reaction with duck ovalbumin was similar in all fractions.

6. The findings suggest that different cells, capable of producing slightly different globulins, may predominate in antibody production according to the route of injection and duration of the antigenic stimulus.

We wish to thank Dr A. Isaacs of this Institute for preparing the anti-influenza serum and for carrying out the agglutinin inhibition assays, as well as for his most valuable interest in this work. We are also grateful to Miss J. Morgan for excellent technical assistance, including many hours spent at −7°.

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The Lipids of Fish

6. THE LIPIDS OF COD FLESH*

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Previous papers in this series have dealt with the lipids of haddock flesh, but have referred to similar findings with cod flesh. Cod (*Gadus callarias*) and haddock (*G. aeglefinus*) are closely related species, so that similar lipid patterns might be expected. Both species are of great economic importance, meriting separate study and, in view of the great complexity of the haddock lipids and the finding in them of various novel and as yet inadequately characterized classes of lipid, the complementary data available from the cod studies are of biochemical interest. It seems unnecessary, however, to present this work in the same detail as the haddock investigations since, in general, the experimental techniques were the same.

EXPERIMENTAL

Extraction and purification

A total of 43·74 kg. of cod flesh was extracted successively with acetone, ethanol–ether, ethanol–benzene, chloroform–methanol and pyridine in the manner described for haddock (Lovern, 1953). The initial acetone extraction of each batch was performed at sea immediately after the death of the fish. The successive crude extracts (or in some cases subfractions of them, see below), after removal of solvent, were purified by dissolving in chloroform–methanol (2:1, by vol.), and submitting this solution to aqueous extraction by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951). Material insoluble in chloroform–methanol, or removed by water extraction, was found, as in the haddock studies, to be overwhelmingly non-lipid in nature, with one notable exception. The acetone-soluble portion of the acetone extract was not submitted to the procedure of Folch *et al.* (1951) until after counter-current distribution between ethanol–water (85:15, by vol.) and light petroleum (b.p. 40–60°). Fractions distributed right to the ethanol end (fractions 1–3) were very rich in non-lipid contaminants and the main lecithin peak followed them (fractions 4–10). The technique of Folch *et al.* (1951) applied to fractions 4–10 resulted in appreciable amounts of lecithin (some 17% of the total in the fraction) passing into the water–methanol phase, and also collecting as an interphasial substance. This did not happen with the preceding fractions (1–3), which were also quite rich in lecithin, nor have we ever encountered this behaviour elsewhere. Fractions 4–10 were unusual, however, in having been largely freed from inorganic salts; the latter would already have passed into fractions 1–3 during counter-current distribution. Folch, Lees & Sloane-Stanley (1954) have recently reported the passing of lipids into the methanol–water phase when previous aqueous extraction has removed such salts. Surprisingly small amounts of salts are involved, even 10^{-4} M-$CaCl_2$ having a marked effect.

All extracts were separated into acetone-soluble (A.S.) and acetone-insoluble (A.I.) fractions, the purification

* Part 5: Olley & Lovern (1954).

