

## Invited Review

# Light-induced Damage to the Retina: Role of Rhodopsin Chromophore Revisited

Małgorzata Różanowska\*<sup>1</sup> and Tadeusz Sarna<sup>2</sup>

<sup>1</sup>School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK

<sup>2</sup>Department of Biophysics, Faculty of Biotechnology, Jagiellonian University, Kraków, Poland

Received 13 November 2004; accepted 24 August 2005; published online 24 August 2005 DOI: 10.1562/2004-11-13-IR-371

## ABSTRACT

The presence of the regenerable visual pigment rhodopsin has been shown to be primarily responsible for the acute photodamage to the retina. The photoexcitation of rhodopsin leads to isomerization of its chromophore 11-*cis*-retinal to all-*trans*-retinal (ATR). ATR is a potent photosensitizer and its role in mediating photodamage has been suspected for over two decades. However, there was lack of experimental evidence that free ATR exists in the retina in sufficient concentrations to impose a risk of photosensitized damage. Identification in the retina of a retinal dimer and a pyridinium bisretinoid, so called A2E, and determination of its biosynthetic pathway indicate that substantial amounts of ATR do accumulate in the retina. Both light damage and A2E accumulation are facilitated under conditions where efficient retinoid cycle operates. Efficient retinoid cycle leads to rapid regeneration of rhodopsin, which may result in ATR release from the opsin “exit site” before its enzymatic reduction to all-*trans*-retinol. Here we discuss photodamage to the retina where ATR could play a role as the main toxic and/or phototoxic agent. Moreover, we discuss secondary products of (photo)toxic properties accumulating within retinal lipofuscin as a result of ATR accumulation.

## INTRODUCTION

The primary function of the retina is to detect incident photons of visible light. However, exposure of the retina to excessive fluxes of visible light, although much below levels sufficient to induce thermal damage, results in damage to the retina. This paper will provide a review of literature on light-induced damage to the retina, followed by a short overview of visual pigment cycle and finally a discussion of the role of retinoid cycle in accumulation of isomerized chromophore of visual pigment, all-*trans*-retinal (ATR) and susceptibility to acute photoinduced damage to the retina. Moreover, we will discuss the long-term effects of ATR accumulation—accumulation of lipofuscin, which may lead to retinal dysfunction and further increases the susceptibility of the retina to light-induced damage.

## LIGHT-INDUCED DAMAGE TO THE RETINA

To elicit damage, light needs to reach and be absorbed in the retina. The spectral characteristics and irradiance levels of light that reaches the retina depend on the absorption properties of the interior segments of the eye (1–4). The cornea absorbs light below 300 nm. The lens of the child transmits partly the remaining ultraviolet light with a transmission window centered at about 320 nm but there is a progressive build-up of UV-absorbing chromophores and, by the age of 22, only visible light above 390 nm is efficiently transmitted through the lens. As a result, in the normal phakic adult less than 0.1–1% of near-UV light reaches the retina (1,3,5). The UV and short-wavelength visible light was recognized to cause retinal damage in cases of arc welding without proper eye protection (6,7). Ageing is accompanied by progressive yellowing of the lens leading to a gradual decrease in transmission of short-wavelength visible light to the retina (1,4).

Retinal photodamage in humans has been widely recognized after gazing at the sun while watching a solar eclipse (8–10) or in controlled studies in patients with malignant melanoma who were scheduled to undergo enucleation (11–13). The retinal irradiance in a human eye viewing the mid-day sun was estimated to be between 1.5 and 122 W/cm<sup>2</sup> depending on the diameter of the pupil (14,15) and exposures lasting for several minutes to tens of minutes were sufficient to cause an ophthalmoscopically visible damage.

Studies in monkeys indicate that 5–450 smaller retinal irradiance levels with similar durations of exposure result in retinal damage

\* To whom correspondence should be addressed: School of Optometry and Vision Sciences, Cardiff University, Cardiff CF10 3NB, Wales, UK. Fax: +44-29-2087 4859; e-mail: rozanowskamb@cf.ac.uk

**Abbreviations:** A2E, 2-(2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,4E,7E-octatetraenyl)-1-(2-hydroxyethyl)-4-(4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl)-pyridinium; ABCR, ATP-binding cassette transporter rim protein; CRABP, cellular retinol-binding protein; CRBP, cellular retinol-binding protein; DHA, docosahexaenoic acid; EPR, electron paramagnetic resonance; ER, endoplasmic reticulum; IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; LCA, Leber's congenital amaurosis; LRAT, lecithin:retinol acyltransferase; NRPE, *N*-retinylidene-phosphatidylethanolamine; Meta I, metarhodopsin I; Meta II, metarhodopsin II; Meta III, metarhodopsin III; OS, outer segment; PE, phosphatidylethanolamine; prRDH, photoreceptor-specific NADPH-dependent retinol dehydrogenase; RDH, retinol dehydrogenase; RPE, retinal pigment epithelium.

© 2005 American Society for Photobiology 0031-8655/05

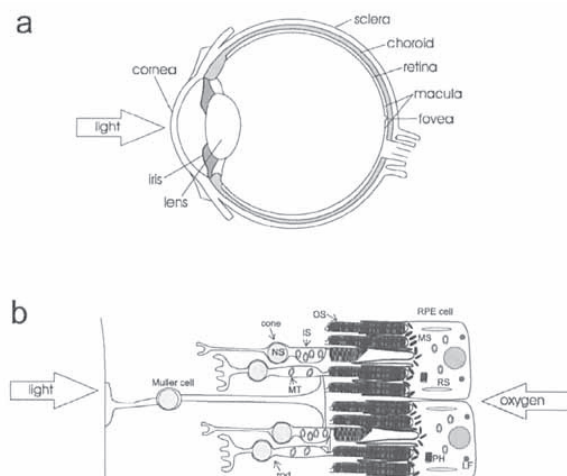


**Figure 1.** Schematic diagrams of the eye (a) and the retina (b). The schemes are not to scale. The diagram of the retina depicts only cells discussed in this review, and all second-order neurons are omitted for clarity. IS, inner segment; NS, nucleus; MT, mitochondrion; MS, melanosome; RS, retinosome; PH, phagosome; LF, lipofuscin.

detectable upon histological examination (14). For instance, exposure of an anesthetized rhesus monkey for 15 min to the retinal irradiance of  $0.27 \text{ W/cm}^2$  from indirect ophthalmoscope (dose of  $243 \text{ J/cm}^2$ ) resulted in severe damage to photoreceptors and changes in the retinal pigment epithelium (RPE) (14). Retinal exposure of cynomolgus monkey to light from an operating microscope ( $3816 \text{ J/cm}^2$ ; irradiance of  $1.06 \text{ W/cm}^2$  for 1 h) resulted in severe changes in the fovea: disarrangement of photoreceptor outer segments, pyknosis of photoreceptor nuclei, swelling of photoreceptor axons, formation of vacuoles within RPE, which in the center of the fovea became necrotic even though there was no apparent damage visible upon ophthalmoscopic examination (16).

During ocular surgery of humans, the retinal irradiance from an operating microscope can reach up to  $0.97 \text{ W/cm}^2$  and the procedure may take up to 2 h (17). Thus ocular surgery has also been considered to impose a risk of photodamage to the retina (see reviews by Kirkness [17], Lerman [2], Sliney [18] and Michael and Sternberg [19]). Several case studies provide evidence supporting the view that ocular surgery can impose a risk of light-induced damage to the retina (20–23). It may be speculated that even a lengthy ophthalmoscopic examination can impose a risk of retinal damage—an indirect ophthalmoscope usually provides irradiance levels of the retina up to  $0.13 \text{ W/cm}^2$ , whereas slit lamp biomicroscopy provides up to  $0.35 \text{ W/cm}^2$  (17).

Retinal irradiance in daylight has been estimated to vary from approximately  $0.01 \text{ mW/cm}^2$  to  $0.1 \text{ mW/cm}^2$  (24). Ocular geometry and behavioral aversion response to bright light, in particular, to its short-wavelength components, provide protective mechanisms against exposure to excessive fluxes of visible radiation (3,25). However, these mechanisms are not completely sufficient to prevent photodamage to the retina. Chronic exposure to bright sunlight can lead to reduced visual acuity and impairments of dark adaptation and night vision (26). Long-term chronic exposure to solar radiation or its blue component throughout lifetime has been identified as one of the risk factors for development of age-related macular degeneration (AMD), the

major cause of blindness in the elderly (27,28). The risk of developing AMD or progression to the late AMD becomes greater after cataract surgery (29–31). It may be speculated that it is related to the exposure to light from the operating microscope during the surgery (32) and/or due to an increased transmission of short-wavelength light by the implanted intraocular lens (33,34).

Genetic background can further predispose to damage (35,36). For instance, the severity of retinitis pigmentosa in patients with the proline-23-histidine (P23H) rhodopsin mutation has been shown to be related with a history of strong exposure to the sun or other light sources (36). P23H mutations result in poor ability of rhodopsin to fold properly after synthesis and weak binding to 11-*cis*-retinal, which results in a poor thermal stability of mutated rhodopsin (37).

To understand the mechanisms responsible for deleterious effects of light, several animal models of light damage and retinal diseases have been employed. For example, experiments on transgenic animals carrying P23H rhodopsin mutation proved that mutants are more susceptible to photodamage than wild-type animals and confirmed that exposure to light accelerates the progression of degenerative changes of photoreceptors (38–42).

Based on the initial site of damage and the irradiation conditions used, several different outcomes of photodamage to the retina have been identified, where a number of different mechanisms responsible may be involved leading to damage to photoreceptors, RPE, Müller cells (12,43), swelling of mitochondria (44) and formation of vacuoles throughout the all retinal layers (42,45) and eventually destruction of the retinal neurons and RPE (see reviews by Remé and colleagues [46,47], Organisciak and Winkler [48], Lerman [2] and Lanum [49]). It has been demonstrated by monitoring the time course of damaging action of diffuse fluorescent light in the rat retina that the photoreceptors are the first to exhibit the signs of damage—disruption and vesiculation of the outer segments (OS) and pyknotic nuclei immediately after 2 h of exposure to diffuse fluorescent light (3000 lux) (50). Apoptosis in the RPE became visible only after 5 h and the deterioration progressed up to the final checkpoint at 72 h (50). This review focuses on photodamage to the retina that occurs in photoreceptor cells and RPE, even though light needs to pass several layers of neurons in the inner retina before reaching these outer layers (Fig. 1).

Photoreceptor cells (rods and cones) are responsible for absorption of photons, phototransduction and signaling to other neurons. These are one of the most metabolically active cells of the body (51,52). Their OS consist of a stack of flat discs or lamellar membranes formed by lipid bilayers containing high concentrations of visual pigments. Both in rods and cones, visual pigments consist of a transmembrane protein bound *via* protonated Schiff base linkage of lysine to 11-*cis*-retinal. The differences in the amino acid composition in proximity of 11-*cis*-retinal is responsible for spectral “tuning” of the absorption maxima of the visual pigments to different wavelengths in rods and cones.

Photoreceptors are in close association with a monolayer of polarized cuboidal cells, RPE (53). RPE is essential for photoreceptor function and survival and its functions include uptake, transport and storage of retinoids. The numerous microvilli at the apical surface of RPE cells project 5–10  $\mu\text{m}$  into the interphotoreceptor matrix surrounding the OS, which is thought to facilitate retinoid trafficking between those two cell types. RPE plays a critical role in providing nutrients, recycling ATR and the molecular renewal of OS: distal ends of OS are shed daily and

phagocytosed by the RPE, which forms transient pseudopodia involved in these processes.

#### **Wavelength dependence of light-induced damage to the retina**

Effects of exposure of the retina to high fluxes of visible light have been studied most extensively in nocturnal rodents (46,54), which are more susceptible to light damage than primates. In albino rats, the action spectrum of retinal photodamage induced by a lengthy exposure of 6 h to light intensities of about 0.7–0.9 mW/cm<sup>2</sup> and assessed by photoreceptor loss, exhibited a maximum at 500 nm—the wavelength corresponding to the absorption maximum of rhodopsin (55). The threshold for green light-induced damage detectable by electroretinography was estimated for retinal irradiance of about 0.001 mW/cm<sup>2</sup> provided over 3 to 4 h (10.8–14.4 mJ/cm<sup>2</sup>) (56). In this type of damage the initial site of damage is restricted to rods that die through apoptosis (46,54).

There are differences in susceptibility to light damage between nocturnal animals such as rats and diurnal primates. The action spectrum of retinal photodamage peaking at 500 nm was observed exclusively in rats which were adapted to circadian dim light/dark cycles for at least 2 weeks (55,56). Several adaptive mechanisms to the environmental light may play a major role in the determination of the threshold for light damage. They include up-regulation of antioxidant defenses and survival factors (57–63).

Probably the most important mechanism of long-term adaptation to environmental light involves down-regulation of rhodopsin synthesis and increased ubiquitin-mediated rhodopsin degradation within photoreceptor inner segments (64,65). As a result, the rhodopsin concentration in OS is smaller in animals reared in light than in animals reared in dark. It takes about 3 weeks for the rat retina to reach a new plateau level of rhodopsin concentration upon increasing the levels of light at which the animals were reared (64). These adaptive mechanisms to environmental light are effective in preventing light-induced injury to the retina (57,66–68). Rats kept in dark for 2 weeks before the exposure to light exhibit extensive retinal injury while rats kept in cyclic light exhibit very little, if any, signs of retinal damage (68). Comparison of susceptibility to retinal photodamage of rats maintained at two levels of cyclic light indicates that the damage threshold can be further increased by rearing animals in a bright-light environment (57,66). Rats reared at cyclic light exhibit increased susceptibility to retinal photodamage with ageing, while rats reared in dark are equally susceptible to light-induced damage at different ages (67).

In rhesus monkeys, where the rod responses were saturated because of the presence of white background light, the damage can be induced selectively to blue, green or red cones by a series of exposures to narrow-band light centered at 463 nm and 520 nm and broad-band light in the range of 630–720 nm, respectively (69,70). In contrast to damage induced to green and red cones, where the cone function recovered after a few weeks, repeated exposures to blue light resulted in an irreversible loss of sensitivity to blue light and permanent damage to blue cones.

Exposures of the retina to relatively high irradiance levels result in similar action spectra in both rodents and primates showing that the efficiency of induction of the damage rapidly increases below 500 nm and further increases with decreasing irradiation wavelength up to the shortest wavelength studied of 320 nm (71,72). The threshold doses of ultraviolet and short-wavelength blue light (320–440 nm) damage only photoreceptors (71,73). The threshold

damage induced by light above 440 nm causes damage also to the RPE (15,71,74). These types of exposures give similar outcomes across all the species studied and exhibit reciprocity of the duration of exposure and irradiance for a wide range of exposure times (71,72). For example, Ham and colleagues (72) determined that reciprocity holds for exposures to 325 nm light resulting in the same threshold damage dose of 5 J/cm<sup>2</sup> for either a 100 s exposure to retinal irradiance levels of 50 mW/cm<sup>2</sup> or a 1000 s exposure to retinal irradiance levels of 5 mW/cm<sup>2</sup>.

#### **Effects of damaging light on morphology of the retina**

To determine the primary site and the sequence of events in light-induced damage, Busch and colleagues (74) monitored the time course of changes in the rat retina following exposures to narrow-band light centered at 380 nm or 470 nm. Damage visible by fundoscopic examination was the most pronounced 3 days after exposure to damaging light and occurred at doses of 0.6 J/cm<sup>2</sup> and 500 J/cm<sup>2</sup> for 380 nm and 470 nm light, respectively.

Histological examination revealed damage to photoreceptors already at a dose of 0.45 J/cm<sup>2</sup> of 380 nm light (74). As early as 3 h postexposure to 380 nm light RPE cells were loaded with phagosomes but, apart from that, looked normal. After 3 weeks RPE appeared completely normal even for doses exceeding 2.5 times the threshold dose but almost all photoreceptors were lost.

Initial changes observed in RPE in response to threshold doses of 470 nm light included altered distribution of melanosomes, cell swelling and some dark inclusions in the cytoplasm, while some photoreceptors (<1%) exhibited pyknotic nuclei (74). Higher doses, exceeding 1.5 times the threshold dose, resulted in damage to photoreceptors and appearance of phagocytic cells and melanosomes in the OS layer—changes visible between 1 and 3 days after the exposure. Two months after the exposure the retina seemed normal except for thinning of the photoreceptor nuclear layer corresponding to loss of up to 6% of photoreceptors. Doses 2.5 times higher than the threshold doses of 470 nm radiation resulted in photoreceptor degeneration, RPE cell swelling and some interruptions of the RPE monolayer. RPE seemed to be healed after 2 months, in contrast to photoreceptors that exhibited massive loss.

Overall the late stages of damage appeared very similar in injuries caused by exposures to 380 nm and 470 nm light (74). Histological examination was necessary to prove the damaging action of light after 2 months postexposure as changes in the fundus became barely visible. As the doses required to induce damage by 470 nm light were 830 times higher than for 380 nm light, it can be argued that retinal damage induced by white fluorescent light is mainly due to the short-wavelength component of its spectrum (74).

#### **Screening effects within the retina**

Another issue that needs to be considered in comparing the effects of different wavelengths on the initial site of damage is the transmission of light through the retina. Rhodopsin has a broad absorption spectrum peaking in humans at 498 nm. Knowing the specific optical density of rod OS and the length of OS at different areas of the retina, absorption of incident light by the OS and the percentage of light transmitted to the RPE can be estimated. The specific optical density of rod OS at 500 nm is about 0.015  $\mu\text{m}^{-1}$  (75). Lengths of rod OS in the perifoveal area of the retina are about 31.2  $\mu\text{m}$ , while in the periphery, about 23.9  $\mu\text{m}$  (75). These

give absorbance values of 0.47 and 0.36 at 500 nm for perifoveal and peripheral rods, respectively, corresponding to rhodopsin concentration within OS of 3.8 mM. This agrees well with the usually cited concentration of rhodopsin within the dark-adapted OS of about 3 mM (76). Thus 500 nm light incident on the dark-adapted retina will be partially absorbed by OS so only 34% and 44% of light will be transmitted to the RPE in the perifovea and peripheral retina, respectively.

However, exposure of the retina to light in most experiments investigating light-induced damage bleaches all the rhodopsin within the first minutes of exposure (71). The main products of rhodopsin photobleaching, metarhodopsin II (Meta II), metarhodopsin III (Meta III) and ATR, exhibit broad-band absorption spectra with maxima at 380 nm, 470 nm and 380 nm, respectively. The molar extinction coefficients at the wavelengths of maximum absorbance for Meta II, Meta III and ATR are similar to that of rhodopsin (77,78). Assuming that all rhodopsin is converted to a mixture of Meta II, ATR and opsin, more than half of the incident photons of 380 nm light are efficiently absorbed by OS so only about 34% and 44% of this short-wavelength light is transmitted to the RPE in perifoveal and peripheral area, respectively. It indicates that the photoreceptors can partially screen the RPE from short-wavelength light during experiments where most of the rhodopsin is bleached. The screening effect of photoreceptors can affect the observed action spectrum of photodamage to the RPE determined *in vivo* in case the chromophores responsible are localized within the RPE.

#### Search for mechanisms responsible for light-induced damage

Both types of retinal photodamage, induced by exposures to either low- or high-intensity light, are dependent on the presence of regenerable rhodopsin in the retina (56,79–81). Vitamin A deficiency, a precursor of rhodopsin chromophore, 11-*cis*-retinal, protects against photodamage (68). However, as estimated by Gorgels and van Norren (71) under their experimental conditions for determination of the action spectrum for photodamage to the retina, all rhodopsin was bleached within the first minutes of exposure independent of the wavelength used.

