Conversion of Carotenoids into Vitamins A₁ and A₂ in Two Species of Freshwater Fish

By JEANA GROSS AND P. BUDOWSKI

Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel

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1. Examination of two zooplankton species predominating in fish ponds, *Daphnia magna* and *Chironomus* larvae, revealed the presence of α - and β -carotene, echinenone, canthaxanthin and 3-hydroxy-4-oxo- β -carotene in *Daphnia*, and β -carotene and cryptoxanthin ester in *Chironomus*. No specific provitamins A₂ (containing a 3,4-dehydro- β -ionone ring) were detected. 2. Guppies (*Lebistes reticulatus*) and platies (*Xiphophorus variatus*) were found to form vitamin A from β -carotene and from its oxygen-containing derivatives isoeaxanthin, canthaxanthin and astaxanthin. Slight conversion into vitamin A₂ seemed to occur simultaneously. 3,4-Dehydro-3'-hydroxy- β -carotene formed little vitamin A, and the latter was mainly of the A₂ type. Lutein was devoid of provitamin A properties. 3. In addition to vitamin A, β -carotene was detected in fish receiving the 4-oxo- and 4-hydroxy-carotenoids. A reaction scheme for the conversion of carotenoids into retinal and and 3,4-dehydroretinal is presented. 4. It is concluded that natural 4-oxo derivatives of β -carotene may play a significant role as vitamin A precursors for fish.

The provitamin A character of carotenoids has been extensively studied in mammals and birds. The presence of an unsubstituted β -ionone ring in the carotenoid molecule appears to constitute a necessary and sufficient condition for vitamin A activity, though certain epoxides have also been reported to display activity. In fishes, and generally in aquatic animals, the question of the conversion of carotenoids into vitamin A has remained relatively obscure, being, furthermore, complicated by the presence of vitamin A₂, which frequently predominates over vitamin A₁ in animals living or spawning in fresh water. (The chemical designations for vitamins A₁ and A₂ are retinol and 3,4-dehydroretinol respectively. However, because of frequent reference to provitamins A₁ and A₂, we have used the common names throughout the text.)

It has been reported that both vitamins A₁ and A₂ can be formed in some fishes from common precursors, such as β -carotene (Morton & Creed, 1939) or astaxanthin (Grangaud, Vignais, Massonet & Moatti, 1957).

Carotenoids containing at least one unsubstituted 3,4-dehydro- β -ionone ring have been shown to act as specific provitamins A₂ in chicks (Budowski, Ascarelli, Gross & Nir, 1963) and mice (Budowski & Gross, 1965), but no such compounds appear to have been found in Nature and their possible role in the biogenesis of vitamin A₂ is therefore obscure. It has also been suggested (Budowski *et al.* 1963) that the dehydration of lutein to anhydrolutein (3,4-dehy-

dro-3'-hydroxy- β -carotene), a provitamin A₂, might be of possible biological significance in this respect.

In the present work we have studied two aspects of the problem of the origin of vitamins A₁ and A₂ in freshwater fish. First, an attempt was made to characterize the carotenoids of plankton commonly found in local carp ponds. This failed to produce any evidence of the presence of specific vitamin A₂ precursors of the type found to be active in chicks and mice. Subsequently, feeding tests were carried out with small tropical freshwater fish receiving different carotenoids. Particular attention was directed towards the provitamin A₁ or A₂ character of β -carotene, astaxanthin (3,3'-dihydroxy-4,4'-dioxo- β -carotene), canthaxanthin (4,4'-dioxo- β -carotene) and isoeaxanthin (4,4'-dihydroxy- β -carotene). The last-named compound is in an intermediate redox position between β -carotene and the 4-oxocarotenoids and is only one dehydration step removed from 3,4-dehydrocarotenoids. Anhydrolutein (3,4-dehydro-3'-hydroxy- β -carotene), one of the specific provitamins A₂, was also tested, as was the universally occurring lutein.

EXPERIMENTAL

Materials

All solvents used were analytical reagents, except for commercial light petroleum (b.p. 60–90°), which was distilled over KOH pellets. Diethyl ether was freed of peroxides by refluxing over zinc dust and KOH for 1 hr. and

distilling. The same treatment was applied to 95% (v/v) ethanol to obtain an aldehyde-free product.

