XLII. HALIBUT INTESTINAL OIL

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HALIBUT intestines have been found to autolyse very rapidly. The fat present in any but the freshest material may therefore have undergone considerable hydrolysis. Oil from stale tissue contains more free acid than that from comparatively fresh material, but so far neutral oils have not been obtained. Hitherto, it has not been possible to carry out extraction sooner than 2 or 3 days after the death of the fish. Preservation in ice and salt certainly slowed down autolysis, but special experiments will be necessary before the free fatty acid content of the functioning intestine can be determined.

A beginning has been made towards ascertaining, (a) the proportions of free and combined vitamin A, (b) the nature of the fatty acids present in the vitamin esters, (c) the relative speeds of enzymic hydrolysis of vitamin esters and glycerides and, (d) the extent to which autolysis results in contamination of the "fatty" extract.

The method of molecular distillation applied to oils [Hickman, 1937, 1] permits the approximate determination of free and combined vitamin A provided the oil is not excessively acid and some 100 ml. are available. The use of constant yield oil as solvent [Hickman, 1937, 2] extends the method to small quantities of solute.

In 1937 a sample of oil extracted from the ileum of a relatively fresh halibut was subjected to analytical distillation using Hickman's constant yield oil. 0.5 g. was diluted with 75 ml. constant yield oil and 75 ml. of residue oil from a previous molecular distillation. Fractions were collected (10° temperature and 18 min. time intervals) and vitamin yields were determined by assaying the whole series. About 4% of the total vitamin A occurred in the fractions collected over the range 90–140° and 96% was volatile at higher temperatures. The elimination curve over the range 150–240° was tall and narrow with a maximum near 210°. The results indicate that approximately 95% of the vitamin occurred as esters and that the acids which were combined in the greater part of these did not differ much in molecular weight.

An attempt was made in the autumn of 1937 to collect sufficient halibut intestines at Aberdeen for a distillation of the undiluted intestinal oil. Unfortunately, the material as received from the fishing vessels had already undergone considerable autolysis, so that the oil obtained (150 g.) had a high acid value (92.4).

A trial distillation gave the results shown in Table I.

About 20–30% of the vitamin existed in the free state and the free acids accompanied the vitamin in the distillates. The high acid content facilitated cyclization and made it difficult to collect the distillates without overheating the condenser.

Accordingly, the remainder of the oil was washed with aqueous K_2CO_3 , the oil being first dissolved in ether and the amount of K_2CO_3 calculated from the

Table I. *Molecular distillation of 53 g. halibut intestinal oil. B.V. 8300.*
Acid value 92.4

Fraction	Temp. °C.	Wt. (g.)	B.V.	Acid value	Colour test		u.v. absorption
					$E_{1\text{ cm.}}^{1\%}$ 620 m μ	$E_{1\text{ cm.}}^{1\%}$ 580 m μ	$E_{1\text{ cm.}}^{1\%}$ 325 m μ
1	100-120	8.2	6400	156	454	230	113*
2	130-140	12.6	6200	129	400	210	98†
Residue	—	32.2	9000	47	715	365	204‡

* Fine structure due to cyclization very marked.

† Fine structure less marked.

‡ No fine structure.

acid value. The acid value was reduced from 92.4 to 37 and although a lower acidity would have been desirable the oil was distilled at this stage lest further washing with alkali should change the proportion of free to esterified vitamin.

85 g. of washed oil mixed with 15 g. of residual oil were distilled and 15 fractions were collected:

 Table II. *Molecular distillation of washed halibut intestinal oil. B.V. 17,000.*
Acid value 37

Fraction	Temp. °C.	Wt. g.	Carr. Price B.V.	u.v. absorption	Colour test, $E_{1\text{ cm.}}^{1\%}$			Non-sap. %	Recovered acids	
				$E_{1\text{ cm.}}^{1\%}$ 325 m μ	620 m μ	580 m μ	693 m μ		%	Mol. wt.
1	90	2.8	17,300	200*	875	480	<50	—	—	—
2	100	4.4	17,300	280*	1185	616	<47	—	—	—
3	110	4.2	17,700	188†	880	490	<42	—	—	—
4	120	5.0	14,250	255†	1100	577	<58	—	—	—
5	130	4.7	10,500	180†	725	400	<43	—	—	—
6	140	5.2	6,380	112.5	337	172	—	53.5	46.9	316
7	150	3.6	6,120	116.6	306	160	—	65.6	37.45	315
8	160-70	7.6	15,676	300	1140	627	—	—	—	—
9	180	3.1	29,000	500	1900	986	93	—	—	—
10	190	2.4	33,300	530	1880	1000	97	55.5	32.8	276
11	200	4.1	29,000	590	2080	1075	113	51.1	45.6	280
12	210	4.7	33,300	550	2006	1060	125	50.7	45.9	278
13	220	4.3	31,500	482	1875	990	93	48.3	51.25	284
14	230-40	9.4	17,300	440	1200	676	58	48.0	52.3	291
Residue	—	34.5	2,900	90‡	211	156	13.4	28.7	68.05	301

* Fine structure marked.

