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The Lipids of Fish

5. THE LIPIDS REMAINING IN THE FLESH OF THE HADDOCK AFTER EXTRACTION BY ACETONE AND ETHANOL-ETHER

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In the first paper of this series (Lovern, 1953) was described the preparation of a series of extracts from the flesh of the haddock, using solvents of presumed successively increasing effectiveness for lipids. The extracts obtained with acetone and ethanol-ether (3:1 by vol.), respectively, both at room temperature, have been discussed in earlier papers of this series (Lovern & Olley, 1953*a*, *b*; Olley & Lovern, 1953) and their extensive contamination with non-lipids was shown. The later extracts were obtained with ethanol-benzene (2:1 by vol.), chloroform-methanol (1:1 by vol.), both at the boiling point, and finally pyridine at 100°. Examination of these three extracts showed them to be overwhelmingly non-lipid in composition, and it was decided to present the results in a single paper. Moreover, in order to obtain sufficient lipid for detailed study, comparable extracts, available from an earlier series of experiments and since stored at -30° under nitrogen, were combined with the current series of ethanol-benzene and chloroform-methanol extracts.

ANALYTICAL METHODS

In general the analytical methods employed were as described in the previous papers. New features are given below.

Hydrolysis. The unsatisfactory hydrolysis of sphingomyelin with 0.5*N* ethanolic KOH (Lovern & Olley, 1953*b*) led to the use in the present work of 6*N* methanolic HCl for the hydrolysis (8 hr. refluxing) of fractions likely to contain over 5% of sphingosine. This reagent does not permit the separate isolation of unsaponifiable matter and plasmals, and the latter were assayed after such hydrolysis colorimetrically (Feulgen & Grünberg, 1938) on the unsaponifiable matter, separated from the methyl esters by a simplification of the procedure of Klenk & Friedrichs (1952). In all cases, however, only traces of plasmal were found, in contrast to the appreciable yields of supposed plasmal regularly isolated as a separate fraction after alkaline hydrolysis. Methanolic acid hydrolysis of ox-brain phospholipids readily gave the plasmal fraction in the form of dimethylacetals in the unsaponifiable fraction. This finding strengthens the doubts expressed in the last paper (Lovern & Olley, 1953*b*) whether the material isolated gravimetrically was genuinely plasmal. Its real nature remains obscure.

It has been found that unsaponifiable matter isolated after 2 hr. refluxing with 0.5N ethanolic KOH is not in all cases resistant to more prolonged, e.g. 16 hr., refluxing with the same reagent. Thus, that portion of the pyridine extract which was insoluble in boiling chloroform-methanol (see below) yielded 161 and 98 mg. of unsaponifiable matter and fatty acids, respectively, after the customary 2 hr. hydrolysis period. A further 16 hr. hydrolysis of the former material gave 113 mg. unsaponifiable matter and a further 38 mg. of fatty acids. This resistance to hydrolysis of what we have designated 'wax' in earlier papers was noticed too late in the present work for all the unsaponifiable fractions to be so examined, but it was taken into account in assessing the likelihood of wax esters occurring in certain fractions. Such esters would probably give approximately equal amounts of acid and unsaponifiable matter on complete hydrolysis.

There is considerable controversy as to the conditions affording complete hydrolysis of sphingomyelin (Thannhauser, Benotti & Reinstein, 1939; Erickson, Avrin, Teague & Williams, 1940; Carayon-Gentil & Corteggiani, 1942; Artom & Fishman, 1943; Hack, 1946; Brante, 1949; Rennkamp, 1949). We have confirmed Rennkamp's finding that hydrolysis with methanolic HCl does not yield any free choline, but does quantitatively split the fatty acid-sphingosine linkage. We have further confirmed the observation of Brante (1949) that refluxing with saturated aqueous baryta quantitatively liberates the choline of sphingomyelin and we also observed quantitative liberation of inorganic phosphate. By contrast, hydrolysis with 0.5N ethanolic KOH only gave low and variable yields of free choline, even after 48 hr. refluxing. Baryta hydrolysis (8 hr. refluxing) after a preliminary hydrolysis (8 hr. refluxing) with methanolic HCl did not give quantitative choline recoveries (yield of the order of 16% of theoretical), presumably because of the stability of the phosphorylcholine liberated by the acid hydrolysis (Rennkamp, 1949). Our final procedure for the hydrolysis of fractions likely to contain sphingomyelin therefore involved 8 hr. refluxing of one sample with saturated aqueous baryta, for choline determination and 8 hr. refluxing with 6N methanolic HCl of another sample for determination of fatty acids and unsaponifiable matter. In contrast to ethanolic alkali and aqueous HCl (Lovern & Olley, 1953a; Olley & Lovern, 1953), methanolic HCl gave fatty acids (as methyl esters), entirely free from nitrogen.

