RETINOID CHANGES IN THE IN VITRO REGENERATION OF FROG VISUAL PIGMENTS

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SUMMARY
To investigate the regeneration of visual pigment, the changes in composition and quantity of retinoids were measured by high-performance liquid chromatography (HPLC). Eye cups or eye cup sections of dark-adapted frogs were exposed to light (>500 nm) and incubated in the dark for several hours (pH 7.4, 27 ± 1°C). Retinoids extracted by the oxime method before and after illumination were analysed by HPLC.

In the dark, every eye cup or eye cup section contained 11-cis and all-trans isomers of retinyl palmitate (the proportion of 11-cis was nearly 40%). The amount of retinyl palmitate was 1–1.5 mol equiv of visual pigment. After 80% of the visual pigment had been bleached by illumination, eye cups or eye cup sections were incubated in the dark for 10 h. During the incubation, 70% of the bleached pigment was regenerated, and the proportion of 11-cis retinyl ester decreased from 40% to 13%. These results indicate that stored 11-cis retinyl ester is used for the regeneration.

The regeneration rate of A2-pigment (half time = 75 min) was faster than that of A1-pigment (half time = 90 min), consistent with the result of Tsin & Flores’ (1986) in vivo experiment with goldfish.

INTRODUCTION
It is well known that vertebrate rhodopsin can regenerate in the dark in that portion of the retina that is in contact with retinal pigment epithelium (RPE) in the living eye, excised eye or opened eye cup (Peskin, 1942; Reuter, 1964; Bridges, 1973, 1976; Ratzlaff, 1975). In the isolated retina, such a regeneration rarely occurs after large bleaches of rhodopsin (Bauman, 1972; Bridges, 1973; Azuma & Azuma, 1980), but does occur after small bleaches (Donner & Hemila, 1975; Azuma, Azuma & Sickel, 1977). Therefore, the RPE has been considered necessary for regeneration after large bleaches of rhodopsin. In the experiments using retinas in contact with the RPE, analyses of retinoids have been carried out by the Carr–Price method, thin-layer chromatography and high-performance liquid chromatography (HPLC) (Hubbard & Dowling, 1962; Bridges, 1973, 1975, 1976; Zimmerman, 1974; Bridges & Alvarez, 1982). Bridges (1975, 1976) pointed out that frog RPE contained a

Key words: retinoid, visual pigment, regeneration, A1-pigment, A2-pigment, eye cup, HPLC.
considerable amount of 11-cis retinyl ester but it was not used preferentially for the regeneration of rhodopsin. When 11-cis isomers of retinal, retinol and retinyl palmate are exogenously applied to the isolated retina or rod outer segments of the frog, only 11-cis retinal and retinol are utilized for the regeneration but not 11-cis retinyl palmate (Bridges, 1977; Yoshikami & Nöll, 1978; Perlman, Nodes & Pepperberg, 1982; Nöll, 1984). The role of RPE retinyl ester in the regeneration has not yet been elucidated.

Methods have now been developed for the chromophores of visual pigments to be extracted as retinaloximes without thermal isomerization and to be precisely analysed by HPLC (Groenendijk, de Grip & Daemen, 1980; Suzuki & Makino-Tasaka, 1983; Azuma & Azuma, 1984). In the present study, we used these methods to investigate visual pigment regeneration in eye cup sections, by measuring the changes in composition and quantity of retinoids caused by bleaching. This report describes the relationship between the change in amount of retinyl ester and the regeneration of visual pigment. Furthermore, the regeneration rate of vitamin A1-based pigment (A1-pigment) was compared with that of vitamin A2-based pigment (A2-pigment). For this purpose, adult bullfrogs collected in winter and spring were used because these frogs had both A1- and A2-pigments (Reuter, White & Wald, 1971; Makino, Nagai & Suzuki, 1983).

