XLI. THE DISTRIBUTION OF VITAMINS A AND A₂. III

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THE presence of large quantities of vitamin A in the intestines of certain fish was recently recorded [Lovern *et al.* 1937; Edisbury *et al.* 1938]. Its distribution in various parts of the alimentary tract was studied and, in the case of the halibut, the stomach was found to contain only traces of the vitamin whereas the "fatty" material extracted from the pyloric caeca, duodenum and ileum contained up to 70% of vitamin A esters.

The object of the present paper is to record the distribution of vitamin A and its congener vitamin A₂ in the different coats of the intestinal tube.

The problem has been approached in two ways:

- (a) Photomicrography of sections cut transversely.
- (b) Determination by spectroscopic methods of the vitamin content of the different layers of tissue after mechanical separation.

Histological work directed towards ascertaining the distribution of vitamin A demands a departure from normal technique because the solvents which might be used for clearing the sections are capable of dissolving out the vitamin, whilst the usual methods of fat-staining are unlikely to discriminate sharply between glycerides and vitamin A esters. We were therefore led to study the possibilities of ultraviolet photomicrography. Vitamin A absorbs radiations in the near ultraviolet region but transmits freely the longer wave-lengths of visible light. In principle, therefore, localized deposits of vitamin A should be detectable by the comparison of photographs taken by means of ultraviolet light and visible light respectively, using the same section and microscope setting for both experiments.

The ordinary microscope transmits ultraviolet rays in the region 340–400 m μ and although the peak of the vitamin A absorption (328 m μ) falls outside this range, the long-wave side of the broad absorption band includes a sufficient part of the spectrum transmitted.

The special glass used in ultraviolet fluorescence cabinets (Wood's glass or Chance's ultraviolet glass) transmits practically no visible light but is fairly transparent towards near ultraviolet rays. If, after taking a photomicrograph in the usual way with visible light, a sheet of such glass is placed between the light source and the microscope, a second photograph may be taken by means of ultraviolet light. The exposure, of course, will need to be much longer.

A simple test of the method is easily made. If three small drops of oil are placed adjacent to one another on a cover slip as follows:

- Drop 1. Olive oil (no vitamin A),
- Drop 2. Cod liver oil (low vitamin A content),
- Drop 3. Halibut liver oil (high vitamin A content),

and photographed in the ways suggested above it is found that the ordinary negative shows a similar degree of blackening for the three drops, whereas the negative obtained with ultraviolet radiation exhibits a decreasing blackening in the order 1, 2, 3.

EXPERIMENTAL

Preliminary experiments were carried out on the intestines of a large halibut. The fish was dissected at Aberdeen and the intestines were sent to Liverpool, in the frozen state, in a large Dewar flask. The material arrived still frozen and the flask could not be emptied until the contents had been thawed. Sections were cut by hand and mounted in a large vertical enlarging lantern. Photographs taken by projection using screened and unscreened light gave promising results. It was realized, however, that the intestines were not too well preserved and that autolysis had occurred, particularly on the inside. Further, the sections were not clear enough, the magnification was inadequate and the ultraviolet pictures were not in good focus.

In order to cope with these difficulties it was arranged to carry out further work at sea on absolutely fresh material. The only method of clearing the sections which seemed satisfactory was that of MacConaill [1937-8], namely, immersing the freshly cut sections in a homogeneous mixture of glucose and golden syrup. It was found that the ordinary microscope could readily be adapted for use. It was employed horizontally, the mirror being dispensed with. A "pointolite" bulb provided sufficient ultraviolet radiation for the purpose. These preliminary details having been settled, fishing for halibut was undertaken in Shetland waters in May, 1938. Adverse weather conditions unfortunately limited both the catch and the experimental work but enough was done to demonstrate the need for fresh material and for fairly thick sections.

A little later the intestines of a very large halibut became available at Aberdeen in good condition and, as a result of previous experience, much more satisfactory photographs were obtained. The best results were obtained with hand-cut slices, 1-2 mm. thick before clearing, though with such sections it was not possible to obtain a clear picture of the detailed structure. Many pairs of photographs were, however, obtained and in the best of these it was evident that the mucosal coats were much more opaque to ultraviolet light than to visible light, whilst the muscle layers showed little sign of such selective absorption.

The technique is capable of considerable refinement but in the present work it was thought better to approach the problem from a different angle.

Attempts were made, therefore, to isolate the mucosal tissue from the underlying muscle and skin, in order to extract the oils and determine the respective vitamin A potencies.