The small quantities available for analysis sometimes required the determination of components in hydrolysates not primarily produced for that particular estimation. Thus serine, ethanolamine, inositol and glycerol were sometimes liberated by 8 hr. hydrolysis with 6N methanolic HCl, removal of methyl esters of fatty acids and unsaponifiable matter with light petroleum, addition of 0.25 vol. water and a further 3 hr. refluxing. In the case of glycerol, it was necessary to allow for hydrolysis of glycerophosphate, which was found to proceed to the extent of 35% under these conditions. Glycerophosphate itself was always determined on the same (alkaline) hydrolysates as choline, but glycerol determination (Lovern & Olley, 1953a) required a minimum of 5 mg. glycerol, and hence could only be carried out on the main hydrolysis product, i.e. from 0.5N ethanolic KOH or 6N methanolic HCl according to sphingosine content.

Sphingosine. The method of McKibbin & Taylor (1949) is

the only published procedure for the quantitative assay of sphingosine in lipids. As the authors point out, it depends on all non-sphingosine N becoming water-soluble during the hydrolysis. We have routinely assayed our fractions for sphingosine by McKibbin & Taylor's method, but there seems little doubt that in many cases the N found was not all sphingosine N (see below).

Reducing sugar. In the early part of the work reducing sugar was determined by the method of Brand & Sperry (1941) but this was superseded by the anthrone method of Mallov, McKibbin & Robb (1953), which proved far more satisfactory with our extracts.

EXPERIMENTAL PROCEDURE

The general procedure followed closely that described in the previous paper (Lovern & Olley, 1953b), in that the three extracts were freed from the greater part of the non-lipid contaminants by re-extraction with solvent followed by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951) and (except in the case of the pyridine extract) later fractionated by countercurrent distribution between light petroleum (b.p. 40–60°) and 85% (v/v) aqueous ethanol. Additional fractionation steps included precipitation with acetone in the presence of $MgCl_2$ and separation of acidic lipids by washing an ethereal solution with aqueous Na_2CO_3 . The examination of the three extracts will be described individually.

Ethanol-benzene extract

24 g. of older extract (A) and 79 g. of later extract (B) were available. Each of them was re-extracted with light petroleum (b.p. 40–60°) leaving 11.5 and 72.3 g. insoluble material, respectively. Both soluble and insoluble portions were taken up in chloroform-methanol (2:1 by vol.) and submitted twice to the procedure of Folch *et al.* (1951). The fractions insoluble in light petroleum each contained material insoluble in chloroform-methanol (approx. 2 and 10 g., respectively, for A and B), which proved to be entirely non-lipid in nature, since after alkaline hydrolysis it became completely water-soluble and yielded neither fatty acids nor unsaponifiable matter. Treatment according to Folch *et al.* yielded the following amounts of purified lipids: (A) 0.5 and 8.4 g.; (B) 3.1 and 6.4 g. from the petroleum-insoluble and petroleum-soluble fractions, respectively. These four fractions were combined to give 18.4 g. of lipid. The aqueous phases resulting from the four treatments were recovered and hydrolysed and the small yields (about 1% of each) of fatty acids and unsaponifiable matter obtained indicated that this material was mainly non-lipid. Extraction of the aqueous phase with light petroleum yielded a small amount (about 0.6%) of lipid which on hydrolysis yielded 65% of fatty acids and 41% of unsaponifiable matter.

The further treatment of the 18.4 g. of purified lipid is illustrated in Fig. 1a. Aqueous-alkali washing was confined to the acetone-soluble lipids, since any free fatty acids should occur there and, moreover, these fractions do not emulsify so readily as the acetone-insoluble ones. Pooling of fractions, on the basis of appearance, distribution curve, analytical data and spot tests (see previous papers) was as indicated in Fig. 1a. The countercurrent distribution curve is given in Fig. 2a. Cholesterol spot tests (fractions 20, 30 and 40) were all so faint as to be negligible; plasmal spot

Fig. 1. Fractionation scheme for purified lipid extracts. All weights corrected for analytical and manipulative losses.
(a) Ethanol-benzene extract; (b) chloroform-methanol extract.

tests (fractions 1, 10, 30 and 40) were all weak but quite definite. Fraction 1 proved to contain a small amount (0.4 g.) of material insoluble in organic solvents but soluble in water. It is designated 'impurity' in Fig. 1a, since on alkaline hydrolysis it yielded only 0.1% fatty acids and 0.2% unsaponifiable matter.

The acidic lipids did not resemble free fatty acids in appearance and proved to contain large amounts (1.3–2.6%) of phosphorus. They were, therefore, hydrolysed (0.5N ethanolic KOH) for analysis.