† Feeble fine structure.

‡ Anomalous absorption.

From Table II it would seem that about 26% of the total vitamin A in this sample of oil occurred as free alcohol. The ratio $E_{1\text{ cm.}}^{1\%}$ 325 m μ /620 m μ (ultra-violet absorption to absorption for the main band in the colour test) is curiously variable. For the richest preparations of vitamin A prepared by saponification but not subjected to molecular distillation the ratio is about 0.32 and for the purest distillates of the free vitamin perhaps 0.30. In the richest undistilled esters which we have prepared from intestinal oils (60-70% vitamin esters) this ratio varies from 0.29 to 0.32.

Now the cyclization product of vitamin A is itself chromogenic towards SbCl_3 and it is striking that for fractions 1-5 the ratio $E_{1\text{ cm.}}^{1\%}$ 325 m μ /620 m μ varies from 0.21 to 0.25. The cyclized product may well account for the main discrepancies. Fractions 6 and 7 are the first to show no spectroscopic evidence of cyclized vitamin A, and the ratio jumps to 0.33 and 0.38. Fractions 8 and 9 show an unusually low ratio for vitamin esters, whilst Fractions 10, 11 and 12

are much more nearly normal. It is interesting that much of the non-saponifiable other than vitamin A distils mainly between 140° and 180°. The discrepancies may indicate variable proportions of stereoisomerides of vitamin A in the different fractions, but other explanations are equally plausible.

A similar distillation carried out on a fish liver oil affords a useful comparison:

Table III. *Molecular distillation of a sturgeon liver oil**

Frac- tion	Temp. ° C.	Vitamin A %	Ratio $E_{1\text{ cm.}}^{1\%}$ 325 m μ 620 m μ	Vitamin esters %	Non- sap. %	Recovered acids		Cyclized vitamin A %	Non-sap. other than vitamin A and spurious A %
						%	Mol. wt.		
Original oil		7.9	0.305	—	24.7	69.5	278.5	None	17
1 (not retained)									
2	100	11.2	0.26	—	27.4	65.0	266	13-14	c. 16
3	110	15.1	0.26	—	34.7	57.7	272	<14	c. 19.6
4	120	15.8	0.26	—	38.5	56.0	273	<10	c. 23
5	130	16.4	0.30	—	43.5	52.0	277	Very little	27
6	140	14.3	0.29	—	49.8	44.8	278	None	35.4
7	150	11.5	0.31	21	55.0	38.7	287	"	43.5
8	160	9.7	0.30	19	67.7	33.1	293	"	58
9	170	11.8	0.30	23	64.1	33.8	297	"	52.2
10	180	21.4	0.30	40	64.5	37.2	280	"	43.1
11	190	29.5	0.28	57	56.1	44.7	276	"	27
12	200	31.6	0.29	60	51.4	50.8	273	"	20
13	210	29.0	0.28	55	47.8	51.3	270	"	18.8
14	220	29.4	0.29	42	44.2	57.2	279	"	20.9
15	230	15.9	0.31	30	39.5	61.2	278	"	23.6
16	240	9.1	0.30	17.5	32.9	66.4	277	"	23.8
17	250	4.7	0.31	c. 9	25.5	72.3	272	"	20.8
Residue		Very low							

* Part of the vitamin A esters had probably been hydrolysed in the process of extraction.

These results indicate a very constant ratio between ultraviolet absorption and colour test intensities once the free acids and cyclized vitamin A have been eliminated. They also illustrate very clearly the limitations imposed by the potency of the original oil and its high content of sterol and sterol esters. Fractions containing 60 % of vitamin esters are obtained but the overlap which exists between the elimination curves of vitamin esters and sterol esters sets a limit to the efficacy of the process. The chances of preparing richer ester fractions would be greatly enhanced if sufficient neutral intestinal oil of high potency (60-70 % vitamin esters as already described) could be obtained. Unfortunately, the bulk of the halibut recently accessible to us has consisted mainly of smaller fish and it has been impossible to obtain fresh intestines. The next step will be to arrange for better preservation on the fishing vessels.

In the meantime experiments have been carried out on the material available.