It was clear from the earliest trials that the inside coats were richer than the outside ones, both in respect of fat and vitamin, but, owing to autolysis, the results lacked quantitative significance. For work of this kind it is desirable to use the very fresh viscera of a large halibut. The thick gut was opened longitudinally and the various coats were separated. Samples of such materials were (a) placed in alcohol and sent from Aberdeen to Liverpool for examination, and (b) extracted in bulk at Aberdeen. In general, lower values were obtained with the alcohol-treated samples, suggesting some destruction of vitamin.

Terminology. A thorough description of the anatomy and histology of the alimentary tract in the king salmon is given by Greene [1912]. We have already found that salmon, like halibut, contain considerable quantities of vitamin A in the intestines, and, as no comparable work on halibut was available to us, we have adopted the terminology used by Greene and supported by detailed drawings of histological sections.

The different coats in the intestines and pyloric caeca, from the lumen to the outer wall, are described as follows:

- I. *Mucosa*: (a) Epithelium,
(b) *Tunica propria*,
(c) *Stratum compactum*,
(d) *Stratum granulosum*.
- II. *Muscularis*: (a) *Circularis*,
(b) *Longitudinalis*.
- III. *Serosa*: (a) *Serosa* proper,
(b) *Sub-serosa*.

The epithelial coat exhibits much folding but no villi and consists mainly of columnar epithelial cells, with mucous cells and wandering cells of the leucocyte type.

The *tunica propria* is made up of supporting connective tissue extending from the bases of the epithelial cells to the *stratum compactum*. It consists of white, fibrous tissue of the areolar type.

The *stratum compactum* is described by Greene as consisting of dense, non-fibrous connective tissue. The inner surface has projecting strands of the same type and the outer surface possesses two or three times as many of these processes, which form a network extending to the muscle coat. "Nowhere does there seem to be any direct opening or break through the thicker portion of the *stratum compactum* except where blood vessels penetrate this coat."

The *stratum granulosum* consists of granule cells enmeshed in the processes of the *stratum compactum*.

The muscle coats have been taken together in this work, as have those of the *serosa*, which consist of an outer "skin" and supporting connective tissue.

Two large halibut, one caught in May (A) and the other in September, 1938 (B), provided suitable material. In fishes generally the intestinal divisions are not so clearly differentiated as in mammals, but in this work a definite thinning of the intestinal wall was taken to mark the transition of duodenum to ileum.

After washing out food residues, a quantity of slimy matter, consisting of partly broken down mucosal epithelium, could be removed by very lightly scraping the inside of the gut. The wet weight of samples was taken before the addition of alcohol.

Mucosal epithelium. Sample 1a (18.76 g.) from the combined pyloric caeca and duodenum of fish A was covered with absolute alcohol and later ground in an agate mortar with sand and anhydrous sodium sulphate. The whole mass was thoroughly extracted with ether, giving 0.68 g. of fatty material (3.6%). Spectroscopic examination showed a vitamin A content of about 5.3%. The vitamin A content of the wet epithelium was therefore 0.19%, i.e. 1900 p.p.m. The ratio $E_{1\text{ cm.}}^{1\%}$ 617 m μ /693 m μ , representing the proportion of vitamin A to vitamin A₂, was 10.8.

Sample 1b. The whole of the slime collected from the pyloric caeca, duodenum and ileum of fish A was subjected to direct fat extraction. The yield was 2.2% of an oil containing 12% of vitamin A, corresponding to 2680 p.p.m. The ratio $E_{1\text{ cm.}}^{1\%}$ 617 m μ /693 m μ (613/51) was about 12.

Sample 1c. Slime (40.04 g.) from the combined intestines of fish B, after grinding with sand and extraction with alcohol followed by ether, gave 3.01 g. of clear oil (7.5%). The spectroscopic characteristics of the oil:

$$E_{1\text{ cm.}}^{1\%} \left\{ \begin{array}{lll} 693 \text{ m}\mu & \text{c.} & 14 \\ 617 & \text{,,} & 170 \\ 583 & \text{,,} & 92 \\ 325 & \text{,,} & 54 \text{ u.v. absorption} \end{array} \right\} \text{ SbCl}_3 \text{ colour test}$$

were quite normal and corresponded to about 3.45% of vitamin A, or 2590 p.p.m. in the wet slime.

Tunica propria. After removal of the epithelial slime, this coat could be removed by harder scraping.

