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SELECTIVE UTILIZATION OF SERUM VITAMIN A FOR VISUAL PIGMENT SYNTHESIS

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Summary

Two groups of goldfish (*Carassius auratus*) were subjected to light and temperature conditions known to promote a contrast in their scotopic visual pigment compositions. After 3 weeks, the porphyropsin/rhodopsin ratio in the neuroretina of these goldfish ranged from 99 % porphyropsin in one group to 59 % in the other. Samples of blood, liver and retinal pigment epithelium (RPE) were also removed from these animals and analysed by high-performance liquid chromatography (HPLC) for vitamin A composition. There was consistently more vitamin A_2 than vitamin A_1 (over 50 % vitamin A_2) in both vitamin A alcohol and vitamin A esters extracted from the liver and the RPE. In contrast, only 30 % of all vitamin A extracted from the blood was vitamin A_2. These observations suggest that it is mainly vitamin A_1 that is transported in the blood, whereas vitamin A_2 is selectively retained in the liver and in the RPE and used to form porphyropsin in the eye.

Introduction

Goldfish (*Carassius auratus*) possess a pair of scotopic visual pigments: rhodopsin (opsin conjugated to vitamin A_1 aldehyde) and porphyropsin (opsin conjugated to vitamin A_2 aldehyde) (Tsin & Beatty, 1978, 1979; Tsin *et al.* 1981; Tsin & Santos, 1985; Tsin & Flores, 1986). (Vitamin A is used collectively to represent all vitamin A_1 and A_2 compounds. Vitamin A_1 aldehyde = retinal; vitamin A_1 alcohol = retinol; vitamin A_1 esters = retinyl esters; vitamin A_2 aldehyde = 3,4-didehydroretinal; vitamin A_2 alcohol = 3,4-didehydroretinol; vitamin A_2 esters = 3,4-didehydroretinyl esters.) The relative proportions of these two visual pigments in the goldfish neuroretina are dependent on temperature and light (intensity and photoperiod) and can be altered by exogenous thyroxine (Tsin & Beatty, 1978, 1979; Tsin *et al.* 1981; Tsin & Flores, 1986). The mechanism underlying these environmental influences on visual pigments is not known at present (Suzuki *et al.* 1985; Tsin *et al.* 1985; Tsin & Flores, 1986; Tsin, 1986*a*,*b*; Tsin & Chambers, 1988).

Key words: visual pigments, vitamins A_1 and A_2, goldfish.

In studying the biochemical basis for changes in the proportions of these two visual pigments in the goldfish neuroretina, the vitamin A composition (vitamin A_1/A_2 ratio) in the retinal pigment epithelium (RPE; which is located adjacent to the retina) was measured by HPLC (Tsin *et al.* 1983, 1986*b*). These earlier studies show that the proportions of vitamin A in the RPE are not reflected in the proportions of the two scotopic visual pigments (rhodopsin and porphyropsin), suggesting that the stored vitamin A in the RPE does not determine the rhodopsin/porphyropsin ratio in the neuroretina.

An important issue yet to be resolved is the composition of vitamin A in the blood and its relationship to the visual pigments in the eye. Exactly how plasma vitamin A enters the RPE and participates in the visual cycle is not understood (Bok, 1985). However, it has been reported that vitamins A_1 and A_2 are both transported by serum retinol-binding protein (Shidoji & Muto, 1977) which binds with RPE cells to deliver vitamin A to the eye (Bok, 1985). Is it possible that the proportion of retinol and 3,4-didehydroretinol in the blood which supplies vitamin A to the eye for visual pigment formation determines the composition of visual pigments?

In the present study, HPLC and spectrophotometry were employed to measure vitamin A levels in the blood, RPE and liver as well as visual pigments in the retina. Our results suggest that the mechanism regulating changes in the proportion of the two visual pigments may not be related to a selective transport of vitamin A in the blood. Instead, it is possible that visual pigment changes are regulated by biochemical processes located in the neuroretina and the RPE.