Chloroform-methanol extract

20 g. of older extract (A) and 103 g. of later extract (B) were separately re-extracted with chloroform-methanol (2:1 by vol.), leaving 6.45 and 44 g., respectively, of insoluble matter (1.45 and 0.2% unsaponifiable matter, 1.1 and 0.1% fatty acids, respectively). The chloroform-methanol solutions yielded (A) 2.7 g. and (B) 2.4 g. of purified lipids (chloroform phase) and the respective water-soluble impurities yielded 0.8 and 0.06% unsaponifiable matter and 0.6 and 0.07% fatty acids. The further treatment of these purified lipid fractions is shown in Fig. 1b. The material described as 'impurity' in the sodium carbonate washing process was a brown gum which separated when the

acetone-soluble lipids were taken up in ether and which collected at the interface on washing with the aqueous Na_2CO_3 . It yielded 11% unsaponifiable matter and 6% fatty acids on hydrolysis (0.5N ethanolic KOH), the rest of it becoming water-soluble. The acidic lipids were again rich in phosphorus (0.7–1.1%), not at all like free fatty acids in appearance, and were hydrolysed for detailed analysis. The countercurrent distribution curves for the neutral acetone-soluble lipids and the acetone-insoluble lipids are given in Fig. 2b and c respectively. The principle of pooling was the same as for the ethanol-benzene extract. Spot tests showed traces of cholesterol in pooled neutral lipids 3–6, but none in fractions 1 and 2. Traces of plasmal were detectable in both groups of neutral lipids and appreciable amounts in some of the acetone-insoluble lipids (fractions 6–10), but a negative plasmal reaction was obtained on fractions 1–5.

Pyridine extract

65 g. of crude extract were re-extracted with boiling chloroform-methanol (2:1 by vol.), leaving 38 g. of insoluble material (0.3% unsaponifiable matter and 0.4% fatty acids on hydrolysis). The soluble material was treated twice by the procedure of Folch *et al.* (1951) giving 3.4 g. of purified lipids and 23.6 g. of water-soluble impurities, the latter yielding 1.5% unsaponifiable matter and 5.1% fatty acids on hydrolysis. The purified lipids were precipitated with acetone and MgCl_2 giving 3.3 g. of soluble and 0.1 g. of insoluble material. The former, on dissolving in ether and washing with aqueous Na_2CO_3 yielded 2.4 g. of neutral lipids, 0.4 g. of acidic lipids and 0.5 g. of a black tar which was insoluble in ether and water, but soluble in acetone. All fractions were hydrolysed for analysis, there being insufficient material for countercurrent distribution. Plasmal spot tests were negative on the neutral and acetone-insoluble lipids and a cholesterol test on the former was also negative. No spot tests were applied to the acidic lipids.

RESULTS

Analytical data on the various groups of fractions are given in Table 1. Blanks in the table indicate that the determination in question was not made, and question marks an unsatisfactory end-point. The nitrogen ascribed to ethanolamine contains minor proportions of serine nitrogen as shown by paper chromatography. Bound reducing sugar represents merely reducing sugar liberated by acid hydrolysis and there is no proof that it is a lipid component. In addition to the components listed in Table 1, which are all recognized constituents of one lipid class or another, most hydrolysates contained appreciable proportions of other types of fragment, e.g. acid-hydrolysable phosphate and volatile base (ammonia?). Fractions containing bound reducing sugar generally contained comparable proportions of free reducing sugar.

The data in Table 1 have been interpreted, so far as possible, on the lines adopted in previous papers, i.e. attempts were made to equate choline, ethanolamine, glycerophosphate and available fatty acids (in the light of claims for the latter by other com-

Fig. 2. Countercurrent distribution of lipid extracts of haddock flesh. Solvents 85% (v/v) aqueous ethanol and light petroleum (b.p. 40–60°). Ordinates: fraction yields (%). Abscissae: fraction numbers. Ethanol-soluble direction to left, petroleum-soluble to right. (a) Ethanol-benzene extract. Twenty flasks in series, 300 ml. light petroleum in each; lipid in flask 1. Twenty batches, each 300 ml., of ethanol passed from flask 1 to 20 and run off to give fractions 1–20. Petroleum remaining in flasks 20–1, in that order, gave fractions 21–40. (b) Acetone-soluble portion of chloroform-methanol extract. Six flasks in series, 100 ml. light petroleum in each; lipid in flask 1. Ethanol, in batches of 100 ml. passed from flasks 1 to 6, 1 to 5, etc., but no fractions run off. Flasks 6–1 gave fractions 1–6, in that order. (c) Acetone-insoluble portion of chloroform-methanol extract. Procedure as in (b) but with ten flasks in series, flask 10 yielding fraction 1.