Adsorbents for chromatography were as follows. Magnesia Seisorb 43 (West Vaco Division, Food Machinery and Chemical Corp., New York, N.Y., U.S.A.) was mixed with diatomaceous earth (Hyflo Super-Cel; Johns-Manville Corp., Lompoc, Calif., U.S.A.) in equal proportions. Alumina for chromatography (British Drug Houses Ltd., Poole, Dorset) was deactivated by adding 10% (v/w) of water and mixing thoroughly. The weakened adsorbent was allowed to stand for at least 48 hr. before use. Calcium hydroxide powder (Baker and Adamson, New York, N.Y., U.S.A.) was used without treatment.

Carotenoids were as follows. β -Carotene (synthetic crystalline) was from Sigma Chemical Co., St Louis, Mo., U.S.A., and canthaxanthin (crystalline) was from Hoffmann-La Roche and Co., Basle, Switzerland.

Vitamin A₁ was prepared by saponification of synthetic vitamin A palmitate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). Vitamin A₂ (crystalline) was from Hoffmann-La Roche and Co.

Sodium borohydride was from Sigma Chemical Co. Alfalfa (lucerne) lipid extract was purchased from a local company.

Methods

Isolation of lutein from alfalfa lipids. The unsaponifiable matter was prepared and chromatographed on magnesia as described by Budowski *et al.* (1963). The column was developed with 15% (v/v) acetone in light petroleum containing 1–2% (v/v) of ethanol. The adsorbent was extruded and the yellow lutein zone was separated mechanically. The lutein was extracted with diethyl ether-ethanol (9:1, v/v) and purified by multiple rechromatography.

Preparation of anhydrolutein and isozeaxanthin. Anhydrolutein was prepared from alfalfa lipids by treatment with toluene-*p*-sulphonic acid and chromatography on magnesia (Budowski *et al.* 1963). Isozeaxanthin was prepared by reduction of canthaxanthin (Petracek & Zechmeister, 1956) with NaBH₄.

Preparation of astaxanthin. Astaxanthin was prepared from the crab (*Cancer pagurus*) essentially by the method of Goodwin & Srisukh (1949a,b) with some modifications. The pigment was extracted from the carapaces and the hypodermis. On testing the crude pigment extract by thin-layer chromatography by the method of Thommen & Wackernagel (1963), six or seven spots were detected. Column chromatography of the pigments was carried out on alumina. The column was developed with light petroleum containing increasing concentrations of acetone. The first fractions, which included two fluorescent zones eluted with light petroleum, were rejected. The fraction eluted with 5–10% (v/v) acetone contained the ester of astaxanthin. Free astaxanthin was eluted with ethanol containing 1% (v/v) of acetic acid. The astaxanthin, both free and esterified, was identified through its characteristic saponification test: the pigment extracted after acidulation of the saponified solution was astacin, which remained at the top of the chromatographic alumina column and could be removed with ethanol containing KOH (2.5%, w/v). It had λ_{max} 496 m μ in pyridine.

Carotenoid analysis of plankton. Permanent plankton (*Daphnia magna*) and the larvae of *Chironomus* (a 'transitory' plankton) were obtained from local ponds. The fresh

material was extracted with acetone (Krinsky & Goldsmith, 1960). The light-petroleum extracts of the pigments were chromatographed on magnesia-Hyflo Super-Cel both before and after saponification, and the chromatograms were developed with light petroleum containing 2–15% (v/v) of acetone. Each pigment zone was separated mechanically and rechromatographed on alumina, magnesia or calcium hydroxide and its absorption spectrum was determined in a Beckman DU spectrophotometer. The pigments with spectra devoid of fine structure were reduced with NaBH₄ overnight at 4° in 95% ethanol (Critchley, Friend & Swain, 1958). The carotenoid concentrations were determined in a Bausch and Lomb spectrophotometer (Spectronic 20) and expressed as β -carotene.

Fish feeding experiments. The small tropical fish used in the present study were the platy (*Xiphophorus variatus*) and the guppy (*Lebistes reticulatus*) belonging to the Family of Poeciliidae or viviparous tooth carps. These small fish are particularly suitable for the feeding tests intended, as they need only small amounts of substances to give significant vitamin A conversions.

