THE *IN VIVO* REGENERATION OF GOLDFISH RHODOPSIN AND PORPHYROPSIN

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SUMMARY

Goldfish with retinas rich in either rhodopsin or porphyropsin were illuminated with bright light and then placed in the dark room to allow visual pigment regeneration. The kinetics of this *in vivo* pigment regeneration were followed by sampling these animals at regular time intervals. The first-order kinetic rate constant for the initial period of porphyropsin regeneration at 20°C was $8\cdot3\times10^{-3}$ nmol kg^{-1} body weight min^{-1} and the half-life of this reaction was 83 min. At 30°C, the rate constant was increased to $1\cdot4\times10^{-2}$ nmol kg^{-1} body weight min^{-1}, yielding a reduced half-life of 49 min. This suggests that the Q_{10} of porphyropsin regeneration is about $1\cdot7$. In goldfish retinas enriched with rhodopsin (62% rhodopsin and 38% porphyropsin), the initial phase of visual pigment regeneration (at 30°C) proceeded at a slower rate (first-order rate constant: $6\cdot5\times10^{-3}$ nmol kg^{-1} body weight min^{-1}; half-life of reaction = 106 min) than the rate of porphyropsin regeneration. This suggests that the high proportion of rhodopsin in the retina of goldfish held at 30°C is not a direct result of a faster rate of regeneration of rhodopsin than of porphyropsin.

INTRODUCTION

Rhodopsin and porphyropsin are visual pigments possessing different spectral properties such as absorption maxima and extinction coefficients (Knowles & Dartnall, 1977). Many vertebrates possess a mixture of rhodopsin and porphyropsin and the composition of these pigments in their eyes may change in response to light and temperature treatments and to other environmental stimuli (Tsin & Beatty, 1979, 1980). This change in the rhodopsin/porphyropsin ratio is associated with an alteration of the visual sensitivity and this may serve as a mechanism of visual adaptation to the environment (especially in migratory fishes existing in both marine and freshwater environments, Lythgoe, 1984). Therefore it is important to understand the biochemical mechanism(s) underlying visual pigment changes in animals.

Goldfish possess both rhodopsin and porphyropsin in their retinas and the proportion of these pigments responds to an alteration of the temperature and light regimen to which the animal is subjected (Tsin & Beatty, 1979). Higher temperatures (30°C or above) and a 16 h light : 8 h dark photoperiod, in combination with low

light intensity (from a 7·5 W bulb) favour a high rhodopsin proportion (up to 90 % or more). Other combinations of light and temperature treatment generally result in higher porphyropsin proportions in the retina (see Tsin & Beatty, 1979, for details). The mechanism of this visual pigment change is not known.

From the result of some *in vitro* experiments, Suzuki, Makino-Tasaka & Miyata (1985) suggested that rhodopsin is selectively formed in the retina as a result of its faster rate of regeneration (when opsin is provided with 11-*cis* retinal) than the regeneration of porphyropsin (when opsin is provided with 11-*cis* 3,4-didehydro-retinal). They hypothesized that visual pigment composition in the retina is determined by the difference of these rates of pigment regeneration (Suzuki *et al.* 1985; Makino-Tasaka & Suzuki, 1984). However, the nature of visual pigment regeneration *in vitro* is different from that carried out in intact animals (*in vivo* experiments). The kinetics of *in vitro* regeneration of rhodopsin or porphyropsin follow that of a second-order reaction based on the available concentrations of both opsin and retinal (Hubbard & Wald, 1952; Suzuki *et al.* 1985). The rate of the *in vivo* regeneration follows the kinetics of either a first-order (Peskin, 1942; Dowling, 1960; Rushton, 1961; Reuter, 1966; Donner & Reuter, 1967; Baumann, 1972) or a zero-order reaction (Lewis, 1957; Ripps, Mehaffey & Siegel, 1981) and the rate limiting step for these reactions has not been fully established. Therefore, it is inappropriate to extend the result of *in vitro* studies to explain the more complex condition of visual pigment regeneration in the intact animal. In the present study, we used *in vivo* experiments to determine that at 30°C, rhodopsin was regenerated *slower* than porphyropsin in the goldfish retina. This suggests that the higher proportion of rhodopsin associated with the retina of the goldfish held at 30°C is *not* the result of a faster rate of rhodopsin regeneration. The mechanism that favours the formation of rhodopsin at this water temperature remains to be determined.

