CCXV. THE DISTRIBUTION OF THE FATTY ACIDS IN HALIBUT INTESTINAL OIL, WITH A NOTE ON THE PRESENCE OF FREE FATTY ACIDS IN THE INTESTINES OF FISH

BY JOHN ARNOLD LOVERN AND RICHARD ALAN MORTON

*From the Torry Research Station, Aberdeen, and the Chemistry Department,
University of Liverpool*

(Received 29 August 1939)

In a previous communication [Lovern & Morton, 1939] the suggestion was advanced that in halibut (and other species of fish which have large deposits of vitamin A in their intestines) vitamin A assists in the process of fat absorption and transference through the intestinal wall. If this view is correct, one would expect to find vitamin A esterified with all the fatty acids concerned; the amounts of the various esters being in the same proportion as the individual fatty acids of the fat being handled.

With the object of obtaining information as to the mode of esterification of vitamin A in this material, halibut intestinal oil was prepared in quantity. The oil is a complex mixture [Lovern *et al.* 1939] containing fat, free fatty acids, phosphatides, vitamin A and its esters, cholesterol and its esters and other substances. These components were separated as well as possible and the fatty acid composition of each fraction determined.

EXPERIMENTAL

Halibut intestines were collected by three commercial line-fishing vessels during the summer of 1938, and were brought back to Aberdeen in tins, packed among ice and salt. The average duration of a trip was about 10 days. The intestines were stored at -30° at Aberdeen until October, when they were extracted. The extraction process, including thawing, mincing, dehydrating with sodium sulphate and extracting with ether, took about 1 week. These data may be of importance in view of the high free acid content of the oil although further information is desirable as to the free fatty acid content of the functioning intestine (*vide infra*).

33,233 g. of intestines gave 1689 g. of "oil" (5.1 %). The oil was dark brown in colour and showed 4200 blue units. This vitamin content is somewhat lower than had been hoped for, and is attributed to the great preponderance of intestines from relatively small fish. The crude extract was treated with acetone, giving 1353 g. of soluble "fat" (80 %) and 336 g. of insoluble "phosphatides" (20 %). The acetone-soluble portion now showed 6500 blue units. Evidently some inhibiting substance had been removed along with the phosphatide fraction. The acetone-soluble "fat" was sent to Liverpool where it was found to contain about 73 % F.F.A. (mol. wt. taken as 340), about 6 % vitamin A and 21.5 % non-sap. (containing 29-31 % vitamin A).

As the oil was to be subjected to molecular distillation, for which process such a high free acid content is undesirable, it was taken up in ether and washed with the calculated amount of aqueous KOH. The result of this treatment was to yield about 65 % of free acids and 35 % of "neutralized" oil. The latter, however, still contained a considerable amount of F.F.A. This difficulty in removing free acid except with a large excess of alkali has been encountered before [Lovern *et al.* 1939] and is presumably due to the instability of soap in aqueous solution at pH less than about 8. Excess of alkali was, however, avoided as it was undesirable to risk any saponification of vitamin esters.

The "neutralized" oil was of a solid consistency; when it was dissolved in hot acetone much sterol (cholesterol) crystallized out as the solution cooled, and was removed by filtration.

The purified material was now liquid. To free it further from traces of phosphatides and gummy matter of the type previously shown to be present in such oils [Lovern *et al.* 1939] it was dissolved in 2 l. of acetone and cooled to -30° . 36 g. of gummy matter separated out. A repetition of this process, but using 2 l. of light petroleum instead of acetone, caused the removal of a further 6 g. of insoluble matter (not gummy, but solid at room temperature). Of the resulting oil (290 g.) half was analysed directly and half used for molecular distillation, which was carried out for us at the research laboratories of The British Drug Houses, Ltd. Table I gives particulars of the various fractions obtained.

Table I: Details of molecular distillation

Fractions	Wt. g.	Temp. $^{\circ}$ C.	Vitamin A %	Non-sap. %
1	1.3	80	32.8	48
2	2.6	90	32.8	
3	8.3	100	36.8	
4	11.8	110	34.7	
5	11.0	120	30.7	
6	11.7	130	22.4	70
7	8.9	140	13.4	
8	5.5	150	8.7	
9	8.4	160	8.0	
10	6.1	170	10.7	
11	4.3	180	17.8	54
12	3.1	190	24.7	
13	7.3	200	27.3	
14	7.6	210	27.1	
15	7.4	220	23.4	
16	8.6	230	17.1	49
Residue	ca. 30	—	8.3	

Certain fractions were then combined for analysis of the constituent fatty acids, by the methods of ester distillation described previously [Guha *et al.* 1930; Lovern, 1934]. The groups so analysed were: fractions 1–5, fractions 7–10, fractions 11–16 and the residue. Fraction 6 was lost. The quantities of acids available were small and so the compositions arrived at must only be regarded as approximate. In addition to these samples, analyses were made of the free acids recovered in the alkali purification process, of the purified oil used for distillation and of the crude phosphatide fraction. The results of these analyses are given in Table II. The free acids contained 5 % of unsaponifiable matter (presumably entrained by emulsification) and the purified oil 48 %. The phosphatides, in addition to unsaponifiable matter, contained a considerable quantity of water-soluble impurities.

