XXXIX. THE CONVERSION OF CAROTENE TO VITAMIN A_2 BY SOME FRESH-WATER FISHES

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FRESHWATER fish contain a substance akin to, but not identical with, vitamin A, which on account of its distribution, chemical, physical and biological properties may properly be designated vitamin A_2. It is differentiated from vitamin A by means of its ultraviolet absorption spectrum and by the antimony trichloride colour test:

	Vitamin A	Vitamin A_2
U.V.	λ_{max} 328 mμ	350 mμ (284 mμ?)
Colour test	λ_{max} 617 mμ [583 mμ] inflexion	693 mμ [645 mμ] inflexion

The available evidence suggests strongly that some of the functions of vitamin A in salt-water fish are fulfilled in freshwater fish by vitamin A_2. The only circumstances under which vitamin A_2 has been found to occur in mammals or birds are those which indicate the presence of vitamin A_2 in the diet (e.g. when freshwater fish are eaten freely) and there is no evidence of the natural synthesis of vitamin A_2 from provitamins except in fishes.

It is accepted that for the mammal α- and β-carotenes (and a few related substances) act as provitamins A, since the administration of such materials results in the relief of symptoms of vitamin A deficiency. If supplied in relative abundance they markedly increase the vitamin A content of the liver.

The origin of the large amounts of vitamin A found in some fishes has never been determined. It is true that phytoplankton contains carotene, but the transformation of carotene into vitamin A has not been directly demonstrated for fishes, nor is there satisfactory evidence that the amount of carotenoid ingested is sufficient to account for the stored vitamin. The constitution of vitamin A_2 remains somewhat speculative and little is known concerning possible or actual precursor substances.

The object of the present series of experiments is to ascertain something of the effect of carotene added to the food of freshwater fish. The literature affords little guidance in the planning of such work. Search was made for a readily accessible freshwater species having the following characteristics:

(1) the fish should be small enough to allow several to live in an aquarium;

(2) the normal vitamin reserves should not be very high;

(3) both vitamin A and vitamin A_2 should be present in easily detectable amounts in the healthy fish on a normal diet.

The most suitable species for a first experiment seemed to be the perch (*Perca fluviatilis*). A number of live fish were secured from Lake Windermere[1] and transported to Liverpool by road in suitable fish tanks. They were transferred on arrival to large glass tanks shaded from direct light and aerated by

[1] The co-operation of the Staff of the Freshwater Biological Association at Wray Castle is gratefully acknowledged.

circulating tap water carrying with it air bubbles. The inlet water was maintained for a time at a slightly higher temperature than that of the room, but after the fish had become habituated to their new surroundings the precaution was found to be unnecessary. It was realized almost at once that too many fish had been placed in the aquaria and a number were removed with a net and pithed. These were then dissected and the different organs tested for carotenoids, vitamin A and vitamin A_2. A few fish died in the first few days and all refused food. This experience is unfortunately common with fish unused to captivity. Search was made for a suitable live food and it was found that the water shrimp *Gammarus neglectus*, which was fortunately available in quantity (River Weaver, Cheshire) proved tempting. After fasting for nearly a fortnight all the perch took the shrimp with avidity and a plentiful supply for about 14 days sufficed to restore a normal appetite.

There were, however, two objections to continuing to feed the fish on water shrimps. In the first place, much work is needed to obtain a sufficient, regular supply of live shrimps and the task of breeding them in the laboratory threatened to be beyond us. Secondly, it was not possible to regard the diet as satisfactory for the experiments which had been planned.

Thus, 28 g. of dead shrimps were completely decomposed by means of alcoholic potash and the non-saponifiable fraction was extracted. It contained a carotenoid pigment (λ_{max} 484, 453, 428 $\sim$ 407 mμ, $\sim$ denoting inflexion) and gave maxima at 617 mμ and 583 mμ with the $SbCl_3$ reagent.