MATERIALS AND METHODS

Preparation of eye cup sections

Adult bullfrogs (Rana catesbeiana) were dark-adapted overnight at room temperature (20-25°C). Under dim red light, eyes were enucleated and hemisected along the equator. Eye cups or eye cup sections (circular sections of 4 mm diameter) were incubated in the dark in physiological solution containing (in mmol l⁻¹): NaCl, 77; KCl, 2.5; MgCl₂, 1.2; NaHCO₃, 25; Na₂SO₄, 0.6; Hepes, 3; glucose, 26; pH 7.4. Some were illuminated for 15 min in yellow light (>500 nm) and incubated in the dark at 27 ± 1°C for various periods.

Extraction of retinoids

Retinal and retinol were extracted by the oxime method, essentially as reported previously (Groenendijk et al. 1980; Suzuki & Makino-Tasaka, 1983; Azuma & Azuma, 1984). The extraction of retinyl ester was carried out according to the method of Bridges & Alvarez (1982). Each eye cup or eye cup section, which was either maintained in the dark or incubated in the dark after illumination, was immersed in a solution of 1 mol⁻¹ NH₂OH (pH 7.2) and methanol (1:2, vol/vol), shaken, and centrifuged at 2500 rev. min⁻¹ for 15 min. The supernatant was mixed with dichloromethane and n-hexane (1:2, vol/vol), and shaken again. After the mixture had stood for several minutes, the upper layer (dichloromethane/hexane layer) was collected. This extraction was repeated three times. The collected solution (called dichloromethane/hexane extract) contained mainly retinaloxime and retinol with a small amount of retinyl ester (see Figs 1A, 2). The precipitate obtained by
centrifugation was ground with anhydrous Na₂SO₄, and then mixed with acetone. The mixture was centrifuged (2500 rev. min⁻¹, 10 min), and the supernatant (acetone layer) was collected. This extraction was repeated three times. The acetone extract included mainly retinyl ester and a residual amount of retinal oxime (see Fig. 1B). The solvents were evaporated under vacuum. After evaporation, the residues were dissolved in 50 μl of n-hexane/diethyl ether/ethanol (90:10:0.3, vol/vol) and analysed by HPLC. All procedures were carried out under dim red light (>640 nm).

Analysis of retinoids

Extracted retinoids were analysed using a JASCO HPLC system equipped with a Finepak SIL column (JASCO, 4.6×250 mm). A mixture of n-hexane/diethyl ether/ethanol (90:10:0.3, vol/vol) was used as the eluant with a flow rate of 0.9 ml min⁻¹. A 10 μl sample of each extract was injected into the column. The absorbance at 350 nm was measured with a JASCO UVIDEC-100-III apparatus and each peak area was determined by integration with a Shimadzu Chromatopac C-R1B. The peak position of each isomer of retinal oxime, retinol and retinyl ester was determined using authentic compounds. The fractions corresponding to retinyl ester were collected (called retinyl ester fraction), evaporated and re-analysed with the eluant n-hexane/diethyl ether (100:1.5, vol/vol), at a flow rate of 1 ml min⁻¹. The absorbance at 325 nm was then measured. The amounts of retinal and retinol isomers were calculated from the HPLC peak areas of retinal oxime and retinol isomers and their absorption coefficients given by Makino et al. (1983) and Azuma & Azuma (1984). For the estimation of the amount of retinyl palmitate, the absorption coefficients of retinol isomers were used, because they were almost the same as those of retinyl palmitate (Bridges & Alvarez, 1982). All-trans retinal, and all-trans retinyl palmitate, were purchased from Sigma Co. Ltd and all-trans retinal and 11-cis retinyl palmitate, were kindly provided by Dr Suzuki of Hyogo College of Medicine. Authentic isomers of retinal oxime and retinol were prepared as described previously (Azuma & Azuma, 1984).