Materials and methods

Light and temperature control

Goldfish were purchased from Grassyfork Fisheries (Martinsville, Indiana; 10–12·5 cm; common and comets). Upon arrival, one group of goldfish was housed in a rectangular aquarium at 30°C under dim light (16 h:8 h L:D cycle; from a 7·5 W light bulb; see Tsin & Beatty, 1979, for the light intensity) to induce rhodopsin. Another group was subjected to a similar light regimen at 20°C to ensure pure porphyropsin. Animals were killed after 3 weeks of light and temperature treatments. During the experiment, animals were fed daily with TetraFin goldfish food (Echo Products, Ortonville, Michigan).

Analysis of visual pigments and vitamin A

All procedures involving retinal tissue were carried out in a dark room using a dim red light. The eyes of an individual were removed following overnight dark adaptation. Retinae were dissected and combined in cold 4% alum. The preparation was centrifuged slowly, the supernatant buffer discarded, and the remaining tissue rinsed twice with cold distilled water. 900 μl of 2% digitonin in phosphate buffer (pH 7) was added to the retinal tissue which was homogenized in a glass–glass homogenizer. The mixture was then incubated at 4°C for 1 h,

followed by centrifugation at 12000 *g* (SS34, for 30 min). 100 μl of 0·1 mol l^{-1} hydroxylamine (neutralized with NaOH) was added to the supernatant before the absorbance was recorded (at 700–300 nm) in a spectrophotometer. The sample was then exposed to room light (bleached) for 10 min and a subsequent absorbance curve was recorded. The difference in the absorbance of the pigment extract before and after bleaching was then calculated by an Apple computer to derive a difference spectrum (see Fig. 1B). The amount of visual pigment present in the retinal extract and the rhodopsin/porphyropsin ratio were calculated from the difference spectrum according to a method described earlier (Munz & Beatty, 1965; Tsin & Beatty, 1978).

The retinal pigment epithelium/choroid (RPE) and liver were also removed and dehydrated using anhydrous sodium sulphate. Vitamin A in these tissues was extracted with acetone. Acetone was removed with a stream of nitrogen and the sample redissolved in 1 ml of 0·5 % dioxane in *n*-hexane. Each sample was introduced into a 5 % water-deactivated alumina column (1 cm × 2 cm) from which vitamin A esters (eluting with 10 ml of 0·5 % dioxane in *n*-hexane) and alcohols (eluting with 10 ml of 10 % dioxane in *n*-hexane) were collected. These vitamin A fractions were then analysed isocratically by HPLC (Tsin *et al.* 1984, 1985). Vitamin A esters were saponified according to the method of Bridges & Alvarez (1982) before HPLC analysis. Vitamin A was quantified in HPLC by comparing the peak area to that of known standards.

Blood samples were removed from caudal vessels in a 2 ml syringe fitted with a 22 gauge needle and centrifuged for 1 min to separate cells from the plasma. 100 μl of water was added to 100 μl of plasma before 200 μl of ethanol was added to denature plasma proteins, including retinol-binding protein. Vitamin A was extracted in three portions of 800 μl of petroleum ether and the vitamin A extract was then purified on a 5 % water-deactivated alumina column (similar to the method stated above) prior to HPLC analysis.

Results

In agreement with earlier reports (Tsin & Beatty, 1978, 1979; Tsin *et al.* 1981; Tsin & Flores, 1986), the present study shows that goldfish responded to temperature treatment by altering the ratio of the two visual pigments in their neuroretinas. Upon arrival from the supplier, goldfish had pure porphyropsin in their eyes. After 3 weeks, the neuroretinas of those animals subjected to the lower temperature (20°C) remained porphyropsin-rich (98·5 % porphyropsin) whereas those subjected to the higher temperature (30°C) contained a higher proportion of rhodopsin (58·7 % porphyropsin). Fig. 1 shows an example of goldfish photopigment extracted from the retina of fish held at 30°C. The difference spectrum (Fig. 1B), which was derived from the absorbance spectra obtained before and after bleaching (Fig. 1A), was similar to that of a mixture of rhodopsin and porphyropsin with the absorbance maximum (510 nm) located between 499 nm (pure rhodopsin) and 522 nm (pure porphyropsin) (Tsin & Beatty, 1978, 1979).

