CLXVII. A PHYSICO-CHEMICAL AND BIOCHEMICAL STUDY OF VITAMIN A₂

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(Received 29 April 1938)

THE literature concerning the existence of different substances in fish liver oils which give a colour with antimony trichloride has been reviewed recently in detail in this *Journal* by Edisbury *et al.* [1938] and by Gillam *et al.* [1938]. These investigators have established the existence in freshwater fish liver oils of a higher homologue of vitamin A, the "693 m μ chromogen" or "vitamin A₂".

Full details are given below of experiments reported briefly elsewhere [Lederer & Rathmann, 1938], which show that vitamin A₂ gives in the presence of SbCl₃ a second absorption band not previously observed. This band is of importance for the calculation of the relative quantities of vitamins A₁ and A₂ in any given material. Our paper deals further with data obtained from oils of freshwater fish from different parts of Europe as well as from oils or fats from other animals. Experiments dealing with the absorption of vitamin A₂ from the intestine and its accumulation in the liver of the rat and the frog are included.

EXPERIMENTAL

Unsaponifiable fractions were prepared by the usual methods [cf. Gillam *et al.* 1938]. The livers and intestines were hydrolysed with aqueous or alcoholic NaOH (5–10 %) and extracted with ether; the resulting concentrate was saponified. As the alkaline hydrolysis was always accompanied by a partial saponification of the oil, no values for the oil content were available. In Tables I and III are given the weights of the unsaponifiable fractions calculated for 100 g. of fresh organ. All measurements were made on the unsaponifiable fractions. For the determination of absorption coefficients a Hilger-Nutting visual spectrophotometer was used. All readings were repeated at least twice by one of us and controlled by the other. In Tables I and III it was thought sufficient to give the data for $E_{1\text{ cm.}}^{1\%}$ 695 m μ , as the corresponding values for 650 and 620 m μ can be easily calculated from the ratios $E\ 695\text{ m}\mu/E\ 650\text{ m}\mu$ and $E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$ given in the tables.

The inhibitor (Table III, no. 18) was prepared as follows. After saponification of the pike liver oil (Table III, no. 17) the ether-extracted soap was acidified with 5 % H₂SO₄ and extracted three times with ether. The solvent was dried with Na₂SO₄ and distilled off. The colourless residue crystallized incompletely. 20 mg. dissolved in 0.1 ml. CHCl₃ were added to 0.2 ml. of a solution of the pike unsaponifiable no. 17 of Table III.

The 650 m μ band of vitamin A₂

There is evidence that vitamin A₂ is a higher homologue of vitamin A₁, having six conjugated double bonds, a ring of β -ionone and one hydroxyl group [Gillam *et al.* 1938]. It would be surprising if this substance had only one absorption band in presence of SbCl₃, seeing that vitamin A₁ and other natural

carotenoids with hydroxyl groups (zeaxanthin, lutein, violaxanthin) show two bands [Castle *et al.* 1934; Gillam, 1935; Euler & Karrer, 1932; Euler *et al.* 1932]. The hydrocarbons (α - and β -carotene, lycopene and *isocarotene*) show only one band [Karrer & Walker, 1933; Gillam, 1935]. This is also true for synthetic polyenes. Thus the tetradecapentaen-ol of Kuhn & Grundmann [1938] gives two bands with SbCl₃, whereas the tetradecaheptaene prepared from the former by splitting off water gives only one band. The *apocarotinols* of Euler *et al.* [1938] seem to be exceptions to these rules.

We have found that vitamin A₂ has indeed a second band (situated at 645–650 m μ) but this band is usually masked, just as is the second band of vitamin A₁ at 580 m μ ; it can be made visible by adding an inhibitor, in the same way as can the second band of vitamin A₁ [Emmerie *et al.* 1931; Eekelen *et al.* 1931; Castle *et al.* 1934].

Table III shows the intensity of absorption at 695 m μ of concentrates from fish liver and intestines. For those which have a relative absorption intensity $E_{695\text{ m}\mu}/E_{620\text{ m}\mu}$ higher than 2, the intensity of absorption at 650 m μ is about two-thirds of that at 695 m μ , the quotient $E_{695\text{ m}\mu}/E_{650\text{ m}\mu}$ varying from 1.38 to 1.80. This variation is probably not significant, so that it may be concluded that both bands are due to the same substance (see Fig. 1).