$E^{1\%}_{1\,cm.}$	620 mμ	0·037
	583 „	0·029

calculated on the weight of shrimp

Similar tests on a further 23 g. of shrimp gave:

$E^{1\%}_{1\,cm.}$	620 mμ	0·048
	583 „	0·036

and direct absorption spectra confirmed the presence of carotenoid with additional maxima at 344, 316$\sim$286 and $\sim$276 mμ.

These results are certainly an indication that the water shrimp contains small quantities of material giving tests very similar to those of vitamin A. It would be necessary to carry out a chromatographic analysis of the non-saponifiable fraction in order to be certain which carotenoids are present and to give rigorous proof that the colour test was due to vitamin A rather than to carotenoids. In any case, the shrimp was a poor carrier for adding carotene to the diet. Moreover, it probably contains astacene which may be a vitamin A precursor in some fish.

Various foods were then tried. The digestive glands of *Mytilus* proved on the whole unsatisfactory, minced lean beef was only sparingly eaten, but after some initial reluctance all the fish took blow-fly larvae with great readiness.

At the end of a preliminary period of six weeks the perch were accustomed to the aquaria and could be fed without difficulty. The diet consisted of larvae smeared occasionally with herring-body oil (of negligible vitamin A content). On some days finely chopped raw lean beef was provided.

The next problem concerned the administration of carotene. The provision of an exact amount of carotene in the daily diet of the perch seemed impossible. The object of the experiment was to test the effect of a diet substantially enriched with carotene, and it was necessary to become reconciled to a considerable wastage of carotene in order to be sure that a fair quantity would actually be ingested.

The material used was a sample of leaf carotene containing both α- and β-carotenes but little or no "xanthophyll".

Finely ground solid carotene was placed in a glass dish and clean larvae were picked out with a pair of forceps and dropped into the dish. By gently rubbing the live larvae in the powdered carotene they were easily coated with a minute staining layer which adhered fairly strongly. The coated larvae were fed to the perch two or three at a time and were taken at once. Feeding was on each occasion discontinued as soon as the fish lost interest. It was at first thought that one meal every 48 hours would be best but experience showed that the fish were ready to eat once each day. A little extra food provided at noon on Saturdays had been eaten before Monday morning. Care was taken to remove food debris from the aquaria and to ensure constant aeration. A group of fish maintained in a separate tank received a diet as nearly as possible identical with that supplied to the experimental group, except that the controls received no added carotene.

After a period of two months the controls were removed by means of a net and at once killed by pithing. They were then dissected and the various portions assembled for analysis. Each sample was refluxed with alcoholic potash and the non-saponifiable fractions were extracted with ether. The ether extracts were dehydrated by treatment with pure alcohol and nitrogen on a water bath. The non-saponifiable fractions were then subjected to the $SbCl_3$ colour test and the vitamin concentrations estimated by spectroscopic examination.

Experimental data

Perch from Lake Windermere—not fed in captivity

(*a*)	(*b*)
8 fish: 4♂, 4♀	3 fish

Average wt. 38 g., average length 14·9 cm.

Livers: 3·274 g., average 0·42 g.

$E^{1\%}_{1\,cm.}$	693 mμ	0·04
	640 ,,	0·075
	Feeble maxima at 595 and 560 mμ	

1·253 g., average 0·42 g.

$E^{1\%}_{1\,cm.}$	693 mμ	0·018
	640 ,,	0·044
	605 ,,	0·053
	560 ,,	0·043

Viscera (comprising hearts, spleens, gonads, mesenteries): 20·3 g., average 2·2 g.

$E^{1\%}_{1\,cm.}$	695 mμ	0·01
	636 ,,	0·011
	556 ,,	0·09

Eyes	Feeble blue colour
Pyloric caeca	,,
Intestines	,,
Mesenteric fat	,,

Alimentary tracts: 6·98 g., average 0·87 g.