Table II. *Fatty acid analyses of various fractions*

Material	Saturated				Unsaturated				
	C ₁₄	C ₁₆	C ₁₈	C ₂₀	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C _{>22}
Phosphatides	3.3	26.5	9.3	0.2	5.0 (-2.3 H)	18.7 (-2.6 H)	15.6 (-4.0 H)	9.9 (-6.0 H)	11.5 (- ? H)
Free acids	1.1	13.6	1.5	—	6.4 (-2.5 H)	27.2 (-2.7 H)	22.6 (-6.4 H)	20.0 (-8.8 H)	7.6 (- ? H)
Neutralized oil	1.2	10.0	3.6	—	5.3 (-2.0 H)	28.2 (-2.8 H)	26.2 (-5.4 H)	16.0 (-7.3 H)	9.5 (- ? H)
Fractions 1-5	—	13	—	—	3	45 (-2.9 H)	27 (-6.0 H)	12 (-8.0 H)	—
„ 7-10	←	3	→	—	?	20 (-3.0 H)	46 (-5.3 H)	31 (-7.7 H)	?
„ 11-16	2	14	2	—	5	37 (-3.2 H)	23 (-6.0 H)	12 (-7.2 H)	5 (- ? H)
Residue	←	8	→	—	2	33 (-2.9 H)	29 (-5.2 H)	14 (-7.8 H)	14 (- ? H)

DISCUSSION

It can be seen from Table I that all the fractions contained appreciable quantities of vitamin A, but that its distribution was irregular, there being two groups of fractions with maximal contents of vitamin. The first group (say fractions 1-5) would contain predominantly free vitamin A and the second (say fractions 11-16) predominantly esterified vitamin A. At the same time there had evidently been considerable overlap in the fractions 7-10.

It can further be seen from Table I that all the fractions contained much unsaponifiable matter other than vitamin A. This would consist partly of cholesterol and partly of unidentified substances (including probably alcoholic substances which were free in the lower fractions and esterified in the later ones). The cholesterol in the lower fractions would also be free, whereas in the later ones it would be esterified. In fractions 7-10, the high content of non-vitamin unsaponifiable matter was probably largely free cholesterol.

Consideration of Table II reveals several points of significance. The neutralized oil had substantially the same fatty acid composition as the fatty acids washed out from it. Hence autolysis had been practically non-selective. On the other hand, the phosphatide fatty acids were appreciably different in that they contained more palmitic and stearic acids and less C₁₈, C₂₀ and C₂₂ unsaturated acids. This finding seems to suggest that these phosphatides do not play any direct part in the transport of absorbed fat in the intestine.

Turning to the distillation fractions, groups 1-5 and 7-10 both contained acids in different proportions from the original oil. However, in both these groups the acids must have been present largely as free acids and the differences would be expected owing to the molecular distillation process causing a greater preponderance of lower acids in the first fractions and of higher acids in the later fractions. If groups 1-5 and 7-10 were combined, the resultant fat would not be so different from the original fat. The acids of fractions 11-16 (the main vitamin ester fractions) were substantially the same as those of the original oil, as were those of the residue (also esterified matter, but with presumably a bigger proportion of sterol esters). The differences in fatty acid composition between fractions 11-16 and the residue are such as would be expected from the separating action of distillation.

It seems probable that the oil, as prepared in these experiments, contained little, if any, glyceride. Any triglyceride should have accumulated in the residue, but the amount of combined unsaponifiable matter was sufficient to account for all the fatty acid present. This also applies to the other fractions. There remains the large proportion of free acid removed by alkali. This was certainly not all present originally as esters of vitamin, sterol etc., and the probability is that some of it was present in the functioning gut as glycerides, which were completely hydrolysed during the rapid post-mortem autolytic processes.

There is also the possibility, however, of appreciable quantities of free fatty acid being present in the living intestine. Hilditch & Shorland [1937] discuss the occurrence of free fatty acid in animal liver. The freshest halibut intestinal tissues so far extracted by us gave oils with free fatty acid contents ranging from 5.3 to 21.0 % [Lovern & Morton, 1939] and experiments on absolutely fresh halibut intestine have so far been held up by lack of material. Meanwhile, tests made on other species of fish (cod, conger eel, lythe, ling and dogfish) have so far not yielded oils with less than 5.0 % F.F.A. [see note]. Oils extracted only a few minutes after the death of the fish frequently contained up to 30 % F.F.A.