In all cases discussed above, the light-induced damage to the retina occurs *via* photochemical mechanism(s) and depends on oxygen concentration (82–84). Elevated oxygen tension results in a decreased threshold of light damage. Thus it indicates that photodynamic action may be responsible for the damage. The outer retina is exposed to high oxygen tension supplied from the dense vascular bed of the choroid. The oxygen tension in the arteries can be further elevated due to oxygen supplementation during ocular surgery (84). Involvement of photodynamic effect in retinal photodamage is further supported by the findings that retinal photodamage is accompanied by lipid peroxidation (85,86), suggesting that it is mediated by reactive oxygen species. As nitric oxide is inherently produced in the retina, it is not surprising that nitrogen reactive species are also formed and lead to the nitration of retinal proteins (87). A number of enzymatic and low-molecular weight antioxidants are involved in photoprotection and may prevent or reduce the extent of photodamage (48,88,89).

There are several chromophores absorbing visible light in the outer retina and their potential involvement in the photodamage to the retina was previously discussed (90). However, the main chromophore responsible for initiation of photosensitized oxidation has not been identified. Even though most studies indicate that

visual pigment, rhodopsin is the essential for photodamage to the retina to occur, photochemical properties of rhodopsin exclude it as the photosensitizer responsible for retinal photodamage. Thus rhodopsin intermediates or bleaching products were suggested as mediators of photodamage (71,80,81).

The product of rhodopsin bleaching ATR has been suspected to be responsible for the photooxidative damage to the retina by Delmelle since 1977 (91), who proposed a mechanism responsible for retinal photodamage. According to this mechanism ATR photosensitizes generation of singlet oxygen, which in turn induces peroxidation of retinal lipids and damage to the lipid membranes. However, it was questionable whether free ATR exists in the retina, or it is always bound with proteins and therefore inactive as a photosensitizer. While there is an extensive literature on light-induced damage to the retina in humans and animals, Delmelle's work (91,92) was largely ignored or ATR was rejected as a possible culprit responsible for light-induced damage based on belief that upon hydrolysis from opsin, ATR is rapidly reduced by an enzyme, retinol dehydrogenase (RDH), to all-*trans*-retinol (71).

In the following chapters we will discuss several pieces of experimental evidence published within recent years indicating that ATR can accumulate in the OS under certain conditions. First, the rhodopsin must be photobleached and ATR hydrolyzed from the protein. After exposure to light bleaching all the rhodopsin, this alone may account for ATR accumulation reaching the concentration of rhodopsin before the exposure. For accumulation of any additional ATR, rhodopsin chromophore, 11-*cis*-retinal, must be efficiently synthesized in the RPE and delivered to OS to regenerate rhodopsin, which subsequently needs to be photobleached. The hydrolyzed ATR remains noncovalently bound to the opsin "exit site" and only upon binding of 11-*cis*-retinal to opsin, when the rhodopsin is regenerated to the native conformation, ATR can be released to the inner leaflet of the disc membrane before its enzymatic reduction to all-*trans*-retinol (93,94). Second, the rate of accumulation of ATR in the inner leaflet of OS disc membrane must exceed the rate of removal of ATR, where removal refers to its direct enzymatic reduction to all-*trans*-retinol, or formation of a reversible Schiff base linkage of ATR with phosphatidylethanolamine (PE), *N*-retinylidene phosphatidylethanolamine (NRPE) and its transport to the outer leaflet of OS disc membrane, where subsequent reduction of ATR to all-*trans*-retinol can occur. The rate of removal of ATR will be limited by the rates with which these processes occur and may be further delayed, *i.e.* due to the absence or dysfunction of ABCR protein responsible for NRPE transport across the disc membrane and/or dysfunction in enzymatic reduction of ATR. Formation of a retinal dimer (95) and a pyridinium bisretinoid, so called A2E (96–99), provides evidence that these processes occur indeed in the retina. In view of these findings we will discuss data on retinal photodamage where regenerable rhodopsin was a necessary factor for light damage to occur. Finally, we will discuss toxic effects of secondary products accumulating in the retina as a result of ATR accumulation—RPE lipofuscin and one of lipofuscin components, A2E.

#### PHOTOACTIVATION OF RHODOPSIN

Upon absorption of a photon, the chromophore of rhodopsin, 11-*cis*-retinal, undergoes ultrafast isomerization to ATR (100,101). This primary event initiates a cascade of conformational changes within the protein molecule (Fig. 2a). Within milliseconds rhodopsin reaches a metastable state—Meta II in which the original

Schiff base linkage between Lys296 with ATR is still intact but deprotonated. Meta II catalyzes activation of the heterotrimeric G-protein, transducin. Activated transducin dissociates from the Meta II and sets off the biochemical cascade leading to the closure of cGMP-gated cation channels in the plasma membrane, which is followed by neuronal signaling enabling visual perception (Fig. 2b). Meta II can activate hundreds of transducin molecules until it becomes deactivated, which usually occurs within 100 ms and is initiated by phosphorylation of Ser and Thr residues in the C terminus of rhodopsin by rhodopsin kinase. Phosphorylated Meta II is then tightly capped by arrestin or its splice variant, so-called p44 protein, which effectively blocks further activation of transducin (102–104).

Meta II is in a dynamic equilibrium with its precursor, metarhodopsin I (Meta I) (Fig. 2a). However, under physiological conditions, the equilibrium is strongly shifted toward Meta II, so Meta I can be considered as a short-lived intermediate decaying within milliseconds to Meta II and Meta III (105). Lipid environment of rhodopsin strongly affects the ratio of formation of Meta II and Meta III. Decrease of polyunsaturated lipids, such as docosahexaenoic acid (DHA) and/or increase in the cholesterol content have been shown to inhibit the transition of Meta I to Meta II (106–109), while promoting transition of Meta I to Meta III (105). Indeed it has been shown that OS from rats depleted in DHA exhibit a decreased rate of Meta II formation (110).

Meta III is considered as a storage form of photoactivated rhodopsin (77,111). Apart from formation directly from Meta I deactivation (105), Meta III can be formed as a result of flash photolysis of Meta II with blue light ( $\lambda = 400 \pm 10$  nm) (112). Meta III is believed to either slowly decay into opsin and ATR (113), or be converted back to Meta II state (114,115). It has been shown that the latter reaction can be catalyzed by transducin or its C-terminal peptides (114). Flash photolysis of Meta III with 477 nm light results in nonspecific isomerization of ATR and results in generation of Meta I/II, dark state rhodopsin and isorhodopsin, which contains 9-*cis*-retinal bound to its active site (115). Meta I/II, rhodopsin and isorhodopsin account for 40%, 37% and 21% of photolyzed Meta III. Accordingly, continuous illumination of the mixture with green light ( $>475$  nm) results in photoformation of exclusively Meta II (115). Binding of transducin disables photo-induced conversion of photoactivated rhodopsin to ground-state rhodopsin (116,117). Thermal stability of Meta III may suggest that transducin-catalyzed conversion of Meta III to Meta II and/or Meta III photolysis are the major routes of Meta III decay (114,115). It remains to be elucidated whether hydrolysis of ATR directly from Meta III may occur *in vivo* as it was previously suggested (113).

## FATE OF ALL-TRANS-RETINAL AFTER HYDROLYSIS FROM OPSIN

### Clearance of ATR in OS

The decay of the active Meta II proceeds through hydrolysis of the Schiff base linkage and ATR is released from the hydrophobic binding pocket (93,94) (Fig. 3a). Next, ATR is channeled to the “exit site” of the protein where it remains noncovalently bound until rhodopsin is regenerated upon binding of another 11-*cis*-retinal molecule to Lys296 in its active site (93,94). While being bound to the “exit site,” ATR can be reduced to all-*trans*-retinol by NADPH-dependent RDH (93,94,118). While both rod and cone

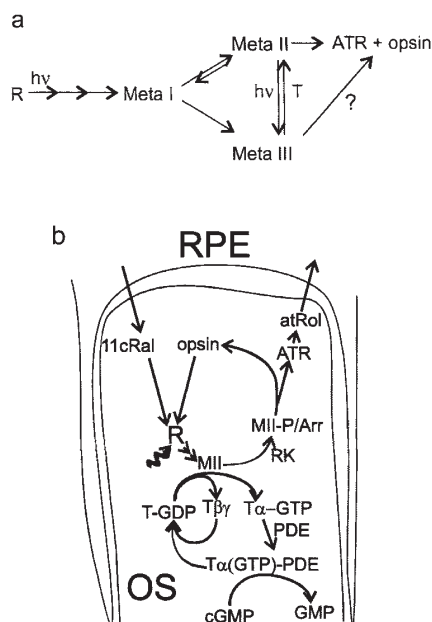
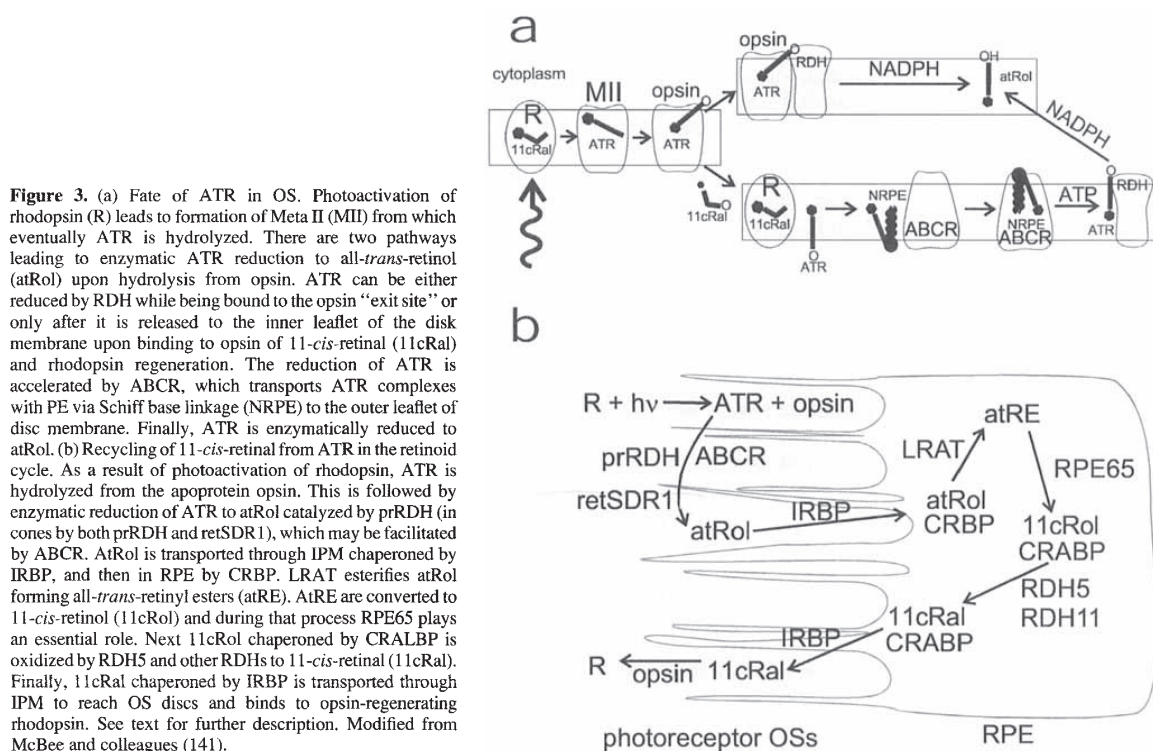


**Figure 2.** (a) Photoactivation of rhodopsin. Absorption of a photon by rhodopsin (R) leads to ultrafast isomerization of the chromophore, 11-*cis*-retinal to ATR, which is followed by conformational changes in the protein leading to formation of a state Meta I. From Meta I state either Meta II state or Meta III state can be formed. Meta III can also be formed as a result of photolysis of Meta II. Transducin (T) catalyzes transformation of Meta III to Meta II. See text for further description. Modified from Ritter and colleagues (112), Vogel and colleagues (105,115) and Zimmermann and colleagues (114). (b) Visual cycle. Photoactivation of rhodopsin (R) leads to formation of biochemically active Meta II (MII) which activates a biochemical cascade leading to visual perception. MII binds a heterotrimeric protein transducin (T) and allows for exchange of GDP nucleotide for GTP in a subunit  $\alpha$  of T. T $\alpha$ (GTP) dissociates from T $\beta\gamma$  and activates phosphodiesterase (PDE). PDE catalyzes hydrolysis of cyclic nucleotide (cGMP). In response to decreased concentration of cGMP, cGMP-gated channels in the OS plasma membranes close (not shown). This results in a reduced influx of sodium and calcium cations. These cations are continuously pumped out of the photoreceptors by ATP-dependent Na/K-pumps in the inner segments; therefore the closure of cGMP-gated channels leads to hyperpolarization of photoreceptor plasma membrane, which, in turn, results in an inhibition of synaptic secretion of glutamate—a neurotransmitter signaling absorption of photons to secondary neurons in the retina. Meta II can catalyze activation of T until it becomes phosphorylated by rhodopsin kinase (RK) and binds arrestin (Arr), which completely prevents further activation of T. Eventually, Meta II hydrolyzes ATR. To regenerate rhodopsin, Arr dissociates, phosphates (P) are removed from opsin by phosphatase, and opsin binds 11-*cis*-retinal (11cRal) delivered from RPE, which completes the rhodopsin cycle. Hydrolyzed ATR is enzymatically reduced to all-*trans*-retinol (atRol) and transported to the RPE where it can be converted back to 11-*cis*-retinal through a multistep process of retinoid cycle. Modified from McBee and colleagues (141).

OS express photoreceptor-specific RDH (prRDH; also known as RDH8) (119), cones express another protein in their OS, which also reduces ATR to all-*trans*-retinol, retSDR1 (120).

The reduction of ATR in rods is a relatively slow process (121–124). In the mouse eye, constant illumination for 60 min leading to an approximately 35% bleach of rhodopsin results in ATR accumulation, which accounts for approximately 30% of total retinoids (122). During a period of dark recovery following the 60 min of steady illumination, the concentration of ATR declines with



a half-life of approximately 5 min, whereas the concentration of ATR after a flash of bleaching light declines with a half-life of approximately 17 min (122).

Upon regeneration of rhodopsin by efficient supply of 11-*cis*-retinal, ATR can be released from the opsin "exit site" before being enzymatically reduced to all-*trans*-retinol (93,94). ATR is believed to be released from the "exit site" to the inner leaflet of the disk membrane where it is inaccessible to RDH until it is flipped over to the outer leaflet.

It has been shown that ATR forms condensation products with a lipid component of the OS membranes, phosphatidylethanolamine (PE), *N*-retinylidene-phosphatidylethanolamine (NRPE) (96,97,125,126). NRPE is a preferred substrate for a protein present in rims of OS discs, ATP-binding cassette transporter rim protein (ABCR) (127–129). ABCR is believed to bind NRPE present in the inner leaflet of the membrane and release it on the cytoplasmic site of OS disc membrane upon binding and hydrolysis of ATP (129). Then ATR becomes available as a substrate for RDH. Lack of *ABCR* gene in *ABCR*<sup>−/−</sup> knockout mice or its deficiency in *ABCR*<sup>+/-</sup> mice leads to delayed clearance of ATR/NRPE from the retina (96,97,130). It may be speculated that in the absence of ABCR the transverse diffusion of ATR/NRPE to the outer leaflet becomes the limiting factor in the reduction of ATR to all-*trans*-retinol.

Mutations in *ABCR* gene have been found in a subpopulation of patients with retinitis pigmentosa (131), rod-cone dystrophy (132) and AMD (133,134) and were identified as a causal factor of Stargardt disease (135). Stargardt disease is a recessive form of macular degeneration with progressive loss of central vision, rapid accumulation of autofluorescent deposits, called lipofuscin, in the

RPE cells followed by atrophy of the RPE (136). RPE lipofuscin is believed to originate mainly from incomplete lysosomal digestion of phagocytosed photoreceptor OS (9,137).

Patients with Stargardt disease and *ABCR* knockout mice have an increased content of PE in their retinas (96,130). The post-mortem retinas from patients with Stargardt disease and fundus flavimaculatus contain 2.1- and 2.9-fold increased PE concentrations, respectively, in comparison with the age-matched controls (97). The concentration of PE in OS of *ABCR* knockout mice is about 1.6–1.9-fold greater than in wild-type mice (96,97). As a result, the *ABCR*<sup>−/−</sup> animals accumulate in their OS about 3 times increased concentrations of NRPE in comparison with wild-type animals (97). NRPE accounts for 24% and 61% of the ATR accumulated in OS of knockout and wild-type animals, respectively (97). The amounts of ATR extracted from the *ABCR* knockout mice OS immediately after photobleaching of about 45% of rhodopsin are similar to the amounts of ATR isolated from the wild-type mice OS (97). The total concentrations of ATR present as free ATR and NRPE are elevated in the *ABCR*<sup>−/−</sup> mice and *ABCR*<sup>+/-</sup> in comparison to the wild-type mice immediately after exposure to light and during subsequent recovery in dark (96,97,130).

The formation of NRPE from ATR and PE is a reversible reaction sensitive to concentration of water (138). Therefore the extraction procedures, evaporation of chloroform and redissolving of the sample in hexane, are likely to affect the equilibrium. The technical difficulties in obtaining reproducible data are apparent from the comparison of the results obtained by the same group on the accumulation of protonated and unprotonated NRPE in wild-type and *ABCR* knockout mice (96,97). First, it was reported that

*ABCR* knockout mice accumulate largely protonated NRPE, while wild-type mice accumulate unprotonated (96). In a subsequent publication, the nonprotonated form was the predominant form of NRPE in both *ABCR* knockout mice and wild-type mice and the protonated form accounted for only 10% and 20% of total NRPE in *ABCR* knockout mice and wild-type mice, respectively (97).

As mentioned earlier, the enzymatic reduction ATR to all-*trans*-retinol is a relatively slow process (122). The reduction is not a limiting factor for rhodopsin regeneration, however; after a flash of light bleaching approximately 80% of rhodopsin, the rate constant of ATR decay of  $0.087 \text{ min}^{-1}$  was 5.8 times greater than the initial rate of regeneration of photobleached rhodopsin,  $0.015 \text{ min}^{-1}$  (76,139). It can be assumed that the initial concentration of ATR released from rhodopsin and the rate of its reduction to all-*trans*-retinol determine the concentrations of ATR transiently accumulating within photoreceptor discs after a pulse of bleaching light. During constant exposure to light, the ability of rhodopsin to regenerate dependent on the supply of 11-*cis*-retinal, is an additional factor determining the steady-state concentrations of ATR, which can be either bound to the opsin "exit site" or completely dissociated and accumulated within photoreceptor OS discs. If the rate of rhodopsin regeneration exceeds the rate of ATR reduction to all-*trans*-retinol, some ATR becomes released from the opsin "exit site" (93). Then the bimolecular rate constants of interaction of ATR and PE and the equilibrium constant of this reversible reaction determine the ratio of free ATR to NRPE. Finally, *ABCR* facilitates the enzymatic reduction of ATR by translocation of NRPE/ATR to the cytoplasmic side of the disk membrane.