The 650 m μ band can be clearly seen on adding to the solution of the unsaponifiable in chloroform the fraction of fatty acids obtained after saponification of freshwater fish liver oils. The absorption at 695 m μ is much diminished, while absorption at 650 and at 620 m μ remains almost unchanged (Table III, nos. 17, 18).

The band at 640 or 645 m μ observed in some natural oils [Heilbron *et al.* 1931; Morton, 1932; Edisbury *et al.* 1938; Gillam *et al.* 1938] seems to correspond to the 645–650 m μ band of vitamin A₂. After saponification of these oils the inhibitor is removed and the 693 m μ band reaches its full strength and masks the second band (cf. Gillam *et al.* [1938], oil of *Huso huso*). The same band is seen in partially saponified oils together with the 693 m μ band and is then located at 645 m μ . On the other hand, the 645 m μ band is sometimes observed in partially decomposed vitamin A₂ concentrates [Gillam *et al.* 1938]; its appearance is due in these cases also to a diminution of the intensity of the 693 m μ band.

In some natural oils full inhibition of the 693 and 650 m μ bands occurs and such oils show only a weak band at 610 m μ (Table III, no. 2). After complete saponification the 610 m μ band disappears, overshadowed by the strong 693 m μ band.

Quantitative determination of vitamins A₁ and A₂

Until quite recently the determination of the relative quantities of vitamins A₁ and A₂ present in an oil was based on two assumptions: (1) that $E_{1\text{ cm.}}^{1\%}$ 693 m μ for vitamin A₂ has the same value as $E_{1\text{ cm.}}^{1\%}$ 620 m μ for vitamin A₁, i.e. about 5000 [Edisbury *et al.* 1938; Gillam *et al.* 1938]; (2) that the absorption at 620 m μ is due solely to vitamin A₁ and that at 693 m μ solely to vitamin A₂.

The first assumption seems to be fairly correct as shown by the following consideration. Vitamin A₂ having one conjugated double bond more than vitamin A₁, its molecular extinction coefficient at 345 m μ should be somewhat higher (about 20 %) than that of vitamin A₁ at 328 m μ . Since, on the other hand, a 1% solution of vitamin A₂ contains about 10% fewer molecules than a 1% solution of vitamin A₁, the $E_{1\text{ cm.}}^{1\%}$ values should be about the same for both in the ultraviolet. Moreover, solutions of vitamins A₁ and A₂ having equal extinction at 328 and 345 m μ respectively show roughly the same $E_{1\text{ cm.}}^{1\%}$ at 620

and 693 m μ , as illustrated by the following figures for vitamin A₂ taken from Gillam *et al.* [1938].

Vitamin A ₂	$E_{1\text{ cm.}}^{1\%}$ 344 m μ = 850	$E_{1\text{ cm.}}^{1\%}$ 697 m μ = 2600
Vitamin A ₁	$E_{1\text{ cm.}}^{1\%}$ 328 m μ = 850	$E_{1\text{ cm.}}^{1\%}$ 620 m μ = 2650 (calculated on the basis of the usual E values for A ₁)

The second assumption is unjustified in so far as it concerns absorption at 620 m μ , for this has been shown to be only partly due to vitamin A₁ [Lederer & Rathmann, 1938].

On measuring the absorption intensities at 620 and 580 m μ of vitamin A₁ concentrates as far as possible free from vitamin A₂ (E 695 m μ / E 620 m μ < 0.17) it was found that E 650 m μ / E 620 m μ for these preparations was approximately 0.25–0.30 (Table I, nos. 2–5). A value of 0.35 was obtained by evaluation of the absorption curve of vitamin A from frog retina measured by Wald [1936].

