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 A_2-pigment (half time = 75 min) was faster than that of A_1-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

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 palmitate 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 palmitate (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 A_1-based pigment (A_1-pigment) was compared with that of vitamin A_2-based pigment (A_2-pigment). For this purpose, adult bullfrogs collected in winter and spring were used because these frogs had both A_1- and A_2-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; MgCl$_2$, 1·2; NaHCO$_3$, 25; Na$_2$SO$_4$, 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 $27 \pm 1 °C$ 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 l^{-1} NH$_2$OH (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 Na_2SO_4, 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 retinaloxime (see Fig. 1B). The solvents were evaporated under vacuum. After evaporation, the residues were dissolved in $50 \mu l$ 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 $10 \mu l$ 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 retinaloxime, 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 retinaloxime 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$_1$ and all-*trans* retinyl palmitate$_1$ were purchased from Sigma Co. Ltd and all-*trans* retinal$_2$ and 11-*cis* retinyl palmitate$_1$ were kindly provided by Dr Suzuki of Hyogo College of Medicine. Authentic isomers of retinaloxime 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* retinaloxime$_1$. Fig. 1B shows a large peak of retinyl ester and small *syn* and *anti* peaks of 11-*cis* retinaloxime$_1$. Therefore, retinaloxime was mostly in the dichloromethane/hexane extract, and retinyl ester in the acetone extract. Peaks of 11-*cis* retinaloxime$_2$ 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
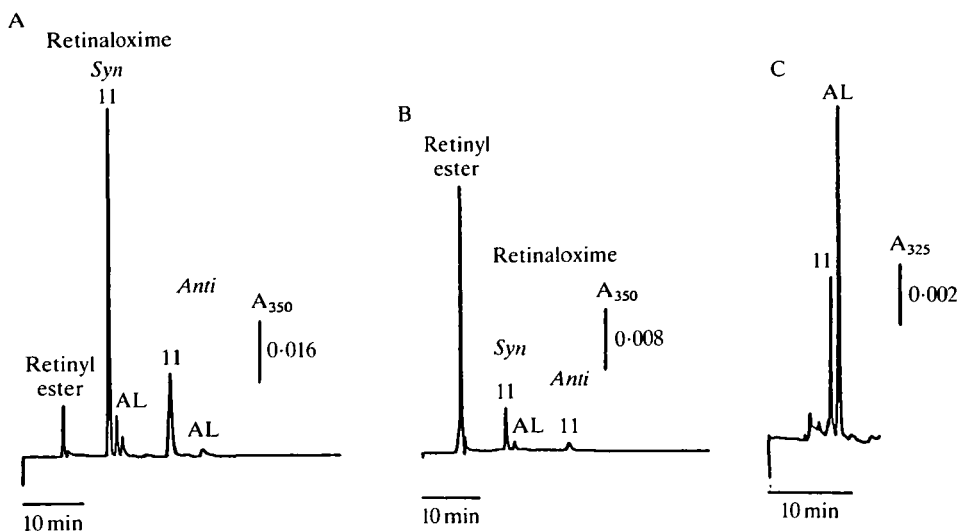
Fig. 1. HPLC chromatograms of retinoids extracted from an eye cup section in the dark. Chromatograms A, B and C show the dichloromethane/hexane extract, acetone extract and retinyl ester fraction, respectively (see text). The names of retinoids and their isomeric forms are expressed in the chromatograms. *Syn, syn* peaks of retinaloxime; *Anti, anti* peaks of retinaloxime. 11, 11-*cis*; AL, all-*trans*. Absorbances are measured at 350 nm (A_{350}) or at 325 nm (A_{325}).

1 week. Such frogs contained scarcely any A_2-pigment in the eyes. These results indicate that 11-*cis* retinal$_1$ 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 palmitate$_1$ and the second is all-*trans* retinyl palmitate$_1$. From the data of each eye cup section, we calculated the amounts of 11-*cis* retinal$_1$ and retinyl palmitate$_1$ (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* retinal$_1$ ranged from 45 to 200 pmol mm^{-2}. The amount of retinyl palmitate$_1$ also varied, corresponding to 1–1·5 mol equiv of 11-*cis* retinal$_1$. As the mole-number of 11-*cis* retinal$_1$ was nearly equal to that of A_1-pigment, the amount of retinyl palmitate$_1$ was 1–1·5 mol equiv of A_1-pigment. The proportion of 11-*cis* isomer in retinyl palmitate$_1$ was 40 % on average.