Table IV. *Fractionation of oil from autolysed intestines*

Fraction	Non-sap. %	Recovered acids		Colour test, $E_{1\text{ cm.}}^{1\%}$			Ultraviolet, $E_{1\text{ cm.}}^{1\%}$	
		%	Mol. wt.	693 m μ	620 m μ	580 m μ	325 m μ	261 m μ
1	21.7	61.7	292	31.5	525	283	158.3	—
2	—	—	—	6.93	110	67.5	38.2	52
3	22.3	65.1	297	29.6	490	286	158	—
4	22.1	70.2	297	32.9	537	302	158.3	—
5	—	—	—	6	27.5	19	11.7	26.6

The following diagram illustrates the scope of the work:

Attention may be called to the following points:

(1) The original extract contained about 25 % of material which was not glyceride, nor vitamin ester, nor even mainly sterol ester.

(2) Even after the acetone treatment further small quantities of material containing relatively little vitamin can be eliminated.

(3) The process, however, is still far from complete. Thus, the "non-sap." and the acids recovered show a large deficit from 100 % in Fraction 1. This is diminished in Fractions 3 and 4 (Table IV).

(4) The gummy residues exhibit a new and well defined absorption band at $261\text{ m}\mu$ (Fractions 2 and 5) which is not shown and may be masked in the other fractions. The new absorbing material remains in the aqueous liquor after acidifying the soaps with H_2SO_4 and extracting with ether. It can be extracted from the evaporated residue by means of alcohol but is accompanied by other products.

The whole problem of the occurrence of substances other than glycerides, vitamin esters and sterol esters in "oils" is under investigation and it seems wise to defer the more detailed discussion of the problems raised above. The molecular distillations provide data for constructing elimination curves for vitamin A and vitamin A_2 [cf. Hickman, 1937, 1, 2].

Fig. 1 shows $E_{1\text{ cm}}^{1\%}$ plotted against temperature for the ultraviolet absorption. The elimination curve shows two maxima, one corresponding with the free vitamin and the other with the esterified vitamin. Fig. 2 shows similar curves for the 693, 620 and $583\text{ m}\mu$ bands in the colour test. It is true that the oil contains much more vitamin A than vitamin A_2 and that the proportions are not altogether favourable for a separation. Nevertheless, the similarity between the elimination curves for the two vitamins is very striking and is a sign that the

Fig. 1.

Fig. 2.

Fig. 1. Halibut intestinal oil. The potency of each molecular distillate as measured by u.v. absorption has been plotted against the temperature of distillation. The smaller maxima are due to free vitamin A and the cyclization product, the large maximum to esterified vitamin A.

Fig. 2. SbCl_5 colour test data for halibut intestinal oil distillates are plotted against temperature. The 693 $\text{m}\mu$ maximum concerns vitamin A_2 , the 620 $\text{m}\mu$ and 583 $\text{m}\mu$ maxima vitamin A.

Fig. 3. The non-saponifiable fractions and the acids recovered from the soaps of halibut intestinal oil distillates were obtained and vitamin A estimated spectrographically. — % non-sap. other than vitamin A. --- % recovered acids.

Fig. 4.

Fig. 5.

Figs. 4 and 5 are comparable with Figs. 1 and 2 and apply to sturgeon liver oil distillates. The dotted portions indicate the course of the curve if no cyclization had occurred.

structure of vitamin A₂ cannot be very different from that of vitamin A. The similarity between the 620 mμ and 583 mμ bands, which both appear to be due to vitamin A, persists after molecular distillation. The elimination curves indicate that both bands are mainly if not entirely due to vitamin A. The halibut intestinal oil which was distilled contained more of the cyclized vitamin than the liver oil used. Cyclization, which is presumably caused by the heating process, is greatly accelerated in the presence of free fatty acid. The elimination curves show quite clearly the extent to which free vitamin A and its cyclization product can be separated. Most of these findings confirm the work of Hickman and his associates. The failure to separate vitamin A from vitamin A₂ is surprising. The work should be repeated on the liver oil from freshwater fish in which vitamin A₂ predominates over vitamin A, and the conditions may prove favourable to the detection of the small differences in the elimination curves if the two vitamins are homologous.

SUMMARY

1. Vitamin A in halibut intestinal oil exists almost entirely (at least 95 %) as esters.
2. Halibut intestines autolyse freely so that the extractable "oil" is a mixture of free fatty acids, free cholesterol and free vitamin A with their esters. The oil is also contaminated with phosphatides and other products. Among these is a water-soluble material showing selective absorption $\lambda_{\max}$ 261 mμ. The study of this substance is being pursued.
3. It is difficult to remove free acids from the autolysed material and cyclization occurs to an appreciable extent on distillation.
4. The role of impurities in affecting the physical assay of vitamin A in rich materials is illustrated and, to some extent, accounted for.
5. The elimination curve of vitamin A₂ appears to resemble that of vitamin A very closely.

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