# Kinetics of Visual Pigment Regeneration in Excised Mouse Eyes and in Mice with a Targeted Disruption of the Gene Encoding Interphotoreceptor Retinoid-Binding Protein or Arrestin†

Krzysztof Palczewski,*,‡,§ J. Preston Van Hooser,‡ Gregory G. Garwin,‡ Jeannie Chen,|| Gregory I. Liou,⊥ and John C. Saari*,‡,#

*Departments of Ophthalmology, Biochemistry, Pharmacology, and Chemistry, University of Washington, Seattle, Washington 98195, Departments of Cellular and Neurobiology and Ophthalmology, University of Southern California, 1333 San Pablo St, Los Angeles, California, and Department of Ophthalmology, Medical College of Georgia, Augusta, Georgia 30912*

*Received March 3, 1999; Revised Manuscript Received June 8, 1999*

ABSTRACT: Photoisomerization of 11-*cis*-retinal to *all-trans*-retinal and reduction to *all-trans*-retinol occur in photoreceptor outer segments whereas enzymatic esterification of *all-trans*-retinol, isomerization to 11-*cis*-retinol, and oxidation to 11-*cis*-retinal occur in adjacent cells. The processes are linked into a visual cycle by intercellular diffusion of retinoids. Knowledge of the mechanistic aspects of the visual cycle is very limited. In this study, we utilize chemical analysis of visual cycle retinoids to assess physiological roles for components inferred from in vitro experiments and to understand why excised mouse eyes fail to regenerate their bleached visual pigment. Flash illumination of excised mouse eyes or eyecups, in which regeneration of rhodopsin does not occur, produced a block in the visual cycle after *all-trans*-retinal formation; constant illumination of eyecups produced a block in the cycle after *all-trans*-retinol formation; and constant illumination of whole excised eyes resulted in a block of the cycle after formation of *all-trans*-retinyl ester. These blocks emphasize the role of cellular metabolism in the visual cycle. Interphotoreceptor retinoid-binding protein (IRBP) has been postulated to play a role in intercellular retinoid transfer in the retina; however, the rates of recovery of 11-*cis*-retinal and of regeneration of rhodopsin in the dark in IRBP-/- mice were very similar to those found with wild-type (wt) mice. Thus, IRBP is necessary for photoreceptor survival but is not essential for a normal rate of visual pigment turnover. Arrestin forms a complex with activated rhodopsin, quenches its activity, and affects the release of *all-trans*-retinal in vitro. The rate of recovery of 11-*cis*-retinal in arrestin-/- mice was modestly delayed relative to wt, and the rate of rhodopsin recovery was ∼80% of that observed with wt mice. Thus, the absence of arrestin appeared to have a minor effect on the kinetics of the visual cycle.

Light absorbed by visual pigments of rod and cone photoreceptor cells causes a photoisomerization that converts 11-*cis*-retinal to *all-trans*-retinal (Figure 1). This photoisomerization triggers the phototransduction cascade and ultimately modifies the rate of neurotransmitter release from photoreceptor cells. A thermally driven, enzymatic reaction produces 11-*cis*-retinal in adjacent retinal pigment epithelial (RPE)[1] cells. Because enzymatic isomerization and photoisomerization occur in different cells, the chromophore must diffuse between RPE and photoreceptor cells (*1*) during visual pigment regeneration. This cycle of bleaching (isomerization) and regeneration of visual pigments, termed the visual cycle (*2*−*5*), occurs continually during light exposure, and constant illumination produces a steady state of bleached visual pigments in which the bleach and regeneration rates are equal (*6*).

In recent studies of the visual cycle, we employed HPLC to analyze retinoids present in the mouse retina during recovery from both flash and constant illumination (*7*). *all-trans*-Retinal was the only retinoid to accumulate in large amounts following a bleach, suggesting that reduction of *all-trans*-retinal occurred slowly relative to other enzymatic and transport reactions of the cycle. Here we extend those studies to excised mouse eyes and to transgenic mice with targeted disruptions of genes thought to be involved in the visual cycle. Processes in the visual cycle dependent on cellular

† This study was supported by National Eye Institute Grants EY01730, EY09339 (K.P.), EY03829 (G.I.L.), EY12155 (J.C.), EY02317 (J.C.S.), by unrestricted awards from Research to Prevent Blindness, Inc. (R.P.B.) to the University of Washington and the Medical College of Georgia, by a grant from the Royalty Research Foundation of the University of Washington, and by the Ruth and Milton Steinbach Fund. K.P., J.C., and J.C.S. are the recipients of a Jules and Doris Stein Professorship, a Career Development Award, and a Senior Scientific Investigator award, respectively, from R.P.B.

* To whom correspondence should be addressed. (J.C.S.) Phone: (206) 543-5633. Fax: (206) 543-4414. E-mail: jsaari@u.washington.edu. (K.P.) Phone: (206) 543−9074. Fax: (206) 543−4414. E-mail: palczews@u.washington.edu.

‡ Department of Ophthalmology, University of Washington.

§ Departments of Pharmacology and Chemistry.

|| Departments of Cellular and Neurobiology and Ophthalmology.

⊥ Department of Ophthalmology, Medical College of Georgia.

# Department of Biochemistry.

[1] Abbreviations: RPE, retinal pigment epithelium; Rho, rhodopsin; wt, wild-type; IRBP, interphotoreceptor retinoid-binding protein.