Sample 2a. This experiment was designed to secure a sample of oil in a state as near as possible to the natural one. The alcohol-preserved tissue from the pyloric caeca and duodenum of fish A was ground in an agate mortar with sharp sand and extracted in the cold with ether, a little alcohol being added occasionally to replace that lost in the successive extractions. 16.18 g. (wet weight) of tissue yielded 1.4 g. of oil, but the extraction was probably not quantitative. It contained 30 % of vitamin A.

Sample 2b. Direct extraction of the main bulk of the *tunica propria* from the pyloric caeca and duodenum yielded about 11 % of oil of similar potency:

$$E_{1\text{ cm.}}^{1\%} \left\{ \begin{array}{ll} 693 \text{ m}\mu & 140 \\ 617 & \text{,,} \quad 1545 \\ 583 & \text{,,} \quad 825 \\ 325 & \text{,,} \quad 460; 445.5 \end{array} \right.$$

Taking these figures to correspond to some 30 % of vitamin A (c. 60 % vitamin A esters) the *tunica propria* contained some 33,000 p.p.m. The vitamin A₂ content is of the order 3000 p.p.m.

Sample 2c. The *tunica propria* scrapings (38 g.) from the ileum of fish A were preserved in alcohol and later, after grinding with sand and sodium sulphate, were exhaustively extracted with alcohol and ether, giving 11 % of pale, clear oil. The spectroscopic data:

$$E_{1\text{ cm.}}^{1\%} \left\{ \begin{array}{ll} 693 \text{ m}\mu & 93.5 \\ 617 & \text{,,} \quad 1650 \\ 583 & \text{,,} \quad 900 \\ 325 & \text{,,} \quad 485 \end{array} \right.$$

correspond to 32.5 % of vitamin A, or 35,750 p.p.m. in the tissue.

Sample 2d. 37.4 g. of the *tunica propria* from the combined intestines of fish B gave 3.85 g. of oil (10.3 %) having the following characteristics:

$$E_{1\text{ cm.}}^{1\%} \left\{ \begin{array}{ll} 693 \text{ m}\mu & 56 \\ 615 & \text{,,} \quad 900 \\ 583 & \text{,,} \quad 500 \\ 325 & \text{,,} \quad 275 \end{array} \right.$$

or 18 % vitamin A, corresponding to 18,500 p.p.m. in the tissue.

Stratum compactum and stratum granulosum. After scraping off the *tunica propria* it was found possible to detach the next coat with a sharp knife and to peel it off in long strips. This coat was taken to include both the *stratum compactum* and the enmeshed *stratum granulosum*.

Sample 3a. A small portion from the pyloric caeca and duodenum of fish A was preserved in alcohol and on extraction gave 3.28 % of oil, containing about 20 % of vitamin A:

$$E_{1\text{ cm.}}^{1\%} \left\{ \begin{array}{ll} 693 \text{ m}\mu & 70 \\ 617 & \text{,,} \quad 1000 \\ 583 & \text{,,} \quad 530 \end{array} \right.$$

corresponding to some 6600 p.p.m. in the tissue.

Sample 3*b*. The bulk sample corresponding to the above yielded 3.1 % of oil containing about 29 % vitamin A:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 122 \\ 617 & \text{,,} & 1446 \\ 583 & \text{,,} & 735 \end{cases}$$

or about 9000 p.p.m. in the tissue.

Sample 3*c*. The coat from the ileum of fish A gave 1.8 % of oil containing 18 % of vitamin:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 56 \\ 617 & \text{,,} & 903 \\ 583 & \text{,,} & 490 \end{cases}$$

or some 3240 p.p.m.

Sample 3*d*. The coat from the combined intestines of fish B yielded 4.8 % of oil containing 28 % vitamin A:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 70 \\ 617 & \text{,,} & 1400 \\ 583 & \text{,,} & 717 \\ 325 & \text{,,} & 432 \end{cases}$$

or 13,440 p.p.m. in the tissue.

Muscle coats. After peeling off the previous coat it was possible to separate with a knife the muscle from the *serosa*. The muscle tissue is not effectively disintegrated by grinding with sand and, as the fat content is low, is best decomposed by refluxing with alcoholic potash.

Sample 4*a*. Using a small sample from the pyloric caeca and duodenum of fish A, preserved in alcohol, the non-saponifiable extract (0.23 %) was tested:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 16 \\ 617 & \text{,,} & 220 \\ 583 & \text{,,} & 135 \end{cases}$$

The vitamin content of 4.5 % corresponds to *c.* 103 p.p.m. in the tissue.

Sample 4*b*. The main bulk of the above material was worked up at once without treatment with alcohol. About 1.1 % of oil was obtained containing 2 % of vitamin A, or 220 p.p.m. of vitamin A in the tissue.