Table 1. *Analytical data on lipid fractions*

% Constituent in fraction. Blank values not determined.

Fraction	Fatty acids	Unsep.	Plasmals	Glycero-phosphate P	Choline	Ethanol-amine N	Sphingosine N	Inositol	Glycerol	Bound reducing sugar
				Ethanol-benzene extract						
Acidic 1	49.0	15.8	3.0	1.64	2.3	0.03	—	Nil	—	—
Acidic 2-8	55.5	12.3	3.0	1.97	7.9	0.07	—	Nil	—	—
Acidic 9-32	47.0	26.4	1.0	0.98	0.3	0.02	—	Nil	—	—
Acidic 33-40	45.3	31.4	10.0	0.58	—	—	—	Nil	—	—
Neutral 1	47.0	←23.6→		2.94	Nil	0.02	0.32	Nil	—	0.07
Neutral 2-8	32.1	←21.0→		0.70	1.8	0.11	0.20	0.33	—	0.39
Neutral 9-32	24.0	69.0	10.0	Nil	—	—	—	—	1.26	Nil
Neutral 33-40	36.0	55.0	4.0	Nil	—	—	—	—	2.07	Nil
Acetone-insol. 1	12.9	←4.8→		2.95	6.3	0.16	0.28	1.9	—	0.42
Acetone-insol. 2-8	51.7	←2.9→		2.16	3.8	0.19	0.12	2.9	2.37	Nil
Acetone-insol. 9-32	20.8	11.0	4.8	1.50	1.7	0.11	0.13	0.33	0.88	Nil
Acetone-insol. 33-40	20.0	←12.2→		2.48	Nil	Nil	0.20	0.43	—	1.21
				Chloroform-methanol extract						
Acidic A	53.2	25.6	5.9	0.78	0.7	0.03	—	Nil	—	—
Acidic B	45.2	37.5	5.9	0.53	0.7	0.03	—	Nil	—	—
Neutral 1-2	53.0	←41.0→		Nil	0.9	0.66	0.58	Nil	6.25	Nil
Neutral 3-6	14.4	←22.8→		0.56	0.3	0.43	0.21	Nil	0.56	Nil
Acetone-insol. 1-5	34.8	6.2	10.5	1.85	1.5	0.27	Nil	5.84	6.55	2.67
Acetone-insol. 6-9	48.5	←22.2→		0.77	0.2	0.12	0.17	1.97	1.40	1.42
Acetone-insol. 10	46.9	←32.0→		0.79	1.0	0.13	0.26	1.40	6.10	0.88
				Pyridine extract						
Acidic	40.7	16.3	7.8	0.22	1.2	—	—	—	—	—
Neutral	16.1	←27.9→		Nil	2.7	Nil	1.03	Nil	—	2.0?
Acetone-insol.	13.1	←23.7→		3.6	2.1	0.23	0.26	1.54	—	?
Tar	17.0	25.0	—	0.10	Trace	—	—	—	—	—

pounds such as glycerol and unsaponifiable matter). In the previous paper (Lovern & Olley, 1953*b*) it was found more difficult to equate the various fragments for the ethanol-ether extract than had been the case for the acetone extract, and this trend is much more in evidence in the present work. Certain fractions clearly contained non-lipid forms of such compounds as choline and glycerophosphate, e.g. acetone-insoluble fraction 1 of the ethanol-benzene extract, where there is a gross deficiency of fatty acids. Similarly, neutral lipid 1-2 of the chloroform-methanol extract contains glycerol equivalent to about 60 % of triglycerides. (There is no inositol, so it cannot be assigned to inositol lipids.) Any triglycerides present should go to the extreme light-petroleum end of the counter-current distribution, i.e. fraction 6. It has been found, however, that monoglycerides (α -monostearin) are concentrated at the ethanol end of the sequence, diglycerides ($\alpha\alpha'$ -distearin)* behaving like triglycerides. The evidence seems insufficient at this stage to conclude that monoglycerides are present in these extracts. In all of such intractable cases, a choice has had to be made in assigning the available fatty acids among the possible lipids present, and where possible this has been influenced by information on countercurrent distribution behaviour and certain analytical values additional to those in Table 1. Thus in some cases, but not in all, there was sufficient unsaponifiable matter available for acetylation and determination of saponification equivalent, permitting an estimate of the relative amounts of alcoholic and non-alcoholic (hydrocarbon?) material. In other cases the unsaponifiable matter was assumed to be entirely alcoholic and, in general, alcoholic material was assumed to have been present as wax esters. An exception will illustrate how an 'informed guess' was sometimes made. Neutral lipid 1 of the ethanol-benzene extract contains glycerophosphate equivalent to about 45 % of fatty acids (found, 47 %), on the lecithin model, although in the absence of choline, ethanolamine and inositol, no known glycerophosphatide is present. This glycerophosphate must, however, be combined in some form of neutral lipid. If the unsaponifiable matter is assumed to require an equal amount of fatty acid to give wax esters, only some 23 % of fatty acids is available to go with the glycerophosphate. In previous experience (see, for instance, the subfractions of S1-5 in Olley & Lovern, 1953) we have noted a tendency for alcoholic unsaponifiable matter at the extreme ethanol end of the countercurrent sequence to be unaccompanied by equivalent amounts of available fatty acids, and it is probably in the free