$E^{1\%}_{1\,cm.}$	693 mμ	0·008
	600 ,,	Transient
	620 ,,	

Gonads	No blue colour
Hearts	,,
Spleens	,,
Stomachs	,,

Eyes and bodies both gave feeble colour tests

Controls: 6 fish fed in captivity, no added carotene

Livers: 3·71 g., average 0·62 g.

$E^{1\%}_{1\,cm.}$	693 mμ	0·064
	620 ,,	0·040

Mesenteries: 3·58 g., average 0·6 g.

$E^{1\%}_{1\,cm.}$	695 mμ	0·0116
	655 ,,	0·0124
	620 ,,	0·0124
	580 ,,	0·0085

Eyes	Very faint blue colour
Pyloric caeca	No blue colour
Hearts	,,
Spleens	,,
Stomachs	,,
Intestines	,,
Gonads	,,

Perch on a diet enriched with carotene

3♀ immature, 3♀ adult, 2♂ adult
Average wt. 36·3 g., average length 15·8 cm.
Livers: 4·28 g., average 0·61

$E^{1\%}_{1\,cm.}$ { 693 mμ 0·206 ; 620 „ 0·086 only 693 mμ band seen

Mesenteries: 4·74 g., average 0·68 g.

$E^{1\%}_{1\,cm.}$ { 645 mμ 0·0086 ; 605 „ 0·0096 ; 592 „ 0·0086

Pyloric caeca: 2·83 g.	Faint blue colour
Intestines: 3·86 g.	Faint blue (greenish blue)
Gonads: 17·43 g.	Faint blue
Eyes: 5·78 g.	„
Stomachs: 2·32 g.	No blue colour
Hearts: 0·47 g.	„
Spleens: 0·28 g.	„

It is clear that vitamin A and vitamin A_2 occur preferentially in the livers. Although in an absolute sense the concentrations are low, the amount of vitamin A_2 is very definitely increased as a result of the administration of dietary carotene.

	$E^{1\%}_{1\,cm.}$ 693 mμ
Windermere fish feeding naturally	0·04
	0·018
Controls	0·064
Carotene-fed perch	0·206

The control fish had, as it turned out, rather larger reserves than those fresh from the open lake. There can be little doubt that the threefold increase in vitamin A_2 and the twofold increase in vitamin A are consequent upon the administration of carotene.

A few months later, perch from a different locality became available after feeding naturally until July, 1938. They had thus had the benefit of the photosynthetic activity in spring and early summer and it would be reasonable to expect that they had obtained full access to the natural provitamins A.

Perch from Aberdeen

20 fish, average wt. 225 g.

Livers 73 g. (average 3·65 g.). After refluxing with alcoholic potash and extraction with ether 0·33 g. of non-saponifiable matter was isolated:

		On non-sap.	On liver	
$E^{1\%}_{1\,cm.}$	693 mμ	132	0·6	Colour test
	617 „	46	0·21	
	351 „	36·4	0·165	U.V. absorption

It will be seen that the ratio $E^{1\%}_{1\,cm.}$ 693/617 mμ is 2·87, showing that vitamin A_2 is present in much larger amount than vitamin A. The absolute quantity, *c.* 120 p.p.m., is not high but is nevertheless three times as great as that shown by the carotene-fed perch and some nine times as great as that shown by the controls. The ultraviolet absorption spectrum shows, in addition to the 351 mμ maximum characteristic of vitamin A_2, a second band at 284 mμ which may be due to a third absorbing entity in the non-saponifiable fraction.

It will be noticed that these perch were on the average much larger (six to seven times by weight) than those obtained from Windermere. The livers from the

present batch of perch averaged 3·65 g. as against 0·6 g. for the experimental fish. The much higher storage reserves of vitamin A_2 in the larger and (probably) older fish is in good accord with experience obtained with the vitamin A contents of cod and halibut livers. It seems likely that in any year the fish tends to show a positive balance in its vitamin A or A_2 economy resulting in greater storage with age.