Regeneration of A_1-pigment

Fig. 2 shows HPLC chromatograms of retinoids extracted from eye cup sections just after illumination (Fig. 2A) and incubated in the dark for 6 h following illumination (Fig. 2B). Both samples for HPLC were dichloromethane/hexane extracts. In chromatogram A, *syn* and *anti* peaks of all-*trans* retinaloxime$_1$ are larger than those of 11-*cis* retinaloxime$_1$, indicating the bleaching of visual pigment by

illumination. The peaks due to 13-*cis* retinaloxime$_1$, 9-*cis* retinaloxime$_1$ and all-*trans* retinol$_1$ are also observed in this chromatogram. In chromatogram B, the peaks of all-*trans* retinaloxime$_1$ and retinol$_1$ are smaller than those of 11-*cis* retinaloxime$_1$. 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$_1$ 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$_1$ initially present, i.e. the amount of visual pigment (A$_1$-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
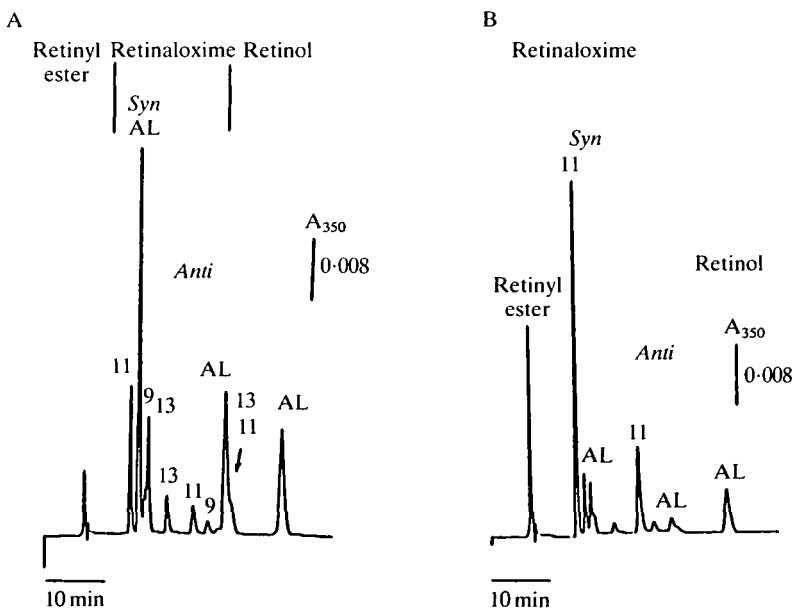

Fig. 2. HPLC chromatograms of retinoids extracted from eye cup sections just after illumination (A) and incubated in the dark for 6 h after illumination (B). 9, 9-*cis*; 13, 13-*cis*. Other details as for Fig. 1.
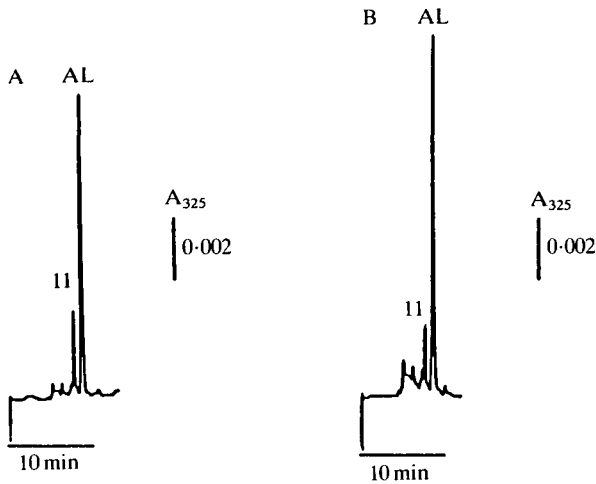
Fig. 3. HPLC chromatograms of retinyl ester fractions obtained from eye cup sections just after illumination (A) and incubated in the dark for 6 h after illumination (B). Other details as for Fig. 1.

further regeneration. Fig. 4B shows the changes in the percentage of 11-*cis* isomer (11-*cis* retinyl palmitate$_1$) present in the total amount of retinyl ester (11-*cis* plus all-*trans* retinyl palmitate$_1$) 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 A$_1$- and A$_2$-pigment

The regeneration rate of A$_1$-pigment was compared with that of A$_2$-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* retinaloxime$_1$ and retinaloxime$_2$ 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* retinaloxime$_1$ and retinaloxime$_2$ increased again (Fig. 5B), corresponding to the regeneration of A$_1$- and A$_2$-pigments. From peak areas and absorption coefficients of both retinaloximes, the percentages of A$_2$-pigment (mol percent of 11-*cis* retinaloxime$_2$ to 11-*cis* retinaloxime$_1$ plus retinaloxime$_2$) 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 A$_1$-pigment (open bars) and A$_2$-pigment (cross-hatched bars) during dark incubation following illumination.