state. This would also be expected from structure and solubility considerations. Hence, in calculating the composition of the neutral lipid 1 in question, we have preferred, in contrast to the general procedure, to assign all the fatty acid to an unidentified lipid of a glycerophosphatidic nature, and to consider the unsaponifiable material as originally unesterified.

Fatty acids in excess of the requirements of other identified lipid fragments in any fraction have, as in previous papers, been assigned to 'unidentified lipid', using an arbitrary conversion value of 75 % fatty acids in the lipid. In some cases this might have contained glycerophosphate, in others it could not have done. It represents a general pool of unidentified, fatty acid-containing material and cannot be directly equated with any of the unidentified lipids A, B and C of the previous paper (Lovern & Olley, 1953*b*).

No direct use has been made of the values for sphingosine and bound reducing sugar. The highest sphingosine values were found in two acetone-soluble products (Table 1), whereas sphingolipids are, in general, considered to be insoluble in cold acetone. On the other hand, ceramides (Thannhauser & Fränkel, 1931; Fränkel & Bielschowsky, 1932), if present, would probably pass into the acetone-soluble material and Brante (1949) unexpectedly found this to be so for brain cerebroside. But sphingomyelin and, probably, ceramides are concentrated toward the ethanol end of the countercurrent distribution sequence (cf. Lovern & Olley, 1953*b*; we have been unable to dissolve ox-brain cerebroside in an 85 % ethanol-light petroleum solvent pair), whereas the sphingosine values in Table 1 show no marked trend in this direction. If the bound reducing sugar is a lipid component, it should show a parallelism to the sphingosine values, so far as is known from the chemistry of animal lipids (this argument would not apply to vegetable lipids). In fact there is no such parallel trend and the highest sugar content of all was found in a fraction (chloroform-methanol acetone-insol. 1-5) with no sphingosine.

The calculated compositions of the various groups of fractions and of the three total extracts are given in Table 2.

DISCUSSION

Non-lipids

The most striking result is the finding that these three extracts are predominantly non-lipid in nature. The subsequent insolubility of some of this material in chloroform-methanol is probably due to the smaller volumes of solvent used for re-extraction than for tissue extraction, but it should be noted that with the ethanol-benzene extract some of the non-lipid was soluble even in light petroleum, and

* Samples of mono- and distearin were kindly supplied by Dr M. L. Meara, of the Department of Organic Chemistry, Liverpool University.

Table 2. *Composition of fractions and total extracts*

As weights (g.) except final percentage for each extract.