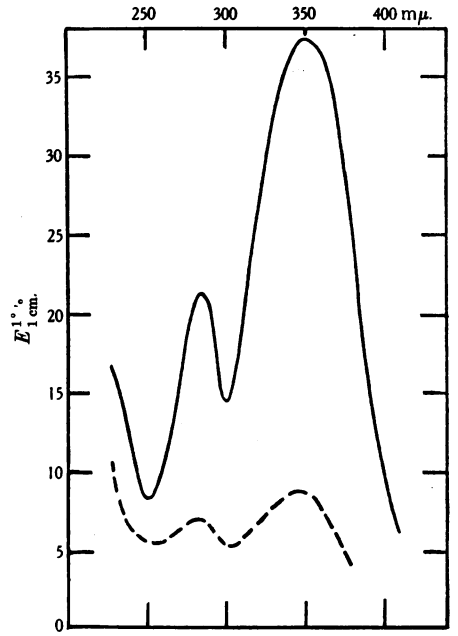

Fig. 1. Ultraviolet absorption spectrum of non-saponifiable fractions from (*a*) perch liver oil ——; (*b*) perch intestinal oil – – –.

Stomachs. 30 g. of tissue yielded 0·1318 g. (4393 p.p.m.) of non-saponifiable matter. A 13·2% solution of the "non-sap." diluted with 10 vol. of the $SbCl_3$ reagent gave a blue solution by means of which it was just possible to recognize the 693 mμ maximum but not the 617 mμ band. The vitamin content of the stomachs must therefore have been negligibly small.

Eyes. The whole eyes from 20 perch weighed 47 g. Prolonged treatment with hot alcoholic potash followed by the usual extraction process gave 0·9563 g. (20,350 p.p.m.) of non-saponifiable matter. This material gave a good blue colour with the $SbCl_3$ reagent:

$E^{1\%}_{1\,cm.}$	693 mμ	Not measurable
	642 „	0·315
	600 „	0·294
	565 „	0·28

Three absorption bands were seen clearly. The eyes evidently contain small quantities of both vitamin A and vitamin A_2 although the rest of the non-saponifiable fraction exerts marked inhibition in the colour test. The eyes probably yielded 2 p.p.m. vitamin A and 3–4 p.p.m. vitamin A_2.

Intestines. 60 g. yielded 0·353 (5900 p.p.m.) of non-saponifiable matter:

		On non-sap.
$E^{1\%}_{1\,cm.}$	693 mμ	23
	617 „	9·3

The ratio $E^{1\%}_{1\,cm.}$ 693 mμ/617 mμ is 2·47, i.e. vitamin A_2 does not preponderate quite so much as in the livers. The ultraviolet absorption shows two maxima, one near 350 mμ and the other near 285 mμ. The relative intensities of these two maxima in the intestinal non-saponifiable fraction are so strikingly different from the relative intensities shown by the liver "non-sap." that little doubt can remain that the two maxima belong to distinct compounds.

Mesenteric fat. A good deal of mesentery could be detached from the ileum of each fish. 68 g. yielded 0·098 g. of "non-sap.":

		On non-sap.
$E^{1\%}_{1\,cm.}$	693 mμ	22·5
	617 „	10·7

corresponding to *c.* 2·5 p.p.m. vitamin A and *c.* 6·5 p.p.m. vitamin A_2.

The fact that carotene can act as provitamin A and provitamin A_2 for the perch seems established from this work, but the failure to raise the storage levels to that observed in larger fish feeding naturally is at first sight disturbing. There is little reason to suppose that carotene is the sole precursor substance. In fact the amounts of carotene available in the natural diet are rather surprisingly small.

Thus a sample of 53·8 g. of zooplankton supplied from Windermere (by courtesy of the Freshwater Biological Research Station, Wray Castle) after saponification yielded a fraction unmistakeably containing carotene but so contaminated with other absorbing substances as to preclude a quantitative assay. The acids recovered from the soaps were deeply coloured and gave numerous absorption bands (λ_{max} 271, 283, 301, 317, 330, 349, 377, 405, 450, 482 mμ) indicating the presence of conjugated polyenes. The quantities available did not permit of fractionation.