#### Conversion of all-*trans*-retinol to 11-*cis*-retinol

Regeneration of 11-*cis*-retinal from all-*trans*-retinol involves a complex system; the isomerized and reduced chromophore is transferred from the OS to the adjacent RPE cells where the multi-step process takes place (Fig. 3b) (see reviews by Imanishi and colleagues [140], Kuksa and colleagues [118], McBee and colleagues [141] and Lamb and Pugh [76]). First, all-*trans*-retinol needs to leave the rod OS disc and cross the OS plasma membrane. The transfer of all-*trans*-retinol through interphotoreceptor matrix (IPM) is facilitated by interphotoreceptor retinoid-binding protein (IRBP) (123), which has been shown to protect all-*trans*-retinol from isomerization and oxidation (142). Within the RPE cells all-*trans*-retinol is chaperoned by cellular retinol-binding protein (CRBP). Next, all-*trans*-retinol is esterified by lecithin:retinol acyltransferase (LRAT) within endoplasmic reticulum (ER) forming predominantly all-*trans*-retinyl palmitate or stearate (143).

All-*trans*-retinyl esters are thought to form aggregates that bud off from the ER as independent structures, forming so called retinosomes or retinyl-ester-storage (REST) particles (140,144). The retinosomes are of cylindrical shape,  $6.9 \pm 1.1 \mu\text{m}$  long and  $0.8 \pm 0.2 \mu\text{m}$  in diameter, distributed perpendicularly to the RPE monolayer. Once formed, retinosomes seem to be stable RPE organelles where all-*trans*-retinyl esters accumulate after rhodopsin bleaching and are depleted during dark adaptation (140,144).

To generate 11-*cis*-retinol, all-*trans*-retinyl esters become isomerized and hydrolyzed (118,141,145,146). The conversion of all-*trans*-retinyl esters to 11-*cis*-retinol requires a RPE-specific 65 kDa protein, RPE65 (147–149). RPE65 has been shown to interact with all-*trans*-retinyl palmitate, all-*trans*-retinol and 11-*cis*-retinol, but only very weakly with 11-*cis*-retinyl palmitate (148). It has been demonstrated recently that RPE65 plays a role in extraction of

all-*trans*-retinyl esters from lipid membranes and is directly responsible for their isomerization (147,150,151). In the absence of RPE65, or if it is dysfunctional, all-*trans*-retinyl esters accumulate in the RPE but there is no production of 11-*cis*-retinal (152,153). Mutations of RPE65 account for about 10% of cases of Leber's congenital amaurosis (LCA), a disease that can cause severe blindness from birth or early childhood (154,155).

#### Oxidation of 11-*cis*-retinol to 11-*cis*-retinal

In the next step, the product of isomerization and hydrolysis, 11-*cis*-retinol, binds to cellular retinal-binding protein (CRABP) (156). CRABP-11-*cis*-retinol complex serves as a substrate carrier for 11-*cis*-RDH (RDH5) (139,156,157). RDH5 with NADH as a cofactor is more efficient in oxidizing retinols than reducing retinals and 9-*cis*-retinol and 11-*cis*-retinol are preferred substrates over all-*trans*-retinol (158). Apart from RDH5 there are at least two other oxidoreductases within RPE that can convert 11-*cis*-retinol to 11-*cis*-retinal (159–162). One of them is RDH11, a membrane-associated protein, which, with NADPH as a cofactor, catalyzes the reduction of retinals (ATR, 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal), which *in vitro* is about 50 times more efficient than oxidation of retinols (163). It also catalyzes the reduction of some aldehydes derived from lipid peroxidation. However, it may be argued that due to the local concentration of substrates within RPE cells, RDH11 may catalyze the reaction in the opposite direction using NADP<sup>+</sup> as a cofactor and oxidize retinols *in vivo* (161). Yet, *RDH11*<sup>−/−</sup> and *RDH5*<sup>−/−</sup> knockout mice are still capable of 11-*cis*-retinal regeneration, which suggest that another oxidoreductase is present within the RPE (159).

Although *RDH11* and *RDH5* knockout mice appear to have normal visual function, mutations of RDH5 in humans, which result in a reduced enzymatic activity, lead to retinal dystrophies, such as recessive fundus albipunctatus, a rare form of stationary night blindness characterized by a delay in the regeneration of visual pigments of cones and rods or recessive cone dystrophy (164–167).

In the final step, the 11-*cis*-retinal needs to cross the RPE and IPM to reach OS membrane. It was suggested that retinoid trafficking may be facilitated by colocalization of several proteins, such as CRABP, RDH5 and IRBP within apical portion of the RPE cells (168–172). 11-*cis*-retinal within the IPM is bound to IRBP (173). However, lack of IRBP does not significantly affect the rates of rhodopsin regeneration, suggesting that its transport function may be replaced by other proteins such as albumin. It appears that the main function of IRBP may be protection of retinoids from photodegradation (142,174).

Transport of 11-*cis*-retinal through the OS plasma membrane to the disc membranes has not been elucidated. It may be speculated that this process may depend on a number of other proteins, probably indirectly involved in retinoid trafficking, such as peripherin, a protein believed to play a structural role in photoreceptor OS coded by *RDS* gene whose mutations can lead to delayed dark adaptation and retinopathies (175–178).

Once in the OS discs 11-*cis*-retinal diffuses to encounter an opsin. Binding of 11-*cis*-retinal to opsin proceeds first through a non-covalent bond at its "entry site," after which a covalent Schiff-base linkage with Lys296 is formed regenerating rhodopsin (93,94). Thus it can be expected that the release of all ATR/retinol from the opsin "exit site" requires the mole equivalent of 11-*cis*-retinal binding to opsin. Currently it is not clear what the regulatory mechanisms

responsible for mobilization of retinyl esters stores are and the upper limit of ATR that can accumulate in the OS remains unknown.

#### Do cones use an additional pathway for regeneration of 11-*cis*-retinal?

The retinoid cycle described above refers to both rods and cones. However, in cone dominant retinas, cones seem to evolve an additional pathway for regeneration of 11-*cis*-retinal (179). It is supported by the fact that cones regenerate visual pigment in isolated retinas detached from the RPE (180,181). It is suggested that cones employ Müller cells, the major glial cells in the retina, instead of RPE cells, to perform isomerization of all-*trans*-retinol to 11-*cis*-retinol and formation of 11-*cis*-retinyl esters stores (179). Müller cells express, similarly to the RPE, retinoid-binding proteins CRBP and CRALBP, which chaperone all-*trans*-retinol and 11-*cis*-retinal/retinol, respectively (171,172,182–184) and can transform all-*trans*-retinol to 11-*cis*-retinol in cell culture (185). Cone-rich retinas of chicken and ground squirrel contain substantial concentrations of 11-*cis*- and all-*trans*-retinyl esters in the neural part of the retina, which are present in the concentrations about 2- to 6-fold greater than in the RPE (179). Interestingly, 11-*cis*-retinyl esters are formed from all-*trans*-retinol and the process is not inhibited by well established inhibitors of LRAT, indicating that there is another enzyme responsible for formation of 11-*cis*-retinyl esters. It is also supported by the fact that 11-*cis*-retinyl esters synthase activity is increased in the presence of palmitoyl, myristoleoyl and stearoyl coenzyme A, but not in the presence of dipalmitoyl phosphatidylcholine, which serves as a fatty acyl donor for LRAT. An essential enzyme for the formation of 11-*cis*-retinol from all-*trans*-retinyl esters RPE65 has been found in cones but not in rods in mammalian retinas (186).

11-*cis*-retinol released from the Müller cells is taken up by the cones, where the final step, NADP<sup>+</sup>-dependent oxidation of 11-*cis*-retinol to 11-*cis*-retinal by 11-*cis*-specific RDH, takes place (179,187,188). It was hypothesized that the main enzyme responsible for the oxidation of 11-*cis*-retinol to 11-*cis*-retinal for regeneration of cone visual pigments is NADPH-dependent RDH12 (141). RDH12 is expressed in the retina in the rod and cone inner segments and cell bodies and exhibits similar activities toward retinals as RDH11; with NADPH as a cofactor it catalyzes the reduction of different isomers of retinals (ATR, 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal) (161). Thus it may be speculated that RDH12 may play a dual role. In both rods and cones RDH12 may regulate the supply of 11-*cis*-retinal for synthesis of visual pigments in the inner segments and in cones may oxidize 11-*cis*-retinol for regeneration of cone visual pigments.

Mutations in *RDH12* cause a severe subtype of LCA, progressive rod and cone dystrophy, leading to severe macular atrophy and vision loss in early adulthood (189,190). Several *RDH12* mutations causing the disease have been identified and their consequences elucidated in COS-7 cells *in vitro* transfected with wild-type or two mutant *RDH12* constructs (190). Cys226 variant caused a decreased protein expression and no detectable activity toward either NADP<sup>+</sup>-dependent all-*trans*-retinol oxidation or NADPH-dependent ATR reduction. On the other hand, Met49 variant exhibited 90% higher activity in conversion of ATR to all-*trans*-retinol and 44% lower activity in conversion of all-*trans*-retinol to ATR in comparison with the wild-type protein. It might be only speculated that, by analogy to RPE65 mutations causing

LCA, the primary cause of the observed phenotype is related to *RDH12* dysfunction leading to deficiency in 11-*cis*-retinal and inability to form visual pigments.

Despite the proposed alternative pathway of regeneration of 11-*cis*-retinal in cones, mutations in *RDH5*, which is expressed exclusively in the RPE, can affect cones more severely than rods leading to recessive cone dystrophy (166,167). Again, the explanation of this can be limited only to speculations. As already mentioned mutations in *RDH5* in humans lead to delayed regeneration of visual pigments of cones and rods (164–167). It may be suggested that delayed regeneration of rhodopsin will lead to a delayed release of ATR/all-*trans*-retinol from the opsin “exit site” and, as a consequence, decreased concentrations of all-*trans*-retinol released from rods. Rods are predominant photoreceptors in the human retina so they are mainly responsible for transient accumulation of all-*trans*-retinol in the IPM. As all-*trans*-retinol released from rods can be used for regeneration of cone visual pigment in the absence of RPE (180,181), therefore a dysfunction of *RDH5* can affect cones both directly, *via* decreased supply of 11-*cis*-retinal from the RPE and indirectly, *via* decreased supply of all-*trans*-retinol from rods.

Cones outside the area responsible for acute vision (fovea) are shorter and rather distant from the apical site of the RPE, so RPE processes need to project for a relatively long distance to reach cone OS. Thus, it is tempting to speculate that peripheral cones in humans are in greater need to use Müller cells for regeneration of their visual pigments than foveal cones present in proximity to the RPE. The alternative pathway of the regeneration of 11-*cis*-retinal may also explain why the visual pigment regeneration in cones is about 20 times faster than in rods, which allows cones to remain responsive at high fluence rates of light reaching the retina (179). Instead of competing with predominant rods for regenerated 11-*cis*-retinal, cones can use the 11-*cis*-retinol produced by rods as a result of rhodopsin photobleaching.

#### What are limiting steps in the synthesis of rhodopsin chromophore?

Thorough analysis of an extensive range of published experimental data on the recovery of rhodopsin and the photosensitivity threshold in humans and experimental animals performed by Lamb and Pugh (76) indicates that the initial rate of recovery of rod rhodopsin is independent of the rate of ATR reduction within rod OS, as previously suggested (122), but it strongly depends on the efficiency of RPE to synthesize 11-*cis*-retinal. It is supported by the fact that, even in the case of patients with *ABCR* mutations in early stages of the disease, where there is a delay in removal of ATR from OS, the rate of the fast component of recovery of photosensitivity remains the same as in normal subjects (191). Also the rate of 11-*cis*-retinal and rhodopsin regeneration is similar in *ABCR*–/– mice and wild-type mice after 5 min exposure to bleaching of about 45% rhodopsin, whereas ATR clearance is significantly delayed in knockout animals compared with wild type (96,130). It may be speculated that the slow component observed in the recovery of rhodopsin in rods, evident upon bleaches of 60% of the pigment and above (76), is due to recycling of ATR released from rhodopsin. If this is the case, the rate of the slow component may be affected by *ABCR* dysfunction.

It has been determined in human eyes postmortem that retinoids present in RPE/choroid, mainly retinyl esters, account for 2.5 mol equiv of rhodopsin (192). In contrast, nocturnal animals, such as

mice and rats, may have no need for accumulation of retinyl esters as, when they are reared under dim light, only a fraction of rhodopsin is bleached. Indeed, retinyl esters account for only a fraction of the mol equivalent of rhodopsin in mice (76).

It may be speculated that rhodopsin photobleaching results in a signaling pathway leading to the mobilization of retinyl ester stores and their conversion to 11-*cis*-retinal. As the retinyl ester stores represent the main pool of retinoids in humans, it would explain the similar rates of rhodopsin recovery observed in patients with Stargardt disease with delayed ATR removal and in normal subjects (191). However, the mechanism responsible for the mobilization of retinyl esters remains unclear. Several publications pointed to the potential role of RPE in this process (193–200). Experiments on fetal human RPE cells in culture demonstrated that RPE cells do play a role in photoreception and respond to light by an increased generation of 11-*cis*-retinal (193). It was shown that on exposure to all-*trans*-retinol RPE cells accumulated all-*trans*-retinyl esters and synthesis of retinyl esters continued in dark after withdrawal of all-*trans*-retinol from the medium. However, cells exposed to light reached 60% lower levels of all-*trans*-retinyl esters compared with cells incubated in the dark and released almost twice greater amounts of 11-*cis*-retinal in the media in the light-compared with the dark-incubated cultures. It was suggested that opsinlike proteins expressed in the RPE cells and Müller cells, such as retinal G-protein-coupled receptor (RGR) or peropsin, may play a signaling role for mobilization of retinyl esters (193).

Isolated RGR from bovine RPE contains mainly ATR, about 85% and small amounts of 11-*cis*-retinal (8%) and 13-*cis*-retinal (7%) (194). ATR is bound to RGR via Schiff base linkage to lysine and RGR exhibits broad absorption spectra with maxima at 370 nm ( $\epsilon = 6.61 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) and 469 nm ( $\epsilon = 6.28 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) for unprotonated and protonated forms, respectively (194). At neutral pH RGR is present mainly in unprotonated form and therefore absorbs UV rather than blue light (195). Irradiation of RGR with UV (370 nm) or blue light (470 nm) *in vitro* leads to isomerization of ATR to 11-*cis*-retinal with a quantum yield of 0.12 (194). RGR copurifies with NADH-dependent 11-*cis* RDH (RDH5) and both proteins form a functional protein complex (196). After photoisomerization of ATR, 11-*cis*-retinal is rapidly reduced to 11-*cis*-retinol and dissociates from RGR (196).

It was proposed that RGR can function as photoisomerase that provides 11-*cis*-retinal for rhodopsin regeneration under exposure to light (197). However, there are several pieces of evidence supporting an opposite view that its role in generating and providing 11-*cis*-retinal to rhodopsin seems unlikely (198). First, RGR is present in a relatively small concentration compared with rhodopsin. Second, it is localized in RPE microsomes and therefore a substantial portion of the short-wavelength light reaching the human retina will be filtered out by photoreceptor outer segments before reaching RPE. Third, its quantum yield of photoisomerization is 5.6 times smaller than rhodopsin. Overall, it can be assumed that RGR is an inefficient photoisomerase of 11-*cis*-retinal and can provide only a minor direct contribution to regeneration of rhodopsin.

Yet it has been shown that RGR does play an important role in regeneration of rhodopsin during constant exposure to white light (196,197). Upon exposure to a constant illuminance of 1400 lux, the steady-state concentration of rhodopsin in RGR-/- mice reached 44% of the dark level, while under the same conditions, rhodopsin in the wild-type mice reached 98% of its level in the dark. The time constant for rhodopsin regeneration was 62 times

greater in knockout animals than in the wild-type animals. Instead of efficient synthesis of 11-*cis*-retinal, the knockout animals accumulated retinyl esters, which were increased by a factor of 14.5 in comparison with wild-type animals. Both wild-type and RGR-/- mice had similar rhodopsin levels in dark and the kinetics of rhodopsin regeneration in dark after a flash of bleaching light was also similar (197,198). Thus, it has been suggested that RGR may stimulate synthesis of 11-*cis*-retinal acting as a G-protein-coupled receptor that becomes activated by absorption of a photon (197).

A comparison of RPE65 knockout mice with double-knockout mice RPE65-/- and RGR-/- demonstrated that specific photoisomerization of ATR to 11-*cis*-retinal occurs both in the neural retina and RPE in both RPE65-/- and RPE65-/-RGR-/- mice (199). This indicates that, apart from RGR, there is another mechanism responsible for photoisomerization of ATR.

Peropsin, another opsinlike protein, is present in the apical part of RPE and microvilli surrounding photoreceptor OS (200). It has been suggested that peropsin may act as a sensor of ATR released by photobleaching of rhodopsin in the adjacent OS (200). Binding of ATR to peropsin may initiate a signaling cascade, independent of light, resulting in generation of 11-*cis*-retinal from retinyl ester pools. Alternatively, the signaling cascade might be activated by a photoisomerization of peropsin chromophore.

Summing up, it can be speculated that the extensive bleaching of rhodopsin accompanied by efficient supply of 11-*cis*-retinal and rhodopsin regeneration may lead to the accumulation of substantial concentrations of ATR within photoreceptor discs. Presently, we do not know what the worst case scenario for accumulation of ATR within OS is. We can only speculate that in the case of exposure to intensive light rapidly bleaching all the rhodopsin resulting in very low steady-state levels of rhodopsin, the rate of removal of ATR, the content of retinyl esters stored in retinosomes and the regulatory pathways responsible for their mobilization will determine the concentrations of ATR transiently accumulating in the OS. It may be further speculated that in case of dysfunction of ATR removal from the OS, the upper limit of accumulation of ATR may be determined by the content of retinoids stored in the RPE. For normal subjects with 3.8 mM rhodopsin and 2.5-fold excess of retinoids stored as retinyl esters, it would give the value of  $(3.8 \text{ mM} + 2.5 \times 3.8 \text{ mM}) = 13.3 \text{ mM}$  ATR in the case of total dysfunction in ATR removal from the OS. Elucidation of the mechanisms responsible for 11-*cis*-retinal synthesis and delivery to the OS and regulatory pathways involved is essential for understanding of the retinoid trafficking in the retina and the design of pharmacological intervention in the case of their dysfunction or inadequacy under bright-light conditions.

## DOES FREE ALL-TRANS-RETINAL EXIST IN THE RETINA?

The inadequacy in the removal of ATR from OS discs leads to accelerated accumulation of a pyridium bisretinoid, so called A2E (Fig. 4), in the RPE. It is evident in particular in patients with Stargardt disease and fundus flavimaculatus caused by mutations in the ABCR gene, or in ABCR-/- or ABCR+/- mice (96,97,130). Study of biosynthetic pathways of A2E indicate that it originates from the condensation of NRPE with another molecule of ATR, which occurs within the OS discs (see papers by Travis and colleagues [96,97] and Nakanishi and colleagues [98,99,201] and review by Lamb and Simon [202]). PE is widely abundant in the OS, where it accounts for 409 to 487 nmol/mg protein (203).