Fraction	Lecithin	Phosphatidyl- ethanolamine	Inositol lipid	Plasmalogen	Unidentified lipid	Waxes and alcohols	Hydrocarbons, etc.	Triglycerides	Non-lipids
Ethanol-benzene extract									
Acidic 1	0.20	0.01	—	0.04	0.07	0.19	—	—	0.09
Acidic 2-8	0.32	0.02	—	0.05	0.14	0.20	—	—	0.07
Acidic 9-32	—	—	—	0.01	0.12	0.21	—	—	0.06
Acidic 33-40	—	—	—	0.04	0.03	0.13	—	—	—
Neutral 1	—	—	—	—	0.69	0.26	—	—	0.15
Neutral 2-8	0.08	0.04	0.01	—	0.04	0.14	0.05	—	0.24
Neutral 9-32	—	—	—	—	—	0.22	0.22	0.06	—
Neutral 33-40	—	—	—	—	—	0.63	0.51	0.31	0.05
Acetone-insol. 1	0.09	0.02	0.02	—	—	0.10	—	—	0.87
Acetone-insol. 2-8	2.38	0.93	1.26	—	1.47	0.52	—	—	2.54
Acetone-insol. 9-32	0.07	0.04	0.02	0.12	0.04	0.29	—	—	0.72
Acetone-insol. 33-40	—	—	0.02	—	0.07	0.19	—	—	0.52
Water-sol. 1	—	—	—	—	—	—	—	—	0.40
Total purified lipid	3.14	1.06	1.33	0.26	2.67	3.08	0.78	0.37	5.71
Total impurities removed	—	—	—	—	—	1.92	—	—	82.78
Total extract (103.1 g.)	3.14	1.06	1.33	0.26	2.67	5.00	0.78	0.37	88.49
Percentage composition	3.0	1.0	1.3	0.2	2.6	4.9	0.8	0.4	85.8
Chloroform-methanol extract									
Acidic 4	0.03	0.01	—	0.04	0.06	0.16	—	—	—
Acidic B	0.01	0.01	—	0.03	0.02	0.22	—	—	0.01
Neutral 1-2	—	—	—	—	0.03	0.17	—	—	—
Neutral 3-6	0.01	0.13	—	—	—	0.13	0.21	—	0.72
Acetone-insol. 1-5	0.02	0.03	0.41	0.30	—	0.19	—	—	0.55
Acetone-insol. 6-9	—	0.02	0.03	—	0.06	0.13	—	—	0.06
Acetone-insol. 10	0.03	0.03	0.03	—	—	0.25	—	—	0.06
Total purified lipid	0.10	0.23	0.47	0.37	0.17	1.25	0.21	—	1.40
Total impurities removed	—	—	—	—	—	0.66	—	—	118.59
Total extract (123.45 g.)	0.10	0.23	0.47	0.37	0.17	1.91	0.21	—	119.99
Percentage composition	0.1	0.2	0.4	0.3	0.1	1.5	0.2	—	97.2
Pyridine extract									
Acidic	0.02	—	—	0.07	0.11	0.13	—	—	0.07
Neutral	—	—	—	—	0.35	0.24	0.55	—	1.26
Acetone-insol.	—	—	—	—	—	0.04	—	—	0.06
Tar	—	—	—	—	—	0.21	—	—	0.29
Total purified lipid	0.02	—	—	0.07	0.46	0.62	0.55	—	1.68
Total impurities removed	—	—	—	—	—	0.38	—	—	61.22
Total extract (65 g.)	0.02	—	—	0.07	0.46	1.00	0.55	—	62.90
Percentage composition	Trace	—	—	0.1	0.7	1.6	0.8	—	96.8

most of it in chloroform-methanol. As in the previous papers, non-lipid material was still present in the purified extracts and in nearly all of the final fractions. We have discussed previously (Lovern & Olley, 1953*b*) the possibility that some of this non-lipid may be present as a complex with one or other of the lipids. Recent reports, (Folch & LeBaron, 1953; Becker, Bode & Schrade, 1953) of the existence of phosphatide-peptide complexes in lipid extracts of various tissues may be noted. Our consistent finding (cf. Lovern & Olley, 1953*b*) of high proportions of (non-sterol) unsaponifiable matter in the small amount of lipid accompanying the non-lipids removed by the purification of Folch *et al.* (1951) suggests that this lipid (wax?) is an important component of lipid-peptide or similar complexes in fish muscle.

Acidic lipids

Another unexpected finding was the range and proportions of the products obtained on hydrolysis of the acidic lipids. These could certainly not have consisted of the usual type of phosphatidic acid. Interestingly, they were completely lacking in inositol and contained little more than traces of non-lipids, possibly owing to the additional possibilities of aqueous extraction. These acidic lipids gave perfectly clear solutions in aqueous sodium carbonate, from which they could only be extracted by ether after acidification. Exactly similar acidic lipids (which in this case were also tested for bound reducing sugar, present to the extent of 2-4%) were obtained from comparable lipid extracts of cod flesh, in other studies now in progress in our laboratory. Again inositol was absent and non-lipids virtually so. It seems probable that complex formation occurs between a number of lipids in such a way that sufficient acidic groupings are free for the whole complex to form a water-soluble sodium salt. Appreciable proportions of both orthophosphate and unidentified phosphorus accompanied the glycerophosphate in the hydrolysates of acidic lipids.

Acetone precipitation

Although acetone precipitation in the presence of magnesium chloride, using optimum concentration of the latter (Sinclair & Dolan, 1942), does effect a marked concentration of the phosphatidic lipids, it by no means results in their quantitative recovery in the case of these particular fish lipids. It is possible that the unusually high degree of unsaturation of the fatty acids, noted in previous papers, is responsible.