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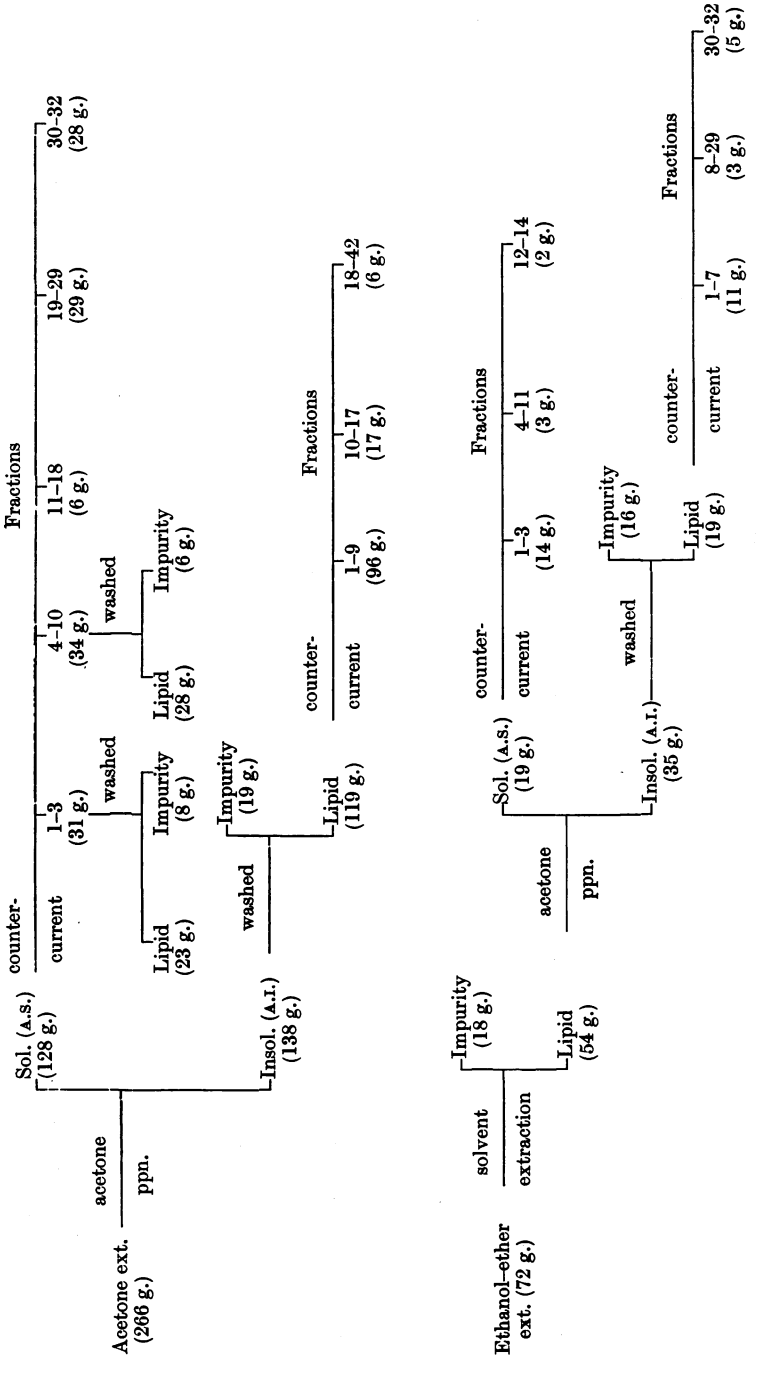
Acetone ext. (266 g.)
acetone
ppn.
Sol. (A.S.) (128 g.)
Insol. (A.I.) (138 g.)
counter-current
Fractions
1–3 (31 g.)
4–10 (34 g.)
11–18 (6 g.)
19–29 (29 g.)
30–32 (28 g.)
washed
Lipid (23 g.)
Impurity (8 g.)
Lipid (28 g.)
Impurity (6 g.)
Impurity (19 g.)
Lipid (119 g.)
1–9 (96 g.)
10–17 (17 g.)
18–42 (6 g.)
Ethanol–ether ext. (72 g.)
solvent extraction
Impurity (18 g.)
Lipid (54 g.)
Sol. (A.S.) (19 g.)
Insol. (A.I.) (35 g.)
1–3 (14 g.)
4–11 (3 g.)
12–14 (2 g.)
Impurity (16 g.)
Lipid (19 g.)
1–7 (11 g.)
8–29 (3 g.)
30–32 (5 g.)