Table I. Vitamin A₁ from marine fish and land animals

No.	Species	Organ	Wt. g.	Nonsap. mg. per 100 g. organ	$E_{1\text{ cm.}}^{1\%}$ 695 m μ	E 650 m μ / E 620 m μ	E 695 m μ / E 620 m μ
1	<i>Gadus merlangus</i>	8 livers	160	400	2.5	0.61	0.21
2	Halibut (<i>Hippoglossus hippoglossus</i>)	—*	—	—	40.0	0.29	0.17
3	<i>Ishinagi sterelepis</i>	—*	—	—	112.0	0.26	0.15
4	Sheep (<i>Ovis aries</i>)	1 liver	1080	430	1.5	0.27	0.03
5	Chick (<i>Gallus domesticus</i>)	1 liver	27	410	1.2	0.35	0.08
6	Frog (<i>Rana esculenta</i>)	14 livers	11	600	8.2	0.52	0.27

* Oil investigated.

If one supposes that the absorption curve of pure vitamin A₂ falls off at the same rate from 650 to 620 m μ as the absorption curve of vitamin A₁ from 620 to 650 m μ , one reaches the conclusion that E 620 m μ / E 650 m μ for pure vitamin A₂ must also be about 0.25–0.30.

The relative intensities of absorption of our richest vitamin A₂ concentrates were: E 695 m μ / E 650 m μ / E 620 m μ = 3/1.8/1. Now if E 620 m μ / E 650 m μ for vitamin A₂ is 0.25, then 0.45 (i.e. 1.8 $\times$ 0.25) or nearly half of the absorption at 620 m μ is due to vitamin A₂.

On the basis of these figures we suppose that E 695 m μ / E 620 m μ for pure vitamin A₂ will be between 6 and 8. (The values previously reported by Lederer & Rathmann [1938] were too low.) Taking 7 as an average value, the relative quantities of vitamins A₁ and A₂ may be calculated by the formula

$$\text{vitamin A}_2/\text{vitamin A}_1 = E \text{ 695 m}\mu / \left(E \text{ 620 m}\mu - \frac{E \text{ 695 m}\mu}{7} \right);$$

Table II gives the relative quantities of the two vitamins calculated directly from the ratio E 695 m μ / E 620 m μ .

Fig. 1 shows the absorption curve of a vitamin A₂ concentrate (E 695 m μ / E 620 m μ = 2.5) in presence of antimony trichloride. The crosses indicate average values from measurements on 10 different unsaponifiable fractions taken from different species of fish with ratios varying from 2.3 to 2.7. The measured curve can be shown to result from 4 separate hypothetical absorption curves with maxima at 693, 650, 620 and 580 m μ respectively (light lines). The figure demonstrates clearly the overlapping of the absorption bands of vitamins A₁ and A₂.

Table II. *Relative quantities of vitamins A₁ and A₂*

$E\ 695\text{m}\mu$ $E\ 620\text{m}\mu$	Vitamin A ₂ Vitamin A ₁	% vitamin A ₂	% vitamin A ₁
0.5	0.54	35	65
1.0	1.16	54	46
1.5	1.88	65	35
2.0	2.80	74	26
2.5	3.90	80	20
3.0	5.30	84	16
4.0	9.30	91	9
5.0	17.00	95	5
6.0	40.00	98	2
7.0	∞	100	0

Of course these figures have to be considered as a first approximation and will have to be revised as soon as more exact values are available. They are independent of the chemical structure of vitamin A₂, which seems to need further investigation, as pointed out by Euler *et al.* [1938].

Fig. 1. Absorption spectrum of vitamin A₂ concentrates ($E\ 695\text{m}\mu/E\ 620\text{m}\mu=2.5$) in presence of SbCl_3 ($\times-\times-\times$). The light lines show the four hypothetical bands with maxima at 695, 650, 620, 580 $\text{m}\mu$.