Composition of haddock-flesh lipids

The very small amounts of lipid available in the various fractions prevented any attempt at sub-fractionation and there is no doubt that the assess-

Table 3. *Composition of the total lipids of haddock flesh*

	% total lipid
Lecithin	42.8
Unidentified lipid	15.2
Waxes and alcohols	10.5
Free cholesterol	6.1
Free fatty acids	6.1
Phosphatidylethanolamine	5.4
Inositol lipids	* 4.4
Cholesterol esters	3.4
Triglycerides	2.4
Hydrocarbons, etc.	2.0
Plasmalogens	1.7

ment of their composition is, in many cases, less satisfactory than for the earlier extracts. Moreover, as already indicated, some of them evidently contained appreciable proportions of incomplete lipid structures, e.g. compounds lacking much of their fatty acid. All the lipids of the present series represent no more than traces in the flesh of the haddock. The older extracts (A) represented 20 kg. of fresh haddock tissue and the later ones (B) 42 kg. Hence the total tissue content of true lipid extracted, for instance, by ethanol-benzene was about 0.02%, and any particular fraction or component represents a negligible proportion of the tissue. It is not, therefore, surprising to find such extracts containing major proportions of the trace constituents of muscle tissue, and there is no reason for regarding say, 'excess' choline or glycerophosphate as post-mortem artifacts.

Because of the negligible concentration of these three extracts in the original tissue, any errors in the assumptions made in this paper will not seriously affect an estimate of the total lipid composition of haddock flesh. A weighted summation of the compositions of the various extracts described in this and the previous papers is given in Table 3. In this summation allowance has been made for the subsequently-determined inositol content of the acetone extract (Lovern & Olley, 1953*b*), all unidentified lipids have been grouped together, and all non-lipids discarded. The outstanding importance of lecithins and, since the unidentified lipids are obviously a mixture, the role of waxes and alcohols as second largest constituent may be noted, while triglycerides are quantitatively among the least important components. The total lipid content of haddock flesh is now seen to be about 0.55%.

Extractability of lipids

An indication of the degree of selectivity of the various solvents is given in Table 4. Whereas virtually all of some lipids, e.g. free and esterified cholesterol, and the greater part of others, e.g. lecithin, is extracted by acetone, this is not so for unidentified lipids, plasmalogens, waxes and hydrocarbons. Unsatisfactory though some of these

Table 4. *Proportion (%) of each lipid removed from tissue by successive solvents*

Lipid	Acetone	Ethanol-ether	Ethanol-benzene	Chloroform-methanol	Pyridine
Lecithin	88.5	9.8	1.6	0.07	0.03
Unidentified lipids	62.4	32.2	3.9	0.2	1.3
Waxes and alcohols	58.0	20.5	14.1	3.3	4.1
Free cholesterol	~ 100	—	—	—	—
Free fatty acids	~ 100?	—	—	—	—
Phosphatidylethanolamine	84.4	10.3	4.4	0.9	—
Inositol lipids	72.5	18.6	6.7	2.2	—
Cholesterol esters	~ 100	—	—	—	—
Triglycerides	96.5	—	3.5	—	—
Hydrocarbons, etc.	61.0	16.8	8.5	2.1	11.6
Plasmalogens	50.3	40.4	3.3	4.3	1.7

classifications may be in the present case, they do represent lipids with a different extraction pattern from the former types. In the case of the unidentified lipids, part of the effect is attributable to the 'unidentified lipid C' of the previous paper (Lovern & Olley, 1953*b*), which appeared as a major component exclusively in the ethanol-ether extract. A precisely similar appearance and distribution pattern of 'unidentified lipid C' has been observed in comparable extracts of cod flesh. The lipids most resistant to complete extraction are rich in non-sterol unsaponifiable matter, as reflected in the figures for waxes and hydrocarbons. Cioffi & Varetto (1952) have noted an increased proportion of unsaponifiable matter in the 'bound' portion of the lipids of *Penicillium javanicum*.

Many points remain unsettled from this series of studies, e.g. the precise nature of the various unidentified lipids and of the unsaponifiable matter. These will be the subject of future research.

SUMMARY

1. Haddock flesh, previously exhaustively extracted with acetone and ethanol-ether (3:1 by vol.) at room temperature, was extracted successively with boiling ethanol-benzene (2:1 by vol.), boiling chloroform-methanol (1:1 by vol.) and pyridine at 100°.

2. The three extracts proved to consist predominantly of non-lipid material, most of which was removed by partition between chloroform and water (Folch *et al.* 1951). The purified extracts were separated into acetone-soluble and acetone-insoluble fractions, and the former further subdivided into neutral and acidic lipids. Countercurrent distribution between light petroleum and 85 % (v/v) aqueous ethanol was also employed in fractionating the ethanol-benzene and chloroform-methanol extracts.

3. Acidic, neutral and acetone-insoluble fractions were all complicated mixtures, and the acidic lipids were neither free fatty acids nor simple phosphatidic acids. The amounts available precluded extensive

subfractionation and estimation of composition from analytical data consequently suffered in precision.

4. It is, nevertheless, evident that the lipid portions of all these extracts differ appreciably from the previous extracts, in particular in the high content of unsaponifiable matter, for the present assigned to waxes and hydrocarbons.