Sample 4*c*. The muscle coats from the ileum of fish A yielded 1 % of oil containing *c.* 0.5 % of vitamin A. The estimated amount lay between 50 and 70 p.p.m.

Sample 4*d* consisted of muscle from the combined intestines of fish B and was decomposed with alcoholic potash. The non-saponifiable matter (0.33 %) gave the following data:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 13.4 \\ 617 & \text{,,} & 283 \\ 583 & \text{,,} & 147 \\ 325 & \text{,,} & 91 \end{cases}$$

corresponding to 5.65 % or 187 p.p.m.

Serosa. This was always carefully freed from adhering mesentery.

Sample 5*a* (12.4 g.), from the pyloric caeca and duodenum of fish A preserved in alcohol, was treated with alcoholic potash and gave 0.3 % of non-saponifiable matter:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 36 \\ 617 & \text{,,} & 470 \\ 583 & \text{,,} & 254 \end{cases}$$

containing *c.* 9.4 % vitamin A or 282 p.p.m. in the tissue.

Sample 5*b*. A bulk extraction of the above material gave 1.2% of oil containing 3.5% of vitamin, or 420 p.p.m.

Sample 5*c*, from the ileum of fish A, contained 1.55% of oil of which about 2% was vitamin A. This corresponds to 310 p.p.m.

Sample 5*d*, from the combined intestines of fish B, was saponified directly and yielded 0.48% of non-saponifiable matter containing 7% of vitamin A, or c. 339 p.p.m. in the tissue.

Mesentery. The mesenteric tissue adhering to the gut was also examined.

Sample 6*a*, composed of tissue from the entire intestines of fish A, was saponified after preservation in alcohol and gave 0.4% of non-saponifiable matter:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 49 \\ 617 & \text{,,} & 693 \\ 583 & \text{,,} & 404 \end{cases}$$

containing about 14% of vitamin A or c. 560 p.p.m. in the tissue.

Sample 6*b*. The corresponding bulk sample yielded 1.25% of oil with c. 5% of vitamin A, or 625 p.p.m. in the tissue.

Sample 6*c*. The mesentery from fish B yielded 2.2% of oil:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & \text{Feeble} \\ 617 & \text{,,} & 169 \\ 583 & \text{,,} & 90 \\ 328 & \text{,,} & 53 \end{cases}$$

containing 3.25% of vitamin, or 734 p.p.m. in the tissue.

The data are collected for reference in Tables I and II, the most probable values being given.

Table I. *Data for fish A*

Coat	Pyloric caeca and duodenum			Ileum		
	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.
Mucosal epithelium*	920	2.2	2,680	—	—	—
<i>Tunica propria</i>	235	11.0	33,000	38	11.0	35,750
<i>Stratum compactum</i>	164	3.1	9,000	61	1.8	3,240
<i>Stratum granulosum</i> }						
Muscle	105	1.1	220	32	1.0	70
<i>Serosa</i>	124	1.2	480	27	1.55	310
Mesentery*	419	1.25	625	—	—	—

* Material from the combined pyloric caeca, duodenum and ileum.

Table II. *Data for fish B*

Coat	Combined pyloric caeca, duodenum and ileum			
	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total vitamin A g.
Mucosal epithelium	468	7.5	2,590	1.21
<i>Tunica propria</i>	232	10.3	18,540	4.30
<i>Stratum compactum</i>	101	4.8	13,440	1.36
<i>Stratum granulosum</i> }				
Muscle	92	1.2	187	0.017
<i>Serosa</i>	67	1.6	339	0.022
Mesentery	197	2.2	734	0.144

It is important to know how much of the vitamin A located in the intestines exists as free alcohol and how much as ester. The only satisfactory approach to

this question is to determine the elimination curve of vitamin A in the process of molecular distillation. For this experiment to be worth while, a relatively large quantity of oil must be accumulated from fresh tissues in which autolysis has not occurred to any great extent. Our first attempts to inhibit autolysis (by storage and transport in tins surrounded by ice and salt) have not been successful and at this stage we shall confine ourselves to reporting the free fatty acid contents of various preparations:

Extract from mucosal epithelium	...	...	...	...	21.0 % F.F.A.
„ <i>tunica propria</i>	...	...	...	...	7.5 % „
„ <i>tunica propria</i> (ileum)	...	...	...	...	5.3 % „
„ <i>stratum compactum</i> and <i>stratum granulosum</i>	...	...	...	...	13.6 % „

It is desirable to obtain such data on preparations from absolutely fresh tissue in order to distinguish between post-mortem hydrolytic products and such free acid as may exist in the naturally functioning tissue.