MATERIALS AND METHODS

Goldfish (*Carassius auratus*, about 25 g body weight) were obtained from Grassyfork Fisheries (Martinsville, IN, USA). They were held in 120-l aquaria either at room temperature (20°C) or at 30°C (attained using a 150-W aquarium heater wrapped with aluminium foil to prevent emission of light) under room light illumination for 2 weeks before use. To induce rhodopsin in the retina, one group of goldfish was held at 30°C under reduced illumination for 30 days (light came from a 7·5-W light bulb located about 60 cm from the water surface; the light bulb was connected to a timer to give a 16 h light and 8 h dark photoperiod; the spectral irradiance was $6·0 \times 10^{13}$ photons cm^{-2} s^{-1}, 400–750 nm, see Tsin & Beatty, 1979).

Our *in vivo* experiments on visual pigment regeneration followed the method described by Baumann (1971). Goldfish were first subjected to bright illumination to bleach a significant portion of their scotopic pigments (two 300-W, two 100-W, one 150-W bulbs; fish were held in a 120-l aquarium). They were then placed in the dark room and sampled at regular time intervals for the amount of visual pigment in their retinas.

In experiment 1, 27 goldfish were held at 30°C for 2 weeks before they were exposed to the bleaching light for 1 h. They were then transferred to the dark room and sampled after 0, 30, 60, 90, 120, 180, 240, 300 and 600 min of dark adaptation. Three fish were sampled at each time point and their retinas were pooled for the extraction and analysis of visual pigments (see below). Experiment 2 was carried out at room temperature (20°C) with 36 fish and the procedure was similar to that of the first experiment. From three to six fish were sampled at each time point during the dark adaptation period subsequent to light bleaching. Experiment 3 was carried out at 30°C upon goldfish with rhodopsin dominated retinas (see above). From three to six fish were sampled at regular intervals (0, 30, 60, 120, 180, 300 and 600 min) during the dark adaptation period (subsequent to bleaching) and a total of 36 fish was used in this experiment.

To prevent pigment regeneration during dissection and pigment extraction, hydroxylamine ($0\cdot1$ mol l^{-1}) was added (1:1) to the phosphate buffer ($0\cdot1$ mol l^{-1}, pH = 7) used to store retinal tissues and to prepare the digitonin extract. After enucleation, the retina was quickly dissected out and placed in 3 ml of cold phosphate buffer containing hydroxylamine. Samples were then frozen overnight in light-tight canisters before visual pigment extraction, which followed standard procedures (Tsin & Beatty, 1979). Visual pigment extracts were analysed by a Bausch & Lomb model 2000 spectrophotometer connected to an Apple II plus computer and an Epson FX 80 dot matrix printer. Absorbance values were recorded between 700 and 300 nm before and after bleaching (room light, for 10 min) to derive the difference spectrum (Tsin & Santos, 1985). The relative proportions of rhodopsin and porphyropsin were obtained from template curves previously established (Tsin & Beatty, 1978, 1979). The quantity of pigment was estimated from the maximum density change using the difference spectrum and the molar extinction coefficients of 40 600 (for rhodopsin) and 30 000 (for porphyropsin) (Hubbard, Brown & Bownds, 1971).

To show that the initial rate of regeneration followed the kinetics of a first-order reaction, the logarithm of the amount of visual pigment, adjusted by the body weight of the animal (nanomole of visual pigment per kilogram body weight), was plotted against time. The slope of the line that best fits all points was estimated by the least squares method (Sokal & Rohlf, 1969). The first-order reaction rate constant was calculated as: slope $\times$ $2\cdot303$ (Frost & Pearson, 1961). The half-life was estimated from the time taken to reach 50 % regeneration from the 25 % point. In first-order reactions, the half-life of the reaction is related to the first-order rate constant by this relationship: first order rate constant $\times$ half-life = $0\cdot693$ (Frost & Pearson, 1961) and our results seem to be in good agreement with this equation.