A further experiment was made on dace fed on blow-fly larvae with and without added carotene:

Experiments on dace

Controls			Diet enriched with carotene		
5 fish			4 fish		
Livers: 8·78 g.			7·4 g.		
$E^{1\%}_{1\,cm.}$	693 mμ	0·19	$E^{1\%}_{1\,cm.}$	693 mμ	0·73 Colour
	620 „	0·19		620 „	0·65 test
				345 „	0·28 u.v. test
Vitamin A	*c.* 38 p.p.m.		Vitamin A	*c.* 130 p.p.m.	
Vitamin A_2	*c.* 38 p.p.m.		Vitamin A_2	*c.* 146 p.p.m.	
Stomachs: 1·45 g.			1·5 g.		
$E^{1\%}_{1\,cm.}$	693 mμ	0·011	$E^{1\%}_{1\,cm.}$	693 mμ	0·35
	620 „	0·027		620 „	0·70
				583 „	0·38
Vitamin A	5·4 p.p.m.		Vitamin A	140 p.p.m.	
Vitamin A_2	2·2 p.p.m.		Vitamin A_2	70 p.p.m.	
Intestines: 1·25 g.			1·14 g.		
$E^{1\%}_{1\,cm.}$	620 mμ	0·027	$E^{1\%}_{1\,cm.}$	693 mμ	0·07
				620 „	0·225
Vitamin A	5·4 p.p.m.		Vitamin A	45 p.p.m.	
			Vitamin A_2	14 p.p.m.	
Rest of viscera: 8·1 g.			5·3 g.		
Vitamin A	2·4 p.p.m.		Vitamin A	4 p.p.m.	
Vitamin A_2	2·0 p.p.m.		Vitamin A_2	3·2 p.p.m.	
Gonads: 27·4 g.			40·8 g.		
Vitamin A	0·84 p.p.m.		Vitamin A	1 p.p.m.	
Vitamin A_2	0·74 p.p.m.		Vitamin A_2	1 p.p.m.	
Eyes: Trace of vitamins A and A_2			Trace of vitamins A and A_2		

The work on dace shows a substantial increase in the amounts of both vitamins A and A_2 as a result of adding carotene to the diet. The marked increase in the alimentary tract is striking and suggests that the possibility of conversion of carotene in the stomach and intestines requires further investigation.

An attempt was made to extend the experiments to chub. The controls (2) on the carotene-free diet were unsatisfactory, as they could not be induced to eat freely. They may have been injured before arrival, but when it was seen that they were not doing well they were removed by means of a net, pithed and examined. Their vitamin reserves were low. The fish receiving a diet enriched with carotene compared badly with later specimens feeding naturally:

	Chub fed on blow-fly larvae plus carotene			Freshly caught chub (Mar. 1938)		
	Wt. g.	P.p.m. vitamin A	P.p.m. vitamin A_2	Wt. g.	P.p.m. vitamin A	P.p.m. vitamin A_2
Livers	8·4	39	33	6·9	120	50
Intestines	3	4·3	1·5	1·33	75	43
Stomachs	4·2	3·2	1·6	1·31	110	6
Rest of viscera	4·1	0·2	1·9	4·73	94	42
Eyes		Traces			Traces	
Gonads		Traces			Traces	

The chub did not take kindly to captivity, and this experiment is unsatisfactory. The striking difference between the contents of the alimentary tracts in respect of vitamins A and A_2 is worth emphasis.

Summary

Perch and dace in captivity appear to thrive for a considerable period on a diet of blow-fly larvae. When this diet is enriched with carotene for a few weeks the store of vitamins A and A_2 increases considerably. From this it is concluded that carotene acts as provitamin for the formation *in vivo* of both vitamins. Whatever the constitution of vitamin A_2 may be it cannot be very different from that of vitamin A.

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