DISCUSSION

The outstanding experimental finding is that the vitamin A occurring in the intestines of the halibut is sharply localized in certain coats. In fish A the *tunica propria* is far richer than the other mucosal coats, whilst the latter in turn are much richer in vitamin than the muscle and *serosa*. In fish B the separation is not quite so sharp, but here also the *tunica propria* is the tissue having the highest concentration of vitamin A.

In an earlier paper [Edisbury *et al.* 1938] the view was advanced that vitamin A might assist in absorption processes (especially of fat), in view of its localization in the absorptive portions of the intestine and its relative absence from the rest of the alimentary tract. The bearing of the present results on this hypothesis may now be considered.

The parallelism between the presence of vitamin and of fat in the various tissues, noted for the alimentary tract as a whole, can be seen to hold equally well in the various intestinal coats. Even when account is taken of the fact that the "fat" percentages recorded include vitamin A esters it is true that the glycerides and the vitamin A esters exhibit parallel distributions.

Greene [1913], again working with the king salmon, has shown that the *tunica propria* and the *stratum compactum* are of peculiar significance in the process of fat absorption in fish. The intestine of a fish is less complex than that of a mammal and fat absorption appears to proceed firstly by diffusion from the lumen into the mucosal epithelium. Possibly bile acids assist in making fatty acids water-soluble, as is the current conception in the case of mammals [Verzár & McDougall, 1936; Frazer, 1938], but absorption by villi and the lymph system does not seem to occur.

In the epithelium the fat appears as droplets which stain as neutral fat. The next stage in absorption is the temporary storage of this fat in the spaces of the *tunica propria*, which acts as a kind of temporary reservoir. The *stratum compactum* forms a definite and continuous boundary to this reservoir. "It is a continuous membrane with no discernible openings other than at the points where blood vessels enter. Any fat passing through the *stratum compactum* would have to pass through in solution or else be carried within in the lumen of the blood vessel. In either case no definite fat globules as such get by this membrane from the *tunica propria*."

Thus, the fat globules in the *tunica propria* must be dispersed and rendered diffusible through the walls of the blood vessels or else through the *stratum*

compactum itself. It is noteworthy that fat leaves the *tunica propria* relatively slowly. It is unlikely that bile acids are present in the *tunica propria* and that fatty acids are rendered water-soluble again for diffusion. It is, however, conceivable that the large quantities of vitamin A present in these tissues may make possible the diffusion of fatty acid either through the blood vessel wall or through the *stratum compactum*.

There is evidence that vitamin A may form a complex with protein, as do certain carotenoids (e.g. astacene). Such a complex exists in visual purple and probably also in liver [Edisbury *et al.* 1938]. Such complexes may well form an integral part of the cell structure of these mucosal tissues and the vitamin A, by repeated esterification and hydrolysis, could thus assist in the transport of fatty acid.

With the evidence at present available it would be unwise to speculate further but should later evidence support this hypothesis there will still be many points to be cleared up. It will be necessary to know whether the fat leaves the *tunica propria* by the blood vessels or directly through the *stratum compactum*. The presence of large quantities of vitamin in the latter coat itself (as well as of appreciable, if much smaller amounts in the muscle, *serosa* and mesentery) suggests that at least a part of the fat may diffuse directly through the *stratum compactum*. It would also be of interest to know whether fat is transported to the liver as vitamin esters or as reformed glycerides. In those fish which do not have large quantities of vitamin A in the intestines, some other substance (e.g. cholesterol or phosphatide) may fulfil a similar function. The hypothesis we have tentatively advanced accounts for the presence of very large amounts of vitamin A in the alimentary tract of certain fishes and also suggests one way in which fat may be transported through the intestinal wall.

SUMMARY

The large vitamin A deposits in the intestines of many species of fish are shown by two methods, applied to halibut, to be sharply localized in the *mucosa* and in particular in the *tunica propria*.

The distribution of fat in the intestine runs parallel with that of vitamin A.

Absorption of fat by fish differs from that obtaining in mammals, proceeding directly through the mucosal epithelium and not by a lymphatic system. The existence of a dense, continuous layer of connective tissue (*stratum compactum*) forms a barrier to fat transport as droplets, which accumulate in the adjacent *tunica propria*.

The hypothesis is advanced that vitamin A esters, in combination with protein, may assist in the dispersion of these droplets and in the removal of fat from the *tunica propria*.

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