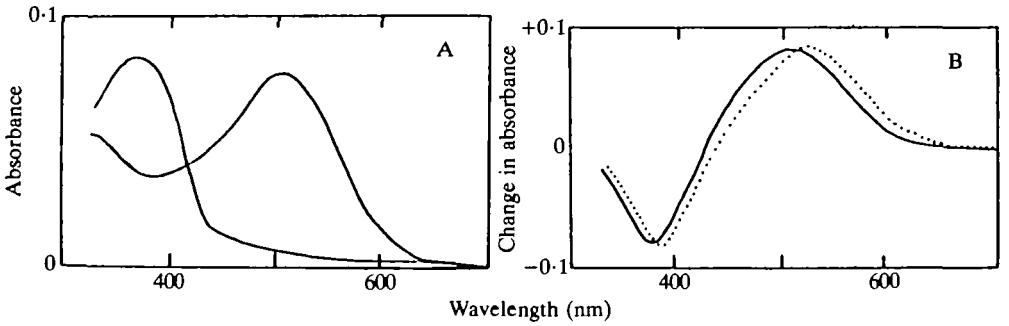

Fig. 1. Absorbance (A) and difference (B) spectra of visual pigment extract prepared from goldfish subjected to 16 h:8 h L:D (30°C) for 3 weeks. The difference spectrum (maximum absorbance at 510 nm) was calculated by an Apple computer from absorbance spectra obtained before and after light bleaching. This sample contained 4 nmol of visual pigments and 57·1 % porphyropsin. For comparison, the dotted line in B represents the difference spectrum of visual pigment (100 % porphyropsin; maximum absorbance 522 nm) extracted from goldfish held in water at a lower temperature (20°C).

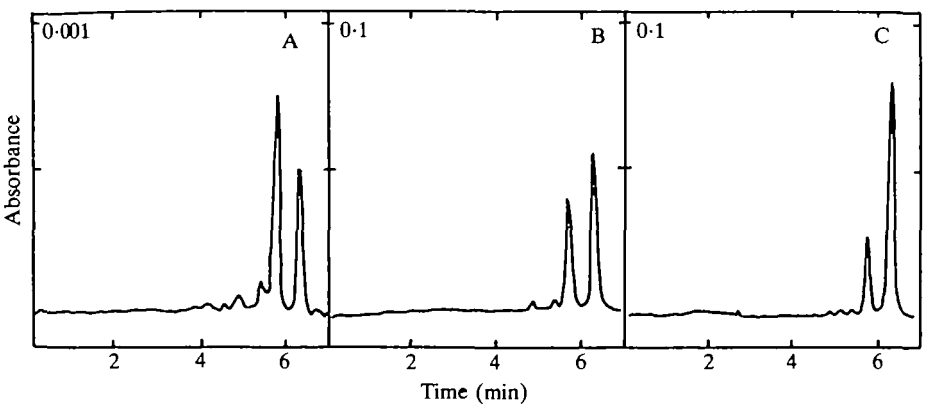

Fig. 2. HPLC elution profiles of retinol and 3,4-didehydroretinol extracted from blood (A), liver (B) and retinal pigment epithelium (RPE; C) from goldfish subjected to 16 h:8 h L:D (20°C) for 3 weeks. The column was Water's Resolve spherical silica (5 μm, 46 mm × 150 mm). The eluant was 10 % dioxane in *n*-hexane at 1 ml min^{-1}. Detection was at 340 nm. The retention time of retinol was 5·7 min, and that of 3,4-didehydroretinol was 6·3 min. There was 20·7 % 3,4-didehydroretinol in the blood (A), 53·5 % in the liver (B) and 70·9 % in the RPE (C).