10.1021/bi990504d CCC: $18.00 
Published on Web 08/18/1999

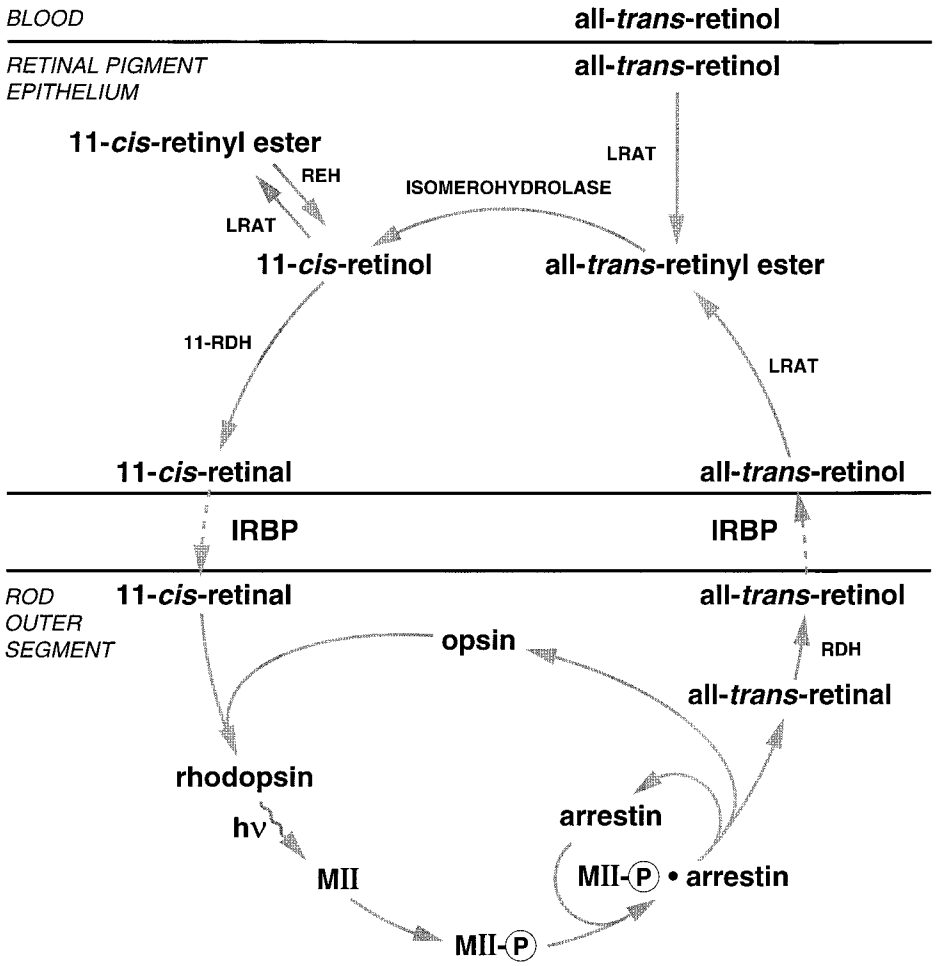


FIGURE 1: Reactions of the mammalian rod visual cycle. Absorption of light by Rho results in the conversion of 11-*cis*-retinal to *all-trans*-retinal and generates the active photoproduct, metarhodopsin II (MII). The G-protein-stimulating activity of MII is quenched by phosphorylation and by the binding of arrestin. MII decays to late photoproducts, the Schiff base linking *all-trans*-retinal and opsin is hydrolyzed by an unknown mechanism, and *all-trans*-retinal is reduced to *all-trans*-retinol by *all-trans*-retinol dehydrogenase (RDH). *all-trans*-Retinol leaves the photoreceptor cell and enters the retinal pigment epithelium (RPE), where it is esterified by lecithin:retinol acyltransferase (LRAT). *all-trans*-Retinyl esters are converted to 11-*cis*-retinol by an isomerohydrolase. 11-*cis*-Retinol can be esterified and stored or oxidized to 11-*cis*-retinal by 11-*cis*-retinol dehydrogenase (11-RDH). 11-*cis*-Retinal diffuses into the photoreceptor rod outer segment where it associates with opsin to regenerate the visual pigment. IRBP is found in the extracellular compartment between RPE and rod outer segments. *all-trans*-Retinol can be taken up from the blood and esterified by LRAT in RPE cells. REH, retinyl ester hydrolase.

metabolism are examined with excised mouse eyes and eyecups and different bleaching conditions. The mechanism of translocation of retinoids between photoreceptor cells and RPE is explored in more detail in mice with a targeted disruption of the gene encoding interphotoreceptor retinoid-binding protein (IRBP), a protein that has been implicated in retinoid transport since its discovery (*8*−*11*). Finally, the intersection of visual cycle and phototransduction pathways is examined in mice bearing a targeted disruption of the gene encoding arrestin, a protein that binds to phosphorylated opsin, quenches the activity of metarhodopsin II (MII), and appears to control the release of *all-trans*-retinal into the visual cycle in vitro (*12*).

## MATERIALS AND METHODS

*Materials.* All experiments involving animals employed procedures approved by the University of Washington Animal Care Committee and The American Veterinary Medical Association Panel on Euthanasia (*13*). Animals employed in these experiments were pigmented (agouti) males and females, approximately 45 days old. IRBP-/- animals were those described in ref *14* and arrestin-/- mice were those described in ref *15*. Wt mice used in these studies were a mixture of strains, ages, and sexes. Wt mice of strains BALB/c (albino), 129svj (albino), C57Bl/6 (pigmented), and C57B2/6 were also examined.

*Methods. Flash or Constant Illumination.* Breeding pairs of IRBP-/- and arrestin-/- mice were maintained in cages in the dark. Pups were born and raised in the dark and were allowed food and water ad libitum. Animals were maintained on a normal mouse diet (Harlan Teklad Rodent Diet, #8604). The genotype of each set of parents and their offspring was determined by PCR (see below). The protein pellet obtained after extraction of retinoids was analyzed by SDS−PAGE and immunoblotting using a polyclonal anti-IRBP (*9*) or anti-arrestin (*16*). The retinas of selected animals were examined by immunocytochemistry with anti-IRBP or anti-arrestin. The methods used for constant and flash illumination of mice, recovery in the dark and the determination of Rho concentrations were described previously (*7*). A single flash was employed in these studies with a SunPak Thyristor 433D flash unit set at maximum power (2.5 ms flash duration) (SunPak Division, Hackensack, NJ).

*Extraction of Retinoids and HPLC Analysis.* Retinoids were extracted with a modification of the method previously described (*7*). Frozen mouse eye backs were placed in a Duall glass-glass homogenizer in 2 mL of 50 mM Mops (3-[*N*-morpholino]propanesulfonic acid), pH 6.5, 10 mM $NH_2OH$ (freshly neutralized $NH_2OH$·HCl), 50% in ethanol and homogenized with six passes of the pestle. Homogenates were incubated at room temperature for 30 min to allow formation of retinal oximes. Retinyl acetate in ethanol was added (1.5−2 nmol/tube) as an external standard for quantitative analysis. Following addition of 4 mL of hexane, the homogenate was drawn up and down in a Pasteur pipet and centrifuged in the homogenizer tube at 900*g* for 2 min to produce a clean phase separation. The upper phase was removed and the extraction repeated following addition of another 4 mL of hexane. The combined upper phases from three extractions were dried with flowing argon, dissolved in 200 $\mu$L of hexane and analyzed with a silica HPLC column (Supelcosil LC-Si, 150 × 4.5 mm, 3 $\mu$m particle size, Supelco Inc., Bellfonte, CA) equilibrated with hexane. Following injection of the sample, the following program was run with a flow rate of 1 mL/min: 0−10 min, 100:0 (hexane:ethyl acetate, v/v); 10−20 min, 99.5:0.5; 20−35 min, 90:10; 35−45 min, 100:0. Retinoids were quantified as described (*7*) except that *all-trans*-retinyl acetate was used to determine the extraction yield instead of [$^3$H]*all-trans*-retinol. Two or four eyes were used for each analysis. The results are expressed as percent of total retinoid because of the variation in absolute amounts observed between individual animals. Saponification and HPLC analysis of the retinols as described previously (*7*) was used to determine the stereoisomeric composition of isolated retinyl esters.