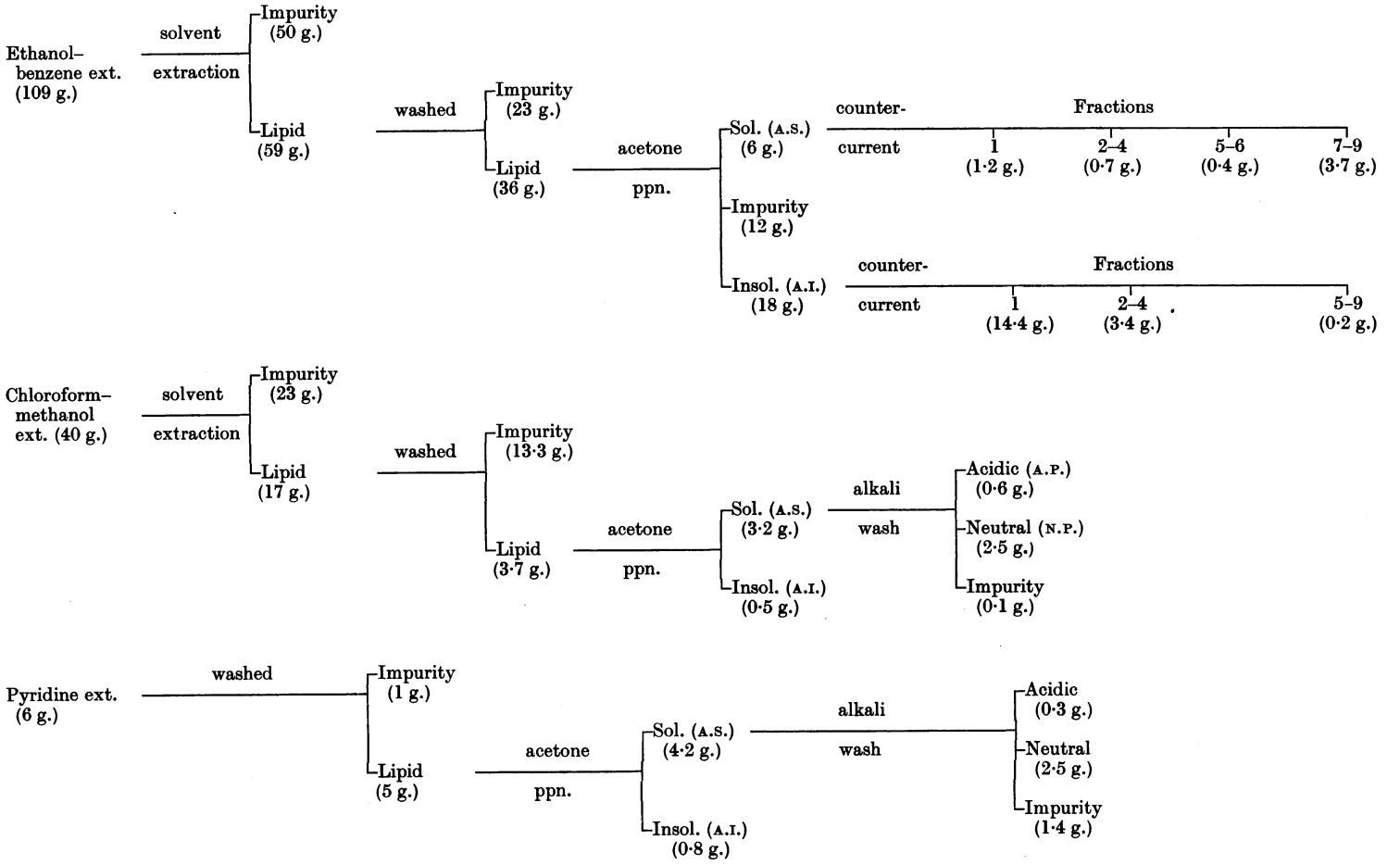

Fig. 1. Fractionation scheme for cod-flesh lipids. All weights corrected for analytical and manipulative losses. Ethanol-soluble direction to left in counter-current data. Impurity on acetone precipitation of ethanol–benzene extract was material left in acetone solution but found to be insoluble in ether and soluble in water. Impurity on alkali washing of chloroform–methanol and pyridine extracts remained in aqueous phase after acidification and re-extraction by ether. Solvent extraction by chloroform–methanol (2:1, by vol.), the pyridine extract being completely soluble. Washing by procedure of Folch *et al.* (1951).

procedure of Folch *et al.* (1951) being applied subsequently in the case of the acetone and ethanol–ether extracts, but previously with the other three extracts. The purified fractions still contained some non-lipid material, as determined subsequently from the analytical data, as was the case with the haddock lipids. The extraction and purification data are summarized in Table 1, where some comparison with haddock is made.

Fractionation and analysis

Counter-current distribution between ethanol–water (85:15, by vol.) and light petroleum (b.p. 40–60°) was used to fractionate the acetone-soluble and acetone-insoluble portions respectively of the acetone, ethanol–ether and ethanol–benzene extracts. The other two extracts were too small to permit of this, but the acetone-soluble portion of

Table 1. *Extraction and purification of the lipids of cod flesh*

All data as g./100 g. fresh tissue. Water washing according to Folch *et al.* (1951).

Solvent	Crude extract	N.L.* removed by re-extraction with $CHCl_3$–methanol	N.L.* removed by water washing	N.L.* remaining in lipids	True lipid	True lipid (haddock)
Acetone	0·61	Nil†	0·10	0·03	0·48	0·48
Ethanol–ether	0·16	0·04	0·03	0·03	0·06	0·07
Ethanol–benzene	0·25	0·14	0·05	0·04	0·02	0·03
Chloroform–methanol	0·09	0·05	0·03	0·004	0·006	0·007
Pyridine	0·01	Nil	0·002	0·003	0·005	0·004
Total	1·12	0·23	0·21	0·11	0·57	0·59

* N.L. = non-lipids.
† The acetone extract had already been purified by re-extraction with light petroleum (cf. Lovern, 1953).

Table 2. *Analytical data on pooled fractions of cod-flesh lipids*

All values as g./100 g. A dash indicates that the particular determination was not made.