The HPLC elution of vitamin A extracted from the goldfish contained only two major components: all-*trans* retinol (vitamin A_1 alcohol) and all-*trans* 3,4-didehydroretinol (vitamin A_2 alcohol). The identity of these vitamins A was confirmed by their absorbance spectra and by comparison of their elution characteristics with authentic retinol and 3,4-didehydroretinol. Fig. 2 shows

Table 1. *Composition of vitamin A_1 and A_2 in blood, liver and retinal pigment epithelium (RPE)*

	Percentage of vitamin A as A_2			
	20°C		30°C	
	Vitamin A alcohols	Vitamin A esters	Vitamin A alcohols	Vitamin A esters
Blood	30·3 ± 4·5 (N = 8)		32·9 ± 7·4 (N = 6)	
Liver	56·6 ± 5·9 (N = 9)	52·1 ± 1·9 (N = 10)	75·9 ± 3·7 (N = 5)	62·8 ± 1·5 (N = 11)
RPE	70·9 ± 7·8 (N = 5)	64·1 ± 4·7 (N = 6)	62·8 ± 9·3 (N = 8)	60·9 ± 2·0 (N = 11)

The mean and standard error (number of determinations) are indicated.

Goldfish held at the lower temperature (20°C) had 98·5 ± 0·3 % porphyropsin (N = 10) whereas those held at the higher temperature (30°C) had only 58·7 ± 3·1 % porphyropsin (N = 11).

All goldfish were maintained under a 16 h:8 h L:D regime.

Total amount of vitamin A in the blood: 0·46 nmol per ml plasma; in the liver: 34·5 nmol (alcohol/ester = 0·16); in the RPE: 0·99 nmol (alcohol/ester = 0·04).

typical HPLC elution profiles of retinols extracted from the blood, liver and RPE of goldfish held at 20°C. In the normal phase adsorption chromatography employed in our study, retinol eluted from the HPLC within 5·7 min whereas 3,4-didehydroretinol had a slightly longer retention time of 6·3 min (Fig. 2). The level of retinol exceeded that of 3,4-didehydroretinol in all blood samples (Fig. 2A; 20·7 % 3,4-didehydroretinol), whereas the reverse was observed in liver and RPE samples (Fig. 2B,C).

Table 1 summarizes vitamin A_1 and A_2 compositions in the blood, liver and RPE. Under both light and temperature regimens, the goldfish possessed mainly vitamin A_1 alcohol (30 % and 33 % as vitamin A_2) in the blood and no significant difference (Student's t-test; $P \leq 0{\cdot}05$) was noted in the vitamin A composition between the two groups. In contrast, the percentage of vitamin A_2 (52–76 %) in liver and RPE was significantly higher (by about 20–50 %) than in the blood. Although the percentage of vitamin A_2 in the RPE appeared to be slightly higher than that in the liver in fish held at 20°C, this relationship was not noted in fish held at 30°C.

Discussion

This study is one of the few on the vitamin A composition of fish blood. The level of total vitamin A in the goldfish blood estimated in our HPLC analysis (46 pmol 100 μl^{-1} of plasma or 13 μg 100 ml^{-1}) agrees well with published data for carp (8·6 μg 100 ml^{-1}; Field *et al.* 1943), trout (17·5 μg 100 ml^{-1}; Field *et al.* 1943) and cod (3–12 μg 100 ml^{-1}; Plack & Woodhead, 1966). It is lower than the

accepted value of blood retinol in humans (40 μg 100 ml^{-1}; Thompson *et al.* 1971; Neeld & Pearson, 1963; Drujan, 1971).