Our results are of importance in considering the biological assay of vitamin A₂ reported by Gillam *et al.* [1938]. A final conclusion as to the activity of vitamin A₂ was difficult to draw at that time as the assayed preparation was thought to contain only two parts of vitamin A₂ to one part of vitamin A₁ ($E\ 695\text{m}\mu/E\ 620\text{m}\mu=2$). Since the activity of this concentrate was somewhat less than that of a halibut oil fed in equal doses, it could have been argued that the activity was due solely to the vitamin A₁ present. As the value of 2 for the ratio $E\ 695\text{m}\mu/E\ 620\text{m}\mu$ had been measured more than 2 weeks after the preparation of the oil, we suppose that the real value for the fresh oil was at least 2.3, as for all *Lucioperca lucioperca* concentrates measured immediately after preparation (*vide infra*). In the light of the conclusions reached above the assayed preparation contained only about 20–25 % vitamin A₁. This seems to prove that the 693 $\text{m}\mu$ chromogen deserves the name vitamin A₂ (cf. the critical remarks

of Euler *et al.* [1938]). Exact assays of our preparations have now been begun by Dr Th. Moore to determine more precisely the biological activity of vitamin A₂.

Ultraviolet absorption spectra of vitamin A₂ concentrates

Gillam *et al.* [1938] as well as Edisbury *et al.* [1938] stated that vitamin A₂ concentrates have two absorption bands in the ultraviolet, one at 345m μ and the other at 280m μ . The former authors are inclined to think that both bands are due to vitamin A₂ because of the more or less constant relative intensities of the two bands in concentrates from different sources, whereas Edisbury *et al.* seem to have separated the two bands by treating vitamin A₂ concentrates with 83 % alcohol. The insoluble fraction is shown to have the band at 280m μ whereas the soluble fraction, representing vitamin A₂, has a band at 345m μ . Edisbury *et al.* [1938] suppose that the insoluble fraction is identical with that isolated before by Pritchard *et al.* [1937] from a mammalian concentrate in the same way and that it represents a lower (C₁₈) homologue of vitamin A₁.

These conclusions seem rather improbable, because it is difficult to believe that the C₁₈ homologue of vitamin A₁ should be quite insoluble in 83 % ethyl alcohol in which vitamin A₁ itself is easily soluble. Moreover, in our experiments we were unable to confirm the findings of Edisbury *et al.* concerning the separation of the two bands in the ultraviolet.

2.2 g. of a concentrate from 50 pike livers (Table III, no. 14) were dissolved in methanol and freed from sterols (1.6 g.) by freezing at -12°. The filtrate was then evaporated to dryness *in vacuo* and dissolved in 83 % ethyl alcohol; after standing for an hour at -12° an amorphous precipitate settled out which was filtered off. The filtrate was evaporated to dryness *in vacuo* and the residue was completely soluble in 77 % ethyl alcohol at room temperature. After standing for an hour at -12° a second (oily) precipitate could be separated. The filtrate contained practically the whole of the vitamin A₂ (320 mg. with $E_{1\text{ cm.}}^{1\%}$ 695m μ = 1060) and showed two bands in the ultraviolet (345 and 280m μ) with relative intensities practically the same as those of the original material, as far as could be judged from the photographs taken with an Yvon and Jobin spectrograph of small dispersion.

It seems therefore that both bands in the ultraviolet belong to vitamin A₂. It may be mentioned here that Gillam [1935] found a 268–280m μ band in the ultraviolet part of the absorption spectrum of all the carotenoids which he investigated.

The alcohol treatment resulted in a slight increase of the ratio $E_{695\text{ m}\mu}/E_{620\text{ m}\mu}$, i.e. from 2.65 for the original material (Table III, no. 14) to 3.0 for the fraction soluble in 77 % ethyl alcohol. The insoluble fraction had a ratio of 2.0.

Colour reactions of vitamins A₁ and A₂

In the hope of finding some reagent which would enable one to distinguish more specifically between the two vitamins than is possible with SbCl₃, a number of other substances were studied. Rosenheim & Drummond [1925] reported that trichloroacetic acid, dimethyl sulphate, perchloric acid, benzoyl chloride, acetyl chloride and arsenic trichloride gave blue solutions in the presence of vitamin A, but they recorded no spectroscopic observations, except for arsenic trichloride.

The following table gives the results of measurements made on a marine and a freshwater fish liver concentrate ($E_{695\text{ m}\mu}/E_{620\text{ m}\mu}$ = 2.5 for the latter).