The fish were purchased from local dealers. In Expts. 1 and 2 the fish were kept on a diet of commercial dried *Daphnia* for a preliminary period of 2 weeks. The *Daphnia* did not contain any detectable vitamin A and their total carotenoid content was 3–5 $\mu\text{g./g.}$ of dry matter. In Expt. 3 the *Daphnia* were exhaustively extracted with ether and ethanol to remove all traces of carotenoids and were then similarly fed to the fish during 2 weeks. At the end of this period the fish were distributed into small aquaria (30 cm. $\times$ 17 cm. $\times$ 20 cm.) fitted with filtering and aeration devices. The test diet was then given during the period indicated. Carotenoids were prepared for feeding by impregnating the dried *Daphnia* with the test compound dissolved in light petroleum with the addition, when necessary, of benzene or ethanol. After evaporation of the solvents under vacuum the diets were mixed thoroughly. Fresh test diets were prepared daily.

In Expts. 1, 2 and 3 the amounts of carotenoids supplied were respectively 1, 3 and 0.6 mg./g. of dried *Daphnia*.

Detection and determination of vitamins A and carotenoids in fish. The fish were homogenized in acetone (50 ml. for ten fish) in an Ultra-Turrax homogenizer (Jankel and Kunkel K.-G. Staufen, Germany). The acetone extract was filtered through Hyflo Super-Cel and the residue was washed with additional amounts of acetone until a total of about 100 ml. was obtained. The acetone was evaporated under vacuum and the residue was saponified in the cold (Plack & Kon, 1961). The unsaponifiable matter was chromatographed on a magnesia-Hyflo Super-Cel column (1.2 cm. $\times$ 12 cm.). The non-polar carotene-like fraction was eluted with 2–4% (v/v) acetone in light petroleum, the vitamin A fraction was eluted with approx. 70 ml. of 10% (v/v) acetone in light petroleum and the remaining xanthophylls were eluted with ethanol-light petroleum (1:1, v/v). The elution of the fluorescent vitamin A fraction was observed by brief irradiation with u.v. light. Occasionally the vitamin fraction was still coloured. It was then further purified by chromatography on alumina 'weakened' with 10% of water and eluted with 2–20% (v/v) ether in light petroleum.

For identification purposes the 'carotene' fraction was submitted to thin-layer chromatography on calcium hydroxide-silica gel G (6:1, w/w) with light petroleum-benzene (49:1, v/v) as the developing solvent. The R_f of

pure all-*trans*- β -carotene in this system was 0.50, rather than 0.84 (Bollinger, 1962). α -Carotene gave R_F 0.56.

Thin-layer chromatography of vitamin A was carried out on light magnesia with light petroleum-acetone (17:3, v/v) as the developing solvent. Spots were located under u.v. light or by spraying with $\text{SbCl}_3\text{-CHCl}_3$. In this system pure vitamins A_1 and A_2 had R_F 0.44 and 0.36 respectively.

Ultraviolet- and visible-absorption spectra were determined in a Beckman DU spectrophotometer. Absorption spectra of the Carr-Price reaction products in Expt. 3 were also recorded in the Beckman DU instrument, fitted with a spectral energy-recording attachment (Beckman 92300) connected to a Sargent model SRL Recorder. Scanning speed 3 was used, giving a scanning time of 28 sec. for the range 710–600 $m\mu$. The chart paper was driven at a speed of 12 in./min. With this single-beam spectrophotometer it was necessary to record the transmittance curves for the reaction product and the reagent separately. After conversion of transmittance values into extinctions and subtraction of the reagent readings from the reaction product readings the final absorption curves were obtained.

Where vitamin A_1 and A_2 values were determined from Carr-Price readings at 620 and 693 $m\mu$ a Bausch and Lomb Spectronic 20 spectrophotometer was used. The amounts of vitamins A_1 and A_2 were calculated by comparison with pure reference samples by following the principle of Plack & Kon (1961). It is realized that this method is not very accurate, particularly when the amounts of vitamins A involved are small.

RESULTS

Carotenoid pigments of Daphnia magna. The following carotenoids were identified: β -carotene (11 $\mu\text{g./g.}$ of dry matter), λ_{max} in ethanol 420 (inflexion), 451 and 476 $m\mu$; echinenone (32 $\mu\text{g./g.}$), λ_{max} in ethanol 462 $m\mu$; after reduction with sodium

borohydride, a fine structure appeared with λ_{max} 420 (inflexion), 451 and 476 $m\mu$; canthaxanthin (10–20 $\mu\text{g./g.}$), λ_{max} 472 $m\mu$; on reduction, λ_{max} 451 and 478 $m\mu$.