5. The composition of the total lipids of haddock flesh, amounting, after purification, to 0.55 % of the fresh tissue, is assessed. Lecithins are outstandingly the major component (43 %) and triglycerides (2.4 %) quantitatively among the least important.

6. The solvents used showed a varying efficiency for the different lipids. Some, e.g. cholesterol, were almost entirely removed by acetone. Curiously, lecithin was removed by acetone to the extent of nearly 90 %. Some lipids were more resistant to acetone, and one component of the unidentified group was confined almost entirely to the ethanol-ether extract. The lipids most resistant to extraction are rich in non-sterol unsaponifiable matter, as are the traces of lipids associated with the water-soluble impurities removed during the purification processes.

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The Reaction of Haemoglobin and some of its Derivatives with *p*-Iodophenylhydroxylamine and *p*-Iodonitrosobenzene

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Evidence has recently been presented that *p*-iodophenylhydroxylamine ($\text{I.C}_6\text{H}_4\text{.NHOH}$) and *p*-iodonitrosobenzene ($\text{I.C}_6\text{H}_4\text{.NO}$) are produced during the metabolism of *p*-iodophenylurethane and *p*-iodoaniline in the rat (Crick & Jackson, 1952, 1953). These metabolites combine firmly with haemoglobin so that erythrocytes become effectively labelled when radioactive iodo-compounds are used. Concurrently, methaemoglobinaemia develops but disappears in the course of an hour or two without loss of radioactivity from the cells.

Phenylhydroxylamine ($\text{C}_6\text{H}_5\text{.NHOH}$) and nitrosobenzene ($\text{C}_6\text{H}_5\text{.NO}$) are known to induce methaemoglobin formation in red cells, and combination occurs between haemoglobin and nitrosobenzene (Heubner, Meier & Rhode, 1923; Jung, 1940). The reactions of haemoglobin and some of its derivatives with phenylhydroxylamine and nitrosobenzene have been studied spectroscopically by Keilin & Hartree (1943) who concluded that the complexes formed were unstable and easily dissociated.

Radio-isotope work described in this paper using $p\text{[}^{131}\text{I]}$ -iodophenylhydroxylamine and $p\text{[}^{131}\text{I]}$ -iodonitrosobenzene has shown that stable complexes are formed with oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin, and that reaction may occur without apparent change in the absorption spectrum of the pigment. It seems likely that similar interpretations may apply to the reactions with phenylhydroxylamine and nitrosobenzene.

These reactions may be of importance in relation to the known toxic effects of aromatic nitro- and amino-compounds used in industry and medicine, for such substances may be metabolized to hydroxylamino- and nitroso-compounds which could be responsible for the observed toxic effects.

EXPERIMENTAL

The preparation of $p\text{[}^{131}\text{I]}$ -iodophenylhydroxylamine (IPhNHOH) and $p\text{[}^{131}\text{I]}$ -iodonitrosobenzene (IPhNO) has been described elsewhere (Crick & Jackson, 1953).

Crystalline rat oxyhaemoglobin. A modification of the method of Drabkin (1946) was used. The stroma from washed, haemolysed cells (1 vol. of cells to 10 vol. of water) was first removed by filtration through filter paper (Whatman, no. 30, double) and through a Seitz filter. Crystallization was induced by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to opalescence, followed by refrigeration, and the product further purified by recrystallization three times from phosphate buffer (Sørensen, 0.067 M, pH 6.6).

Alkaline haematin. This was prepared by the method of Anson & Mirsky (1929–30) and twice recrystallized. For use it was dissolved in a mixture of acetone (1 vol.) and 0.5 M- K_2HPO_4 (3 vol.).

Reactions with IPhNHOH and IPhNO. Both IPhNHOH and IPhNO are virtually insoluble in water, and for use were dissolved in a minimal amount of absolute ethanol. The hydroxylamine is unstable in solution, especially in aqueous alcohol, and must be freshly prepared; the pure nitroso-compound is more stable. The technique used in studying their reactions with the pigments is illustrated by the following typical example:

A solution of oxyhaemoglobin in phosphate buffer (10 ml. of solution containing 580 mg. of pigment) was treated with a solution of IPhNHOH (25 mg. in 0.5 ml. of ethanol) and the mixture shaken for a few minutes. The violet solution was centrifuged to remove a small amount of debris and any excess reagent, and the supernatant treated with solid $(\text{NH}_4)_2\text{SO}_4$ added in small portions, to cause partial separation of the pigment. The latter was removed by centrifuging, dissolved in phosphate buffer (pH 6.6) and again partially precipitated. The separated pigment was again removed and dissolved in a suitable volume of the same buffer. From the radioactivity present in the solution (75 000 counts/ml.) and the specific activity of the IPhNHOH (104 counts/ $\mu\text{g.}$), the amount of this latter substance associated with the pigment