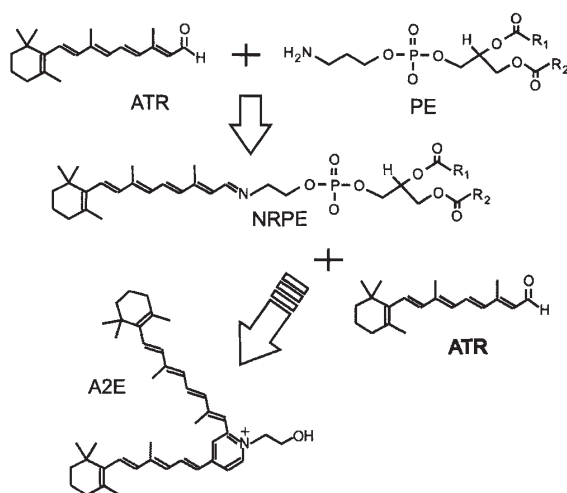


**Figure 4.** Initial reactions in biosynthesis of A2E. ATR hydrolyzed from opsin binds to a highly abundant OS phospholipid, PE, forming NRPE. Binding of another molecule of ATR is required to form a precursor for a pyridinium bisretinoid, A2E. See text for further description.

Assuming that all protein in the OS is rhodopsin, it allows the calculation of an upper limit of the molar ratio of ATR to PE, which is at most 6.1% in normal retinas after a flash of light photobleaching all rhodopsin. Thus the molar ratio of ATR to PE in photoreceptor outer segments is similar to the ratios employed for efficient NRPE synthesis *in vitro* (138,204).

*In vitro*, in chloroform or chloroform/ethanol mixture (2:1, v/v), the bimolecular rates of interaction of ATR with PE leading to the formation of NRPE are within the range of  $(3.0\text{--}4.5) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ , whereas the rate constant for the backward reaction is within the range of  $(5.9\text{--}9.9) \times 10^{-6} \text{ s}^{-1}$  (138). Assuming the bimolecular rate of interaction of ATR with PE in OS discs is similar as in solution, the initial rate of formation of NRPE from interaction of 3 mM ATR and 50 mM PE is expected to be about  $4.5\text{--}6.7 \mu\text{M s}^{-1}$ . This implies that 3 mM ATR rapidly released from opsin may be transformed into NRPE within tens of minutes. Yet, A2E synthesis requires NRPE to interact with another ATR. Thus it may be speculated that OS discs membranes favor generation of NRPE and the interaction of ATR with NRPE is a rare event requiring local excess of ATR concentration over PE concentration.

A2E has also been identified in substantial quantities in normal human RPE from mature donors (205,206). Eldred and Lasky obtained almost 0.1 mg of A2E from RPE cells from at least 250 eyes of human donors of 40 years of age or older, which allows obtaining a lower estimate of the average content of A2E in RPE for about 400 ng/eye (0.675 nmol/eye) (205) and the A2E concentration within RPE cells. Assuming that A2E is distributed evenly throughout the whole RPE overlying the retina of surface of about 1000 mm<sup>2</sup> and the thickness of RPE monolayer is 0.014 mm, the average concentration of A2E within the RPE layer is 48  $\mu\text{M}$ . Measurements of A2E content in individual RPE isolations from 10 healthy human donors indicated that there are considerable variations between individuals (206). The highest content of A2E of 134 ng/10<sup>5</sup> cells was noted in a 64-year-old person, whereas the lowest, 34 ng/10<sup>5</sup> cells in a 79-year-old person. Assuming a cuboidal shape of a RPE cell with 0.014 mm thickness, it gives concentrations of A2E in RPE cells falling within a range of

209–824  $\mu\text{M}$ , which is 4 to 17 times greater than the concentration estimate obtained from the Eldred and Lasky's data (205).

A2E fluorescence allows monitoring of the distribution of A2E in the retina. The highest A2E fluorescence intensities were identified in the perifovea and decrease toward the center of the fovea and periphery (207,208). Thus it may be expected that the concentrations of A2E in the retina can largely exceed the average concentrations in the perifovea and fall down to much lower levels outside this area.

A2E levels can be greatly increased in some retinal dystrophies and retinopathies. Patients with Stargardt disease and fundus flavimaculatus have 6.22-fold and 11.5-fold increased levels of A2E in the RPE, respectively, in comparison with RPE from age-matched control eyes (97). The levels of A2E in *ABCR*<sup>−/−</sup> mice are increasing with age at the rate approximately 9 to 13 times greater than in wild-type mice when animals are kept in cyclic light, but when animals are kept in dark the rate of A2E accumulation is greatly reduced and similar in both groups (97,130). Also *ABCR*<sup>+/−</sup> mice exhibit about 4 times increased accumulation of A2E over wild-type mice (130). It indicates that accumulation of ATR in OS and light exposure play major causative roles in A2E accumulation.

On the other hand, impairment in the regeneration pathways of 11-*cis*-retinal within RPE, caused either by defective or absent RPE65 protein, leads to inhibition in the accumulation of A2E (152,209). 13-*cis*-retinoic acid (Isotretinoin; Accutane) slows down 11-*cis*-retinal synthesis because of competitive binding to RPE65 and 11-*cis*-RDH (210,211) and also blocks the slower, age-dependent accumulation of A2E/lipofuscin in wild-type mice (212), suggesting that the accumulation depends also on the supply of 11-*cis*-retinal to the OS.

Recently, another molecule, so called retinal dimer and its conjugate with PE has been identified in isolated bovine OS irradiated with white light, although in much smaller quantities than the precursor of A2E, A2-PE (95). Products of similar UV-visible absorption spectra and HPLC retention times as retinal dimer-PE conjugate were also extracted from RPE/choroids and neural retinas of *ABCR* knockout mice and human RPE cells from 31- to 40-year-old donors. It has been suggested that retinal dimer is derived from an interaction of ATR with NRPE in the OS followed by elimination of PE. It was demonstrated that the retinal dimer can easily form a conjugate with PE *via* Schiff base.

Accumulation pattern of A2E in the retina and the discovery of its biosynthetic pathway and identification of retinal dimer provide evidence that substantial amounts of ATR must be present in the OS to form a condensation product of two molecules of ATR with PE.

## PHOTOSENSITIZING PROPERTIES OF ATR AND ITS DERIVATIVES

Delmelle postulated as early as in 1977 that the isomerized chromophore of visual pigment ATR is responsible for initiation of photosensitized damage to the retina (91,92). ATR is a potent photosensitizer whose absorption spectrum with an absorption maximum at about 380 nm extends into the visible (Fig. 5a). Like most biological molecules, except for molecular oxygen, ATR is present in its singlet state in the ground state with all electrons in molecular orbitals being paired. Photoexcitation of ATR with 353 nm light results in formation of an excited singlet state followed by an efficient intersystem crossing from an excited singlet state to an excited triplet state with quantum yields dependent on solvent

polarity (213) and varying from 0.05 to 0.12 in methanol to 0.50 in cyclohexane (213–215). 11-*cis*-retinal exhibits similar quantum yields of intersystem crossing: 0.11 in methanol and 0.51 in hexane. The ability to undergo intersystem crossing and form an excited electronic state of multiplicity different from the ground state is a good indication of potential photosensitizing properties of a molecule, as then the excited state is relatively long lived, in the range of microseconds, which increases the probability of interaction with other molecules (214). Triplet state of ATR decays with a rate constant  $0.6 \times 10^5 \text{ s}^{-1}$  and  $1.1 \times 10^5 \text{ s}^{-1}$  in methanol and hexane, respectively (214).

In the presence of molecular oxygen, the triplet state of ATR is efficiently quenched by oxygen (216,217) and the excess of electronic energy is transferred to oxygen forming an electronically excited state, singlet oxygen with quantum yields ranging from 0.05 in methanol (92,217) to 0.30 in benzene (218). Triplet state of ATR can also interact with a number of electron donors and acceptors (219). Photoexcitation of ATR in methanol or DMSO results in generation of superoxide radical (215,220). Photoexcitation of ATR in liposomes containing unsaturated fatty acids and cholesterol leads to lipid peroxidation which is mediated mainly by singlet oxygen (220).

In the retina, ATR can be bound by a number of proteins or phosphatidylethanolamine (76,126) and the binding may dramatically change its photochemical properties. In rhodopsin and its intermediates formed by photolysis, including Meta II and Meta III, 11-*cis*-retinal or ATR is bound *via* Schiff base linkage to lysine. All Schiff bases of ATR studied so far, such as *N*-retinylidene butylamine or *N*-retinylidene ethanolamine, exhibit substantially smaller quantum yields of intersystem crossing than free ATR. Quantum yields of triplet state formation range from below 0.001 in alcohol and 0.003–0.008 in hydrocarbon solvents for *N*-retinylidene butylamine (213–215,221) to 0.055 for *N*-retinylidene ethanolamine in acetonitrile (222).

Photophysical studies on rhodopsin and its photointermediates do not indicate any photosensitizing potential. Photoexcitation of rhodopsin leads to isomerization of its chromophore with a quantum yield of 0.65, whereas the photoexcitation by the remaining 35% of photons is followed by deactivation *via* internal conversion (223). Photoexcitation of Meta II results in formation of Meta III (112). Elucidation of photochemical pathways following photoexcitation of Meta III (115) indicate that 98% of photolyzed intermediates of Meta III account for well-known products (Meta I/II, rhodopsin and isorhodopsin) and therefore only the remaining 2% might be considered as potential products of photosensitizing properties. The elucidated interactions of rhodopsin intermediates with light and the fact that in both cases ATR is still bound *via* Schiff base linkage to opsin exclude Meta II and Meta III as effective photosensitizers.

As already mentioned, ATR released from opsin in photoreceptor OS binds to PE *via* Schiff base linkage forming NRPE (96,97,125,126). Therefore, it may be speculated that the presence of increased concentrations of PE observed in retinas in *ABCR*–/– mice or patients with Stargardt disease with mutations in the *ABCR* gene (96), plays a protective role facilitating the formation of NRPE. Indeed, we have shown that susceptibility to photooxidation of liposomes containing ATR in the presence of PE is dramatically reduced compared with liposomes in the absence of PE (224). This effect is due to the decreased quantum yields of singlet oxygen generation of ATR bound to PE in comparison with free ATR (224).

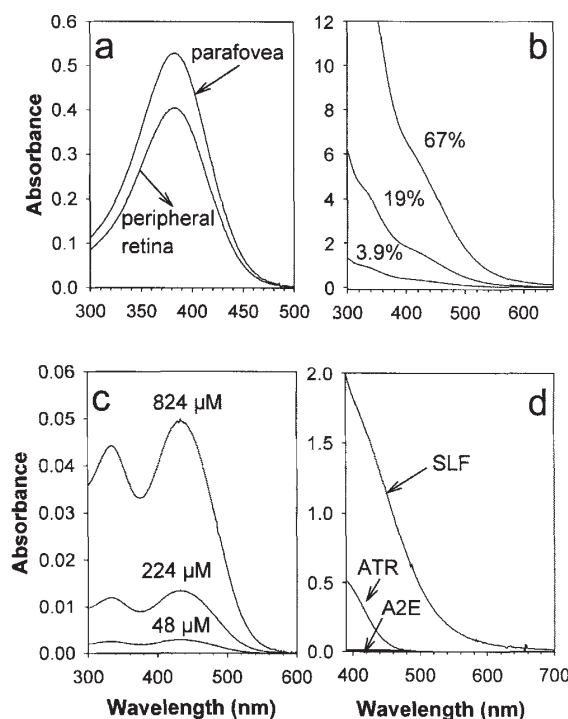


**Figure 5.** Contribution of ATR (a), chloroform-soluble components of lipofuscin (b) and A2E (c) to the absorption of light in the outer retina, OS and RPE layers. Absorption spectra of 3.8 mM ATR accumulated in perfovea and periphery (a) are based on the extinction coefficient of ATR of  $48\,000 \text{ M}^{-1}\text{cm}^{-1}$  (198) and thickness of OS layer of 31.2 μm and 23.9 μm in perfovea and periphery (75), respectively. Absorption spectra of chloroform-soluble components of lipofuscin (b) are based on (i) absorption spectra of measured dry weight of these components solubilized in benzene (283); (ii) the content of chloroform-soluble components of lipofuscin per lipofuscin granule, 0.093 pg/granule (283); (iii) an estimated contribution of lipofuscin to RPE cell volume, where a lipofuscin volume was calculated based on an average diameter of lipofuscin granule of 0.5 μm; and (iv) thickness of RPE layer of 14 μm (286). Volume of 3.9% occupied by lipofuscin in RPE was obtained based on (i) data of Eldred and Lasky (205) providing a lower limit of average content of A2E in RPE cells from donors above 40 years old and (ii) data of Davies and colleagues (284), on A2E content per lipofuscin granule,  $7.8 \times 10^{-20} \text{ mol/granule}$ ; (iii) surface of the retina of  $1000 \text{ mm}^2$  and thickness of RPE layer of 14 μm (286). Volume of 19% occupied by lipofuscin in RPE was obtained directly based on (i) data of (285) on the upper limit of 19% of RPE cell volume occupied by lipofuscin in the macula and (ii) cuboidal shape of RPE cells of the side length of 14 μm (286). Volume of 67% occupied by lipofuscin in RPE was obtained based on (i) data of Sparrow and colleagues (206) on the upper limit of A2E content per RPE cell of 1.34 pg/cell reported in RPE cells from a healthy retina, (ii) the content of A2E per lipofuscin granule (284) and (iii) cuboidal shape of RPE cells of a side length of 14 μm (286). These data lead to an extremely high value of lipofuscin content in RPE cells, which may be due to an underestimated size of RPE cells in this individual. As with age there is a progressive loss of RPE cells, the neighboring cells may spread and occupy a larger area, which results in an increased cell volume. (c) Absorption spectra of A2E in RPE layer are based on extinction coefficient of A2E at absorption maximum of  $36\,900 \text{ M}^{-1}\text{cm}^{-1}$  and correspond to A2E concentrations in RPE calculated based on the data as in (b). Concentrations of 48, 224 and 824 μM A2E correspond to 3.9%, 19% and 67% of cell volume occupied by lipofuscin and 1706, 7966, 29000 lipofuscin granules per cell, respectively. (d) Comparison of the contribution to absorption of visible light in the perfovea by 3.8 mM ATR, chloroform-soluble components of lipofuscin, which occupies 19% of cell volume, and 224 μM A2E present in that lipofuscin.

Binding of retinals to opsin "entrance/exit sites," CRABP or IRBP involve noncovalent interactions (93,157,174,225). It remains to be elucidated how these noncovalent bonds affect photochemical and photosensitizing properties of retinals. Many studies have shown that retinal can induce photodamage to proteins it interacts with (174,226,227).

Photooxidation of SH groups in rhodopsin has been shown *in vitro* (226). However, mass spectroscopic analysis of rhodopsin isolated from rats subjected to light-induced retinal damage did not reveal any oxidatively damaged residues except for tryptophan, whose oxidation was ascribed to the CNBr used for protein cleavage (228).

Photoexcitation of ATR in the IRBP-ATR complex *in vitro* results in damage to the protein detected as loss of tryptophan fluorescence, decreased thiol groups and retinol-binding activity, while the ATR remains (174). CRABP on the other hand, does not prevent isomerization of complexed 11-*cis*-retinal though the quantum yield of photoisomerization, 0.07, is relatively small (157,225). Photoexcitation of CRABP-11-*cis*-retinal complex with blue light ( $\lambda_{\text{max}} = 425$  nm) results in a release of ATR. Thus it may be speculated that CRABP may then become a potential target for photosensitized damage mediated by photoexcited ATR.

Irradiation of photoreceptor proteins (peripherin/RDS, ROM-1, or ABCR) *in vitro* with 366 nm light or visible light  $>410$  nm in the presence of ATR under aerobic conditions results in oxidative damage to the protein and their aggregation through the formation of covalent cross-links. In addition, ATPase activity of ABCR is lost (227). Thus it may be speculated that if photooxidative damage to ABCR occurs *in vivo*, it would lock NRPE within the protein and disable further catalytic removal of ATR to the cytoplasmic site of the OS membrane resulting in accumulation of the photosensitizer within the membrane.

Overall, it is clear that accumulation of free ATR can impose a risk of photooxidative damage to the retina. However, in studies on light damage to the retina, ATR was not considered as a mediator of photodamage to the retina as it was believed that after hydrolysis from opsin it was rapidly reduced to all-*trans*-retinol (71). Only in the recent years has a sufficient body of evidence accumulated to indicate that indeed ATR does transiently accumulate in the retina as a result of photobleaching of rhodopsin (122) and the biosynthetic pathway of A2E formation indicates that substantial concentrations of free ATR must be present in the OS to react with NRPE instead of with abundant PE (96–99,122). The study on the mechanism of the release of ATR from opsin (93,94) indicates the importance of efficient synthesis and delivery of 11-*cis*-retinal to the OS for ATR accumulation.

## ROLE OF ATR IN RHODOPSIN-DEPENDENT LIGHT-INDUCED DAMAGE TO THE RETINA

### Rhodopsin as an essential factor for photodamage to occur

Rhodopsin has been shown to be an essential factor for photodamage to the retina to occur (56,79–81). As mentioned earlier, the action spectrum of retinal photodamage in rats reared at dim cyclic light and exposed for a relatively long periods to low-intensity light coincides with the absorption spectrum of rhodopsin (55,56). In these experiments demonstrating the action spectrum of retinal photodamage peaking at 500 nm, rhodopsin reached a steady-state concentration of about 35% of the value in dark-adapted animals. Under these conditions ATR cannot act as

photosensitizer as the contribution of ATR to the absorption of photons of 500 nm wavelength is negligible. It may be speculated that in these experiments, the damaging action was due to the constant fluxes 11-*cis*-retinal/ATR to and from OS and retinals exerted their toxic effects through imposing oxidative stress in OS without involvement of photosensitization. Indeed, it has been shown that ATR can impose oxidative damage to DNA (229) and toxicity to cells in culture (230) in dark at concentrations as low as 0.001 mM and 0.100 mM, respectively. It may be assumed that this type of damage occurs exclusively in retinas with high levels of rhodopsin, such as in nocturnal animals reared in dark or under dim cyclic light.

The action spectra of retinal photodamage observed in primates and rodents at higher irradiance levels exhibited an increase in susceptibility to photodamage with decreasing wavelength down to 320 nm (71,72). ATR is a labile compound that on exposure to light under aerobic conditions undergoes rapid degradation and products absorbing at shorter wavelengths are formed (231). It may be speculated that the photodegradation products of ATR retain some of the photosensitizing properties and may propagate the damage initiated by ATR. It may be further speculated that ATR can initiate oxidation of other compounds, such as polyunsaturated fatty acids highly abundant in the OS and form products of photosensitizing properties absorbing short-wavelength light. Indeed, we have shown that oxidation of DHA, which accounts for 30–35% of fatty acids in the human OS (203), leads to the formation of chromophores that photosensitize singlet oxygen and free radicals generation upon photoexcitation with short-wavelength light (232). Thus it may be speculated that irradiation with blue light initiates photosensitized damage as long as ATR is not degraded, while during irradiation with UV light generates photosensitized damage, which is initially mediated by ATR and later by the oxidation products formed.

Indeed, we have shown that wavelength dependence of initial rates of photooxidation of isolated dark-adapted bovine OS exhibits spectral characteristics similar to liposomes containing ATR and made of phosphatidylcholine or a mixture of phosphatidylcholine and PE (65:35 mol/mol) (224,233), with the maximum at 370 nm. Photobleaching of OS by exposure to room light before measurements results in an action spectrum exhibiting a steady increase of the rates of photooxidation with decreasing wavelength (233). This action spectrum is similar to the action spectrum of acute retinal photodamage (71,72). These data support a view that ATR undergoes rapid photodegradation and induces formation of photosensitizing products absorbing at shorter wavelengths than ATR.