Besides these carotenoids, which were also reported by Thommen & Wackernagel (1964), we have identified α -carotene, λ_{max} 420 (inflexion), 446 and 475 $m\mu$, eluted slightly before β -carotene, and 3-hydroxy-4-oxo- β -carotene, λ_{max} 462 $m\mu$. This latter carotenoid, situated above canthaxanthin on the magnesia column, was identified by its conversion into 3,4-dioxo- β -carotene on saponification: this results in a bathochromic shift of the absorption maximum from 462 to 473 $m\mu$ (Krinsky & Goldsmith, 1960).

Carotenoids of Chironomus larvae. The main pigments detected were β -carotene (0.9 $\mu\text{g./g.}$ of fresh material), λ_{max} 450 and 478 $m\mu$, and a second, more polar, pigment (2.3 $\mu\text{g./g.}$ of fresh material), with the same absorption maxima (450 and 478 $m\mu$) as β -carotene. When co-chromatographed with cryptoxanthin isolated from *Zea mays* the unknown pigment was eluted first. After saponification its spectrum remained unchanged, but the pigment no longer separated from cryptoxanthin on co-chromatography. It appears therefore that the second predominating pigment of *Chironomus* larvae is cryptoxanthin ester. Not enough material was available for further tests.

Conversion of carotenoids in fish. Expts. 1 and 2 involved platies (*Xiphophorus variatus*) and guppies (*Lebistes reticulatus*) respectively. In Expt. 3, involving guppies, an attempt was made to characterize more fully the type of vitamin A formed.

Table 1. *Expt. 1: effect of carotenoids on the content of vitamins A_1 and A_2 in the platy (Xiphophorus variatus)*

The depletion period was 14 days and the test period 24 days. Carotenoids were supplied at 1 mg./g. of dried *Daphnia*.

Dietary carotenoid supplied	No. of fish	Mean wt. of fish (g.)	Vitamin A_1 ($\mu\text{g./g.}$ of fish)	Vitamin A_2 ($\mu\text{g./g.}$ of fish)	Carotene ($\mu\text{g./g.}$ of fish)	Xanthophylls ($\mu\text{g./g.}$ of fish)
None (control)	11	0.37	0.50	0.34	0.36	18.2
β -Carotene	9	0.41	2.72	0.75	6.79	18.6
	10	0.33	1.62	0.90	5.00	22.0
	10*	0.61	1.95	0.63	4.30	24.3
Astaxanthin†	10	0.41	1.70	0.45	0.54	38.0
	11	0.43	1.86	0.81	0.31	31.0
Canthaxanthin	10	0.34	1.91	0.84	2.13	30.0
	10	0.35	3.00	1.60	2.00	33.4
Anhydrolutein	11	0.44	0.13	0.74	0.61	32.4‡
	11	0.40	0.42	0.57	0.65	36.5‡
	10*	0.42	0.61	1.65	0.45	26.7‡

* These fish were purchased from a different supplier.

† A mixture of the free and esterified forms.

‡ About one-third of these pigments is anhydrolutein.

Table 2. *Expt. 2: effect of carotenoids on the content of vitamin A₁ and A₂ in the guppy (Lebistes reticulatus)*

The depletion period was 14 days and the test period 24 days. Carotenoids were supplied at 3 mg./g. of dried *Daphnia*. All fish except the control contained the corresponding dietary carotenoid. With hydrolutein this amounted to 40% of the total xanthophylls of the fish and in the canthaxanthin group to 73% of the total. N.D., Not detectable.

Dietary carotenoid supplied	No. of fish	Mean wt. of fish (g.)	Vitamin A ₁ (μg./g. of fish)	Vitamin A ₂ (μg./g. of fish)	Carotene (μg./g. of fish)	Xanthophylls (μg./g. of fish)
None (control)	14	0.13	0.63	0.58	N.D.	10.4
	12	0.15	1.17	0.53	N.D.	10.9
Lutein	8	0.32	1.09	0.72	N.D.	20.8
	8	0.28	0.75	0.75	N.D.	18.5
	8	0.27	0.54	0.79	N.D.	15.3
β-Carotene	10	0.20	6.5	1.58	45.9	9.8
	9	0.19	7.1	2.10	71.0	7.3
	10	0.18	6.2	1.77	51.5	10.0
Isozeaxanthin	11	0.17	8.0	1.1	4.2	30.4
	11	0.16	9.5	1.6	6.3	37.2
Canthaxanthin	46	0.19	6.6	0.76	6.81	28.1
Anhydrolutein	10	0.16	0.8	2.6	N.D.	15.6
	10	0.18	1.8	3.9	N.D.	11.7
	10	0.23	1.03	3.5	N.D.	13.3

