. The loss of lipid is small but selective, since although only 2·5 % of the total fatty acids were removed, 38 % of the total unsaponifiable matter was lost. Folch *et al.* (1951) also noted a selective lipid loss with brain extracts, the feature most directly relevant to the present work being a selective loss of diphosphoinositide. We had not commenced our microbiological inositol assays when we used the Folch process, and had no reason from paper-chromatographic data to look for inositol. It is possible, therefore, that our reported inositol values are too low. However, even if all the fatty acid lost by the Folch treatment represented inositol lipids, these would still be no more than a minor constituent of the total extract.

The procedure of Folch *et al.* appears to be the simplest effective purification technique yet reported, particularly when relatively large amounts of material are involved. It does not suffer from one possible disadvantage of purification by acid-regenerated cation-exchange resins, the risk of hydrolysis of lipids if contact is prolonged (Sussman, 1946).

The material removed as insoluble in light petroleum, and on the ion-exchange resin, likewise contained small proportions of lipids rich in unsaponifiable matter. This suggests that such lipid is present in combination with amino acids or similar compounds. Similarly, the manner in which 'extra' nitrogen and phosphorus accompany phospholipids during countercurrent distribution suggests the presence of complexes of phospholipids with other substances.

SUMMARY

1. Haddock flesh previously extracted with acetone was extracted with ethanol:ether (3:1, v/v). The crude product, although entirely soluble

in light petroleum, contained over 50 % of non-lipid impurities. A large part of this impurity was readily removed by the method of Folch *et al.* (1951) with only small accompanying loss of lipids. This loss was, however, selective. Some additional non-lipid was removed with an ion-exchange resin.

2. The purified extract was examined by counter-current distribution between light petroleum and 85 % ethanol. The component lipid classes found were lecithin, phosphatidylethanolamine, plasmalogens, waxes, hydrocarbons, inositol lipids and unidentified material. This last included possibly three different types of lipid.

3. The plasmalogen could not be satisfactorily characterized and much of it probably did not correspond to the established formula. The inositol lipids included two and possibly three components. Certain unidentified lipids were accompanied by a nitrogenous base previously reported (Olley & Lovern, 1953) and another fraction by a different nitrogenous compound or compounds.

4. The lecithin contained a fatty acid mixture generally similar to that of other lecithins previously isolated from haddock flesh. The unidentified lipids contained fatty acids showing certain variations from the lecithin pattern, but all similar in containing 20–30 % of saturated components and having C_{20} acids, arachidonic acid or an isomer, as the most plentiful constituent.

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Zinc and Carbonic Anhydrase in Human Semen

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The prostate glands of the rabbit and of man, and the dorsolateral prostate of the rat, contain a very high concentration of zinc (Mawson & Fischer, 1951, 1952*a*). The source of the zinc found in human semen by Bertrand & Vladesco (1921) and in ram sperm and seminal plasma by Mann (1945) is not known, but it seemed possible that this could be investigated in man by collecting the first, middle and last portions of each ejaculate separately and by estimating the zinc content of the separate fractions. Gutman & Gutman (1941) showed that acid phosphatase, which can be assumed to originate in the prostate gland, occurs mainly in the first fraction. Lundquist (1949) and Pryde (1950) estimated fructose in separate fractions, and found most of the fructose in the last fraction which they assumed to come from the seminal vesicles.

Any attempt to explain the presence of zinc in tissues must take into account the fact established by Keilin & Mann (1940) that zinc is the prosthetic metal of carbonic anhydrase. Mawson & Fischer (1952*b*) have shown that the high concentration of