Many studies have shown that the degree of retinal photodamage positively correlates with the rhodopsin content in the retina before light exposure (48,234). In view of the above-discussed conditions allowing for accumulation of ATR within OS, the content of rhodopsin plays the major role—the more rhodopsin photobleached the more ATR released. Rhodopsin knockout mice are completely protected against light damage to photoreceptors, but the rods are devoid of OS to begin with, so they are no longer photosensitive (80).

Concentrations of rhodopsin in OS is inversely related to the light levels under which the animals are reared (235). Rearing rats and mice over several weeks in bright cyclic light protects the animals against acute photodamage induced by high-intensity light (66–68, 236). Although there are several other adaptive responses elicited by light that can play a protective role, such as increased concentrations of low-molecular-weight antioxidants, ascorbate and  $\alpha$ -tocopherol (59–61), up-regulation of antioxidant

enzymes (58,59) and survival factors (57,62,63), the reduced content of rhodopsin may play a critical role in the protective effect observed.

#### Effects of lipid composition of OS on susceptibility to light-induced damage

Lipid environment in OS affects the susceptibility to retinal photodamage (237). It has been suggested that it is due to its effect on the rate of rhodopsin regeneration. A substantial depletion of DHA in rats distinctly reduces the regeneration rate of rhodopsin and prevents white light-induced lesions (237), even though the amount of rhodopsin in dark-adapted eyes is increased in comparison with rats fed with normal diet (238).

However, as mentioned earlier, lipid environment in OS strongly affects the ratio of formation of MetaII and MetaIII (105–110). Thus the decreased content of DHA in the retina may decrease the quantum yield of photoactivation of rhodopsin to Meta II and therefore the quantum yield of ATR hydrolyzed from opsin.

This effect is important with regard to the fact that adaptation to environmental light includes changes in the lipid composition of the OS membranes. Rearing animals in bright light results in an increase in the cholesterol content and a decrease in polyunsaturated lipid content, such as DHA (60,239). Thus it may be speculated that the adaptive changes in the OS membrane lipid composition in response to rearing in bright light serve as another protective mechanism by providing conditions facilitating Meta III formation. In the dark-adapted retinas there is a high concentration of transducin within the OS (240) and therefore, after a flash of bleaching light, Meta III may be efficiently converted to Meta II by transducin. It may imply that, in case of exposure to a pulse of light, the modified OS lipid composition results only in a delay of activation of the phototransduction cascade and subsequent hydrolysis of ATR from opsin, which will be determined by the bimolecular rate constant of interaction of Meta III with transducin and concentration of transducin.

Under conditions of continuous exposure to light most transducin translocates into the photoreceptor inner segment (240) and therefore transducin-catalyzed conversion of Meta III to Meta II may be less efficient due to low concentration of transducin in the OS. However, under these conditions absorption of a photon by Meta III can convert it to Meta II (115). As consecutive absorptions of two or more photons are required to generate a biochemically active form, Meta II, under conditions favoring initial formation of Meta III over Meta II from Meta I, it may be speculated that the decreased susceptibility to light damage in DHA-depleted animals is related not only to its inhibitory effect on rhodopsin regeneration but also to the decreased quantum yields of ATR hydrolyzed from opsin.

#### Role of 11-*cis*-retinal availability for regeneration of rhodopsin in light damage

Several pieces of evidence indicate that it is the ability to regenerate rhodopsin and the rate of this process that are the major factors determining the susceptibility of the retina to photodamage. In animals with inefficient rhodopsin regeneration, the susceptibility to photodamage is decreased even if animals with defects in rhodopsin regeneration have initially a higher rhodopsin content in their dark-adapted retinas than animals with normal rhodopsin regeneration (79,237,238).

As mentioned earlier, rhodopsin regeneration depends on the binding of a new molecule of 11-*cis*-retinal to the opsin. Thus, to provide OS discs with new molecules of 11-*cis*-retinal, the stores of retinyl esters need to be mobilized and transformed into visual pigment chromophores (76). Currently, it is not clear what the signaling pathways for mobilization of the retinyl esters and synthesis of 11-*cis*-retinal are, or what limits the synthesis of 11-*cis*-retinal and its delivery to the OS. Synthesis of 11-*cis*-retinal requires functional RPE65 protein which is essential for binding of all-*trans*-retinyl esters and enables their isomerization and hydrolysis to form 11-*cis*-retinol (147–149,151). In the absence or dysfunction of RPE65, there is no ligand available to regenerate rhodopsin and therefore hydrolyzed ATR can be safely reduced to all-*trans*-retinol by RDH at the “exit site” of opsin (93). In contrast, under conditions where there is efficient regeneration of rhodopsin, ATR may be released from the protein before its enzymatic reduction (93), which may result in accumulation of substantial concentrations of ATR within the OS.

Indeed, *RPE65*<sup>−/−</sup> mice are completely protected against light-induced damage in comparison with the wild-type mice (80). Mutations of *RPE65* that result in a slow metabolic regeneration of rhodopsin result in greater resistance to light damage in comparison with mouse strains with fast regeneration kinetics (79,241). In particular, it was found that a variation of RPE65 at position 450 where Leu is replaced by Met is a strong genetic modifier of susceptibility to light-induced damage in mice (241–243). Experiments on different strains of mice without and with Met450Leu RPE65 mutation demonstrated that the susceptibility to light-induced damage correlates positively with the rates of rhodopsin regeneration (79). As expected, Met450Leu RPE65 mutation leads to a decreased accumulation of A2E (209). Met450Leu RPE65 mutation in *ABCR*<sup>−/−</sup> also slows down A2E accumulation in comparison to *ABCR*<sup>−/−</sup> mice with functional RPE65, but still the levels of accumulated A2E are about 3-fold higher in mutant knockouts than in wild-type mice (209).

Treatment with 13-*cis*-retinoic acid (known as Isotretinoin or Accutane) slows rhodopsin regeneration due to inhibition of RPE65 and 11-*cis*-RDH (158,210,211), inhibits accumulation of A2E (212,244,245) and was also found to protect rat photoreceptors from light-induced damage (245). CRABP has been identified as another protein whose dysfunction leads to impairment of 11-*cis*-retinal production. Albino mice with a nonfunctional CRABP gene exhibit 10-fold decreased rhodopsin regeneration due to delayed 11-*cis*-retinal production and are protected from light damage in comparison with the wild type (139).

It can be speculated that an efficiently operating retinoid cycle allows for build-up of substantial steady-state concentrations of ATR in OS membranes during exposure to bright light, where it can elicit photosensitized damage. Thus, slowing down the retinoid cycle may offer an option for pharmacological intervention to prevent photooxidative damage. This can be particularly useful during intraocular surgery involving exposure of the retina to high irradiance levels. It is tempting to speculate that inhibition of the mobilization of stores of all-*trans*-retinyl esters may be the key target of this preventive treatment. Although using 13-*cis*-retinoic acid may prove adequate for use during ocular surgery, its daily systemic administration to patients with Stargardt disease or AMD imposes a risk of systemic toxicity and teratogenicity (245). Therefore there is a need to develop other safer synthetic retinoids and analogues that may prove useful for long-term therapy. It has been demonstrated *in vitro* and *in vivo* in mice that retinylamine is

a more effective inhibitor of isomerization and regeneration of 11-*cis*-retinal than 13-*cis*-retinoic acid, while being ineffective in activation of retinoic acid-dependent transcription and does not exhibit systemic toxicity (146). It may be expected that understanding pathways responsible for retinoid trafficking upon photobleaching of visual pigments will open new venues for pharmacological interventions to prevent light-induced damage to the retina.

#### **Binding of 11-*cis*-retinal in rhodopsin active site as a modifier of susceptibility to light damage**

To release ATR from the opsin "exit site," a molecule of 11-*cis*-retinal needs to bind to Lys296 in the active binding site (93). Halothane is an anesthetic that is thought to compete with 11-*cis*-retinal for the binding to opsin (246) and efficiently prevents rhodopsin regeneration (247). Mice and rats anesthetized with halothane were completely protected against white light-induced damage in comparison with animals anesthetized with other drugs (81,247). Halothane did not protect against blue light-induced damage in rats, where 403 nm light induced condensation of nuclear chromatin in photoreceptors and severe changes within RPE, such as swelling and numerous inclusions (81,247). The results of these experiments were discussed in terms of toxicity related to partial rhodopsin photoregeneration (80,81). Grimm and colleagues (80,81) proposed that photoexcitation of rhodopsin intermediates by blue light results in regeneration of rhodopsin, so called photoreversal of bleaching. This claim was based on measurements of rhodopsin contents after exposure to blue (403  $\pm$  10 nm) or green light (550  $\pm$  10 nm), which demonstrated that rhodopsin was almost completely bleached by green light but the exposure to blue light resulted in reaching a plateau at approximately 20% of the dark value (80). Grimm and colleagues (81) suggested that blue light may exert its toxic effect by photoisomerization of metastable rhodopsin intermediates such as Meta II so to regenerate the rhodopsin and this was postulated as a casual factor in light damage. However, the exact mechanism by which regeneration of rhodopsin through photoreversal was supposed to mediate toxicity was not discussed.

As previously mentioned, pathways of photolysis of rhodopsin intermediates have been recently elucidated and indicate that photolysis of Meta II leads to formation of Meta III (112) and photolysis of Meta III results in nonspecific isomerization of ATR and results in generation of Meta II, dark-state rhodopsin and isorhodopsin, which contains 9-*cis*-retinal bound to its active site (115). Thus analysis of retinal isomers after exposure to blue light can provide a definitive answer whether the photoreversal of rhodopsin occurs *in vivo* and contributes to rhodopsin regeneration under experimental conditions employed by Grimm and colleagues (80,81).

Although the postulated toxic effects of rhodopsin intermediates and the mechanism of their toxic action remain mysterious, the observations made by Grimm and colleagues (80,81) can be explained in an alternative way. During the exposure to continuous light the steady-state level of rhodopsin concentration depends on the rates of rhodopsin bleaching and regeneration. In the experiments described, rats were exposed to 1.86 times more photons of green light (0.409 mW/cm<sup>2</sup>) than blue light (0.300 mW/cm<sup>2</sup>) (80). Moreover, the molar extinction coefficient of rhodopsin at 403 nm light is about twice smaller than that at 550 nm. Thus the rate of absorption of photons and therefore the rate of rhodopsin bleaching, may be expected to be about 3.7 times smaller in case

of blue light irradiation than for irradiation with green light under experimental conditions discussed (80). It agrees well with the ratio of the rates of rhodopsin bleaching reported by Grimm and colleagues (80), which were evaluated under conditions where the impact of rhodopsin regeneration on the concentration of measured rhodopsin was negligible. The rate of bleaching by green light was determined during the 5 min exposure to green light, while the bleaching of rhodopsin by blue light was monitored in the presence of hydroxylamine that binds free retinals and intercepts ATR from photoactivated rhodopsin intermediates but does not interact with 11-*cis*-retinal bound to the rhodopsin active site. Under these conditions, blue light in the presence of NH<sub>2</sub>OH induced rhodopsin bleaching with a rate of about 0.61/min, while green light in the absence of NH<sub>2</sub>OH induces rhodopsin bleaching with a rate of about 2.1/min (80). Thus the experimentally determined rate of photobleaching induced by green light was 3.4 times greater than the rate of photobleaching induced by blue light, which is similar to the value of 3.7 calculated above.

Thus, the presence of substantial concentrations of rhodopsin in retinas exposed to blue light may result in part from a decreased rate of rhodopsin photobleaching. It may be further speculated that the damaging effect of blue light under halothane anesthesia was induced by ATR released from opsin and/or regenerated 11-*cis*-retinal transported into the OS but unable to bind to opsin. Accumulated retinals could act as photosensitizers responsible for blue light-induced damage.

It may be speculated that compounds acting in a similar way as hydroxylamine could efficiently protect against light-induced damage. In contrast to halothane, hydroxylamine removes retinals, the culprit responsible for blue light-induced photodamage by efficient binding and formation of retinal oximes, which absorption spectrum is shifted toward shorter wavelengths in comparison with ATR so their absorption cross-section of blue light is substantially smaller than for ATR. Moreover, as Schiff base linkage is formed, photosensitizing properties are also expected to be substantially diminished.

While retinals are potent photosensitizers, they are also susceptible to photoinduced isomerization and degradation under aerobic conditions (231). The latter is frequently used to deplete retinoids from the isolated retina or its components (145). Thus it may be speculated that the degradation of retinals due to irradiation with blue light contributed to the delayed rhodopsin regeneration observed by Grimm and colleagues (80,81). Again, thorough analysis of retinal isomers could provide an answer whether or not photoactivation of free retinals played a significant role under the conditions employed. Photoisomerization of free ATR is expected to yield 13-*cis*-retinal, which is not generated by photoisomerization of protein-bound retinals (213,214).

The role of degradation of retinoids in dysfunction of rhodopsin regeneration is further supported by the study of Rapp and Ghalayini (248). They have shown that an exposure of rats to UV light (312–398 nm) results in a decreased recovery of rhodopsin (248). The exposure led to a gradual decline of rhodopsin level to 8% after 1 h. The rhodopsin level increased only to 23 and 27% after 1 and 2 h postexposure, respectively, in comparison with the nonexposed eye. Rhodopsin level further increased to 45–57% the next day postexposure and remained within that range up to 7 days, while the level of total opsin remained unchanged at 2 days following the UV exposure. Further increase in rhodopsin level was noted after 2 weeks when it reached 80% of the control value. However, the exposure led to structurally evident damage to

photoreceptors: swelling, disorganization and shortening of the outer segments in the central inferior retina. Radiation used in the experiment covered the range of 312–398 nm and thus led to photoexcitation of both rhodopsin and retinoids. Because no measurements of retinoids were undertaken, it may be only speculated that the delayed recovery of rhodopsin was initially due to retinoid depletion as a result of their photodegradation, whereas later the structural damage to photoreceptors disabled retinoid trafficking in the retinoid cycle. It may be further speculated that initial accumulation of ATR could be responsible for the damage observed.

As mentioned previously, patients and animals with P23H rhodopsin mutation are particularly susceptible to light-induced damage (36,38–42). P23H rhodopsin cannot fold properly after synthesis and most of it becomes marked by ubiquitin for proteasomal degradation. Biosynthesis of P23H rhodopsin and its incorporation into membrane can be rescued through the supply of 11-*cis*-retinal or its analogues, such as 9-*cis*-retinal or synthetic 11-*cis*-7-ring retinal (37,249). All three retinoids, 11-*cis*-7-ring retinal, 11-*cis*-retinal or 9-*cis*-retinal, bind to P23H opsin *via* Schiff base linkage to Lys296 and allow for proper folding of mutant protein. P23H rhodopsin with 11-*cis*-retinal or 9-*cis*-retinal as the chromophore responds to photon absorption, which results in a decrease of the absorption at 500 nm and formation of intermediates absorbing at shorter wavelengths (37). In contrast to 11-*cis*-retinal and 9-*cis*-retinal, 11-*cis*-7-ring retinal forms locked, nonphotoisomerizable and nonbleachable form of P23H rhodopsin in cell membranes (249).

However, the binding of all retinals to P23H opsin is very weak and results in very poor thermal stability (37,249). P23H rhodopsin with either 11-*cis*-retinal or 11-*cis*-7-ring retinal loses its chromophore within 10–20 min during incubation at 37°C. It may be speculated that the increased susceptibility of P23H patients and animals to light-induced damage to the retina is related to poor thermal stability of P23H rhodopsin and release of 11-*cis*-retinal that then may exert its (photo)toxicity. Thus, while these studies are promising in terms of pharmacological intervention in rescuing P23H rhodopsin, supplementation with retinoids requires a particular caution as it may increase the risk of light-induced damage. Because of the poor thermal stability of P23H rhodopsin, there is a need to develop 11-*cis*-retinal analogues that would allow for formation of a stable protein in dark as well as functional phototransduction.

In conclusion, there are several factors that affect the susceptibility of the retina to light-induced damage. They include rhodopsin content, the rate of rhodopsin photoactivation to Meta II, the rate of 11-*cis*-retinal synthesis and the rate of rhodopsin regeneration. All of these factors directly or indirectly affect the rate of ATR hydrolysis and release from opsin and therefore its transient accumulation in OS discs. In some cases discussed above it may be also 11-*cis*-retinal that induces (photo)toxicity upon accumulation in photoreceptors being unable to bind to opsin due to the presence of a competitor, such as haloethane, or hydrolysis from thermally unstable mutated opsin, such as P23H opsin. Accumulated retinals can induce oxidative damage in dark, but the efficiency of this process is substantially increased upon photoexcitation of retinals with UVA or blue light (230). This may provide the mechanism responsible for all types of photodamage observed in rodents and primates affecting photoreceptors and possibly also RPE due to its proximity to accumulation site of the photosensitizers.

## SECONDARY DAMAGE MEDIATED BY RETINAL: CYTOTOXIC AND PHOTSENSITIZING PROPERTIES OF LIPOFUSCIN

Availability of ATR in the retina and exposure to light are the main factors responsible for accumulation of lipofuscin granules within RPE (152,250,251). Retinal lipofuscin consists mainly from lipids and proteins (203,252) and contains a number of fluorophores (253), one of which has been identified as A2E (205,254). Progressive accumulation of lipofuscin occurs with ageing (9,255) and is even further elevated in patients with Stargardt disease (256,257), AMD (258–260), Best disease (261) and some cases of retinitis pigmentosa (262,263), as well as in the *ABCR*<sup>−/−</sup> and *ABCR*<sup>+/−</sup> mice (96,97,130).

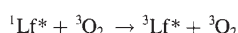
### (Photo)toxic properties of lipofuscin

The biological consequences of lipofuscin accumulation in the human RPE remain largely unknown; however, it has been postulated that excessive levels of the age pigment could compromise essential RPE functions and even contribute to the pathogenesis of AMD, the leading cause of blindness in people over 60 in developed Western countries (264–270). The spatial topography, age relationship and racial distributions of lipofuscin exhibit remarkable similarity to patterns seen in AMD (208). Perhaps the best association between lipofuscin accumulation and retinal degeneration was delineated in case of Stargardt macular dystrophy (271). A recent study has shown that mutations in *ABCR* result in accumulation of lipofuscin before slowing of the retinoid cycle (191). Thus, lipofuscin accumulation could be viewed as a key and early component of the expression of Stargardt disease.

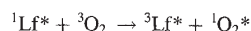
Although the exact mechanism of lipofuscin-dependent cytotoxicity in the RPE is not known, it was hypothesized that oxidative stress, mediated by the photoexcited lipofuscin, could play an important role (272,273). Significantly, it has been shown that lipofuscin contributes to age-related increase of susceptibility of RPE cells to photooxidation (274). Aerobic irradiation of purified lipofuscin granules with short-wavelength visible light brings about generation of reactive oxygen species including superoxide anion, hydrogen peroxide, lipid hydroperoxides and singlet oxygen (274–278). Photoinduced reduction of oxygen to superoxide and hydrogen peroxide is only a minor process for lipofuscin-dependent oxygen utilization, accounting for less than 3% of oxygen used, whereas most oxygen is used for oxidation of lipofuscin components (278). Action spectra of photoinduced generation of hydrogen peroxide and oxidation of lipofuscin exhibit a monotonic increase with decreasing wavelength (274). Photooxidation of RPE cells and lipofuscin occurred on timescales of minutes to tens of minutes using irradiation levels of 5–30 mW/cm<sup>2</sup> for the shortest and the longest wavelengths tested, respectively. It was demonstrated that lipofuscin mediates photoinduced oxidation of extragranular lipids and proteins (274,276–278) and inhibits several lysosomal and antioxidant enzymes (276,279).