The results of Expt. 1 (Table 1) show that β-carotene, astaxanthin and canthaxanthin produce a pronounced increase in the vitamin A content of the fish, as measured by the Carr-Price reaction at 620 mμ. Readings at 693 mμ indicate slight increases in vitamin A₂ as well, but in the absence of fuller characterization no definite conclusion in this respect appears possible. In this and other feeding experiments the vitamin A fraction investigated was free of carotenoids. Feeding with anhydrolutein produced a slight increase in vitamin A₂, but not in vitamin A₁.

The results of Expt. 2, involving guppies, are presented in Table 2. As in Expt. 1, canthaxanthin causes a considerable increase in the vitamin A₁ content. Similarly, β-carotene and isozeaxanthin act as provitamins A in these fish, whereas lutein is without activity. Relatively slight increases in vitamin A₂ seem to accompany the formation of vitamin A₁. Feeding with anhydrolutein results in a clear increase in the blue colour readings at 693 mμ, in accordance with the provitamin A₂ character of this carotenoid. Again, no significant increase in vitamin A₁ is found.

Feeding with β-carotene caused a considerable amount of 'carotene' to be deposited in both species of fish, particularly in *L. reticulatus* (Table 2). This may be due, to some extent, to the intestinal contents, which presumably contain the dietary carotenoid. But feeding with other carotenoids seemed to increase the 'carotene' content of the fish

as well. This was true for *X. variatus* receiving canthaxanthin (Table 1) and for *L. reticulatus* being supplied canthaxanthin and isozeaxanthin (Table 2). The fish 'carotene', on rechromatography on columns of magnesia, calcium hydroxide and alumina, yielded a single band that coincided with that of authentic β-carotene. The pigment had maxima at 450 and 478 mμ in ethanol and an inflexion at 425 mμ.

In Expt. 3, canthaxanthin and anhydrolutein were fed to guppies, whereas carotenoid-free *Daphnia* served as the control diet. The canthaxanthin-fed group comprised 48 guppies with an average weight of 0.21 g., the anhydrolutein-fed group included 46 fish weighing 0.19 g. on the average, and the control group was made up of 28 fish with an average weight of 0.24 g. After chromatography of the unsaponifiable extracts, as described in the Methods section, the ultraviolet-absorption spectra of the vitamin A fractions were determined and are presented in Fig. 1.

Conversion of canthaxanthin into vitamin A₁ is indicated by the marked increase in light-absorption in the 325 mμ region. The absorption curve obtained from the anhydrolutein-fed group had a weak peak in the same region. The only indication for the possible presence of vitamin A₂ was the broader shape of the peak decreasing more slowly toward the visible region. No specific absorption occurred in the control group.

Thin-layer chromatography of the extract from

Fig. 1. Ultraviolet-absorption spectra in ethanol of the vitamin A fractions from guppies receiving astaxanthin (○), anhydrolutein (●) and no supplement (control) (Δ). The concentration was vitamin A fraction from 0.5 g. of fish/ml.

Fig. 2. Absorption spectra of the Carr-Price reaction products given by the vitamin A fractions from guppies. —, Canthaxanthin-fed group (vitamin A fraction from 0.75 g. of fish/3 ml. of reaction mixture); — —, anhydrolutein-fed group (vitamin A fraction from 2.87 g. of fish/3 ml.); — · —, control group (vitamin A fraction from 2.80 g. of fish/ml.); · · ·, control group (vitamin A fraction from 0.75 g. of fish/ml.). Curves for the control group were obtained at two concentrations for purposes of comparison with the canthaxanthin-fed and anhydrolutein-fed groups.

the canthaxanthin-fed group revealed one spot corresponding to vitamin A₁ (R_f 0.44). Attempts to detect vitamin A₂ in the extract from the anhydrolutein-fed group by the same method were unsuccessful.