*Eyecup and Whole Eye Preparations.* Eyes were removed from dark-adapted animals under red illumination, rinsed, and used for whole eye experiments. For eyecup experiments, the anterior segment including the cornea and lens was removed, a process requiring ∼2 min. The eyecup was placed posterior pole down and incubated at room temperature (∼22 °C) in 10 mM Hepes, pH 7.5, containing 120 mM NaCl, 3.5 mM KCl, 10 mM glucose, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, and 1 mM EDTA. The buffer was neither oxygenated nor changed during the experiments. A single flash was delivered with the photographic flash unit described above. Constant illumination (54 ft cd) was provided by one 60 W incandescent bulb 32 cm from the eyes.

*Experimental Protocol.* A typical experiment consisted of bleaching (via a flash or constant illumination) the visual pigment in dark adapted mice (or eyes derived from dark adapted animals) and returning the animals to the dark for a recovery period. Retinoids (or rhodopsin) were extracted from the animals before the bleach and during the recovery period in the dark. The point marked $t = 0$ in our figures is actually ∼30 s after the bleach. All results shown are the averages of the mean with bars indicating the standard deviation from the mean ($n \geq 8$).

*PCR.* Tail biopsies were collected at the time of weaning. Murine DNA for genotype analysis was obtained from proteinase K tissue digests of 2 cm tail biopsy specimens using a Qiagen Genomic Tip 20/G kit (Qiagen Inc., Valencia, CA). The arrestin genotype was determined as described (*15*). The following primers were used to characterize the IRBP genotype: *Neo*, 5′-CTT GCC GAA TAT CAT GGT GG-3′; *IRBP*, 5′-TGC TGC GTC ACT GTG GGA CT-3′; *IRBPF*, 5′-CCT GGA TAA CTA CTG CTT CCC TGA G-3′; *IRBPR*, 5′-GAG ATC TAG CAC CAA GGA GGA GGT G-3′. The knockout PCR product results from the *Neo/IRBP* primers (530 bp). The wt PCR product results from the *IRBPF/IRBPR* primers (403 bp). One microgram of genomic DNA was combined with 150 ng of each oligonucleotide primer in 50 mM KCl, 10 mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 0.1% Triton X-100; 500 $\mu$M each of dATP, dCTP, dGTP, and dTTP; and 0.5 $\mu$L of Taq DNA polymerase (Promega, Madison, WI) in a total volume of 50.5 $\mu$L. The reaction was assembled on ice and processed as follows: IRBP, 94 °C, 4 min; 40 cycles, 94 °C, 15 s, 54 °C, 30 s, 72 °C, 1 min; 72 °C, 7 min.

## RESULTS

*Eyecup and Excised Whole Eye Experiments*. Eyecup preparations and excised whole eyes provided insight into aspects of the visual cycle dependent on cellular metabolism. Eyecups from dark-adapted, wt mice were illuminated either with a flash that bleached ∼35% of the visual pigment followed by incubation in the dark (Figure 2), or with constant light. The flash produced the decrease in 11-*cis*-retinal and the corresponding increase in *all-trans*-retinal expected from photoisomerization (Figure 2A). Further incubation in the dark did not result in an increase in 11-*cis*-retinal, and Rho did not regenerate, as previously noted by others in mice (*17*). *all-trans*-Retinal was not reduced to *all-trans*-retinol during the subsequent incubation in the dark, and there was no increase in the amount of retinyl ester. However, when eyecups were subjected to constant illumination (Figure 2B), *all-trans*-retinal disappeared with time concomitant with the appearance of *all-trans*-retinol, indicating that reduction had taken place. There was no increase in the amount of retinyl ester, suggesting that transport of *all-trans*-retinol to RPE, where LRAT is found (*18, 19*), had not occurred.

Because retinal detachment could have occurred during the prolonged incubation of mouse eyecups in buffer, we applied the same experimental paradigm to excised, intact mouse eyes. The results of these experiments are shown in Figure 3. Flash illumination of excised eyes produced the same result as observed with flashed eyecups, namely, production of *all-trans*-retinal from 11-*cis*-retinal, but no reduction of all-*trans*-retinal to *all-trans*-retinol (Figure 3A). Constant illumination of excised mouse eyes resulted in a decrease in the amount of 11-*cis*-retinal and a concomitant increase in the amount of *all-trans*-retinal, resulting from photoisomerization of Rho. With increasing illumination time, we also observed a steady decrease in the amount of *all-trans*-retinal, and increases in the amounts of *all-trans*-retinol and *all-trans*-retinyl esters (Figure 3B). Therefore, the absence of esterification observed with constant illumination of eyecups is likely to have resulted from retinal detachment during the incubation period. We repeated these experiments and analyzed dissected neural retina and RPE/choroid. The results indicated that *all-trans*-retinal and *all-trans*-retinol accumulated in neural retina (photoreceptor cells) and retinyl ester accumulated in RPE (results not shown). We also determined that the retinyl esters that accumulated were ∼90% *all-trans*-retinyl esters.