RESULTS

Amounts of retinoids in eye cup section

The amounts of retinoids were measured in dark-adapted eye cups or eye cup sections. Typical chromatograms of retinoids extracted from a dark-adapted eye cup section are shown in Fig. 1. Chromatograms A and B show the dichloromethane/hexane extract and the acetone extract (see Materials and Methods), respectively. Fig. 1A shows a small peak of retinyl ester and large syn and anti peaks of 11-cis retinal oxime. Fig. 1B shows a large peak of retinyl ester and small syn and anti peaks of 11-cis retinal oxime. Therefore, retinal oxime was mostly in the dichloromethane/hexane extract, and retinyl ester in the acetone extract. Peaks of 11-cis retinal oxime were not observed in these chromatograms, because the extracts were obtained from a frog collected in summer or one maintained at about 26°C for over
Fig. 1. HPLC chromatograms of retinoids extracted from an eye cup section in the dark. Chromatograms A, B and C show the dichloromethane/hexane extract, acetone extract and retinyl ester fraction, respectively (see text). The names of retinoids and their isomeric forms are expressed in the chromatograms. Syn, syn peaks of retinaloxime; Anti, anti peaks of retinaloxime. 11, 11-cis; AL, all-trans. Absorbances are measured at 350 nm (A350) or at 325 nm (A325).

1 week. Such frogs contained scarcely any A2-pigment in the eyes. These results indicate that 11-cis retinal1 is a major component of retinal isomers in the dark-adapted eye. The isomeric composition of retinyl ester fractions separated from both extracts is shown in Fig. 1C. This chromatogram shows two large peaks; the first one, which is smaller than the second, is due to 11-cis retinyl palmitate1 and the second is all-trans retinyl palmitate1. From the data of each eye cup section, we calculated the amounts of 11-cis retinal1 and retinyl palmitate1 (11-cis plus all-trans). The calculated values were different and depended on the position of the section in the eye cup and also varied from frog to frog. The amount of 11-cis retinal1 ranged from 45 to 200 pmol mm⁻². The amount of retinyl palmitate1 also varied, corresponding to 1–1.5 mol equiv of 11-cis retinal1. As the mole-number of 11-cis retinal1 was nearly equal to that of A1-pigment, the amount of retinyl palmitate1 was 1–1.5 mol equiv of A1-pigment. The proportion of 11-cis isomer in retinyl palmitate1 was 40% on average.

**Regeneration of A1-pigment**

Fig. 2 shows HPLC chromatograms of retinoids extracted from eye cup sections just after illumination (Fig. 2A) and incubated in the dark for 6h following illumination (Fig. 2B). Both samples for HPLC were dichloromethane/hexane extracts. In chromatogram A, syn and anti peaks of all-trans retinaloxime1 are larger than those of 11-cis retinaloxime1, indicating the bleaching of visual pigment by
illumination. The peaks due to 13-cis retinal oxime, 9-cis retinal oxime, and all-trans retinol are also observed in this chromatogram. In chromatogram B, the peaks of all-trans retinal oxime and retinol are smaller than those of 11-cis retinal oxime. This result indicates that the visual pigment chromophore, 11-cis retinal, is formed during dark incubation after illumination, which corresponds to the regeneration of visual pigment. Fig. 3 shows the isomeric forms of retinyl ester fractions obtained from eye cup sections just after illumination (Fig. 3A) and incubated in the dark for 6 h following illumination (Fig. 3B). The peaks due to 11-cis and all-trans retinyl palmitate are distinct in both chromatograms, but the relative peak height of 11-cis to all-trans in chromatogram B is smaller than that in chromatogram A. The data indicate that the relative amount of 11-cis retinyl palmitate decreases during dark incubation following illumination.

As described above, the amount of 11-cis retinal initially present, i.e. the amount of visual pigment (A pigment), was different in each eye cup section. To compare the amount of regenerated pigment among different sections, the ratio of the amount of 11-cis retinal to the total amount of retinal and retinol was calculated for each eye cup section. The ratio corresponded to the relative amount of visual pigment, and the changes in this ratio induced by illumination and during dark incubation after illumination are shown in Fig. 4A. About 80% of the visual pigment initially present was bleached by the illumination, and about 70% of bleached pigment could regenerate after dark incubation for 10 h. Additional incubation did not promote
Fig. 3. HPLC chromatograms of retinyl ester fractions obtained from eye cup sections just after illumination (A) and incubated in the dark for 6 h after illumination (B). Other details as for Fig. 1.