The absorption spectra of the fish extracts after application of the Carr-Price reaction are presented in Fig. 2. A typical vitamin A₁ spectrum, with a peak at 620 mμ, is obtained from the canthaxanthin-fed group. No indication of the presence of vitamin A₂ is apparent. The spectrum from the anhydrolutein-fed group exhibits two peaks at 627 and 687 mμ, which may be taken as evidence that both vitamins A₁ and A₂ are present. Compared with the carotenoid-fed groups, the control group yields a much weaker absorption over the range tested.

Further identification of the vitamin A formed from canthaxanthin was obtained by its conversion into anhydrovitamin A. The difference spectrum corresponding to this reaction is presented in Fig. 3. It is identical with that obtained from authentic vitamin A (Budowski & Bondi, 1957), with maxima at 399.5 and 378 mμ and a minimum at 389 mμ. There is an inflexion at 360–365 mμ. Below 246.5 mμ, the difference in absorption between anhydrovitamin A and vitamin A becomes negative.

As in the previous feeding experiments, the canthaxanthin-fed group yielded a 'carotene' fraction, whereas the corresponding chromatographic fraction obtained from the control group was colourless. The 'carotene' fraction was carefully rechromatographed on magnesia and alumina. The absorption spectrum in hexane is presented in

Fig. 3. Difference spectrum of the dehydration reaction for the vitamin A fraction obtained from guppies receiving canthaxanthin. The concentration was vitamin A fraction from 2.5 g. of fish/5 ml. of reaction mixture.

Fig. 4. Absorption spectrum in *n*-hexane of the 'carotene' fractions obtained from guppies receiving canthaxanthin (O). The concentration was 'carotene' fraction from 2.6g. of fish/ml. The lower curve (●) was obtained from authentic all-*trans*- β -carotene (2.29 μ g/ml.) under the same conditions.

Fig. 4, together with that of authentic β -carotene similarly chromatographed. Maxima were found at 451 and 478 $m\mu$, and a shoulder at about 420 $m\mu$. In ethanol, the same maxima were found, but the peaks were less sharp. Further confirmation of the identity of this pigment was obtained by thin-layer chromatography. Crystalline β -carotene (all-*trans*) and the fish carotene gave the same R_F , namely 0.50, and a mixture of the two pigments gave a single spot. On the basis of its absorption spectrum and chromatographic behaviour, the fish pigment was identified as β -carotene.

DISCUSSION

In the first part of this work, examination of the carotenoids of two plankton species quantitatively important as natural fish food revealed the presence of β -carotene and crytoxanthin ester in *Chironomus* larvae, whereas *Daphnia magna* contained, in addition to a little β -carotene, mainly 4-oxocarotenoids. No provitamins A₂, characterized by the presence of a 3,4-dehydro- β -ionone ring, were detected. Unlike most plant carotenoids, such compounds exhibit an absorption peak devoid of fine structure (Zechmeister, 1962). Treatment with sodium borohydride produces no change in the absorption spectrum, in contrast with the behaviour of 4-oxocarotenoids, which also exhibit a single absorption peak in the visible region, but are reduced by borohydride to 4-hydroxy compounds with fine-structured absorption spectra.

The role of 4-oxocarotenoids as precursors of vitamin A is revealed in the second part of this study. Astaxanthin, canthaxanthin, as well as isoeaxanthin and β -carotene, increase the vitamin A content in one or both of the species of fish investigated, compared with the control groups receiving unsupplemented food. Lutein was com-

pletely inactive. Thus there is no support for the hypothesis (Budowski *et al.* 1963) that lutein might conceivably undergo biological dehydration to yield anhydrolutein, a provitamin A₂.

In Expt. 3 evidence is provided showing that the vitamin A formed in guppies (*L. reticulatus*) on feeding with canthaxanthin is essentially vitamin A₁. This is shown by thin-layer chromatography, by the increase in the absorption peak at 325 $m\mu$, by the formation of a pronounced absorption peak at 620 $m\mu$ after application of the Carr-Price reaction, and by the conversion of vitamin A into anhydro-vitamin A, yielding a characteristic difference spectrum (Budowski & Bondi, 1957). The last-named reaction is the most specific one known for vitamin A₁. The ultraviolet-absorption curve and the absorption spectrum of the antimony trichloride reaction product obtained from the control group under the same conditions indicated that very little vitamin A₂ was present in that group, in confirmation of the results obtained in Expts. 1 and 2.