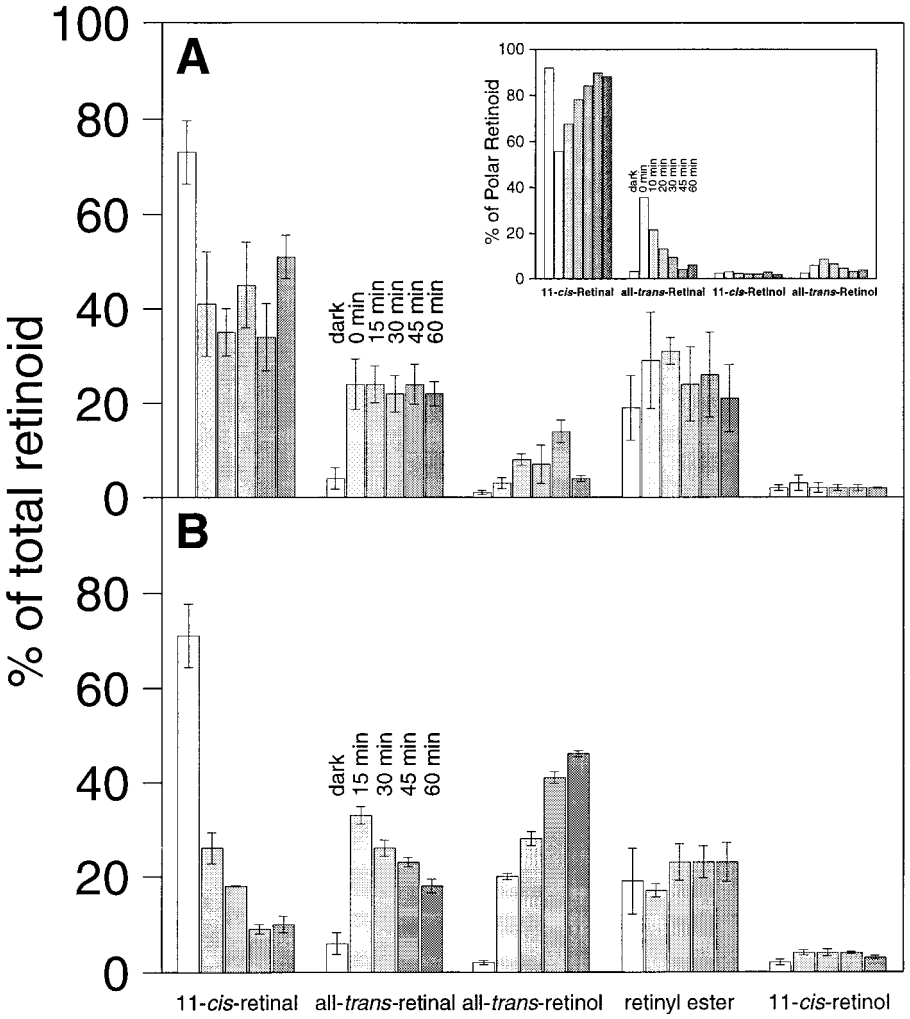


FIGURE 2: Changes in retinoid composition in wt mouse eyecups. Retinoids in mouse eyecups were extracted and analyzed before and after a flash or during constant illumination. (A) Flash. Eyecups were prepared in red light and exposed to a flash. Retinoids were extracted and analyzed before the flash (dark) and after 0, 15, 30, 45, and 60 min in the dark following the flash. (B) Constant illumination. Eyecups were prepared in red light and exposed to constant light. Retinoids were extracted and analyzed before (dark) and after 15, 30, 45, and 60 min of illumination. The bars show the mean values and standard deviations from the mean ($n \geq 8$). For comparison, the insert shows the profile obtained with wt living mice subjected to a flash and regeneration in the dark.

*IRBP-/- Mice*. IRBP-/- mice were born and raised in the dark until they were ~45 days old, a time sufficient for normal retinal maturation (*20*). The genotypes of all IRBP-/- animals used in the experiments were confirmed using PCR. In addition, IRBP was absent in the retina, as judged by SDS−PAGE and immunoblotting with anti-IRBP (*9*) and by immunocytochemistry employing the same antibody. Histologic examination revealed that there were ~7 nuclei in the outer nuclear layer of retinas from IRBP-/- mice, compared to the 11−12 nuclei found in wt mice, similar to the results reported for animals raised in cyclic lighting (*14*). Thus, the photoreceptor degeneration observed in these animals appears to occur in the dark.

IRBP-/- mice were dark adapted and exposed to a flash that bleached ~35% of their visual pigment. Animals were sacrificed before the flash (dark adapted) and at 0 (~30 s), 15, 30, 45, and 60 min in the dark after the flash. Retinoids were extracted from the posterior poles of the eyes as described previously (*7*) and analyzed by HPLC as described in the Materials and Methods. The results of four experiments are summarized in Figure 4A. Retinyl esters in IRBP-/- mice represented a smaller fraction of the total retinoids than observed with wt mice (~5% compared to ~20%). The flash produced an immediate 35% decrease in the amount of 11-*cis*-retinal and a concomitant, proportional increase in the amount of the photolysis product, *all-trans*-retinal. Recovery in the dark resulted in an increase in the amount of 11-*cis*-retinal and a corresponding decrease in the amount of *all-trans*-retinal. Small, transient increases of *all-trans*-retinol and retinyl ester (from ~2 to ~10% of total, and from ~5 to ~10% of total, respectively) were observed during the return to the dark-adapted state (Figure 4A). The recovery kinetics resulting from four experiments with IRBP-/- mice are shown in Figure 4B, along with the recovery curve obtained with wt mice. IRBP-/- mice regenerated their 11-*cis*-retinal with a rate of 0.8%/min, compared to a value of 1.1%/min for a mixed population of wt mice. We noted similar small differences in the rates of regeneration of 11-*cis*-retinal with other pure strains of wt mice also examined (BALB/c albino; 129svj albino; C57Bl/6 pigmented; C57Bl2/6). Thus, the relatively modest difference in rates of recovery (28%) shown in Figure 4 is not consistent with an obligatory requirement for IRBP in regeneration of visual pigments.

Because translocation of retinoids from photoreceptor cells to RPE was also observed in excised eyes with constant illumination (Figure 3B), we applied the same protocol to