The percentage of A_2-pigment in the dark and just after illumination was 23 % in both cases. During dark incubation following illumination, the relative amount of A_2-pigment increased to 29 % after 1 h, 37 % after 2 h and 32 % after 4 h. These results suggest that the A_2-pigment regenerates faster than the A_1-pigment.

A comparison between regeneration rates of A_1- and A_2-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 A_1- and A_2-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 A_1- and A_2-pigments, respectively, and t is the incubation time in the dark. The half times of A_1- and A_2-pigment regeneration are 90 min and 75 min, respectively, indicating that the regeneration of the A_2-pigment is faster than that of the A_1-pigment.
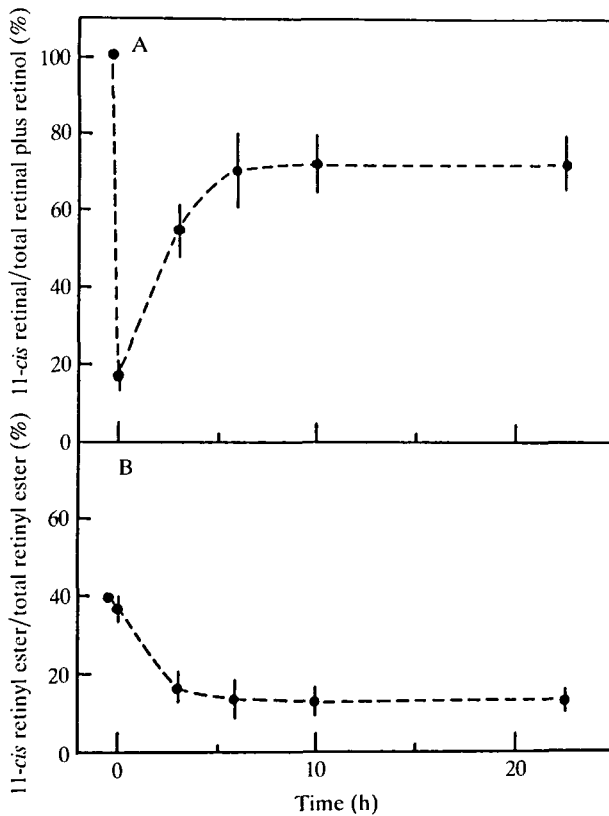

Fig. 4. Regeneration of visual pigment and change in the proportion of 11-*cis* retinyl ester. The means of four experiments are plotted with standard deviations against the time in the dark after illumination.
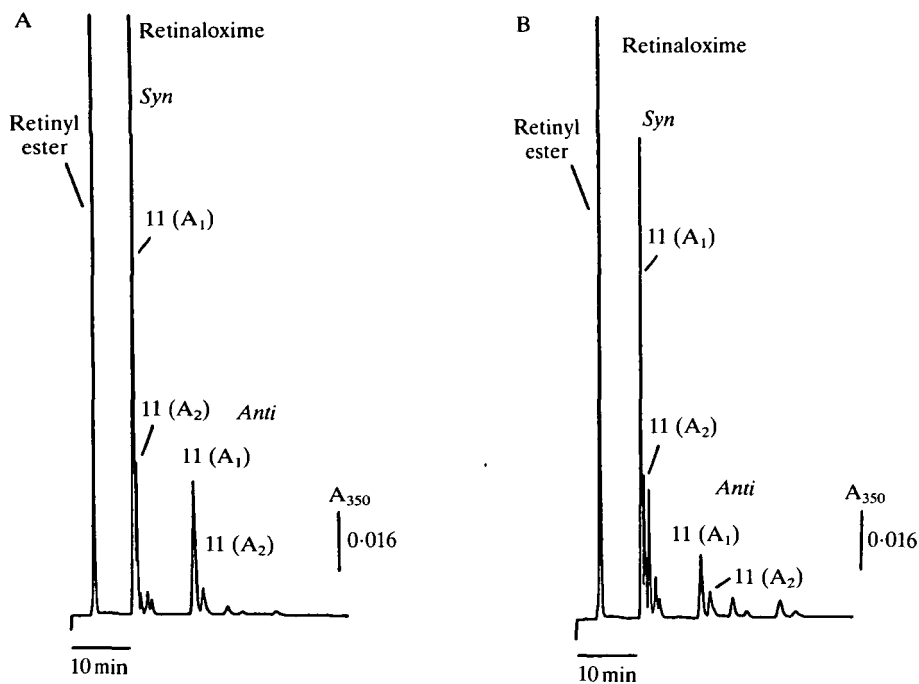
Fig. 5. HPLC chromatograms of retinoids extracted from two eye cups containing A_1-
and A_2-pigments. The two eye cups were obtained from the same frog. One was dark-
adapted (A) and the other was after 3 h of dark incubation following illumination (B).
$11(A_1)$, 11-*cis* retinaloxime$_1$; $11(A_2)$, 11-*cis* retinaloxime$_2$. A_{350}, 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 homogen-
ized 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 *in vitro* experiment using 11-*cis* $retinal_1$ or $retinal_2$ and opsin solution made by
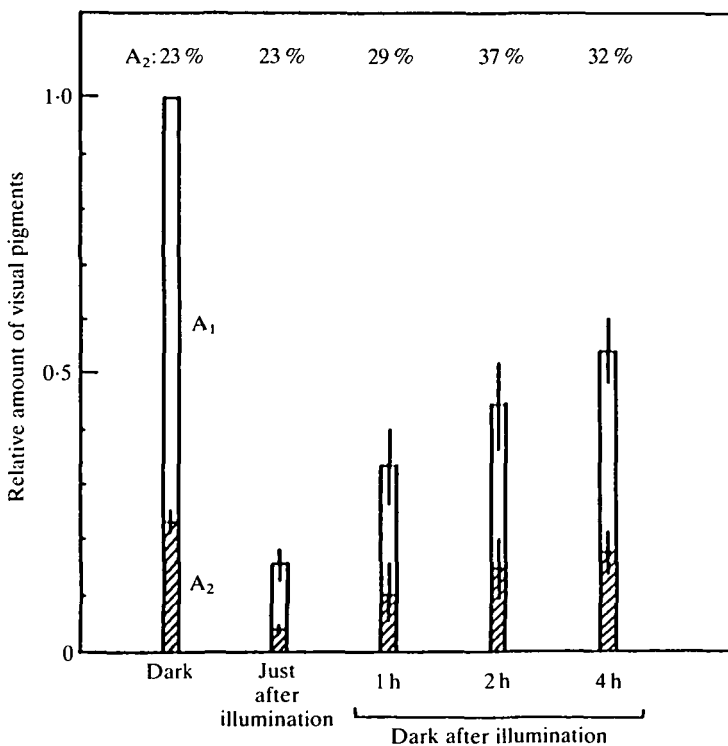