A fraction of lipofuscin chromophores can be extracted by organic solvents and their properties studied in solution indicate that it includes potent photosensitizer(s) (218,280,281). Photoexcitation of lipofuscin extract solubilized in nonpolar solvents, such as hexane or benzene, leads to photogeneration of a triplet state exhibiting an absorption maximum at about 440 nm decaying with a rate constant of 10<sup>5</sup> s<sup>−1</sup>, corresponding to lifetime of 10.5 μs, in the absence of oxygen (218). In the presence of oxygen, the

lipofuscin triplet state interacts with oxygen with a bimolecular rate constant of  $1.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  and, as a result of energy transfer, singlet oxygen is formed. The quantum yields of singlet oxygen generation by lipofuscin extract depends on the excitation wavelength, being 0.08 for 355 nm excitation and 0.05 for 420, 430 and 440 nm in air-saturated benzene. It may indicate that there are different photosensitizers contributing to absorption at 355 nm and blue light and/or the ratio of photosensitizers to other chromophores varies at different wavelengths. The quantum yields of singlet oxygen generation were substantially increased to 0.15 for 355 nm and 0.09 for 420, 430 and 440 nm upon saturation of the solution with oxygen. It may indicate that lipofuscin extract includes photosensitizers that form shorter-lived triplets than the one observed. Alternatively, an increased concentration of oxygen may increase the efficiency of intersystem crossing through the interaction with excited singlet states (282). Gaillard and colleagues (281) measured fluorescence lifetimes in lipofuscin extracts and determined that apart from fluorophores of 0.06 ns and 0.32 ns lifetimes, there are fluorophores of fluorescence lifetimes of 1.2 ns and 4.8 ns, which are long enough to allow for interaction of the excited singlet states of lipofuscin fluorophores ( $^1\text{Lf}^*$ ) with oxygen, leading to the formation of lipofuscin triplet state  $^3\text{Lf}^*$ :



If the energy difference between the first excited singlet state and triplet state of lipofuscin is greater than the energy of the singlet oxygen (94 kJ/mol), as a result of the above reaction, singlet oxygen may be formed:



These processes can increase the quantum yield of singlet oxygen generation.

Photoexcitation with 355 nm laser pulse of lipofuscin extract solubilized in polar solvents, such as methanol, leads to formation of a triplet state of 7  $\mu\text{s}$  lifetime and another species, possibly a radical (281). The photoformation of free radicals by photoexcited lipofuscin extract was confirmed by electron paramagnetic resonance (EPR) spin-trapping experiments (274,280). In methanol, upon photoexcitation with 355 nm, lipofuscin extract photo-generates singlet oxygen with a quantum yield of 0.05 equal to that of ATR (280,281).

It appears that aging increases the risk of lipofuscin-dependent oxidative stress in the outer retina due to both accumulation of lipofuscin and an increase in its aerobic photoreactivity. Indeed, a detailed analysis of aerobic photoreactivity of purified lipofuscin granules, isolated from donors of different age groups, revealed that the rate of oxygen photo-consumption was 1.6–2.2 times faster for lipofuscin from donors 80 years and older than from donors younger than 40 years (283). Consistently with this observation, lipofuscin granules from older donors photogenerated superoxide anion at a faster rate than those from younger donors (283). To determine whether the age-related changes of human retinal lipofuscin granules arise from changes in photochemical properties of any lipofuscin components that can be isolated, the efficiency and action spectra of photogeneration of reactive oxygen species for chloroform-soluble and chloroform-insoluble fractions of the granules, were compared (283). The data clearly show that, although both fractions exhibit substantial photoreactivity, neither exhibits significant age-dependent changes and the only variable

that consistently increased with age was the content of chloroform-insoluble fraction in lipofuscin granules. The insoluble fraction of lipofuscin includes as well powerful photosensitizers and its contribution to lipofuscin photoreactivity is similar to that of chloroform-soluble fraction. Both fractions photogenerate singlet oxygen, superoxide, induce lipid and protein oxidation and undergo photooxidation themselves.

The phototoxic potential of retinal lipofuscin has been demonstrated in an *in vitro* study, in which human RPE cell cultures were fed lipofuscin granules and exposed to either 390–550 nm (2.8 mW/cm<sup>2</sup>) or 550–800 nm light (2.8 mW/cm<sup>2</sup>) (284). Only cells that were fed lipofuscin granules and exposed to 390–550 nm light for 12 h (dose of 121 J/cm<sup>2</sup>) exhibited significant morphological changes, loss of lysosomal integrity, enhanced peroxidation of lipids and greatly reduced survival. In this study RPE cells in culture were exposed to 300 lipofuscin granules per cell, part of which was phagocytosed. Lipofuscin accumulation *in vivo* can greatly exceed that value. Morphometric study of lipofuscin content in human RPE demonstrated that lipofuscin can occupy as much as 19% of cell volume in macular RPE from 81- to 90-year-old donors (285). Assuming 0.5  $\mu\text{m}$  as an average diameter of lipofuscin granule and cuboidal shape of RPE cells of side length of 14  $\mu\text{m}$  (286), 19% of cell volume occupied by lipofuscin corresponds to 7966 lipofuscin granules per cell.

Lipofuscin content in RPE can be also estimated based on the content of A2E in RPE cells and content of A2E in lipofuscin granules. The content of A2E/iso-A2E was estimated for about  $7.8 \times 10^{-20}$  mol per lipofuscin granule isolated from 60- to 70-year-old donors (284). Thus, a retina accumulating 400 ng (0.675 nmol) of A2E (205) will have about  $8.7 \times 10^9$  lipofuscin granules. It allows the estimation of the average number of lipofuscin granules per RPE monolayer volume unit, or assumption of the cuboidal shape of RPE cells of the side length of 14  $\mu\text{m}$  and their densely packing, in a single RPE cell. This rough estimate gives 5.1 million RPE cells per 1000 mm<sup>2</sup> of retinal surface, which gives about 1706 lipofuscin granules per cell from donors over the age of 40.

Based on data of A2E content in RPE cells from individual healthy aged human donors determined by Sparrow and colleagues (206), it can be estimated that RPE cells with 34–134 ng of accumulated A2E per  $10^5$  cells contain 7400 to 29 000 lipofuscin granules per cell, respectively. These estimates give a range of 17% to 69% of RPE cell volume occupied by lipofuscin granules.

It needs to be kept in mind that these estimates refer to content of lipofuscin averaged over a great number of RPE cells and retinas. There is a great deal of heterogeneity in lipofuscin accumulation in different RPE cells within the same retina and even greater for different retinas, in particular, in cases of retinal dystrophies (137,207,285,287,288).

#### Contribution of A2E to (photo)toxic properties of lipofuscin

A2E attracted a great deal of attention as a potential photosensitizer responsible for photodamaging effects of lipofuscin (see review by Sparrow and colleagues [99]). A2E is extracted from lipofuscin along with other chloroform-soluble components (205). For the wavelength corresponding to A2E absorption maximum ( $\epsilon = 36\,900 \text{ M}^{-1} \text{ cm}^{-1}$  at 439 nm [254]) and the 0.014 mm thickness of RPE monolayer, the absorbance of A2E can be estimated. This leads to values of A2E absorbance at 439 nm in the RPE layer varying from 0.0028 to 0.048 for 48  $\mu\text{M}$  and 824  $\mu\text{M}$  A2E concentrations, estimated based on data of Eldred and Lasky (205) and Sparrow and colleagues (206), respectively (Fig. 5c).

A2E concentrations of 48  $\mu\text{M}$  and 824  $\mu\text{M}$  correspond to the minimal and maximal estimates of 1706 and 29000 lipofuscin granules per cell, respectively. It allows calculation of the absorption of light by soluble components of lipofuscin throughout the RPE monolayer and a contribution of A2E to that absorption (Fig. 5b–d). Chloroform-soluble fraction of lipofuscin contributes about 0.093 pg dry weight to lipofuscin granule (283), thus 1706 and 29000 lipofuscin granules per cell correspond to 159 pg and 2697 pg of chloroform-soluble fraction of lipofuscin per cell, or in terms of concentrations, 39 mg/mL and 658 mg/mL. Chloroform-soluble fraction of lipofuscin of 0.24 mg/mL concentration exhibits absorbance of 0.125 at 439 nm for 1 mm pathlength (283). Therefore, absorbance of chloroform-soluble components of lipofuscin in RPE monolayer of 14  $\mu\text{m}$  thickness is 0.284 and 4.791 at 439 nm for RPE with 1706 and 29000 lipofuscin granules per cell, respectively (Fig. 5b). It needs to be kept in mind that the calculations of the expected absorption of light by lipofuscin extracts and A2E refer to chromophores in solutions. Under physiological conditions, all chromophores are encapsulated within lipofuscin granules and therefore the absorption cross-section at the RPE may be substantially different than that in solution.

The comparison of absorption properties of A2E and all chloroform-soluble components of lipofuscin indicate that the contribution of A2E to the absorption of visible light by lipofuscin is only minor (Fig. 5d). The integrated absorption in the visible (>390 nm) of lipofuscin-soluble components is 120 times greater than for A2E (Fig. 5d). For lipofuscin occupying 19% of RPE volume, absorption of visible light by soluble components of lipofuscin is 8.6 times greater than by 3.8 mM ATR in perifoveal OS (Fig. 5d). In this case, ATR exhibits 14 times greater integrated absorption in the visible than 224  $\mu\text{M}$  A2E contained within lipofuscin occupying 19% of RPE volume. It needs to be stressed that soluble components of lipofuscin account only for half or less of total dry mass of lipofuscin granules and the remaining components also absorb light and contribute to photodynamic action of lipofuscin (283). With ageing the yellowing lens absorbs more and more short-wavelength light and the probability of photoexcitation of ATR and A2E progressively decreases. Lipofuscin, however, still efficiently absorbs light above 460 nm and this long-wavelength blue and green light can still induce photosensitized generation of singlet oxygen and photooxidation (218,274).

In low concentrations, A2E was shown to actually protect against UV-induced DNA damage in RPE cells *in vitro* (289). In experiments performed by Roberts and colleagues (289), RPE cells were incubated for 2 h in culture medium without or with A2E, after which the cells were washed and exposed UV light. Cells preincubated in the presence of 5  $\mu\text{M}$  A2E were significantly protected against DNA damage in comparison with the control cells. However, at high concentration, A2E can exert several deleterious effects on cells in dark and mediate blue-light induced damage to the RPE cells *in vitro* (reviewed by Sparrow and colleagues [99], Boulton and colleagues [270], Sparrow and Boulton [290] and Lamb and Simon [202]).

A2E was shown to cause membrane permeabilization (291,292), inhibit lysosomal ATPase activity (293), inhibit cytochrome *c* oxidase and decrease mitochondrial activity (294) and induce release of proapoptotic proteins from mitochondria (295), leading eventually to apoptosis (206). A2E has been shown to act as a competitive inhibitor of organic anion-transporting protein (OATP) present at the apical microvilli of the rat RPE and to

a lesser degree in small retinal vessels (296). OATP, which is also expressed in the brain and liver, transports amphiphilic compounds across the plasma membrane in a sodium-independent manner and in the retina is suspected to be involved in retinoid transport.

Most studies on A2E toxicity were done on cells in culture, which were exposed to A2E provided as a solution (206,295,297–301). Under physiological conditions A2E is believed to be present mainly within the lipofuscin granule (99) and therefore its toxic action *in vitro* when it was delivered in solution cannot be extrapolated directly to the situation *in vivo*. Encapsulation of A2E within the lipofuscin granule, where it accounts for only 0.019% to 0.024% dry weight (283,284), is likely to prevent A2E from exerting its deleterious effects on mitochondria, DNA and transporter proteins. Thus it may be argued that the biologically relevant studies on the cytotoxicity of A2E were those where the physiological compartmentalization of A2E in lipofuscin was modeled by allowing cells to phagocytose LDL-bound A2E (293,302,303).

As A2E is believed to be formed within the phagolysosomal compartment of the cell, the effects of A2E on lysosomes are of particular importance. A2E-induced inhibition of ATP-driven proton pump located in the lysosomal membrane results in inhibition of proton transport to the lysosomal lumen and leads to an increase of the lysosomal pH with subsequent inhibition of lysosomal hydrolases (293). This may explain why A2E inhibits degradation of phagocytosed low-density lipoproteins and OS lipids. In RPE cells *in vitro* A2E was shown to substantially inhibit catabolism of endogeneous proteins (302) but did not affect the rate of degradation of phagocytosed OS proteins (304). Altogether, A2E is likely to contribute to lipofuscin accumulation by its inhibitory effect on degradation of phagocytosed OS. Although A2E does not inhibit directly lysosomal enzymes (305), lipofuscin inhibits directly several hydrolytic enzymes in dark and the effect is exacerbated during exposure to light (276,279).

The phototoxicity of lipofuscin to RPE cells in culture cannot be explained by the action of A2E. Lipofuscin exerted its toxic effects to the RPE cell *in vitro* where the content of A2E in cells was at most 1.3 ng/10<sup>5</sup> cells (284), so it was at least two orders of magnitude lower than that required to elicit detectable damaging effects mediated by A2E in dark (206) and 17 times lower than that required to elicit A2E-mediated phototoxicity induced by 60 s exposure to blue light of 7.5 W/cm<sup>2</sup> irradiance (dose of 450 J/cm<sup>2</sup>) (297).

A comparison of photophysical and photochemical properties of A2E with lipofuscin indicate that A2E may play only a minute role in photosensitized damage mediated by lipofuscin (see review by Lamb and Simon [202]). It has been shown that this pyridinium bis-retinoid very efficiently converts energy of the absorbed photons into heat with quantum yield of 0.96 in ethanol (306). As a result, no long-lived triplet excited state, a key intermediate for generation of singlet oxygen or other reactive oxygen species, is formed in substantial yields (220,289,307). As quantum yield of A2E fluorescence is 0.01 in ethanol, the upper limit for triplet state generation in this solvent is 0.03 (306). Using cholesterol in liposomal membranes as a mechanistic reporter molecule and direct detection of singlet oxygen phosphorescence at 1270 nm, the quantum yield of photogeneration of singlet oxygen by A2E has been determined to be at most 0.02 (220,289,307). This implies that an equal number of photons absorbed by lipofuscin will generate at least 2.5 times more singlet oxygen than when they are absorbed by A2E. Considering a minor contribution of A2E into

the absorption of visible light by lipofuscin, it can be estimated that A2E contributes less than one singlet oxygen molecule to more than 300 photogenerated by lipofuscin. Based on the quantum yield of A2E fluorescence of 0.01 (306), it can be estimated that during fundoscopic examination of the retina by autofluorescence, every photon that is emitted by photoexcited A2E is accompanied by the formation of at least 600 singlet oxygen molecules photosensitized by lipofuscin.

EPR spin-trapping experiment revealed that, although the photoexcited A2E could also generate superoxide anion, its quantum yield was even lower than for singlet oxygen, about 0.03%, which was twice smaller than quantum yield determined for soluble components of lipofuscin (220). Also action spectra for photoinduced oxygen uptake by A2E in  $\beta$ -oleoyl- $\gamma$ -palmitoyl-L- $\alpha$ -phosphatidylcholine liposomes, compared with those by lipofuscin granules or lipofuscin extracts, indicate that A2E cannot be a major contributor to aerobic photoreactivity of lipofuscin (308).

A2E has been shown to be an efficient acceptor of energy from excited states which results in fluorescence emission from A2E even though blue light is absorbed by many other chromophores as well (309). It may be speculated that if A2E is so efficient in accepting energy from short-lived excited singlet states it may be even more efficient in accepting the energy from the triplet states and therefore it may deactivate photosensitizers before they can activate oxygen or other molecules *via* energy or electron transfer. However, laser flash photolysis of chloroform soluble extracts of lipofuscin leads to the formation of a triplet state with an absorption maximum at 440 nm methanol (281) and benzene (218) and no transient products can be observed with spectral characteristics of A2E triplet, which exhibits absorption maximum at about 540 nm in benzene (310) and 510–550 nm in methanol (310,311). This confirms a minor contribution of A2E into lipofuscin photochemistry.

Similarly to its precursor, ATR, A2E is very susceptible to oxidation (312,313). It was determined that A2E quenches singlet oxygen with a bimolecular rate constant in the range of  $(0.3\text{--}2) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  (289,312). Thus it is likely that singlet oxygen photogenerated by A2E will be in part quenched by A2E itself. As a result of chemical quenching, a variety of polyepoxides are formed (312). These epoxides, called oxiranes and its derivatives are formed as a result of photooxidation of A2E *in vitro* (300,312–315). Epoxides and bis-furanoid oxides have been identified in RPE suggesting that they are formed *in vivo* (314,316,317). As contribution of A2E to singlet oxygen generation by photoexcited lipofuscin is small, it may be speculated that A2E-derived oxiranes are generated by other photosensitizers present in lipofuscin. It has been shown that products of A2E oxidation delivered in solution can induce oxidative damage to DNA and cytotoxicity (315). However, being formed within the lipofuscin granule, their toxicity under physiological conditions remains to be shown.

As mentioned earlier ATR plays an essential role in the accumulation of A2E and lipofuscin; however, it was not detected as one of lipofuscin components. ATR quenches singlet oxygen with a bimolecular rate constant of  $3.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  and 20% of interacting ATR molecules become oxidized during that process. It may be speculated that retinals and retinols also form epoxides in the retina, but, in contrast to A2E, they may more easily diffuse out of the lipofuscin granule and damage cellular components.

While it is clear that A2E does not contribute significantly to lipofuscin photoreactivity, the molecular nature of major lipofuscin

constituent(s) responsible for the photochemical reactivity has yet to be determined.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

There is a great deal of circumstantial evidence implicating ATR released from opsin as a mediator of acute photodamage to the retina as well as accumulation of (photo)toxic components in the RPE. Upon photobleaching of rhodopsin, the rate of removal of hydrolyzed ATR depends on the rate of regeneration of rhodopsin and the activity of RDH that reduces ATR to all-*trans*-retinol. In case the rate of regeneration exceeds the rate of reduction, ATR is released to the lipid membrane before being reduced (93), presumably to the inner leaflet of the disc membrane where ATR is not accessible for RDH. There ATR forms a condensation product with PE, NRPE and becomes a substrate for ABCR protein to be flipped over to the outer leaflet (129) and become accessible for RDH-mediated reduction. Because the formation of NRPE is not instantaneous, substantial concentrations of (photo)toxic ATR may build up in the retina during exposure to high levels of visible light and efficient rhodopsin regeneration. This is a likely mechanism responsible for retinal damage induced by exposure to high irradiance levels during ocular surgery.

Elevated chronic exposure to light has been identified as a risk factor for development of AMD and it may be speculated that photooxidative damage to the retina mediated by ATR and/or lipofuscin may play a role. It is tempting to speculate that light may be particularly toxic in the case of a subset of AMD patients with ABCR mutations or in Stargardt disease. *ABCR* knockout mice provide an excellent model to test this hypothesis. *ABCR*<sup>−/−</sup> mice raised in the dark do not accumulate lipofuscin, so lipofuscin-mediated phototoxicity can be excluded as a mediator of the damage in experiments where susceptibility to acute light-induced damage of dark-reared animals is compared between knockout and wild-type mice. It also allows the evaluation of the effects of vitamin A depletion or efficacy of retinoid derivatives slowing down the retinoid cycle in prevention of light-induced damage. Moreover, it would be of interest to make these comparisons separately for damage induced by blue light, which may lead to photodynamic damage mediated by ATR and green light, where high steady-state concentrations of ATR may prove damaging due to dark toxicity of ATR.