Fraction	Fatty acid	Unsaponifiable matter	N	P	Glycerophosphate P	Choline	Ethanolamine N	Glycerol	Inositol
Lecithin concentrate	64	8·4	1·3	3·2	3·1	7·8	0·15	—	0·45
Acetone ext.									
A.S. 1–3 (after washing)	53	2·3	1·5	2·7	1·15	15·6	Nil	0·6	Nil
A.S. 11–18	48	20	—	—	0·47	0·25	—	0·7	Nil
A.S. 19–29	32	67	Nil	Nil	—	—	—	0·5	Nil
A.S. 30–32	49	53	—	—	—	—	—	1·8	Nil
A.I. 10–17	71	3·9	1·5	3·5	2·4	1·25	0·64	0·7	0·15
A.I. 18–42	34	32	—	—	0·64	Nil	—	1·3	0·02
Ethanol–ether ext.									
A.S. 1–3	22	3·5	1·1	1·3	0·8	19·5	Nil	3·9	Nil
A.S. 4–11	17	23	—	—	0·59	—	—	7·2	—
A.S. 12–14	42	46	—	—	0·04	—	—	Nil	—
A.I. 8–29	28	11	—	—	1·8	2·1	0·21	3·41	4·10
A.I. 30–32	69	7·2	—	—	1·8	1·6	0·16	5·91	1·04
Ethanol–benzene ext.									
A.S. 1	14	28	7·1	1·1	0·54	1·6	0·07	Nil	Nil
A.S. 2–4	54	22	3·2	0·43	0·40	0·95	Nil	Nil	Nil
A.S. 5–6	53	20	0·5	Trace	—	Nil	—	—	Nil
A.S. 7–9	41	42	0·2	Trace	—	Nil	Nil	1·65	Nil
A.I. 1	0·4	3·3	10·3	0·46	—	—	—	—	—
A.I. 2–4	12	4	5·4	2·5	1·35	0·4	0·16	7·9	5·05
A.I. 5–9	56	25	0·5	2·9	1·0	0·4	0·02	4·65	—
Chloroform–methanol ext.									
A.S. neutral	38	50	0·9	0·07	—	Nil	0·01	1·85	2·5
A.S. acidic	47	33	—	0·76	0·61	1·5	—	—	—
A.I.	Lost	25	3·0	2·53	1·14	1·9	0·18	6·2	6·7
Pyridine ext.									
A.S. neutral	6·8	84	4·6	Nil	—	Nil	Nil	Nil	Nil
A.S. acidic	62	25	—	0·22	0·14	Nil	—	—	—
A.I.	8·4	9·5	5·3	1·06	0·13	Nil	Nil	Nil	Nil

each was separated into a neutral and an acidic fraction by washing an ether solution with aqueous sodium carbonate (cf. Olley & Lovern, 1954). Fractions were pooled on the basis of distribution behaviour, appearance and analytical data, as in previous papers. The entire fractionation scheme is summarized in Fig. 1. The counter-current distribution curves showed the same forms as those for the corresponding fractions of haddock-flesh lipids and are not reproduced here.

The final groups of fractions were analysed, by the methods described previously (Lovern & Olley, 1953*a*, *b*; Olley & Lovern, 1953, 1954), for fatty acids, unsaponifiable matter, nitrogen, phosphorus, choline, ethanolamine (with the exception of one fraction, serine was absent), glycerophosphate, glycerol and inositol. Only after the completion of most of these studies did we become aware of the complex nature of the supposed 'glycerophosphate' in certain of the fractions (see Olley, 1956). Only two fractions were subsequently available for detailed studies of glycerophosphate-like esters, namely A.S. 1–3 and A.I. 10–17 of the acetone extract. The former of these contained a total of 2·4% of material reacting as glycerophosphate with acid periodate, but only 48% of this material was insoluble in ethanol. Fractions A.I. 10–17 contained mainly ethanol-insoluble glycerophosphate, presumably genuine, but there was a small proportion of ethanol-soluble phosphate esters (Olley, 1956). Certain fractions were also analysed for free and total cholesterol and for free fatty acids (or acidic lipids). Three groups of fractions, which were main lecithin concentrates, were pooled for further analysis. These were fractions A.S. 4–10 and A.I. 1–9 of the acetone extract, and fractions A.I. 1–7 of the ethanol–ether extract. Plasmal, determined by the method of Feulgen & Grünberg (1938), was present to the extent of about 1·5%. The main analytical results are given in Table 2. Cholesterol determinations on the fractions where it was concentrated (A.S. 19–29 and 30–32 of the acetone extract) gave values of 64 and 71% free and total respectively for 19–29, and 10·1 and 33·7% for 30–32. Fractions A.S. 11–18, A.S. 19–29 and A.S. 30–32 of the acetone extract contained 43·5, 25·2 and 14·0% respectively of acidic lipids, essentially free fatty acids. Fractions A.S. 1–3, A.S. 4–11 and A.S. 12–14 of the ethanol–ether extract contained 13·8, 48·5 and 2·1%, respectively, of acidic lipids, which were not free fatty acids, but complex substances like the acidic lipids found in the chloroform–methanol and pyridine extracts (and in corresponding haddock-flesh extracts).