Reagent	Vitamin A ₁	Vitamin A ₂	Remarks
Trichloroacetic acid	610 mμ	690 mμ	30% reagent in CHCl ₃
Dimethyl sulphate	615 „	680 „	20% reagent in CHCl ₃
Perchloric acid	605 „	680 „	5–10% reagent in CHCl ₃
Benzoyl chloride	Very weak	590 „	20% reagent in CHCl ₃
Acetyl chloride	572 mμ	No band	A ₁ , very unstable violet colour; A ₂ , first blue, then violet
Arsenic trichloride	628, 577 mμ	710, then 590 mμ	A ₂ , first green, then violet
Reaction of Rosenthal & Erdélyi [1934]	558, 480, 450 mμ	685 mμ	A ₂ , no colour change on heating

These results were not encouraging for further studies, as the bands were more or less the same as those which occur in the Carr and Price reaction.

Determination of the ratio E 695 mμ/E 620 mμ in liver oils of freshwater fish from different sources

A striking difference seemed to exist between the results of Edisbury *et al.* [1938] on English fish and those of Gillam *et al.* [1938] on Russian fish. The former authors found in general a ratio $E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$ varying from 0.8 to 1.5 (for the livers of *Salmo salar*, *Salmo trutta* v. *fario*, *Salmo irideus* and *Perca fluviatilis*), whereas the livers of Russian freshwater fish (*Lucioperca lucioperca*, *Esox lucius*, *Silurus glanis*, *Abramis brama*, *Huso huso*, *Stenodus leucichthys nelma*, *Coregonus lavaretus pidschian* etc.) had ratios varying from 1.6 to 2.6. Only a few varieties of salmon and carp had low values (0.25–0.88).

We decided to investigate this point and measured the relative absorption intensities at 695 and 620 mμ of liver concentrates from a number of fish from various parts of Europe (Austria, France, Holland, Hungary and Yugoslavia). The results showed (Table III) that there was no geographical difference but a very marked species specificity as regards these relative intensities. Specimens of pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*) and catfish (*Silurus glanis*) from all over Europe had a ratio of 1.9–2.7, whereas carp (*Cyprinus carpio*), trout (*Salmo irideus*), salmon (*Salmo salar*) and sturgeon (*Acipenser sturio*) had much lower values (0.4–0.8). Marine fish from the Atlantic coast of France (*Gadus merlangus*, *Hippoglossus hippoglossus*) and from Japan (*Ishinagi sterelepis*) had still lower values (0.17–0.21). These measurements agreed well with those of Edisbury *et al.* [1938] and of Gillam *et al.* [1938] on English and Russian fish and the “striking difference” between the results of these two groups of investigators was simply due to the fact that in the two laboratories different species of fish were used.

We have observed that the beginning of oxidation in concentrates of vitamin A₂ is indicated by a decrease of the ratio $E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$, due probably to the more rapid destruction of vitamin A₂. Some of the lower values for this quotient reported in the literature ([Gillam *et al.* 1938] and Table III of this paper) may be explained in this way.

Comparison of liver oils and intestinal oils

Edisbury *et al.* [1938] made the interesting observation that the intestinal oils of some fish contained great quantities of vitamin A, comparable with those found in the liver. They also recorded measurements of the ratios vitamin A₁/vitamin A₂ in the intestinal oils of freshwater fish, and found that as a rule they were lower than in the corresponding liver oils.

Our measurements confirmed the findings of Edisbury *et al.* and showed that in some species (carp, Table III, nos. 30–33) $E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$ of the intestinal oils was even higher than the corresponding ratio in liver oils from the same fish.