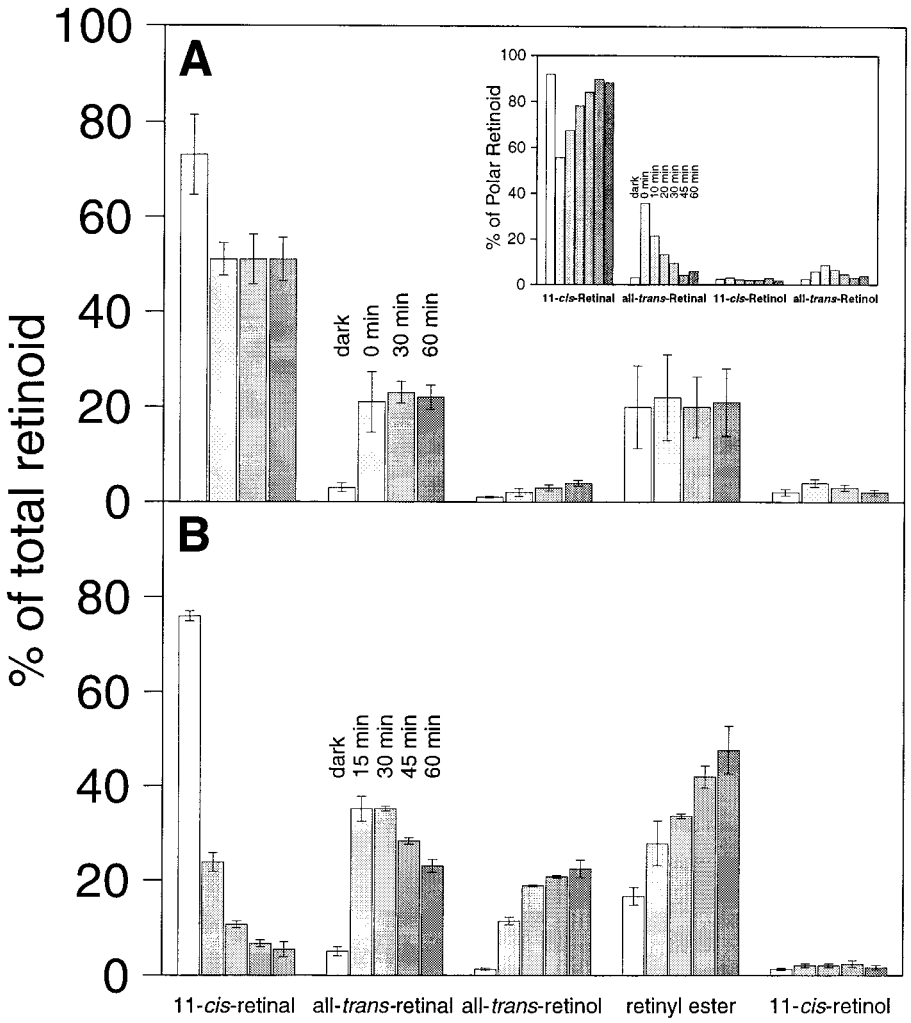


FIGURE 3: Changes in retinoid composition in wt excised, whole mouse eyes. Retinoids were extracted and analyzed before and after a flash or during constant illumination. (A) Flash. Eyes were removed from dark-adapted mice in red light and exposed to a flash. Retinoids were extracted from the posterior half of the eyes and analyzed before the flash (dark) and after 0, 30, and 60 min in the dark following the flash. (B) Constant illumination. Eyes were removed from dark-adapted mice as described above and exposed to constant light. Retinoids were extracted as above and analyzed before (dark) and after 15, 30, 45 and 60 min of illumination. The bars represent the means and standard deviations from the mean ($n \geq 8$). For comparison, the insert shows the response of living wt mice to a flash and recovery period in the dark.

eyes removed from IRBP-/- mice. The pattern of retinoid distribution was identical to that observed with wt mice, providing further indication that the absence of IRBP did not affect the flow of retinoid between neural retina and RPE.

Regeneration of Rho in IRBP-/- mice was examined using the same experimental paradigm. Results of four experiments are shown in Figure 4C. Rho levels in dark-adapted IRBP-/- mice were lower than those found in wt animals (~450 pmol/eye compared to ~600 pmol/eye), in keeping with the loss of ~40% of their photoreceptor cells. The initial rate of regeneration of Rho in the dark (0.8%/min) was equal to the rate of regeneration of 11-*cis*-retinal and 80% of the rate observed with wt mice (1%/min).

*Arrestin-/- Mice*. Arrestin-/- mice were born and raised in the dark and subjected to the same experimental paradigm as described above. Figure 5A presents the distribution of visual cycle retinoids observed before and after a flash. The recovery of 11-*cis*-retinal in the dark is shown in Figure 5B for wt and arrestin-/- mice, and the recovery of Rho is shown in Figure 5C. Arrestin-/- mice regenerated 11-*cis*-retinal in the dark at a rate of 0.6%/min compared to 1.1%/min for wt mice (Figure 5B). However, the rate of regeneration of Rho in arrestin-/- mice (0.8%/min) was only modestly different from that observed with wt mice (1%/min). The retinyl ester content of arrestin-/- mice was approximately equal to that found in wt mice (~25% of total retinoids), in contrast to the lower value found in IRBP-/- mice (~5%).

## DISCUSSION

The vertebrate visual cycle involves reactions in two different cells coupled by intercellular transport. *all-trans*-Retinol, produced in photoreceptor cells, must diffuse into the adjacent RPE cells and then back into the photoreceptor cells after transformation into 11-*cis*-retinal (*1*) (Figure 1). We explored mechanistic aspects of the mouse visual cycle with high-resolution HPLC analyses of retinoids (*7*). The results here demonstrate that the cycle can be blocked at different sites with accumulations of different retinoids and validate the general experimental approach. The results also provide insight into the functions of IRBP and arrestin.

*Excised Eyes and Eyecups.* Studies from other laboratories have established that excised mouse eyes do not regenerate