The unsaponifiable matter was in some cases acetylated and the saponification equivalent of the acetates determined, thus permitting a rough estimation to be made of the proportion of alcoholic and non-alcoholic (hydrocarbon?) material present. Values obtained were as follows: ethanol–ether extract, A.S. 1–3, 414; A.S. 4–11, 560; A.S. 12–14, 1437; ethanol–benzene extract, A.S. 7–9, 1340; chloroform–methanol extract, A.S. neutral, 1277. This is in agreement with findings on haddock lipids, that the saponification equivalents rise steeply towards the light-petroleum end of a counter-current distribution.

Fraction A.S. 30–32 of the acetone extract was submitted to chromatography (from light-petroleum solution) on silica gel (Borgström, 1952). Elution was achieved with light petroleum (b.p. 40–60°) containing increasing proportions of benzene, as described by Borgström. The most readily eluted material consisted of unsaponifiable matter other than cholesterol, followed by cholesterol esters, triglycerides plus free fatty acids, and finally free cholesterol. Separation was sufficiently sharp to permit a rough comparison of the composition of fractions 30–32 as calculated from the analytical data (see Table 4) and as observed chromatographically. This is shown in Table 3.

Table 3. *Comparison of chromatographic and calculated composition of fraction* A.S. 30–32 *of the acetone extract*

	Chromatography		Analysis
	Fractions	(%)	(%)
Unsaponifiable matter other than cholesterol	1–4	16	19
Cholesterol esters	5–12	35	40
Triglycerides plus free fatty acids	13–20	39	31
Free cholesterol	21–23	10	10

Chromatographic fractions 1–4 proved to consist entirely of a mixture of hydrocarbons, partial separation by vacuum distillation indicating a molecular weight range of at least 240–414. The unexpectedly low degree of unsaturation (iodine values 7–28) suggested contamination of the crude extracts with material derived from rubber. Rubber bungs extracted with organic solvents, e.g. light petroleum, yielded an extract consisting almost entirely of unsaponifiable matter, which was predominantly a mixture of hydrocarbons. Chromatographic analysis, infrared spectroscopy and analytical data all showed that these markedly resembled the material found in the fish extracts.

DISCUSSION

Calculation of composition

The analytical data given in Table 2 have been interpreted as outlined in previous papers. No attempt has been made, however, to estimate separately the different types of 'unidentified lipid' in the way followed for haddock. Nor have plasmalogens been calculated, the plasmal found in the main lecithin fraction being treated as equivalent to twice its weight of fatty acids for purposes of calculation. Hence plasmalogens have been 'absorbed', on a *pro rata* basis, into the other various lipid classes of this fraction. The impurities removed at different stages (cf. Fig. 1) were all hydrolysed and examined for fatty acids, unsaponifiable matter and, for the material from fractions A.S. 1–3 and 4–10 of the acetone extract, also for choline and glycerophosphate. The calculated composition of the fractions and of the total lipids of cod flesh is given in Table 4, the data on the various impurities being summarized for this purpose.

For comparison the composition of haddock-flesh lipids (Olley & Lovern, 1954) has been added at the foot of Table 4, the small proportion of plasmalogens (1·7%) being incorporated *pro rata*

Table 4. *Composition of cod-flesh lipids*

All values as wt. (g.) except final % composition. A dash indicates that the particular determination was not made.