RESULTS

The regeneration of porphyropsin in retinas of goldfish held at 30°C is shown in Fig. 1 (experiment 1). The light exposure bleached only 50 % of the porphyropsin in the retina. Regeneration was slow during the first 30 min but then became faster and was completed within 3 h of dark adaptation. The mechanism that causes the delay is
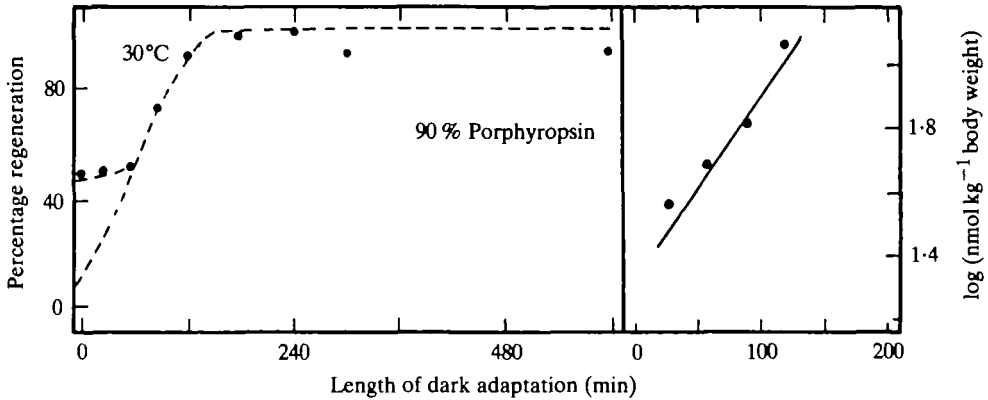
Fig. 1. The *in vivo* regeneration of porphyropsin at 30 °C (experiment 1). Twenty-seven goldfish were held at 30 °C for 2 weeks before use. They were then exposed to a bright illumination for 1 h and transferred to the dark room. At regular time intervals, three animals were sampled for visual pigment in their retinas. The half-life of the regeneration was estimated to be 49 min. The line indicated in the semi-logarithmic plot was calculated by the least squares method. It has an intercept of 1·23 nmol kg^{-1} body weight and a slope of 6·22×10^{-3} nmol kg^{-1} min^{-1}. The first-order rate constant was calculated from the slope of this line as described in Materials and Methods.

not known but similar delays have been reported for the *in vivo* regeneration of visual pigment in other species (Peskin, 1942; Reuter, 1966), including the regeneration of porphyropsin in the crucian carp (*Carassius carassius,* Baumann, 1971). The semi-logarithmic plot of initial time points (30, 60, 90 and 120 min) suggests that the initial rate of the regeneration of porphyropsin proceeded as a first-order reaction with a kinetic rate constant of 1·4×10^{-2} nmol porphyropsin kg^{-1} body weight min^{-1}. The half-life of the reaction was estimated to be 49 min. As the regeneration approached completion (120, 240, 300 and 600 min), the rate of this regeneration exhibited that of a zero-order reaction.

When this experiment was repeated with goldfish held at 20 °C (experiment 2, Fig. 2), a much slower rate of regeneration of porphyropsin was observed. The light exposure bleached about 90 % of the porphyropsin in these retinas and a steady regeneration was observed during subsequent dark adaptation. This regeneration was completed at the fifth hour of dark adaptation. The semi-logarithmic plot shows that the initial rate of this regeneration (30, 60, 90, 120, 180 min) followed a first-order reaction with a kinetic rate constant of 8·3×10^{-3} nmol porphyropsin kg^{-1} body weight min^{-1} and a half-life of 83 min. This suggests that in this temperature range (20–30 °C), the Q_{10} of porphyropsin regeneration is 1·7.