It seems that avoiding exposures of the retina to bright short-wavelength light is the simplest preventive measure against light damage. Filtering out infrared and short-wavelength light below 450 nm in ophthalmic instruments was suggested to decrease the risk of retinal damage due to ocular surgery or ophthalmic examination (19,318). Yellow intraocular lenses may mimic protection offered by natural lens in the elderly patients after cataract surgery. Long-term adaptation to the environmental lighting conditions plays an important role in modulating susceptibility to photooxidative damage (57,66,67) and include decreased concentration of rhodopsin and increased concentration of low-molecular antioxidants (59–61,239). To enable this protective mechanism to operate, appropriate dietary intake of antioxidant vitamins and micronutrients is necessary. It may also indicate that a lifestyle may predispose the retina to light damage. It may be speculated that spending long hours in the office with little exposure to sunlight leads to adaptive response and increased rhodopsin concentrations within OS. Then escape to the sun during the weekend may impose

a greater risk of photodamage than in people constantly being outdoors. If this effect takes place, it may be even more pronounced in people escaping rainy and cloudy cities in winter searching for sunny beaches or ski slopes. Then, yellow glasses may offer protection against overexposure to blue light.

Complete elucidation of the retinoid cycle may provide alternative strategies for prevention of photodamage to the retina. For instance, understanding regulatory mechanisms responsible for mobilization of retinyl esters and their conversion to 11-*cis*-retinal may provide more options for pharmacological intervention than toxic 13-*cis*-retinoic acid. As retinyl esters, in contrast to retinols and retinals, are anchored in the lipid membrane and can accumulate in large quantities without toxic effects (152), targeting the pathway of mobilization of retinyl esters seems to be the most promising strategy. Another pathway to diminish accumulation of ATR within OS would be to increase activity of prRDH. PrRDH activity decreases upon exposure to intensive green light but may be prevented with an antioxidant, 1,3-dimethylthiourea (319). The effect of blue light on prRDH activity has not been studied, but as blue light is more efficient in inducing oxidative damage than green light, it may be speculated that it may also affect prRDH and, as a result, contribute to delays in ATR reduction to retinol.

Accumulation of RPE lipofuscin may be viewed as a marker of damaging action of light exerted in the retina and a rising threat throughout ageing. Identification and characterization of main photosensitizers present in lipofuscin is required to design therapeutic strategy for their detoxification.

**Acknowledgements**—We thank anonymous reviewers for their constructive criticism. We thank Ania Różanowska for help in figures preparation. This work was supported by State Committee for Scientific Research (KBN; grant PB 4PO4A 044 23) and Cardiff University.

## REFERENCES

- Boettner, E. A. and J. R. Wolter (1962) Transmission of the ocular media. *Investig. Ophthalmol.* **1**, 776–783.
- Lerman, S. (1987) Effects of sunlight on the eye. In *Photomedicine* (Edited by E. Ben Hur and I. Rosenthal), pp. 79–121. CRC Press, Boca Raton.
- Sloney, D. H. (2002) How light reaches the eye and its components. *Int. J. Toxicol.* **21**, 501–509.
- Dillon, J., L. Zheng, J. C. Merriam and E. R. Gaillard (2004) Transmission of light to the aging human retina: possible implications for age related macular degeneration. *Exp. Eye Res.* **79**, 753–759.
- Sloney, D. H. (2001) Photoprotection of the eye—UV radiation and sunglasses. *J. Photochem. Photobiol. B-Biol.* **64**, 166–175.
- Naidoff, M. A. and D. H. Sloney (1974) Retinal injury from a welding arc. *Am. J. Ophthalmol.* **77**, 663–668.
- Romanchuk, K. G., V. Pollak and R. J. Schneider (1978) Retinal burn from a welding arc. *Can. J. Ophthalmol.* **13**, 120–122.
- Schatz, P., U. Eriksson, V. Ponjavic and S. Andreasson (2004) Multifocal electroretinography and optical coherence tomography in two patients with solar retinopathy. *Acta Ophthalmol. Scand.* **82**, 476–480.
- Young, R. W. (1988) Solar radiation and age-related macular degeneration. *Surv. Ophthalmol.* **32**, 252–269.
- Ham, W. T., H. A. Mueller, J. J. Ruffolo and D. I. Guerry (1980) Solar retinopathy as a function of wavelength: its significance for protective eyewear. In *The Effects of Constant Light on Visual Processes* (Edited by T. P. Williams and B. N. Baker), pp. 319–346. Plenum Press, New York.
- Tso, M. O. and F. G. La Piana (1974) The human fovea after sunbathing. *Trans. Sect. Ophthalmol. Am. Acad. Ophthalmol. Otolaryngol.* **79**, 788–795.
- Green, W. R. and D. M. Robertson (1991) Pathologic findings of photic retinopathy in the human eye. *Am. J. Ophthalmol.* **112**, 520–527.
- Hope-Ross, M. W., G. J. Mahon, T. A. Gardiner and D. B. Archer (1993) Ultrastructural findings in solar retinopathy. *Eye* **7**, 29–33.
- Friedman, E. and T. Kuwabara (1968) The retinal pigment epithelium. IV. The damaging effects of radiant energy. *Arch. Ophthalmol.* **80**, 265–279.
- Ham, W., Jr, J. Ruffolo, Jr, H. Mueller, A. Clarke and M. Moon (1978) Histologic analysis of photochemical lesions produced in rhesus retina by short-wavelength light. *Investig. Ophthalmol. Vis. Sci.* **17**, 1029–1035.
- Parver, L. M., C. R. Auken and B. S. Fine (1983) Observations on monkey eyes exposed to light from an operating microscope. *Ophthalmology* **90**, 964–972.
- Kirkness, C. M. (1986) Do ophthalmic instruments pose a hazard of light-induced damage to the eye? In *Hazards of Light: Myths & Realities; Eye and Skin*, 1st edition (Edited by J. Cronly-Dillon, E. S. Rosen and J. Marshall), pp. 179–186. Pergamon Press, Oxford.
- Sloney, D. H. (1997) Optical radiation safety of medical light sources. *Phys. Med. Biol.* **42**, 981–996.
- Michels, M. and P. Sternberg (1990) Operating microscope induced retinal phototoxicity—pathophysiology, clinical manifestations and prevention. *Surv. Ophthalmol.* **34**, 237–252.
- McDonald, H. R. and A. R. Irvine (1983) Light-Induced maculopathy from the operating microscope in extracapsular cataract-extraction and intraocular-lens implantation. *Ophthalmology* **90**, 945–951.
- McDonald, H. R. and M. J. Harris (1988) Operating microscope-induced retinal phototoxicity during pars plana vitrectomy. *Arch. Ophthalmol.* **106**, 521–523.
- Poliner, L. S. and P. E. Tornambe (1992) Retinal pigment epitheliopathy after macular hole surgery. *Ophthalmology* **99**, 1671–1677.
- Michels, M., H. Lewis, G. W. Abrams, D. P. Han, W. F. Mieler and J. Neitz (1992) Macular phototoxicity caused by fiberoptic endoillumination during pars-plana vitrectomy. *Am. J. Ophthalmol.* **114**, 287–296.
- Sloney, D. H. (1983) Eye protective techniques for bright light. *Ophthalmology* **90**, 937–944.
- Stringham, J. M., K. Fuld and A. J. Wenzel (2003) Action spectrum for photophobia. *J. Opt. Soc. Am. A-Opt. Image Sci. Vis.* **20**, 1852–1858.
- Marlor, R. L., B. R. Blais, F. R. Preston and D. G. Boyden (1973) Foveomacular retinitis, an important problem in military medicine: epidemiology. *Investig. Ophthalmol.* **12**, 5–16.
- Tomany, S. C., K. J. Cruickshanks, R. Klein, B. E. Klein and M. D. Knudson (2004) Sunlight and the 10-year incidence of age-related maculopathy—the Beaver Dam Eye Study. *Arch. Ophthalmol.* **122**, 750–757.
- Taylor, H. R., S. West, B. Munoz, F. S. Rosenthal, S. B. Bressler and N. M. Bressler (1992) The long-term effects of visible-light on the eye. *Arch. Ophthalmol.* **110**, 99–104.
- Klein, R., T. Peto, A. Bird and M. R. Vannewkirk (2004) The epidemiology of age-related macular degeneration. *Am. J. Ophthalmol.* **137**, 486–495.
- van Leeuwen, R., C. C. W. Klaver, J. R. Vingerling, A. Hofman and P. de Jong (2003) Epidemiology of age-related maculopathy: a review. *Eur. J. Epidemiol.* **18**, 845–854.
- de Jong, P. and J. Lubsen (2004) The standard gamble between cataract extraction and AMD. *Graefes Arch. Clin. Exp. Ophthalmol.* **242**, 103–105.
- Khwarg, S. G., F. A. Linstone, S. A. Daniels, S. J. Isenberg, T. A. Hanscom, M. Geoghegan and B. R. Straatsma (1987) Incidence, risk factors, and morphology in operating microscope light retinopathy. *Am. J. Ophthalmol.* **103**, 255–263.
- Mainster, M. A. and J. R. Sparrow (2003) How much blue light should an IOL transmit? *Br. J. Ophthalmol.* **87**, 1523–1528.
- Peyman, G. A., R. Zak and H. Sloane (1983) Ultraviolet-absorbing pseudophakos: an efficacy study. *J. Am. Intraocul. Implant. Soc.* **9**, 161–170.
- Dowling, J. E. and R. L. Sidman (1962) Inherited retinal dystrophy in the rat. *J. Cell Biol.* **14**, 73–109.
- Heckenlively, J. R., J. A. Rodriguez and S. P. Daiger (1991) Autosomal dominant sectoral retinitis-pigmentosa—2 families with transversion mutation in codon 23 of rhodopsin. *Arch. Ophthalmol.* **109**, 84–91.
- Noorwez, S. M., R. Malhotra, J. H. McDowell, K. A. Smith, M. P. Krebs and S. Kaushal (2004) Retinoids assist the cellular folding

- of the autosomal dominant retinitis pigmentosa opsin mutant P23H. *J. Biol. Chem.* **279**, 16278–16284.
38. Naash, M. L., N. S. Peachey, Z. Y. Li, C. C. Gryczan, Y. Goto, J. Blanks, A. H. Milam and H. Ripps (1996) Light-induced acceleration of photoreceptor degeneration in transgenic mice expressing mutant rhodopsin. *Investig. Ophthalmol. Vis. Sci.* **37**, 775–782.
  39. Organisciak, D. T., R. A. Darrow, L. Barsalou, R. K. Kutty and B. Wiggert (2003) Susceptibility to retinal light damage in transgenic rats with rhodopsin mutations. *Investig. Ophthalmol. Vis. Sci.* **44**, 486–492.
  40. Vaughan, D. K., S. F. Coulbaly, R. M. Darrow and D. T. Organisciak (2003) A morphometric study of light-induced damage in transgenic rat models of retinitis pigmentosa. *Investig. Ophthalmol. Vis. Sci.* **44**, 848–855.
  41. LaVail, M. M., G. M. Gorin, D. Yasumura and M. T. Matthes (1999) Increased susceptibility to constant light in *nr* and *pcd* mice with inherited retinal degenerations. *Investig. Ophthalmol. Vis. Sci.* **40**, 1020–1024.
  42. Wang, M., T. T. Lam, M. O. M. Tso and M. I. Naash (1997) Expression of a mutant opsin gene increases the susceptibility of the retina to light damage. *Vis. Neurosci.* **14**, 55–62.
  43. Tso, M. O. M. and B. J. Woodford (1983) Effect of photic injury on the retinal tissues. *Ophthalmology* **90**, 952–963.
  44. Lawwill, T. (1982) Three major pathologic processes caused by light in the primate retina: a search for mechanisms. *Trans. Am. Ophthalmol. Soc.* **80**, 517–579.
  45. Irvine, A. R., I. Wood and B. W. Morris (1984) Retinal damage from the illumination of the operating microscope—an experimental study in pseudophakic monkeys. *Arch. Ophthalmol.* **102**, 1358–1365.
  46. Reme, C. E., F. Hafezi, A. Marti, K. Munz and J. J. Reinboth (1998) Light damage to retina and retinal pigment epithelium. In *The Retinal Pigment Epithelium* (Edited by M. Marmor and T. Wolfensberger), pp. 563–586. Oxford University Press, Oxford.
  47. Reme, C. E., C. Grimm, F. Hafezi, A. Marti and A. Wenzel (1998) Apoptotic cell death in retinal degenerations. *Prog. Retin. Eye Res.* **17**, 443–464.
  48. Organisciak, D. T. and B. S. Winkler (1994) Retinal light damage: practical and theoretical considerations. *Prog. Retin. Eye Res.* **13**, 1–29.
  49. Lanum, J. (1978) The damaging effects of light on the retina. Empirical findings, theoretical and practical implications. *Surv. Ophthalmol.* **22**, 221–249.
  50. Hafezi, F., A. Marti, K. Munz and C. E. Reme (1997) Light-induced apoptosis: differential timing in the retina and pigment epithelium. *Exp. Eye Res.* **64**, 963–970.
  51. Yu, D. Y. and S. J. Cringle (2001) Oxygen distribution and consumption within the retina in vascularised and avascular retinas and in animal models of retinal disease. *Prog. Retin. Eye Res.* **20**, 175–208.
  52. Wangsa-Wirawan, N. D. and R. A. Linsenmeier (2003) Retinal oxygen—fundamental and clinical aspects. *Arch. Ophthalmol.* **121**, 547–557.
  53. Bok, D. (1993) The retinal-pigment epithelium—a versatile partner in vision. *J. Cell Sci.* **17**, 189–195.
  54. Wenzel, A., C. Grimm, M. Samardzija and C. E. Reme (2005) Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog. Retin. Eye Res.* **24**, 275–306.
  55. Williams, T. P. and W. L. Howell (1983) Action spectrum of retinal light-damage in albino rats. *Investig. Ophthalmol. Vis. Sci.* **24**, 285–287.
  56. Noell, W. K., V. S. Walker, B. S. Kang and S. Berman (1966) Retinal damage by light in rats. *Investig. Ophthalmol.* **5**, 450–473.
  57. Li, F., W. Cao and R. E. Anderson (2003) Alleviation of constant-light-induced photoreceptor degeneration by adaptation of adult albino rat to bright cyclic light. *Investig. Ophthalmol. Vis. Sci.* **44**, 4968–4975.
  58. Ohira, A., M. Tanito, S. Kaidzu and T. Kondo (2003) Glutathione peroxidase induced in rat retinas to counteract photic injury. *Investig. Ophthalmol. Vis. Sci.* **44**, 1230–1236.
  59. Penn, J. S., M. I. Naash and R. E. Anderson (1987) Effect of light history on retinal antioxidants and light damage susceptibility in the rat. *Exp. Eye Res.* **44**, 779–788.
  60. Wiegand, R. D., C. D. Joel, L. M. Rapp, J. C. Nielsen, M. B. Maude and R. E. Anderson (1986) Polyunsaturated fatty acids and vitamin E in rat rod outer segments during light damage. *Investig. Ophthalmol. Vis. Sci.* **27**, 727–733.
  61. Hunt, D. F., D. T. Organisciak, H. M. Wang and R. L. C. Wu (1984) Alpha-tocopherol in the developing rat retina—a high pressure liquid chromatographic analysis. *Curr. Eye Res.* **3**, 1281–1288.
  62. LaVail, M. M., K. Unoki, D. Yasumura, M. T. Matthes, G. D. Yancopoulos and R. H. Steinberg (1992) Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci. USA* **89**, 11249–11253.
  63. Faktorovich, E. G., R. H. Steinberg, D. Yasumura, M. T. Matthes and M. M. Lavail (1992) Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J. Neurosci.* **12**, 3554–3567.
  64. Schremser, J. L. and T. P. Williams (1995) Rod outer segment (ROS) renewal as a mechanism for adaptation to a new intensity environment. I. Rhodopsin levels and ROS length. *Exp. Eye Res.* **61**, 17–23.
  65. Reme, C. E., U. Wolfrum, C. Imsand, F. Hafezi and T. P. Williams (1999) Photoreceptor autophagy: effects of light history on number and opsin content of degradative vacuoles. *Investig. Ophthalmol. Vis. Sci.* **40**, 2398–2404.
  66. Li, F., W. Cao and R. E. Anderson (2001) Protection of photoreceptor cells in adult rats from light-induced degeneration by adaptation to bright cyclic light. *Exp. Eye Res.* **73**, 569–577.
  67. Organisciak, D. T., R. M. Darrow, L. Barsalou, R. A. Darrow, R. K. Krishnan, G. Kutty and B. Wiggert (1998) Light history and age-related changes in retinal light damage. *Investig. Ophthalmol. Vis. Sci.* **39**, 1107–1116.
  68. Noell, W. K. (1979) Effects of environmental lighting and dietary vitamin A on the vulnerability of the retina to light damage. *Photochem. Photobiol.* **29**, 717–723.
  69. Harwerth, R. S. and H. G. Sperling (1975) Effects of intense visible radiation on the increment-threshold spectral sensitivity of the rhesus monkey eye. *Vision Res.* **15**, 1193–1204.
  70. Sperling, H. G., C. Johnson and R. S. Harwerth (1980) Differential spectral photic damage to primate cones. *Vision Res.* **20**, 1117–1125.
  71. Gorgels, T. and D. Vannorren (1995) Ultraviolet and green light cause different types of damage in rat retina. *Investig. Ophthalmol. Vis. Sci.* **36**, 851–863.
  72. Ham, W. T., H. A. Mueller, J. J. Ruffolo, D. Guerry and R. K. Guerry (1982) Action spectrum for retinal injury from near-ultraviolet radiation in the aphakic monkey. *Am. J. Ophthalmol.* **93**, 299–306.
  73. Rapp, L. M. and S. C. Smith (1992) Morphologic comparisons between rhodopsin-mediated and short-wavelength classes of retinal light damage. *Investig. Ophthalmol. Vis. Sci.* **33**, 3367–3377.
  74. Bush, E. M., T. G. M. F. Gorgels and D. van Norren (1999) Temporal sequence of changes in rat retina after UV-A and blue light exposure. *Vision Res.* **39**, 1233–1247.
  75. Rodieck, R. W. (1998) *The First Steps in Seeing*. Sinauer Associates, Inc., Sunderland, MA.
  76. Lamb, T. D. and E. N. Pugh (2004) Dark adaptation and the retinoid cycle of vision. *Prog. Retin. Eye Res.* **23**, 307–380.
  77. Kolesnikov, A. V., E. Y. Golobokova and V. I. Govardovskii (2003) The identity of metarhodopsin III. *Vis. Neurosci.* **20**, 249–265.
  78. Garwin, G. G. and J. C. Saari (2000) High-performance liquid chromatography analysis of visual cycle retinoids. *Methods Enzymol.* **316**, 313–324.
  79. Wenzel, A., C. E. Reme, T. P. Williams, F. Hafezi and C. Grimm (2001) The RPE65 Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. *J. Neurosci.* **21**, 53–58.
  80. Grimm, C., A. Wenzel, F. Hafezi, S. Yu, T. M. Redmond and C. E. Reme (2000) Protection of RPE65-deficient mice identifies rhodopsin as a mediator of light-induced retinal degeneration. *Nat. Genet.* **25**, 63–66.
  81. Grimm, C., A. Wenzel, T. P. Williams, P. O. Rol, F. Hafezi and C. E. Reme (2001) Rhodopsin-mediated blue-light damage to the rat retina: effect of photoreversal of bleaching. *Investig. Ophthalmol. Vis. Sci.* **42**, 497–505.
  82. Ruffolo, J., Jr, W. Ham, Jr, H. Mueller and J. Millen (1984) Photochemical lesions in the primate retina under conditions of elevated blood oxygen. *Investig. Ophthalmol. Vis. Sci.* **25**, 893–898.