Further regeneration. Fig. 4B shows the changes in the percentage of 11-cis isomer (11-cis retinyl palmitate) present in the total amount of retinyl ester (11-cis plus all-trans retinyl palmitate) in each eye cup section during dark incubation after illumination. The proportion of 11-cis retinyl ester was about 40% in the dark and decreased to 13% after 10 h of dark incubation following illumination. This decrease seems to indicate the utilization of 11-cis retinyl ester for the regeneration of visual pigment.

Comparison of the regeneration of A1- and A2-pigment

The regeneration rate of A1-pigment was compared with that of A2-pigment using eye cups containing both pigments. Fig. 5 shows typical chromatograms of retinoids extracted from left and right eye cups of the same frog. Each sample for HPLC was the sum of the dichloromethane/hexane and the acetone extract. In the dark, there were syn and anti peaks of 11-cis retinaloxime1 and retinaloxime2 together with the peak of retinyl ester (Fig. 5A). Just after illumination, the peaks of 11-cis retinaloximes decreased and those of all-trans retinaloximes and retinols increased (not shown). After 3 h of dark incubation following illumination, the peaks due to 11-cis retinaloxime1 and retinaloxime2 increased again (Fig. 5B), corresponding to the regeneration of A1- and A2-pigments. From peak areas and absorption coefficients of both retinaloximes, the percentages of A2-pigment (mol percent of 11-cis retinaloxime2 to 11-cis retinaloxime1 plus retinaloxime2) were calculated as 19% initially in the dark and 28% after 3 h of dark incubation following illumination. Fig. 6 shows the change in relative amounts of A1-pigment (open bars) and A2-pigment (cross-hatched bars) during dark incubation following illumination.
Retinoid changes in visual pigment regeneration

The percentage of A2-pigment in the dark and just after illumination was 23% in both cases. During dark incubation following illumination, the relative amount of A2-pigment increased to 29% after 1 h, 37% after 2 h and 32% after 4 h. These results suggest that the A2-pigment regenerates faster than the A1-pigment.

A comparison between regeneration rates of A1- and A2-pigments is shown in Fig. 7. As the regeneration of both pigments proceeded no further after 10 h of dark incubation following illumination, maximum regeneration of each pigment corresponded to the difference between the amount of each pigment after 10 h of darkness and that just after illumination. The time courses of A1- and A2-pigment regeneration are expressed by the equations of $y(A_1) = 1 - e^{-0.46t}$ (solid line) and $y(A_2) = 1 - e^{-0.56t}$ (dotted line), respectively, where $y(A_1)$ and $y(A_2)$ are percent regeneration of the A1- and A2-pigments, respectively, and $t$ is the incubation time in the dark. The half times of A1- and A2-pigment regeneration are 90 min and 75 min, respectively, indicating that the regeneration of the A2-pigment is faster than that of the A1-pigment.

![Fig. 4. Regeneration of visual pigment and change in the proportion of 11-cis retinyl ester. The means of four experiments are plotted with standard deviations against the time in the dark after illumination.](image-url)
Fig. 5. HPLC chromatograms of retinoids extracted from two eye cups containing A1- and A2-pigments. The two eye cups were obtained from the same frog. One was dark-adapted (A) and the other was after 3 h of dark incubation following illumination (B). 11(A1), 11-cis retinaloxime; 11(A2), 11-cis retinaloxime2. A350, absorbance at 350 nm.

DISCUSSION

In this study, the regeneration of visual pigments in frog eye cup sections was investigated quantitatively using HPLC. We detected a change in composition and amount of retinoids in relation to visual pigment regeneration. The HPLC method would be more valid using homogeneous materials, but regeneration in a homogenized mixture of RPE and retina has not yet been reported. Therefore, eye cups or eye cup sections were used as an in vitro regeneration system.