The regeneration of visual pigments in goldfish retinas containing a significant proportion of rhodopsin (62 % rhodopsin; experiment 3, Fig. 3; this experiment was performed at 30 °C) proceeded slower than the regeneration of porphyropsin at the same temperature (experiment 1, Fig. 1). The light exposure bleached about 80 % of the visual pigments in these retinas and the complete regeneration took about 6 h to accomplish. As for the regeneration of porphyropsin at 30 °C (Fig. 1), a similar delay of about 30 min was also observed in this rhodopsin regeneration. The initial rate of

this regeneration also followed the kinetics of a first-order reaction, as revealed by the straight line connecting the initial time points (30, 60, 120 and 180 min) in the semi-logarithmic plot. The first-order reaction rate constant was determined to be $6 \cdot 5 \times 10^{-3}$ nmol visual pigment kg^{-1} body weight min^{-1} and the half-life was estimated as 106 min.

DISCUSSION

The present study constitutes the first report on the *in vivo* regeneration of goldfish porphyropsin. The regeneration of porphyropsin in a similar species, *Carassius carassius* (crucian carp, also a cyprinid) has been reported earlier by Baumann (1971). Although a direct comparison of Baumann's result on the crucian carp to our results on the goldfish is inappropriate due to species and temperature differences, there are similarities between the regeneration of porphyropsin at 20 °C observed in the present study (Fig. 2) and the results obtained by Baumann at 16–17 °C (Baumann, 1971; fig. 2). In both cases, the regeneration of porphyropsin required about 5 h to complete and rates of regeneration of these porphyropsins appeared similar.

The present study shows that the kinetics of rhodopsin and porphyropsin regeneration were complex. In all three experiments, an initial delay ('lag period') of 30–120 min was observed (see Figs 1–3). This was followed by rapid regeneration with kinetics of a first-order reaction. Using the semi-logarithmic plot, a rate constant was derived and used to compare quantitatively the difference of regeneration of three experiments carried out in the present study. After these initial time points, the regeneration of porphyropsin and rhodopsin approached the asymptotic condition of (near) completion. During this period, minimal change of visual pigment was observed over time.
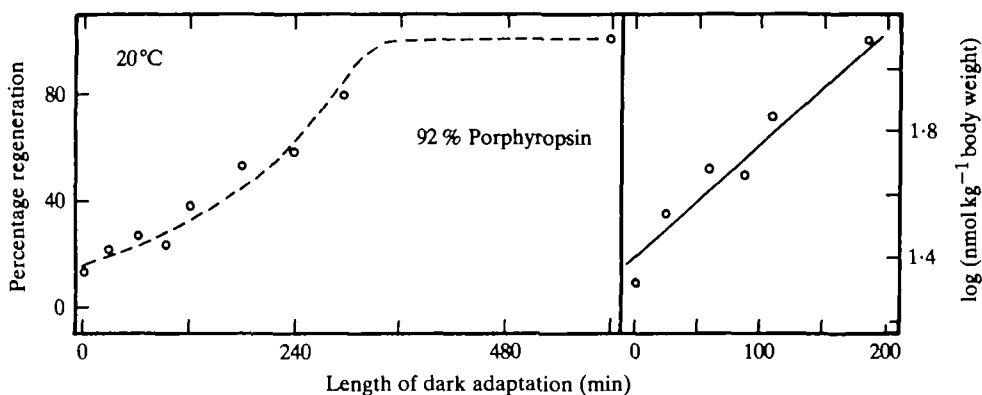