Whether some vitamin A₂ formation accompanies the production of vitamin A₁ cannot be ascertained unambiguously from the results obtained. The increase in the Carr-Price readings in the 690 $m\mu$ region indicates that some vitamin A₂ is probably being formed: at this wavelength vitamin A₁ does not interfere, and the absence of carotenoids from the vitamin A fractions also eliminates any possible interference from this source.

Anhydrolutein, which was less effective as a vitamin A precursor in the fish tested, did, however, form vitamin A₂, together with some vitamin A₁, as shown in Fig. 2 by the two absorption peaks at 627 and 687 $m\mu$ of the Carr-Price reaction product. In Expts. 1 and 2 formation of vitamin A₂ from anhydrolutein was also clearly indicated by the Carr-Price readings at 693 $m\mu$, though formation of vitamin A₁ was not evident. Detection of vitamin A₂ by thin-layer chromatography was unsuccessful, presumably because of the low vitamin concentrations and the accompanying impurities.

The formation of β -carotene from astaxanthin, canthaxanthin and isoeaxanthin, revealed in the present investigation, is of particular interest: vitamin A and β -carotene were detected in vitamin A-depleted *Gambusia holbrooki* after administration of astaxanthin (Grangaud *et al.* 1957). According to these authors, the probable steps leading from astaxanthin to vitamins A₁ and A₂ are as follows: (a) reduction of astaxanthin to β -carotene; (b) conversion of β -carotene into vitamin A₁ (or retinal); (c) dehydrogenation of vitamin A₁ to A₂ (or of retinal to 3,4-dehydroretinal).

Our evidence for the presence of β -carotene and the provitamin A properties of canthaxanthin and isoeaxanthin provide strong support for such a scheme. Moreover, we suggest the reaction mechan-

Scheme 1. Suggested mechanism for the formation of retinal and 3,4-dehydroretinal from carotenoids in fish.

ism shown in Scheme 1 at C-3 and C-4 of the β -ionone ring. This Scheme postulates the existence of an intermediate 3,4-dehydro- β -ionone structure in the reaction sequence through which oxygen is removed from C-4. This compound may provide a means by which the relative proportion of vitamins A₁ and A₂ is regulated, since it may also undergo direct conversion into 3,4-dehydroretinol, the main site being presumably the intestinal mucosa. Thus dietary anhydrolutein, a representative of the group of 3,4-dehydro compounds, yielded mainly vitamin A₂. (The relatively low activity of this carotenoid may well be due to the fact that it contains only one provitamin A moiety, in contrast with the symmetrical provitamins A.)

Scheme 1 includes an alternative path for the formation of 3,4-dehydroretinal, involving desaturation of retinal. The latter process has been postulated to occur in the visual pigments of some fish (Dartnall, Lander & Munz, 1961). Naito & Wilt (1962), working with labelled compounds, adduced direct evidence for the conversion of vitamin A₁ into 3,4-dehydroretinal in fish eyes. Such conversions are, however, difficult to demonstrate by feeding experiments with preformed vitamins A, especially when massive doses are supplied, because storage of the dietary type of vitamin A usually masks any interconversions that may take place. Thus Morcos & Salah (1951) reported that two species of Nile fish, which normally contain vitamin A₂, stored only vitamin A₁ on prolonged feeding of the latter. Our own (unpublished) feeding trials with guppies, platies and rosy barbs (*Barbus conchoniensis*) receiving preformed vitamins A₁ and A₂ have confirmed the storage of the dietary type of vitamin A, but evidence for some slight conversion of vitamin A₁ into A₂ was also found.

Clearly, much additional work is required to substantiate the proposed mechanism of conversion of carotenoids into vitamins A. There is no doubt, however, about the significance of the main finding of the present investigation, namely that 4-oxo-carotenoids, such as astaxanthin and canthaxanthin, which abound in lower aquatic life forms, are readily utilized as provitamins A, at least by some fish species.

Note added in proof. Dartnall *et al.* (1961) and Bridges (1965) have shown that the ratio of vitamin A₁- and A₂-based photoreceptors in certain fish is influenced by the length of the daily illumination period. We have not found any such effect on the vitamin A content of fish: repetition of Expt. 3, modified to include extreme light regimes, showed that guppies kept under bright illumination, and those kept in the dark, converted canthaxanthin into retinol to the same extent, without appreciable vitamin A₂ formation. This, however, does not exclude the possibility of a shift in the relative proportion of the corresponding visual pigments.

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