Table III. *Vitamins A₁ and A₂ in liver and intestine of freshwater fish*

No.	Name of fish	Origin	Organ	Wt. g.	Unsap. mg. per 100 g. fresh organ	E ¹ %, cm., 695 mμ	E 695 E 650	E 695 E 620	Remarks
1	Pike-perch (<i>Lucioperca lucioperca</i>)	France (Saône)	5 livers	52	340	40	1.80	2.63	Gillam <i>et al.</i> [1938] for Russian fish $E 695 m\mu = 2.18-2.34$
2	"	"	5 livers	52	(8 g. oil)	0.5	1.00	0.58	Oil from no. 7 before saponification Whole intestine with stomach and fat Same fish
3	"	"	4 intestines	180	220	16	1.27	1.60	
4	"	"	14 livers	93	300	88	1.65	2.40	
5	"	"	7 caeca	47	430	78	1.80	2.25	
6	"	"	Exterior caecal fat from 7 caeca	110	600	5	1.18	1.00	
7	"	"	10 stomachs	70	250	2	1.00	1.00	
8	"	Austria (Bodensee)	1 liver	27	500	110	1.60	2.60	
9	"	"	1 intestine	56	300	115	—	2.35	
10	"	Hungary	1 liver	25	360	100	1.46	2.00	Measured 2 months after preparation of unsap.
11	"	Holland	1 liver	18	560	15	1.55	2.35	
12	Pike (<i>Esox lucius</i>)	France	7 livers	65	300	84	1.66	2.00	Measured 7 days after preparation of unsap.
13	"	"	7 intestines	125	420	8	1.30	1.80	
14	"	"	50 livers	440	530	180	1.56	2.65	Gillam <i>et al.</i> [1938] for Russian fish: $E 695 m\mu = 1.90$ $E 620 m\mu = 1.90$
15	"	Austria (Danube)	4 livers	40	250	112	1.38	2.00	Measured 13 days after preparation of unsap.
16	"	"	3 intestines	120	230	24	1.48	1.88	
17	"	Holland	1 liver	12	610	185	1.54	2.45	
18	"	"	1 liver	12	610	55	0.43	0.58	Unsap. no. 17 + inhibitor
19	Catfish (<i>Silurus glanis</i>)	Austria (Danube)	2 livers	100	750	40	1.41	2.00	Gillam <i>et al.</i> [1938] for Russian fish: $E 695 m\mu = 2.0$ $E 620 m\mu = 2.0$
20	"	"	3 intestines	210	360	7	1.28	1.64	
21	"	Hungary	1 liver	75	670	156	1.56	2.20	
22	Perch (<i>Perca fluviatilis</i>)	Holland	5 livers	30	675	32	1.52	2.20	Young fish
23	" (<i>Tinca tinca</i>)	France	5 livers + intestines	8	520	35	1.52	1.52	Young fish
24	Bream (<i>Abramis brama</i>)	"	10 livers + intestines	5	460	14	—	1.40	
25	" (<i>Abramis alburnus</i>)	"	80 livers + intestines	75	175	18	1.13	1.25	Young fish
26	Trout (<i>Salmo trutta</i>)	"	10 livers + intestines	11	500	8	1.20	0.75	Edisbury <i>et al.</i> [1938] for English fish: $E 695 m\mu = 0.8$ $E 620 m\mu = 0.8$
27	"	"	22 livers	31	530	9	1.05	0.54	Same fish
28	"	"	25 caeca	80	370	9	0.84	0.42	$E 695 m\mu = 0.25$ $E 620 m\mu = 0.25$
29	"	"	25 intestines	126	220	4	0.87	0.44	
30	Carp (<i>Cyprinus carpio</i>)	"	1 liver	30	300	7	0.67	0.42	Gillam <i>et al.</i> [1938] for Russian fish: $E 695 m\mu = 0.25$ $E 620 m\mu = 0.25$
31	"	"	1 intestine	30	360	3	—	0.62	
32	"	Yugoslavia	2 livers	100	260	20	0.95	0.50	
33	"	"	2 intestines	160	400	5	1.20	0.70	
34	"	Hungary	3 livers	190	380	32	1.10	0.69	
35	"	Holland	1 liver	13	685	105	0.95	0.50	
36	Sturgeon (<i>Acipenser sturio</i>)	France (Atlantic coast)	1 liver	400	260	78	1.00	0.60	Gillam <i>et al.</i> [1938] for Russian fish: $E 695 m\mu = 1.32$ $E 620 m\mu = 1.32$
37	Salmon (<i>Salmo salar</i>)	Holland	1 liver	75	210	144	1.10	0.72	Edisbury <i>et al.</i> [1938] for English fish: $E 695 m\mu = 0.8-1.0$ $E 620 m\mu = 0.8-1.0$

More detailed determinations were made on the distribution of vitamins A₁ and A₂ in the intestinal tract of the pike-perch (*Lucioperca lucioperca*). The pyloric caeca of this fish are surrounded by great quantities of fat which can be separated mechanically from the caeca themselves. For stomach and exterior caecal fat $E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$ was 1.0 (Table III, nos. 6, 7). The corresponding quotient for the caeca themselves and for the liver was 2.4 (Table III, nos. 4, 5).