Fig. 2. The *in vivo* regeneration of porphyropsin at 20 °C (experiment 2). This experiment was carried out in the same way as experiment 1 (Fig. 1), except that 36 fish were used and from 3–6 fish were sampled at each time point of dark adaptation. The half-life of regeneration was estimated to be 83 min and the slope and the intercept of the line appearing in the semi-logarithmic plot were calculated by the method of least squares as $3 \cdot 6 \times 10^{-3}$ nmol kg^{-1} min^{-1} and $1 \cdot 37$ nmol kg^{-1}, respectively.
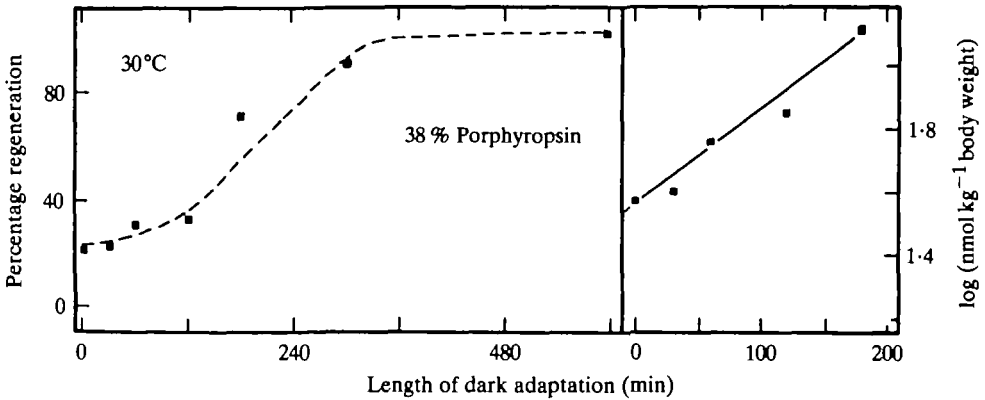
Fig. 3. The *in vivo* regeneration of visual pigments at 30 °C (experiment 3). Thirty-six goldfish were placed at 30 °C in a photoperiod of 16 h light and 8 h dark (under dim light, see Materials and Methods) for 30 days to induce higher concentrations of rhodopsin in their retina. They were then subjected to bleaching and dark adaptation as in experiment 1 (Fig. 1). The half-life of this regeneration was estimated to be 106 min and the slope and the intercept of the line appearing in the semi-logarithmic plot were calculated by the method of least squares as $2 \cdot 8 \times 10^{-3}$ nmol kg^{-1} min^{-1} and $1 \cdot 55$ nmol kg^{-1}, respectively.

There has not been any report in the literature concerning the effect of temperature on the *in vivo* regeneration of porphyropsins, although Suzuki *et al.* (1985) suggested a temperature influence on the regeneration of porphyropsin, *in vitro*. Estimated from fig. 2 of the Suzuki paper, the Q_{10} of porphyropsin regeneration between 10 and 20 °C was $2 \cdot 7$. This is significantly higher than the value of $1 \cdot 7$ reported in the present study. The Q_{10} obtained for porphyropsin in the present study is, however, similar to the Q_{10} of $1 \cdot 8$ for the *in vivo* regeneration of rhodopsin in the frog of 15–20 °C (Peskin, 1942).

We are also the first to report the *in vivo* regeneration of both rhodopsin and porphyropsin in a single species. It is important to point out that in experiment 3 we have only recorded the regeneration of visual pigments in a goldfish retina possessing a significant proportion of rhodopsin, and not pure rhodopsin. However, by comparing results in our experiments 1 and 3, it is possible to reveal the nature of rhodopsin regeneration. The significant presence of rhodopsin resulted in a *slower* rate of regeneration of visual pigment in these retinas. This suggests that higher proportions of rhodopsin associated with the retina of goldfish held at 30 °C cannot be attributable to a higher rate of rhodopsin than porphyropsin regeneration.

Suzuki *et al.* (1985) found that the *in vitro* rate of rhodopsin regeneration is higher than that of porphyropsin. This is not in direct contradiction to the result of our study. It is because the *in vitro* regeneration of visual pigments follows the kinetics of a second-order reaction (based on the concentration of the available opsin and retinal, Hubbard & Wald, 1952) whereas the *in vivo* regeneration does not (Peskin, 1942; Dowling, 1960; Rushton, 1961; Reuter, 1966; Baumann, 1972; Lewis, 1957; Ripps *et al.* 1981; and this study). The rate determining factor of this *in vivo* regeneration has yet to be identified and it may be related to the transport of retinoids between the retina and the pigment epithelium (as suggested by Reuter, 1966) or to

the availability of chromophores, which is in turn related to the rate of hydrolysis of retinyl and 3,4-didehydroretinyl esters (Blaner, Flood, Bilek & Gouras, 1984). Alternatively, the environmental condition favouring rhodopsin synthesis may also (or independently) alter systemic properties of the regeneration of both rhodopsin and porphyropsin, resulting in the slower kinetics observed in experiment 3.

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