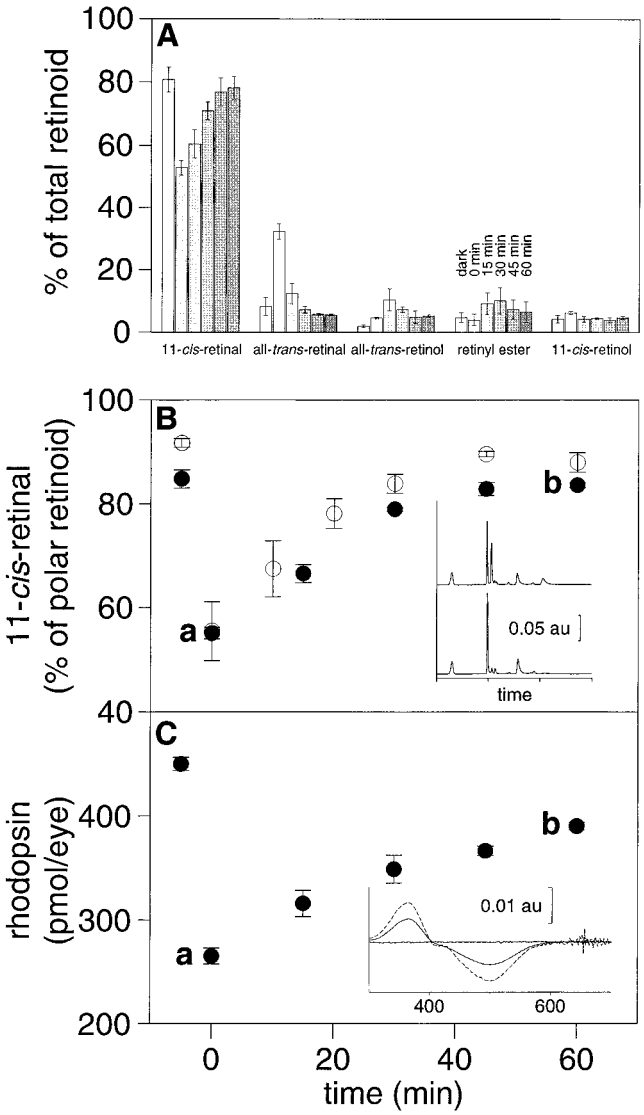


FIGURE 4: Kinetics of retinoid and rhodopsin recoveries in IRBP-/- mice after a flash. (A) Retinoid composition in RPE/retina. Mice were dark-adapted overnight. Animals (2 per analysis, $n = 4$) were sacrificed in red light before (dark) or after a flash ($t = 0$, 15, 30, 45, or 60 min in the dark) that bleached ~35% of the visual pigment. Retinoids were extracted and analyzed by HPLC as described in the Materials and Methods. (B) Kinetics of 11-*cis*-retinal formation in the dark. The open circles were derived from wt mice, the filled circles from IRBP-/- mice. (Inset) HPLC traces obtained during retinoid analyses of mice immediately after the flash (point a) (upper trace, 11-cis- and *all-trans*-retinal oximes the major retinoids) and after 60 min in the dark (point b) (lower trace, 11-*cis*-retinal oximes the major retinoids). (C) Bleaching and recovery of Rho. Rho concentrations were measured before and after a flash that bleached ~35% of the visual pigment. (Inset) Rhodopsin bleaching difference spectra obtained from mice immediately after the flash (point a) (solid curve), and after 60 min of recovery (point b) (dashed curve). (B and C) The error bars indicate the standard error of the mean ($n = 4$). The abscissa indicates the time in the dark after the flash. The points at −5 min are from dark-adapted mice.

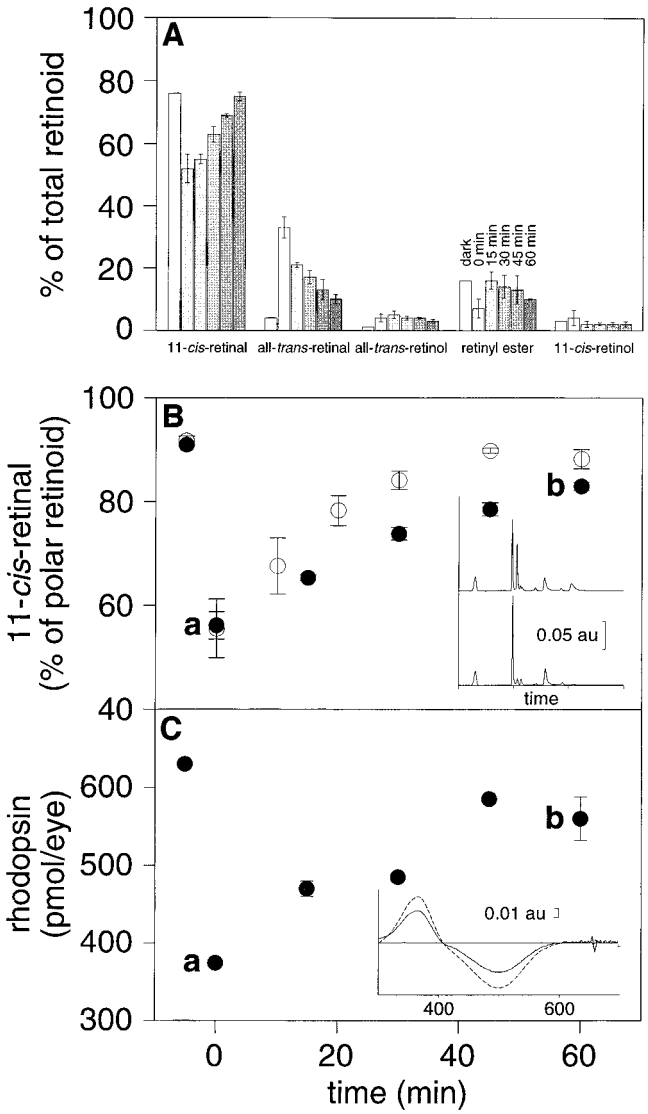


FIGURE 5: Kinetics of retinoid and rhodopsin recoveries in arrestin-/- mice after a flash. The experimental paradigm is as explained in Figure 4. (A) Retinoid composition in RPE/retina. (B) Kinetics of 11-*cis*-retinal formation in the dark. The open circles were derived from wt mice, the filled circles from arrestin-/- mice. Inset. HPLC traces obtained during retinoid analyses of mice immediately after the flash (point a) (upper trace, 11-*cis*- and *all-trans*-retinal oximes the major retinoids) and after 60 min in the dark (point b) (lower trace, 11-*cis*-retinal oximes the major retinoids). (C) Bleaching and recovery of Rho. Rho concentrations were measured before and after a flash that bleached 40% of the visual pigment. (Inset) Rhodopsin bleaching difference spectra obtained from mice immediately after the flash (point a) (solid curve) and after 60 min of recovery (point b) (dashed curve). (B and C) The abscissa indicate the time in the dark after the flash. The error bars indicate the standard error of the mean ($n = 4$). The points at −5 min are from dark-adapted mice.

their visual pigment unless provided with oxygen and glucose (*17*). However, the step in the visual cycle that is blocked in these eyes has not been identified. Energy is required to drive the production of the hindered 11-*cis*-retinal from *all-trans*-retinal (*21*, *22*); however, the proposed mechanism for this reaction would not appear to require the input for metabolic energy. We examined this question with mouse eyecup preparations in unoxygenated buffers. Studies with rabbit retina have shown that a light-evoked compound action potential disappeared completely in 2.5 min (*23*), attributed to a loss of high-energy phosphate compounds. Thus, ATP stores are likely to have been depleted in excised mouse eyecups during the relatively long incubations (up to 60 min) employed in our experiments.

Flash illumination of mouse eyecups or whole eyes produced the expected immediate loss of 11-*cis*-retinal and gain of *all-trans*-retinal. However, the failure to observe

reduction of *all-trans*-retinal to *all-trans*-retinol was unanticipated. The results obtained with a flash are in contrast to those observed with constant illumination of either eyecups or whole eyes, where *all-trans*-retinal was reduced to *all-trans*-retinol. While the explanations for these differences are uncertain, it is possible that a flash fails to activate the pentose phosphate pathway and that the retina under these conditions lacks sufficient NADPH for reduction of *all-trans*-retinal.

Constant illumination of excised whole mouse eyes resulted in an accumulation of *all-trans*-retinyl ester in RPE, a phenomenon not observed in living animals or in eyecup preparations. This observation suggests that the failure to observe retinyl ester synthesis with mouse eyecups with the same experimental paradigm was most likely caused by retinal detachment during the prolonged incubation. The accumulation of *all-trans*-retinyl esters in excised eyes with constant illumination suggests that the cycle is blocked at the isomerization reaction. Rando and associates (*3, 24*) have proposed that the energy for formation of the 11-*cis*-configuration (+4 kcal/mol) is provided by the hydrolysis of the carboxylic ester bond of *all-trans*-retinyl esters (−5 kcal/mol) in a concerted reaction catalyzed by a putative isomerohydrolase (Figure 1). If conversion of *all-trans*-retinyl ester to 11-*cis*-retinol proceeds thermodynamically downhill, why should this compound accumulate when cellular metabolism and energy production have been disrupted? Recent observations document that conversion of *all-trans*-retinol to 11-*cis*-retinol in vitro does not proceed by way of the bulk retinyl ester pool (*25*). Further experimentation will be required to resolve this issue, but the observations raise the intriguing possibility that the visual cycle requires the direct input of energy derived from cellular metabolism.