This difference may be explained as follows. The vitamin A of the food is composed of about equal parts of vitamins A₁ and A₂ ($E\ 695\text{ m}\mu/E\ 620\text{ m}\mu = 1$ for the stomach contents). After absorption, a part of the fat of the food is deposited in the fat depots around the caeca, a process which leaves the ratio vitamin A₁/vitamin A₂ unchanged. In the liver and the caeca, on the other hand, there seems to be a constant coming and going of fat resulting in a greater accumulation of vitamin A₂ owing to its more unsaturated character. This would be in agreement with the observations of Zechmeister & Tuszone [1935, 1] that the more unsaturated carotenoids lycopene and capsanthine are more easily stored in human fat than carotene or lutein, as well as with the experiments of Thorbjarnarson & Drummond [1938] which show that fat leaving the liver may carry away some vitamin A.

In one experiment the caeca of a pike-perch were emptied of their contents and the caecal skin and the contents were analysed separately, with the following results:

	Wt. g.	Nonsap. mg.	$E_{1\text{ cm.}}^{1\%}$ 695 m μ	$E\ 695\text{ m}\mu/E\ 620\text{ m}\mu$	Mg. vit. A ₂ per 100 g.*
Caecal skin	2.1	27†	50.5	2.4	12.5
Caecal contents	1.9	12†	24.0	2.1	3.4

* Calculated on the assumption that $E_{1\text{ cm.}}^{1\%}$ 695 m μ of pure vitamin A₂ is 5000.

† Not entirely saponified.

The pyloric caecal skin had a high absolute content of vitamin A₂ which was more than twice as great as that of the corresponding liver (Table III, no. 4; 5.3 mg. vitamin A₂ per 100 g. fresh organ). It is reasonable to suppose that the accumulation of this vitamin in the caeca is not fortuitous but is related to the mechanism of fat absorption discussed by Edisbury *et al.* [1938] in connexion with vitamin A₁.

E 695 m μ /E 620 m μ of liver fat of animals other than fish

The liver fat of sheep and chicken contains no vitamin A₂ (Table I, nos. 4, 5), that of the green frog only small quantities (Table I, no. 6).

These observations confirm and extend the findings of Castle *et al.* [1934], Edisbury *et al.* [1938] and Gillam *et al.* [1938] concerning the absence of the 693 m μ chromogen from mammalian liver fat and seem to indicate that vitamin A₂ is a specific product of the liver metabolism of certain species of freshwater fish.

Exact measurements of the vitamin content of invertebrates have often been impossible because of the minute quantities of chromogenic material isolated or because of the presence of substances causing the formation of a turbidity with SbCl₃. Qualitative observations indicate that the intestines of the cephalopod *Loligo vulgaris* contain vitamin A₁ without any trace of vitamin A₂.

Accumulation of vitamin A₂ in the liver of the rat and the frog

The absence of vitamin A₂ from mammalian liver fat might be due to a differential absorption of vitamins A₁ and A₂ from the intestine (cf. the differential absorption of carotene and xanthophyll by the horse intestine observed by

Zechmeister & Tuszon [1935, 2]) or to a more rapid destruction of vitamin A₂ after absorption.

Qualitative feeding experiments have shown that the rat accumulates vitamin A₂ in its liver when receiving orally a concentrate from freshwater fish liver oil in the same way that it accumulates vitamin A₁. The absence of vitamin A₂ from mammalian liver fat can therefore be explained by the absence of vitamin A₂ from the food.