Fig. 6. Changes in amounts of A_1- and A_2-pigments induced by illumination and in the dark after illumination. Open and cross-hatched bars show relative amounts of A_1- and A_2-pigments, respectively. Each value is the mean of four experiments. Percentages indicated in the figure compare A_2-pigment to total A_1- plus A_2-pigments.
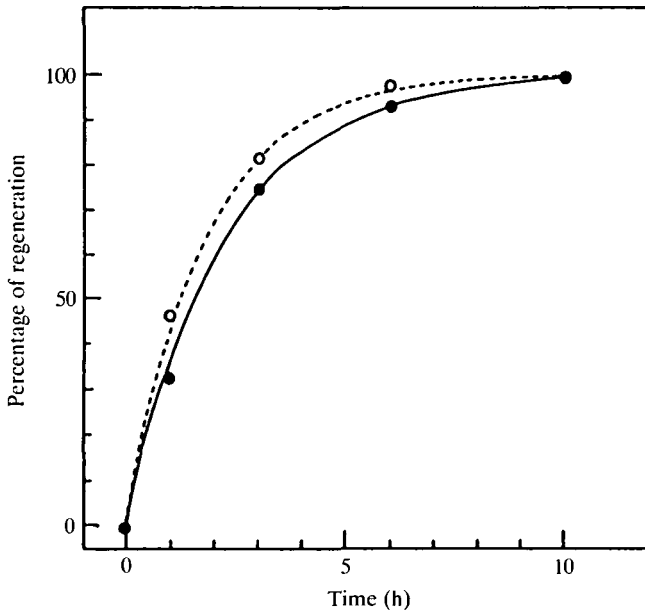
Fig. 7. Time courses of A_1- and A_2-pigment regeneration. Filled and open circles indicate percentages of regeneration of A_1- and A_2-pigments, respectively. Each value is calculated (see text) from four experimental results, some of which are shown in Fig. 6.

Suzuki, Makino-Tasaka & Miyata (1985). In their experiment, the formation of rhodopsin (A_1-pigment) was faster than that of porphyropsin (A_2-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 A_2-pigment than of A_1-pigment was also detected in *Xenopus* isolated eyes (unpublished observation). The percentage of A_2-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 A_2-pigment than of A_1-pigment seems to be independent of the relative amount of A_2-pigment initially present. The reason A_2-pigment regenerates faster than A_1-pigment remains to be clarified.

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