Whether or not the main part of the fatty acids in the functioning gut (and present as free acids in this sample) was present as glycerides or as free acids, the fact remains that vitamin A (and also cholesterol) was esterified with fatty acids in approximately the same proportions as the acids were present in the total extract. Whilst not a proof that vitamin A assists in the processes of fat absorption in halibut, this evidence supports such a hypothesis. If vitamin A (or cholesterol) does participate in fat transference, a certain amount of free fatty acid must be present at certain stages of the process, although, depending on the rapidity of some of the reactions, the concentration may never need to rise to a high level. With present evidence, there seems no reason to doubt the presence (or potential presence) of adequate concentrations of free fatty acid in the living gut.

A NOTE ON THE OCCURRENCE OF FREE FATTY ACID IN THE INTESTINES OF FISH [J. A. L.]

As all samples of intestinal oils so far examined have contained appreciable (and often large) proportions of free fatty acid, experiments have been made to ascertain whether such acid is normally present in the functioning intestine, or is entirely a post-mortem product. It would have been highly desirable to include halibut amongst the species studied, and a visit to the Shetland Isles on the research vessel attached to the Torry Research Station was made for this purpose. Unfortunately, the presence of very large numbers of dogfish on the fishing grounds prevented the catching of any other species in these waters. The other fish studied were caught near Aberdeen.

In these experiments various procedures have been followed. In the first series, on cod, the fish were brought back ungutted by local yawls and were only just dead when landed. The fish were gutted as rapidly as possible, the stomachs removed from the intestines and the food residue washed out, either by forcing water through the gut, or by slitting it open and washing in water. The pyloric caeca were sometimes worked up separately but gave values identical with those of the intestines. Following the washing, the intestines were minced, desiccated with anhydrous sodium sulphate and extracted with ether. The whole batch of intestines was collected and treated together, involving a certain time lag for some of them.

In the next experiment, intestines were removed from live cod on the research vessel, washed, and at once placed in a large volume of ethyl alcohol. On return to Aberdeen they (and the alcohol) were worked up for fat and the fat tested for free acid.

Following on this, experiments were carried out wholly at sea. In the first of these, on dogfish, the intestines were removed from living fish, a number collected, slit open and washed, then minced, desiccated with sodium sulphate and extracted with ether. As it was becoming evident that great speed was of the utmost importance, the method was now altered. The intestine from one fish at a time was dealt with, instead of accumulating a number before washing and mincing. After mincing the intestine was at once mixed with a large volume of acetone, which was thought to be a more rapid method of dehydrating. The effect of this more rapid technique can be seen from Table III but it still appears that there must be some free acid in the living gut, although there may, of course, be differences in amount from species to species.

Table III. *Free fatty acid in the intestines of fish*

Species	Time between death and beginning of desiccation	Treatment	F.F.A. in oil %
Cod	2-3 hr.	Bulk washing and sodium sulphate	30.0
"	2 hr. 15 min.	" "	30.1
"	1 hr. 20 min.	" "	27.8
"	5-10 min.	Single washing and alcohol before mincing	23.2
Dogfish	5 min.	Bulk washing and sodium sulphate	23.6
"	10 min.	" "	18.3
"	1-2 min.	Single washing and acetone after mincing	7.4
Conger eel	1 min.	" "	5.0
"	1 min.	" "	6.1
Lythe	1 min.	" "	7.7
Ling	1 min.	" "	9.6

SUMMARY

A large quantity of halibut intestines was extracted with ether and the resulting oil was partially separated into its components, the processes used being alkali-washing, acetone precipitation and crystallization and molecular distillation. The products separated for examination included free fatty acids, phosphatides, vitamin and cholesterol esters and a "neutralized" oil.

The fatty acids recovered from these products were quantitatively analysed and it is shown that both vitamin A and cholesterol were esterified with all the various fatty acids present, the amounts of the different esters being proportional to the relative amounts of the acids present in the main fatty extract. This evidence is in harmony with the hypothesis that vitamin A assists in fat absorption processes.

The fatty acids of the phosphatide fraction were appreciably different from those of the main fat fraction and it seems unlikely that this phosphatide was directly concerned in fat absorption.

The probable presence of free fatty acid in the living intestine is indicated and the bearing of this on vitamin-assisted fat absorption is discussed.

Our thanks are due to the Medical Research Council for a grant in aid of the expenses of the work (R. A. M.) and to Dr F. H. Carr and Dr T. H. Mead, of The British Drug Houses, Ltd., for kindly carrying out the molecular distillation.

REFERENCES

- Guha, Hilditch & Lovern (1930). *Biochem. J.* **24**, 266.
Hilditch & Shorland (1937). *Biochem. J.* **31**, 1499.
Lovern (1934). *Biochem. J.* **28**, 394.
—— Mead & Morton (1939). *Biochem. J.* **33**, 325.
—— & Morton (1939). *Biochem. J.* **33**, 330.