We observed previously that regeneration of 11-*cis*-retinal is more rapid after constant illumination than after flash illumination (*7, 26*). Others noted that recovery of Rho and visual sensitivity after flash illumination took as long as recovery from prolonged light adaptation that bleached twice as much visual pigment (*27, 28*). It is possible that these phenomena are related to the absence of reduction of *all-trans*-retinal after a flash, as observed in our studies with eyecups and whole eyes.

*IRBP-/- Mice.* The low solubility of retinoids in aqueous media seems at odds with their postulated intercellular diffusion as part of the visual cycle. In the early 1980s, several laboratories discovered IRBP in extracts of retina (*8, 10, 11*). Several lines of evidence suggested a role for IRBP in the transport of retinoids between photoreceptors and RPE. First, the protein possessed two high-affinity binding sites for retinoids (*29*−*31*). Second, the amount of *all-trans*-retinol bound to IRBP increased following illumination and the amount of 11-*cis*-retinal increased in the dark (*31, 32*). Third, removal of IRBP slowed the regeneration of visual pigment in skate eyecups, and its replacement partially restored the normal regeneration rate (*33*). Fourth, bleached salamander photoreceptors resensitized when presented with 11-*cis*-retinal (rods and cones) or 11-*cis*-retinol (cones) bound to IRBP (*34*). Finally, IRBP was found precisely in the extracellular compartment separating photoreceptor and RPE cells (*9*).

Mechanistic aspects of IRBP's role in diffusion of retinoids between photoreceptors and RPE cells have remained unclear and controversial. Several lines of experimentation suggested that IRBP plays a passive rather than an active role in retinoid transport. First, physical studies revealed that IRBP is a high molecular weight protein with an axial ratio of ∼7 to 1 (*31, 35*), an unusual shape for a diffusible protein carrier. Second, a number of in vitro studies established that polar retinoids diffused through the aqueous phase relatively rapidly and that IRBP actually retarded the rate of retinoid transfer between populations of vesicles (*36*). However, other investigators found that release of 11-*cis*-retinal from RPE cells required IRBP (*37, 38*), suggesting a molecular interaction between IRBP and components of the RPE cell.

If IRBP is required for efficient transport of retinoids between RPE and photoreceptor cells, the absence of the protein should result in a buildup of *all-trans*-retinol following a visual pigment bleach and a delayed rate of visual pigment regeneration (Figure 1). In this study, we tested this hypothesis with mice lacking a functional gene for IRBP. The results demonstrate that IRBP does not appear to be necessary for retinoid transfer during bleaching and regeneration of visual pigments in mice. The rates of regeneration of 11-*cis*-retinal and decay of *all-trans*-retinal were only modestly slower than those observed for these processes in wt mice. In addition, only small, transient increases in the relative amounts of *all-trans*-retinol were observed following either a flash that bleached ∼35% of the visual pigment or 2 h of constant illumination (results not shown), similar to the results with wt mice (*7*). Constant illumination would be expected to exhaust endogenous stores of 11-*cis*-retinoid [although none appear to be present in the mouse retina (*7*)] and to ensure that regeneration was due to de novo synthesis of 11-*cis*-retinoids. While it is possible that effects of the absence of IRBP only come into play at low fractional bleaches, our results appear to diminish the importance of IRBP as a necessary component of the retinoid transport system through the photoreceptor RPE extracellular space.

The loss of photoreceptor cells observed in IRBP-/- mice (*14*) suggests that the protein plays an essential role in maintaining the integrity of these cells. Because IRBP is a major protein in the interphotoreceptor matrix (*39, 40*), it is possible that its absence causes a disruption of the extracellular matrix with resulting death of the photoreceptor cells. It is also possible that a function of IRBP is to minimize the exposure of the retina to transient increases in extracellular retinoid concentration (*36*) and prevent their deleterious effects (*41*).

*Arrestin-/- Mice*. Photoactivated Rho is quenched in a two-step process involving opsin phosphorylation by Rho kinase (reviewed in ref *42*) and binding of arrestin to phosphorylated opsin (reviewed in ref *43*). The absence of arrestin in humans results in Oguchi's disease in which the inability to quench activated Rho produces a decrease in the sensitivity of the visual system, manifested as night blindness (*44*).

In vitro experiments suggested a role for arrestin in reactions of the visual cycle. First, addition of arrestin to photolyzed Rho stabilized MII (*45*). Second, addition of *all-trans*-retinal promoted arrestin binding to phosphorylated opsin (*46*). Third, addition of NADPH resulted in dissociation of arrestin from its complex with phosphorylated, photolyzed Rho (*46*) (Figure 1), suggesting that reduction of *all-trans*-retinal to *all-trans*-retinol promotes arrestin dissociation. Fourth, addition of ATP to unwashed rod outer segments

substantially reduced the rate of reduction of *all-trans*-retinal generated by a flash (*12*). These results suggested that arrestin stabilizes *all-trans*-retinal in the binding pocket of MII or MII-like forms of Rho and that mice lacking arrestin would show abnormalities in the kinetics of photopigment regeneration. The rate of recovery of 11-*cis*-retinal was 1.8 times faster for wt mice than for arrestin-/- mice; however, the rate of visual pigment regeneration was equal to that observed with IRBP-/- mice and only modestly slower than that of wt (0.8%/min compared to 1%/min). These observations are counterintuitive to experiments in vitro, described above (*12*), which would predict accelerated reduction of *all-trans*-retinal in the absence of arrestin. Perhaps the in vitro experiments poorly reflect the in vivo conditions because of altered ratios of key components in a rod outer segment (ROS) preparation, or because of the dilutions involved.

*Conclusions.* The results of this study resolve issues regarding the visual cycle and raise additional, interesting questions. First, the results suggest that cellular metabolism is required for conversion of *all-trans*-retinol to 11-*cis*-retinoid, a finding not predicted from consideration of the isomerohydrolase reaction. Second, the absence of reduction of *all-trans*-retinal to *all-trans*-retinol in flashed eyecups or excised eyes suggests that control of this step may be more complex than considered previously. Third, the absence of arrestin had little effect on retinoid processing kinetics, in contrast to the results from experiments in vitro. Finally, IRBP apparently plays an important role in photoreceptor physiology but its function is not related to the kinetics of retinoid processing.