Fraction	Lecithin	Phosphatidyl ethanol-amine	Inositol lipids	Unidentified lipids	Free fatty acids	Tri-glycerides	Waxes and alcohols	Free cholesterol	Cholesterol esters	Hydro-carbons (see text)	Non-lipids
Main lecithin	72·15	10·33	2·82	28·97	—	—	17·57	—	—	—	2·28
Acetone											
A.S. 1–3	7·20	—	—	9·52	—	—	0·53	—	—	—	5·90
A.S. 11–18	0·10	—	—	—	2·60	—	0·39	1·01	—	—	1·88
A.S. 19–29	—	—	—	—	6·76	1·39	—	17·73	2·53	—	—
A.S. 30–32	—	—	—	—	3·80	4·90	0·32	2·74	11·13	5·07	—
A.I. 10–17	1·50	7·74	0·12	6·68	—	—	1·36	—	—	—	—
A.I. 18–42	—	—	0·01	1·04	—	—	2·62	—	—	0·63	1·75
Ethanol–ether											
A.S. 1–3	2·94	—	—	1·20	—	—	0·48	—	—	—	8·99
A.S. 4–11	—	—	—	0·51	—	—	0·54	—	—	0·34	1·79
A.S. 12–14	—	—	—	0·96	—	—	0·44	—	—	0·81	0·01
A.I. 8–29	0·45	0·36	0·61	—	—	—	0·34	—	—	—	1·38
A.I. 30–32	0·53	0·42	0·24	2·75	—	—	0·70	—	—	—	0·17
Ethanol–benzene											
A.S. 1	0·13	0·05	—	—	—	—	0·37	—	—	—	0·64
A.S. 2–4	0·04	—	—	—	0·18	—	0·29	—	—	—	0·15
A.S. 5–6	—	—	—	—	0·14	—	0·17	—	—	—	0·12
A.S. 7–9	—	—	—	—	0·62	0·61	0·70	—	—	1·21	0·58
A.I. 1	—	—	—	—	—	—	0·53	—	—	—	13·82
A.I. 2–4	—	—	0·83	—	—	—	0·27	—	—	—	2·34
A.I. 5–9	—	—	0·03	0·06	—	—	0·10	—	—	—	0·01
Chloroform–methanol											
A.S.–N.P.	—	—	0·20	0·42	—	—	0·41	—	—	0·68	0·06
A.S.–A.P.	0·06	—	—	0·05	—	—	0·38	—	—	—	0·09
A.I.	0·06	0·05	—	0·04	—	—	0·25	—	—	—	0·10
Pyridine											
A.S.–N.P.	—	—	—	—	—	—	2·21	—	—	—	0·24
A.S.–A.P.	—	—	—	0·14	—	—	0·15	—	—	—	—
A.I.	—	—	—	—	—	—	0·14	—	—	—	0·64
Combined impurities	3·86	—	—	0·75	—	—	2·51	—	—	—	203·83
Total wt.	89·02	18·95	4·86	53·09	14·10	6·90	33·77	21·48	13·66	8·74	246·77
% composition excluding non-lipids and hydrocarbons	34·8	7·4	1·9	20·8	5·5	2·7	13·2	8·4	5·3	—	—
Haddock lipids (excluding hydrocarbons)	45·0	5·5	4·5	15·9	6·2	2·5	10·7	6·2	3·5	—	—

into the two main fatty acid-containing lipids. The similar lipid pattern is clear. There is marked discrepancy between these results and those obtained for cod flesh by Kaucher, Galbraith, Button & Williams (1943) by calculation from certain analytical data on an unpurified extract.

Unidentified lipids

The unidentified lipids of cod, like those of haddock, include a phospholipid only partially separable from lecithin by counter-current distribution, e.g. in the main lecithin fraction, and another phospholipid easily separable from lecithin in that it passes to the extreme light-petroleum end of the counter-current sequence. In both haddock and cod this second lipid was found predominantly in the ethanol–ether extract (A.I. 30–32 in Tables 2 and 4). These substances correspond to the 'unidentified lipids A and C' of the haddock extracts. It is uncertain to what extent the 'unidentified lipid B' of haddock (Lovern & Olley, 1953*b*) was present in cod.

In haddock, unidentified lipids A and C were found to have a fatty acid : 'glycerophosphate' P ratio considerably in excess of the 2:1 of the normal phosphatidyl esters (Olley & Lovern, 1953; Lovern & Olley, 1953*b*). The same is true for the corresponding lipids of cod flesh. Taking fractions relatively rich in unidentified lipid A, e.g. A.I. 12–25 of the acetone extract of haddock (Olley & Lovern, 1953) and the corresponding fraction from cod (A.I. 10–17 of the acetone extract), a ratio of 4·7 was obtained for both, assuming fatty acids of average molecular weight of 300. For unidentified lipid C, the main concentrate of this from the ethanol–ether extract of both haddock (*L.* 19–20, Lovern & Olley, 1953*b*) and cod (A.I. 30–32) again show a ratio of 4·7 for haddock and 4·4 for cod. Bearing in mind the various assumptions that have to be made in assigning the total fatty acids and 'glycerophosphate' of the fractions, this agreement suggests that at least part of the structure is the same in the two types of unidentified lipid, which may have four or five fatty acid molecules per atom of phosphorus.

The 'glycerophosphate' in question gives a water-soluble barium salt, i.e. it does not include the 'false' glycerophosphate fraction of Olley & Lovern (1953). Nevertheless, it is certainly not all true glycerophosphate in unidentified lipid A (Olley, 1956), and it is possible that in unidentified lipid C, not yet studied in this respect, the 'glycerophosphate' includes other phosphate esters estimated as glycerophosphate by the acid periodate method. In fractions A.I. 10–17 of the acetone extract of cod flesh some 71% of the 'glycerophosphate' P can be accounted for by identifiable lipids. The ratio of 'excess' glycerol liberated by complete hydrolysis of these phosphate esters (i.e. total glycerol liberated minus glycerol attributable to identified phospholipids) to excess 'glycerophosphate' P is about 2·3 (Olley, 1956). Phosphate esters with a high glycerol:P ratio were also found in a haddock preparation containing unidentified lipid A (Olley, 1956).