The amount of retinoids in an eye cup section (circular section of 4 mm diameter) was enough to be analysed by HPLC. The amount of retinoids varied among sections, but the proportion of retinyl ester to 11-cis retinal was less variable: 1–1.5 mol equiv. This value is a little smaller than that (1.2–2.3 mol equiv) reported by Bridges (1976). As indicated in Fig. 4B, the proportion of 11-cis isomer in retinyl ester (11-cis plus all-trans) falls from about 40% to 13% during the regeneration. If this decrease were due only to an increase in the amount of all-trans retinyl ester formed from all-trans retinal released from bleached pigment, the proportion after regeneration should be 22–26%. This value was estimated from the result that about 80% of visual pigment initially present was bleached by the illumination (Fig. 4A) and from the ratio of retinyl ester to visual pigment (1–1.5). Because the value decreased to 13%, 11-cis retinyl ester must be utilized for the regeneration in eye cup
sections. The finding that 11-cis retinyl ester is left in spite of the imperfect regeneration (see Fig. 4) suggests that the amount of 11-cis retinyl ester stored in RPE is not the only limiting factor in the regeneration. Bridges (1976) reported a slow increase in the proportion of 11-cis retinyl ester left after the termination of pigment regeneration in the dark in the living frog. This increase was considered to be due to the slow transfer of 11-cis retinoid formed in the retina to the RPE. We could not detect such a slow increase in the regeneration system of eye cups or eye cup sections. The discrepancy may indicate that the retinoid shuttle mechanism between the retina and RPE works more efficiently in living eyes than in eye cup sections.

This study includes a comparison between the regeneration of the \( A_1 \) and \( A_2 \)-pigments of frog. The experiments were carried out with eye cups, but not with eye cup sections, because the distribution of pigments was not uniform in the retina: the amount of porphyropsin was larger in the dorsal than in the ventral part (Reuter et al. 1971; Tsin & Beatty, 1980; Makino et al. 1983). As shown in Figs 6 and 7, \( A_2 \)-pigment regenerated faster than \( A_1 \)-pigment. This result is inconsistent with that of the \emph{in vitro} experiment using 11-cis retinal or retinal or opsin solution made by

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Changes in amounts of \( A_1 \) and \( A_2 \)-pigments induced by illumination and in the dark after illumination. Open and cross-hatched bars show relative amounts of \( A_1 \) and \( A_2 \)-pigments, respectively. Each value is the mean of four experiments. Percentages indicated in the figure compare \( A_2 \)-pigment to total \( A_1 \) plus \( A_2 \)-pigments.}
\end{figure}
Fig. 7. Time courses of A₁- and A₂-pigment regeneration. Filled and open circles indicate percentages of regeneration of A₁- and A₂-pigments, respectively. Each value is calculated (see text) from four experimental results, some of which are shown in Fig. 6.

Suzuki, Makino-Tasaka & Miyata (1985). In their experiment, the formation of rhodopsin (A₁-pigment) was faster than that of porphyropsin (A₂-pigment). However, Tsin & Flores (1986) reported that pigment regeneration was faster in living goldfish with retinas rich in porphyropsin than in those with retinas rich in rhodopsin. Their results suggest that porphyropsin regenerates faster than rhodopsin. This is consistent with our result, although the species and the experimental conditions are different. A faster regeneration of A₂-pigment than of A₁-pigment was also detected in Xenopus isolated eyes (unpublished observation). The percentage of A₂-pigment was 20–30 % in bullfrog eyes used in this study and 85–95 % in Xenopus eyes. Tsin & Flores (1986) used goldfish containing 90 %, 92 % or 38 % porphyropsin. Therefore, the faster regeneration of A₂-pigment than of A₁-pigment seems to be independent of the relative amount of A₂-pigment initially present. The reason A₂-pigment regenerates faster than A₁-pigment remains to be clarified.

REFERENCES