## REFERENCES


1. Dowling, J. E. (1960) *Nature 188*, 114−118.
2. Bok, D. (1993) *J. Cell Sci.* (Suppl.) *17*, 189−195.
3. Rando, R. R. (1996) *Chem. Biol. 3*, 255−262.
4. Saari, J. C. (1994) in *The Retinoids: Biology, chemistry and medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) 2nd ed., pp 351−385, Raven Press, Ltd., New York.
5. Wald, G. (1968) *Science 162*, 230−239.
6. Alpern, M., Maaseidvaag, F., and Ohba, N. (1971) *Vision Res. 11*, 539−549.
7. Saari, J. C., Garwin, G. G., Van Hooser, J. P., and Palczewski, K. (1998) *Vision Res. 38*, 1325−1333.
8. Adler, A. J., and Martin, K. J. (1982) *Biochem. Biophys. Res. Commun. 108*, 1601−1608.
9. Bunt-Milam, A. H., and Saari, J. C. (1983) *J. Cell Biol. 97*, 703−712.
10. Lai, Y. L., Wiggert, B., Liu, Y. P., and Chader, G. J. (1982) *Nature 298*, 848−849.
11. Liou, G. I., Bridges, C. D. B., Fong, S.-L., Alvarez, R. A., and Gonzalez-Fernandez, F. (1982) *Vision Res. 22*, 1457−1467.
12. Palczewski, K., Jager, S., Buczylko, J., Crouch, R. K., Bredberg, D. L., Hofmann, K. P., Asson-Batres, M. A., and Saari, J. C. (1994) *Biochemistry 33*, 13741−13750.
13. Andrews, E. J., Bennett, B. T., Clark, J. D., Houpt, K. A., Pascoe, P. J., Robinson, G. W., and Boyce, J. R. (1993) *J. Am. Vet. Med. Assoc. 202*, 229−249.
14. Liou, G. I, Fei, Y., Peachey, N. S., Matragoon, S., Wei, S., Blaner, W. S., Wang, Y., Liu, C., Gottesman, M. E., and Ripps, H. (1998) *J. Neurosci. 18*, 4511−4520.
15. Xu, J., Dodd, R. L., Makino, C. L., Simon, M. I., Baylor, D. A., and Chen, J. (1997) *Nature 389*, 505−509.
16. Palczewski, K., and Smith, W. C. (1996) *Exp. Eye Res. 63*, 599−602.
17. Ostroy, S. E, Friedmann, A. L., and Gaitatzes, C. G. (1992) *Exp. Eye Res. 55*, 419−423.
18. Barry, R. J., Canada, F. J., and Rando, R. R. (1989) *J. Biol. Chem. 264*, 9231−9238.
19. Saari, J. C., and Bredberg, D. L. (1989) *J. Biol. Chem. 264*, 8638−8640.
20. Carter-Dawson, L., Alvarez, R. A., Fong, S.-L., Liou, G. I., Sperling, H. G., and Bridges, C. D. B. (1986) *Dev. Biol. 116*, 431−438.
21. Hubbard, R. (1966) *J. Biol. Chem. 241*, 1814−1818.
22. Rando, R. R., and Chang, A. (1983) *J. Am. Chem. Soc. 105*, 2879−2882.
23. Ames, A., III, and Gurian, B. S. (1963) *J. Neurophysiol. 26*, 617−634.
24. Deigner, P. S., Law, W. C., Canada, F. J., and Rando, R. R. (1989) *Science 244*, 968−971.
25. Stecher, H, Gelb, M. H., Saari, J. C., and Palczewski, K. (1999) *J. Biol. Chem. 274*, 8577−8585.
26. Ohguro, H., Van Hooser, J. P, Milam, A. H., and Palczewski, K. (1995) *J. Biol. Chem. 270*, 14259−14262.
27. Dowling, J. E., and Hubbard, R. (1963) *Nature 199*, 972−975.
28. Rushton, W. A. H. (1963) *Nature 199*, 971−972.
29. Fong, S.-L., Liou, G. I., Landers, R. A., Alvarez, R. A., and Bridges, C. D. (1984) *J. Biol. Chem. 259*, 6534−6542.
30. Chen, Y., Saari, J. C., and Noy, N. (1993) *Biochemistry 32*, 11311−11318.
31. Saari, J. C., Teller, D. C., Crabb, J. W., and Bredberg, L. (1995) *J. Biol. Chem. 260*, 195−201.
32. Adler, A. J., and Spencer, S. A. (1991) *Exp. Eye Res. 53*, 337−346.
33. Duffy, M., Sun, Y., Wiggert, B., Duncan, T., Chader, G. J., and Ripps, H. (1993) *Exp. Eye Res. 57*, 771−782.
34. Jones, G. J., Crouch, R. K., Wiggert, B., Cornwall, M. C., and Chader, G. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9606−9610.
35. Adler, A. J., Evans, C. D., and Stafford, W. F., III (1985) *J. Biol. Chem. 260*, 4850−4855.
36. Ho, M.-T. P., Massey, J. B., Pownall, H. J., Anderson, R. E., and Hollyfield, J. G. (1989) *J. Biol. Chem. 264*, 928−935.
37. Carlson, A., and Bok, D. (1992) *Biochemistry 31*, 9056−9062
38. Okajima, T.-I., Pepperberg, D. R., Ripps, H., Wiggert, B., and Chader, G. J. (1990) *Proc. Natl. Acad. Sci. 87*, 6907−6911.
39. Adler, A. J., and Klucznik, K. M. (1982) *Exp. Eye Res. 34*, 423−434.
40. Pfeffer, B., Wiggert, B., Lee, L., Zonnenberg, B., Newsome, D., and Chader, G. (1983) *J. Cell. Physiol. 117*, 333−341.
41. Dingle, J. T., and Lucy, J. A. (1965) *Biol. Rev. 40*, 422−461.
42. Palczewski, K. (1997) *Eur. J. Biochem. 248*, 261−269.
43. Palczewski, K. (1994) *Protein Sci. 2*, 1355−1361.
44. Fuchs, S., Nakazawa, M., Maw, M., Tamai, M., Oguchi, Y., and Gal, A. (1995) *Nat. Genet. 10*, 360−362.
45. Schleicher, A., Kuhn, H., and Hofmann, K. P. (1989) *Biochemistry 28*, 1770−1775.
46. Hofmann, K. P., Pulvermuller, A., Buczylko, J., Van Hooser, P., and Palczewski, K. (1992) *J. Biol. Chem. 267*, 15701−15706.


BI990504D