In spite of the very mixed nature of the fractions rich in unidentified lipids A and C, there is thus emerging a possible clue to part of the structure of these lipids, e.g. a phosphate radical linked to two glycerol molecules, each of which is esterified with two fatty acid radicals. (P:glycerol:fatty acid = 1:2:4). More complex units of this general type might equally be involved, with varying ratios of these three components. More decisive hydrolytic studies must, however, await the isolation of relatively pure preparations of these particular lipids.

The nitrogenous components of concentrates of unidentified lipids A and C have not yet been identified, nor is it clear that all the nitrogen present is of lipid origin. In earlier papers (e.g. Olley & Lovern, 1953) reference was made to a base, found in preparations of unidentified lipid A but also detectable in most other fractions, which reacts on paper chromatograms with phosphomolybdic acid, but is readily distinguishable from choline. We have never been able to isolate measurable amounts of the substance in question and have now found that it is a compound derived in minute traces from the rubber bungs used until recently during vacuum removal of residual HCl from hydrolysates. It represents an insignificant proportion of the total nitrogen of concentrates of unidentified lipids A or C.

Concentrates of unidentified lipid C yield a mixture of bases, some of which interfere in the assay of choline as reineckate (Lovern & Olley, 1953*b*). The total bases of a hydrolysate of fractions A.I. 30–32 of the ethanol–ether extract of cod flesh was separated into fractions precipitable by reineckate and fractions not precipitable by reineckate, each of which was shown by ion-exchange chromatography and by solubility properties to be a complex mixture.

Inositol lipids

The inositol lipids of haddock flesh consisted mainly of two types of compound which were sharply separated by counter-current distribution to the two ends of the system, with possibly a third component of intermediate partition coefficient (Lovern & Olley, 1953*b*). In cod flesh this intermediate type is well in evidence (cf. fractions A.I. 8–29 of the ethanol–ether extract, Table 2), but the type found at the extreme ethanol end is the predominant one (cf. main lecithin fraction in

Table 4). There is no evidence for the existence in cod of the inositol lipid which is distributed to the light-petroleum end of the counter-current sequence.

Further evidence of a different inositol lipid pattern of cod and haddock flesh is afforded by chromatography. When the main lecithin fraction of cod flesh, representing over half the total inositol lipids, was passed in chloroform–methanol–water (80:20:2·5, by vol.) solution through a cellulose column (cellulose:lipid ratio, 19:1) and the column was washed with the same solvent, only 16% of the total inositol could be eluted. The remaining 84% was eluted with methanol and found to be combined inositol, probably lipid inositol, since fatty acids in more than adequate proportions were found in hydrolysates of this fraction. With the total acetone-insoluble lipid from the acetone extract of haddock flesh, only 19·4% of the inositol present (equally being combined inositol, as was that in the unadsorbed fractions) remained on the column (cellulose:lipid ratio, 25:1) from a chloroform–methanol–water solution.

Smith (1954) found that, with the lipids of rubber latex, inositol remaining on a cellulose column from a similar solvent (containing ethanol instead of methanol) was in the free state, all lipid inositol passing through. Adsorption on cellulose is a useful method of purification in some cases, e.g. for the phospholipids of egg yolk (Lea & Rhodes, 1953) or rubber latex (Smith, 1954), but this method could not be used for these fish-muscle extracts without loss of inositol-containing lipids.

Lipids containing glycerol

The presence of considerable proportions of glycerol in alkaline hydrolysates of fractions, where triglycerides should not be encountered, was reported for haddock, and the possible presence of monoglycerides was mentioned (Olley & Lovern, 1954). Similar occurrences of glycerol were observed in some cod fractions. The glycerol in fractions A.S. 1–3 and A.S. 4–11 of the ethanol–ether extract (Table 2) may again suggest the presence of partial glycerides since it cannot be attributed to inositol lipids or triglycerides. The glycerol in fractions A.I. 30–32 of the same extract is far in excess of the inositol present, but the presence of triglycerides, which would accord with the partition coefficient, is inconsistent with acetone insolubility. A similar phenomenon was observed in the haddock investigations, e.g. in fraction A.I. 10 of the chloroform–methanol extract (Table 1 of Olley & Lovern, 1954). Both species appear to contain very small proportions of glycerol-based lipids, other than the familiar type of glyceride. The presence of considerable amounts of glycerol was in all cases confirmed by paper chromatography, in addition to its analytical determination by the method of Whyte (1946). Fleury & Le Dizet (1952) noted the presence of small proportions of glycerol in hydrolysates of highly purified lecithin. As with haddock, inositol in the cod fractions was always accompanied by glycerol, and in inositol-rich fractions the glycerol can be assigned fairly satisfactorily to inositol lipids, the molar ratio of glycerol to inositol being, as in haddock, about two instead of one as in brain diphosphoinositide.