In experiments involving feeding vitamin A_2 aldehyde to laboratory rats, John *et al.* (1966) reported that the level of vitamin A_2 in the rat blood increased in proportion to the level of vitamin A_2 esters in the liver. However, there is no evidence that the blood level of vitamin A_2 in the goldfish in the present study was influenced by ingested vitamin A_2 aldehyde, since the diet did not contain any vitamin A_2 (unpublished observations). Plack & Woodhead (1966) showed that cod had approximately 20–40 % vitamin A_2 aldehyde in their blood and they estimated that the A_2 form of the vitamin constituted about 15 % of total vitamin A alcohols and vitamin A esters. However, in a comparative study on vitamin A in the blood of non-mammalian vertebrates, Shidoji & Muto (1977) showed that only cyclostomes, such as the lampreys, contain vitamin A esters in their blood, and there was no evidence that other species (from teleosts such as carp and yellowtail to mammals) contain any forms of vitamin A other than retinol and 3,4-didehydroretinol. These studies therefore pose unresolved questions on vitamin A transport in fishes. In this study, we have shown that vitamin A_2 constituted approximately 30 % of the vitamin A alcohols in goldfish blood, and this proportion does not change with temperature. In goldfish blood, we have not investigated any vitamin A components other than retinol and 3,4-didehydroretinol.

In mammals, vitamin A mobilization from the liver and its delivery to the target tissue *via* the blood are highly regulated processes (Goodman & Blaner, 1984). Vitamin A esters in the liver are first hydrolysed by hepatic retinyl ester hydrolase (Tsin *et al.* 1986*a*), and vitamin A alcohol is bound to serum retinol-binding protein (Goodman, 1984) and then transported in the circulation to the basal surface of the RPE where the vitamin A is released (Bok, 1985). Upon entry into the RPE, the vitamin A is esterified (Berman *et al.* 1980; Tsin, 1986*a*,*b*) and stored mainly as vitamin A esters (Lai *et al.* 1985), which later undergo hydrolysis (Blaner *et al.* 1987; Tsin & Lam, 1986) to supply vitamin A for visual pigment synthesis.

In lower vertebrates, where vitamin A_1 and A_2 co-exist, events associated with the supply of vitamin A_1 and A_2 to form visual pigments are far less well understood. The present study shows that vitamin A_1 and A_2 are both found in the liver and the blood, yet the proportion of vitamin A_1 to A_2 differs significantly between these compartments (see Table 1). It is unlikely that the carrier protein in the blood selectively favours the transport of retinol rather than 3,4-didehydroretinol because both vitamins have a similar affinity for the serum retinol-binding protein (Shidoji & Muto, 1971). One possibility is that a selective hydrolysis of vitamin A esters may take place in the liver, resulting in the release of a higher proportion of vitamin A_1. Alternatively, if the eye were an important site of vitamin A_2 synthesis (Naito & Wilt, 1962; Tsin *et al.* 1985) or utilization (Shantz *et al.* 1946), then we would expect to find a significantly higher proportion of vitamin A_2 in the RPE (Table 1). It is clear, however, that the percentage of vitamin A_2 in different body compartments (liver, blood, RPE) does not reflect

the photopigment composition in the retina (Table 1). This suggests that the formation of visual pigment in the retina is not dependent on a simple equimolar transfer of available circulating vitamin A to serve as chromophores for opsin. This suggestion is further substantiated by the observations (Yoshikami *et al.* 1969; Pearlman & Crescitelli, 1971; John *et al.* 1966) that feeding rats with vitamin A_2 and increasing the vitamin A_2 level in the blood did not lead to the formation of porphyropsin in the retina. However, Suzuki & Miyata (1988), using mice injected with vitamin A_2, found high proportions of vitamin A_2 in the liver, blood and neuroretina.

In conclusion, the present study clearly shows that environmental light and temperature exerted a significant influence on the proportions of rhodopsin and porphyropsin in the goldfish neuroretina. Moreover, temperature did not change the visual pigment ratios *via* an alteration of the relative availability of vitamin A_1 and A_2 in the blood. Other body compartments such as liver and retinal pigment epithelium (RPE) also stored vitamin A_1 and A_2 in proportions that seem unrelated to the scotopic visual pigment ratios in the eye. The mechanism of visual pigment changes therefore may lie within the tissue where visual pigments reside, the retina itself. Additional work is currently under way to explore further this process of photopigment conversion in the vertebrate neuroretina (Suzuki *et al.* 1985; Tsin & Chambers, 1988).

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