Feeding experiments on frogs, using concentrates of both vitamin A₁ and vitamin A₂, have shown that the amount of vitamin A₁ as well as the ratio vitamin A₂/vitamin A₁ can be increased in the liver.

*Increase of the ratio E 695mμ/E 620mμ in the liver fat of frogs fed
on a vitamin A₂ concentrate*

No. of frogs	No. of feedings*	E 695mμ/E 620mμ
4	0	0.24
2	4	0.30
2	8	0.30
4	18	0.55

* Two drops of pike-perch liver oil (Table III, no. 2) a day per frog; no other food.

Owing to the slow metabolism of these animals little effect was produced till the feedings were continued for more than a week. Most of the oil fed was found in the intestine of the frogs.

SUMMARY

1. Vitamin A₂ (693mμ chromogen) gives with antimony trichloride a two-banded spectrum (maxima at 693 and 650mμ). In unsaponifiable fractions the band at 650mμ is entirely masked by the 693mμ band; it can be seen in some natural oils and in partly saponified oils owing to the presence of inhibitors which diminish the intensity of the 693mμ band. It can be made visible in unsaponifiable fractions by the addition of such inhibitors.

2. The 650mμ band of vitamin A₂ causes considerable absorption at 620mμ and therefore only a part of the absorption at 620mμ observed in vitamin A₂ concentrates is due to the presence of vitamin A₁. Measurements and calculations lead to the conclusion that in concentrates rich in vitamin A₂ ($E\ 695m\mu/E\ 620m\mu=3$) nearly half of the absorption at 620mμ is due to vitamin A₂. Thus $E\ 695m\mu/E\ 620m\mu$ of pure vitamin A₂ would lie between 6 and 8.

3. The ultraviolet absorption spectrum of our purest vitamin A₂ concentrates, which are entirely soluble in 77 % ethyl alcohol, shows absorption bands at 345 and 280mμ.

4. Trichloroacetic acid, dimethyl sulphate, perchloric acid and other reagents give with both the vitamins coloured solutions having about the same bands as those which occur in the Carr and Price reaction.

5. The relative quantities of vitamins A₁ and A₂ (expressed as $E\ 695m\mu/E\ 620m\mu$) have been measured for a series of livers from freshwater fish from several countries of Europe. It has been found that this ratio is more or less constant for a given species. Thus pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*), catfish (*Silurus glanis*), and perch (*Perca fluviatilis*) have ratios of 2.0–2.7; trout (*Salmo irideus*), carp (*Cyprinus carpio*), sturgeon (*Acipenser sturio*) and salmon (*Salmo salar*) have low ratios (0.4–0.8). Marine fish (*Gadus merlangus*, *Hippoglossus hippoglossus*, *Ishinagi sterelepis*) and land animals such as sheep

(*Ovis aries*), chicken (*Gallus domesticus*) and frog (*Rana esculenta*) have still lower ratios (0.03–0.27).

6. Intestinal oils of freshwater fish have usually a somewhat lower ratio $E\ 695\text{m}\mu/E\ 620\text{m}\mu$ than the liver oils of the same animals. For the carp, on the contrary, this ratio tends to be higher in the intestine than in the liver.

7. The stomach contents and depot fat around the pyloric caeca of the pike-perch have a ratio $E\ 695\text{m}\mu/E\ 620\text{m}\mu = 1.0$, whereas the liver and caeca themselves have a ratio of 2.4. A possible explanation for this difference is discussed.

8. Feeding experiments have shown that the rat and the frog can absorb vitamin A₂ from the intestine and accumulate it in the liver. Thus the absence of vitamin A₂ from the liver of mammals and other land animals can be explained simply by the absence of vitamin A₂ from the food.

Vitamin A₂ seems to be a specific product of the liver metabolism of certain species of freshwater fish.

We wish to express our sincere thanks to Profs. G. Dupont and R. Lévy for working facilities at the École Normale Supérieure and to Prof. P. Langevin for permission to use his Hilger-Nutting spectrophotometer. Our thanks are due also to Prof. L. Wolff, Utrecht, for several specimens of Dutch fish livers and to Dr Vevers of the London Zoological Gardens for a sample of Ishinagi liver oil.

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