Hydrocarbons

The probable presence of very small proportions of hydrocarbons in haddock-lipid extracts was reported previously (Olley & Lovern, 1954). Such compounds have now been isolated chromatographically from lipid extracts of cod flesh and their presence in haddock extracts was confirmed by the same technique. Although rubber bungs were used only during vacuum removal of residual traces of solvents, it appears probable that the hydrocarbons found were largely, if not entirely, derived from these bungs. They have, accordingly, been deleted from the final composition of these fish lipids, as given at the bottom of Table 4.

Acidic lipids

The cod extracts, like the haddock extracts (Olley & Lovern, 1954), contained complex acidic lipids.

SUMMARY

1. Cod flesh was extracted successively with a series of solvents and the various extracts were purified, fractionated and analysed by the procedures used previously for haddock flesh.

2. Cod flesh contains the same amount of total lipids as haddock flesh (about 0·6%) and the lipid mixture is very similar in the two species, that from cod containing approximately: lecithin 35, waxes and alcohols 13, free cholesterol 8, phosphatidyl ethanolamine 7, free fatty acids 6, cholesterol esters 5, triglycerides 3, inositol lipids 2 and unidentified lipids 21%.

3. The unidentified lipids of cod flesh resemble those from haddock in containing at least two types of phospholipid. One type is apparently based on phosphorylated glycerol but not on normal glycerophosphoric acid, and probably has a fatty acid:glycerol:P ratio approximating 4:2:1. The other type also has a fatty acid:P ratio of about 4:1, but its phosphorus:glycerol relationship has not yet been studied. These phospholipids probably contain nitrogen, but the bases in question have not been identified.

4. The inositol lipids of both species include more than one type of compound and in the cod

such compounds are present in considerably different proportions from those found in haddock-flesh extracts.

5. Hydrocarbons found in both cod- and haddock-lipid extracts are probably contaminants derived from rubber.

6. Complex acidic lipids occur in the cod extracts, as in those from haddock.

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The Lipids of Fish

7. PHOSPHATE ESTERS IN THE LIPIDS OF HADDOCK AND COD FLESH

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In the course of investigations of fish lipids in this laboratory, it became evident that in certain cases the phosphate esters present in the aqueous phase from an alkaline hydrolysate could not be accounted for as glycerophosphate. The solubility, chromatographic and hydrolytic properties were not compatible with those of the latter ester. Several methods other than the classical separations of the heavy-metal salts have been used in an attempt to identify these other phosphate esters. They are as follows: (1) Paper chromatography. (2) Solubility in ethanol; Fleury & Le Dizet (1950) found that sodium glycerophosphate has a negligible solubility in absolute ethanol even at the boiling point. (3) Susceptibility to hydrolysis at pH 4. Fleury (1948*b*) used this method to distinguish between glycerophosphoric acid and certain of its derivatives; the latter require a preliminary hydrolysis with N-HCl at 100° to convert them into glycerophosphoric acid which is then hydrolysed at pH 4 to yield inorganic phosphate and glycerol. (4) Estimation of the glycerol:phosphorus ratio of esters susceptible to hydrolysis at pH 4 and also of those hydrolysed at pH 4 only after a preliminary hydrolysis with N-HCl. (5) Separation of phosphate esters according to the solubility of their barium salts in water. 10% of the phosphate esters from the ethanolic-KOH hydrolysates of the total acetone-insoluble phospholipids of the acetone extract of haddock flesh, which reacted with periodate, had been found to have water-insoluble barium salts (Olley & Lovern, 1953). Barium glycerophosphate is water-soluble.

Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) have very recently published a method for distinguishing the glycerylphosphoryl esters by their complete resistance to prostatic and intestinal phosphatases.

EXPERIMENTAL

Two fractions from an acetone extract of cod muscle were examined. These have been referred to (Garcia, Lovern & Olley, 1956; Table 5) as A.S. 1–3 and A.I. 10–17, i.e. the first three most polar fractions in the counter-current distribution of the acetone-soluble (A.S.) lipids of the acetone extract between light petroleum : ethanol–water (85:15, by vol.), and the less polar fractions following the main 'lecithin' peak from the counter-current distribution of the acetone-insoluble (A.I.) lipids of the acetone-soluble fraction, respectively.

These fractions were hydrolysed by refluxing for 2 hr. with 0·5 N-KOH in 95% (v/v) ethanol. They were acidified and the fatty acids and unsaponifiable matter were extracted with ether. The aqueous hydrolysate was taken to dryness and excess HCl was removed under vacuum. The