83. Ham, W. T., H. A. Mueller, J. J. Ruffolo, J. E. Millen, S. F. Cleary, R. K. Guerry and D. Guerry (1984) Basic mechanisms underlying the production of photochemical lesions in the mammalian retina. *Curr. Eye Res.* **3**, 165–174.
84. Jaffe, G. J., A. R. Irvine, I. S. Wood, J. W. Severinghaus, G. R. Pino and C. Haugen (1988) Retinal phototoxicity from the operating microscope—the role of inspired oxygen. *Ophthalmology* **95**, 1130–1141.
85. Kayatz, P., K. Heimann and U. Schraermeyer (1999) Tracing of benzidine-reactive substances in ROS, RPE and choroid after light-induced peroxidation. *Graefes Arch. Clin. Exp. Ophthalmol.* **237**, 763–774.
86. Wiegand, R. D., N. M. Giusto, L. M. Rapp and R. E. Anderson (1983) Evidence for rod outer segment lipid-peroxidation following constant illumination of the rat retina. *Investig. Ophthalmol. Vis. Sci.* **24**, 1433–1435.
87. Miyagi, M., H. Sakaguchi, R. M. Darrow, L. Yan, K. A. West, K. S. Aulak, D. J. Stuehr, J. G. Hollyfield, D. T. Organisciak and J. W. Crabb (2002) Evidence that light modulates protein nitration in rat retina. *Mol. Cell. Proteomics* **1**, 293–303.
88. Ranchon, I., J. M. Gorrand, J. Cluzel, M. T. Droy-Lefaix and M. Doly (1999) Functional protection of photoreceptors from light-induced damage by dimethylthiourea and Ginkgo biloba extract. *Investig. Ophthalmol. Vis. Sci.* **40**, 1191–1199.
89. Ranchon, I., S. Chen, K. Alvarez and R. E. Anderson (2001) Systemic administration of phenyl-N-tert-butyl nitrone protects the retina from light damage. *Investig. Ophthalmol. Vis. Sci.* **42**, 1375–1379.
90. Boulton, M., M. Rozanowska and B. Rozanowski (2001) Retinal photodamage. *J. Photochem. Photobiol. B-Biol.* **64**, 144–161.
91. Delmelle, M. (1977) Retinal damage by light: possible implication of singlet oxygen. *Biophys. Struct. Mech.* **3**, 195–198.
92. Delmelle, M. (1978) An investigation of retinal as a source of singlet oxygen. *Photochem. Photobiol.* **27**, 721–734.
93. Schadel, S. A., M. Heck, D. Maretzki, S. Filipek, D. C. Teller, K. Palczewski and K. P. Hofmann (2003) Ligand channeling within a G-protein-coupled receptor—the entry and exit of retinals in native opsin. *J. Biol. Chem.* **278**, 24896–24903.
94. Heck, M., S. A. Schadel, D. Maretzki and K. P. Hofmann (2003) Secondary binding sites of retinoids in opsin: characterization and role in regeneration. *Vision Res.* **43**, 3003–3010.
95. Fishkin, N. E., J. R. Sparrow, R. Allikmets and K. Nakanishi (2005) Isolation and characterization of a retinal pigment epithelial cell fluorophore: an all-trans-retinal dimer conjugate. *Proc. Natl. Acad. Sci. USA* **102**, 7091–7096.
96. Weng, J., N. L. Mata, S. M. Azarian, R. T. Tzekov, D. G. Birch and G. H. Travis (1999) Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in aber knockout mice. *Cell* **98**, 13–23.
97. Mata, N. L., J. Weng and G. H. Travis (2000) Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc. Natl. Acad. Sci. USA* **97**, 7154–7159.
98. Ben-Shabat, S., C. A. Parish, H. R. Vollmer, Y. Itagaki, N. Fishkin, K. Nakanishi and J. R. Sparrow (2002) Biosynthetic studies of A2E, a major fluorophore of retinal pigment epithelial lipofuscin. *J. Biol. Chem.* **277**, 7183–7190.
99. Sparrow, J. R., N. Fishkin, J. L. Zhou, B. L. Cai, Y. P. Jang, S. Krane, Y. Itagaki and K. Nakanishi (2003) A2E, a by-product of the visual cycle. *Vision Res.* **43**, 2983–2990.
100. Pepe, I. M. (2001) Recent advances in our understanding of rhodopsin and phototransduction. *Prog. Retin. Eye Res.* **20**, 733–759.
101. Okada, T., O. P. Ernst, K. Palczewski and K. P. Hofmann (2001) Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem. Sci.* **26**, 318–324.
102. Schroder, K., A. Pulvermuller and K. P. Hofmann (2002) Arrestin and its splice variant Arr(1-370A) (P-44)—mechanism and biological role of their interaction with rhodopsin. *J. Biol. Chem.* **277**, 43987–43996.
103. Pulvermuller, A., D. Maretzki, M. Rudnicka-Nawrot, W. C. Smith, K. Palczewski and K. P. Hofmann (1997) Functional differences in the interaction of arrestin and its splice variant, p(44), with rhodopsin. *Biochemistry* **36**, 9253–9260.
104. Palczewski, K., J. Buczylo, H. Ohguro, R. S. Annan, S. A. Carr, J. W. Crabb, M. W. Kaplan, R. S. Johnson and K. A. Walsh (1994) Characterization of a truncated form of arrestin isolated from bovine rod outer segments. *Protein Sci.* **3**, 314–324.
105. Vogel, R., F. Siebert, X. Y. Zhang, G. B. Fan and M. Sheves (2004) Formation of meta III during the decay of activated rhodopsin proceeds via meta I and not via meta II. *Biochemistry* **43**, 9457–9466.
106. Niu, S. L., D. C. Mitchell and B. J. Litman (2002) Manipulation of cholesterol levels in rod disk membranes by methyl-beta-cyclodextrin. Effects on receptor activation. *J. Biol. Chem.* **277**, 20139–20145.
107. Litman, B. J. and D. C. Mitchell (1996) A role for phospholipid polyunsaturation in modulating membrane protein function. *Lipids* **31**, S193–S197.
108. Gibson, N. J. and M. F. Brown (1993) Lipid headgroup and acyl chain composition modulate the MI-MII equilibrium of rhodopsin in recombinant membranes. *Biochemistry* **32**, 2438–2454.
109. Mitchell, D. C., M. Straume and B. J. Litman (1992) Role of Sn-1-saturated, Sn-2-polyunsaturated phospholipids in control of membrane-receptor conformational equilibrium—effects of cholesterol and acyl chain unsaturation on the Metarhodopsin-I-Metarhodopsin-II equilibrium. *Biochemistry* **31**, 662–670.
110. Niu, S. L., D. C. Mitchell, S. Y. Lim, Z. M. Wen, H. Y. Kim, N. Salem and B. J. Litman (2004) Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J. Biol. Chem.* **279**, 31098–31104.
111. Heck, M., S. A. Schadel, D. Maretzki, F. J. Bartl, E. Ritter, K. Palczewski and K. P. Hofmann (2003) Signaling states of rhodopsin—formation of the storage form, metarhodopsin III, from active metarhodopsin II. *J. Biol. Chem.* **278**, 3162–3169.
112. Ritter, E., K. Zimmermann, M. Heck, K. P. Hofmann and F. J. Bartl (2004) Transition of rhodopsin into the active metarhodopsin II state opens a new light induced pathway linked to Schiff base isomerization. *J. Biol. Chem.* **279**, 48102–48111.
113. Blazynski, C. and S. E. Ostroy (1984) Pathways in the hydrolysis of vertebrate rhodopsin. *Vision Res.* **24**, 459–470.
114. Zimmermann, K., E. Ritter, F. J. Bartl, K. P. Hofmann and M. Martin Heck (2004) Interaction with transducin depletes Metarhodopsin III: a regulated retinal storage in visual signal transduction? *J. Biol. Chem.* **279**, 48112–48119.
115. Vogel, R., S. Ludeke, I. Radu, F. Siebert and M. Sheves (2004) Photoreactions of metarhodopsin III. *Biochemistry* **43**, 10255–10264.
116. Ernst, O. P., C. K. Meyer, E. P. Marin, P. Henklein, W. Y. Fu, T. P. Sakmar and K. P. Hofmann (2000) Mutation of the fourth cytoplasmic loop of rhodopsin affects binding of transducin and peptides derived from the carboxyl-terminal sequences of transducin alpha and gamma subunits. *J. Biol. Chem.* **275**, 1937–1943.
117. Kisselev, O. G., C. K. Meyer, M. Heck, O. P. Ernst and K. P. Hofmann (1999) Signal transfer from rhodopsin to the G-protein: evidence for a two-site sequential fit mechanism. *Proc. Natl. Acad. Sci. USA* **96**, 4898–4903.
118. Kuksa, V., Y. Imanishi, M. Batten, K. Palczewski and A. R. Moise (2003) Retinoid cycle in the vertebrate retina: experimental approaches and mechanisms of isomerization. *Vision Res.* **43**, 2959–2981.
119. Rattner, A., P. M. Smallwood and J. Nathans (2000) Identification and characterization of all-trans-retinal dehydrogenase from photoreceptor outer segments, the visual cycle enzyme that reduces all-trans-retinal to all-trans-retinol. *J. Biol. Chem.* **275**, 11034–11043.
120. Haeseleer, F., J. Huang, L. Lebiada, J. C. Saari and K. Palczewski (1998) Molecular characterization of a novel short-chain dehydrogenase/reductase that reduces all-trans-retinal. *J. Biol. Chem.* **273**, 21790–21799.
121. Zimmerman, W. F., M. T. Yost and F. J. M. Daemen (1974) Dynamics and function of vitamin A compounds in rat retina after a small bleach of rhodopsin. *Nature* **250**, 66–67.
122. Saari, J. C., G. G. Garwin, J. P. Van Hooser and K. Palczewski (1998) Reduction of all-trans-retinal limits regeneration of visual pigment in mice. *Vision Res.* **38**, 1325–1333.
123. Tsina, E., C. Chen, Y. Koutalos, P. Ala-Laurila, M. Tsacopoulos, B. Wiggert, R. K. Crouch and M. C. Cornwall (2004) Physiological and microfluorimetric studies of reduction and clearance of retinal in bleached rod photoreceptors. *J. Gen. Physiol.* **124**, 429–443.
124. Chen, C., E. Tsina, M. C. Cornwall, R. K. Crouch, S. Vijayaraghavan and Y. Koutalos (2005) Reduction of all-trans retinal to all-trans retinol in the outer segments of frog and mouse rod photoreceptors. *Biophys. J.* **88**, 2278–2287.

125. Poincelot, R. P., P. G. Millar, R. L. J. Kimbel and E. W. Abrahamson (1969) Lipid to protein chromophore transfer in the photolysis of visual pigments. *Nature* **221**, 256–257.
126. Anderson, R. E. and M. B. Maude (1970) Phospholipids of bovine rod outer segments. *Biochemistry* **9**, 3624–3628.
127. Ahn, J., J. T. Wong and R. S. Molday (2000) The effect of lipid environment and retinoids on the ATPase activity of ABCR, the photoreceptor ABC transporter responsible for Stargardt macular dystrophy. *J. Biol. Chem.* **275**, 20399–20405.
128. Sun, H., R. S. Molday and J. Nathans (1999) Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease. *J. Biol. Chem.* **274**, 8269–8281.
129. Beharry, S., M. Zhong and R. S. Molday (2004) N-retinylidene phosphatidylethanolamine is the preferred retinoid substrate for the photoreceptor specific ABC transporter ABCA4 (ABCR). *J. Biol. Chem.* **279**, 53970–53979.
130. Mata, N. L., R. T. Tzekov, X. R. Liu, J. Weng, D. G. Birch and G. H. Travis (2001) Delayed dark-adaptation and lipofuscin accumulation in *abcr*<sup>+/–</sup> mice: implications for involvement of ABCR in age-related macular degeneration. *Investig. Ophthalmol. Vis. Sci.* **42**, 1685–1690.
131. Shroyer, N. F., R. A. Lewis, A. N. Yatsenko and J. R. Lupski (2001) Null missense ABCR (ABCA4) mutations in a family with Stargardt disease and retinitis pigmentosa. *Investig. Ophthalmol. Vis. Sci.* **42**, 2757–2761.
132. Maugeri, A., B. J. Klevering, K. Rohrschneider, A. Blankenagel, H. G. Brunner, A. F. Deutman, C. B. Hoyng and F. P. M. Cremers (2000) Mutations in the ABCA4 (ABCR) gene are the major cause of autosomal recessive cone-rod dystrophy. *Am. J. Hum. Genet.* **67**, 960–966.
133. Michaelides, M., D. M. Hunt and A. T. Moore (2003) The genetics of inherited macular dystrophies. *J. Med. Genet.* **40**, 641–650.
134. Allikmets, R. (1999) Molecular genetics of age-related macular degeneration: current status. *Eur. J. Ophthalmol.* **9**, 255–265.
135. Allikmets, R., N. Singh, H. Sun, N. E. Shroyer, A. Hutchinson, A. Chidambaram, B. Gerrard, L. Baird, D. Stauffer, A. Peiffer, A. Rattner, P. Smallwood, Y. X. Li, K. L. Anderson, R. A. Lewis, J. Nathans, M. Leppert, M. Dean and J. R. Lupski (1997) A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat. Genet.* **15**, 236–246.
136. Besch, D., H. Jagle, H. P. N. Scholl, M. W. Seeliger and E. Zrenner (2003) Inherited multifocal RPE-diseases: mechanisms for local dysfunction in global retinoid cycle gene defects. *Vision Res.* **43**, 3095–3108.
137. Feeney-Burns, L., E. R. Berman and H. Rothman (1980) Lipofuscin of human retinal pigment epithelium. *Am. J. Ophthalmol.* **90**, 783–791.
138. Plack, P. A. and D. J. Pritchard (1969) Schiff bases formed from retinal and phosphatidylethanolamine, phosphatidylserine, ethanolamine or serine. *Biochem. J.* **115**, 927–934.
139. Saari, J. C., M. Nawrot, B. N. Kennedy, G. G. Garwin, J. B. Hurley, J. Huang, D. E. Possin and J. W. Crabb (2001) Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. *Neuron* **29**, 739–748.
140. Imanishi, Y., V. Gerke and K. Palczewski (2004) Retinosomes: new insights into intracellular managing of hydrophobic substances in lipid bodies. *J. Cell Biol.* **166**, 447–453.
141. McBee, J. K., K. Palczewski, W. Baehr and D. R. Pepperberg (2001) Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog. Retin. Eye Res.* **20**, 469–529.
142. Crouch, R. K., E. S. Hazard, T. Lind, B. Wiggert, G. Chader and D. W. Corson (1992) Interphotoreceptor retinoid-binding protein and alpha-tocopherol preserve the isomeric and oxidation-state of retinol. *Photochem. Photobiol.* **56**, 251–255.
143. Batten, M. L., Y. Imanishi, T. Maeda, D. C. Tu, A. R. Moise, D. Bronson, D. Possin, R. N. Van Gelder, W. Baehr and K. Palczewski (2004) Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. *J. Biol. Chem.* **279**, 10422–10432.
144. Imanishi, Y., M. L. Batten, D. W. Piston, W. Baehr and K. Palczewski (2004) Noninvasive two-photon imaging reveals retinyl ester storage structures in the eye. *J. Cell Biol.* **164**, 373–383.
145. Gollapalli, D. R. and R. R. Rando (2003) Specific inactivation of isomerohydrolase activity by 11-cis-retinoids. *Biochim. Biophys. Acta* **1651**, 93–101.
146. Golczak, M., V. Kuksa, T. Maeda, A. R. Moise and K. Palczewski (2005) Positively charged retinoids are potent and selective inhibitors of the trans-cis isomerization in the retinoid (visual) cycle. *Proc. Natl. Acad. Sci. USA* **102**, 8162–8167.
147. Mata, N. L., W. N. Moghrabi, J. S. Lee, T. V. Bui, R. A. Radu, J. Horwitz and G. H. Travis (2004) Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. *J. Biol. Chem.* **279**, 635–643.
148. Gollapalli, D. R., P. Maiti and R. R. Rando (2003) RPE65 operates in the vertebrate visual cycle by stereospecifically binding all-trans-retinyl esters. *Biochemistry* **42**, 11824–11830.
149. Wolf, G. (2005) Function of the protein RPE65 in the visual cycle. *Nutr. Rev.* **63**, 97–1000.
150. Xue, L. L., D. R. Gollapalli, P. Maiti, W. J. Jahng and R. R. Rando (2004) A palmitoylation switch mechanism in the regulation of the visual cycle. *Cell* **117**, 761–771.
151. Jin, M., S. Li, W. N. Moghrabi, H. Sun and G. H. Travis (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell* **122**, 449–459.
152. Katz, M. L. and T. M. Redmond (2001) Effect of RPE65 knockout on accumulation of lipofuscin fluorophores in the retinal pigment epithelium. *Investig. Ophthalmol. Vis. Sci.* **42**, 3023–3030.
153. Redmond, T. M., S. Yu, E. Lee, D. Bok, D. Hamasaki, N. Chen, P. Goletz, J. X. Ma, R. K. Crouch and K. Pfeifer (1998) RPE65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat. Genet.* **20**, 344–351.
154. Marlhens, F., C. Bareil, J. M. Griffioen, E. Zrenner, P. Amalric, C. Eliaou, S. Y. Liu, E. Harris, T. M. Redmond, B. Arnaud, M. Claustres and C. P. Hamel (1997) Mutations in RPE65 cause Leber's congenital amaurosis. *Nat. Genet.* **17**, 139–141.
155. Cremers, F. P. M., J. van den Hurk and A. I. den Hollander (2002) Molecular genetics of Leber congenital amaurosis. *Hum. Mol. Genet.* **11**, 1169–1176.
156. Stecher, H., M. H. Gelb, J. C. Saari and K. Palczewski (1999) Preferential release of 11-cis-retinol from retinal pigment epithelial cells in the presence of cellular retinaldehyde-binding protein. *J. Biol. Chem.* **274**, 8577–8585.
157. Wu, Z. P., Y. W. Yang, N. Shaw, S. Bhattacharya, L. Yan, K. West, K. Roth, N. Noy, J. Qin and J. W. Crabb (2003) Mapping the ligand binding pocket in the cellular retinaldehyde binding protein. *J. Biol. Chem.* **278**, 12390–12396.
158. Gamble, M. V., N. L. Mata, A. T. Tsin, J. R. Mertz and W. S. Blamer (2000) Substrate specificities and 13-cis-retinoic acid inhibition of human, mouse and bovine cis-retinol dehydrogenases. *Biochim. Biophys. Acta* **1476**, 3–8.
159. Kim, T. S., A. Maeda, T. Maeda, C. Heinlein, N. Kedishvili, K. Palczewski and P. S. Nelson (2005) Delayed dark adaptation in 11-cis-retinol dehydrogenase-deficient mice. *J. Biol. Chem.* **280**, 8694–8704.
160. Driessen, C., H. Winkens, F. Haeseleer, K. Palczewski and J. Janssen (2003) Novel targeting strategy for generating mouse models with defects in the retinoid cycle. *Vision Res.* **43**, 3075–3079.
161. Haeseleer, F., G. F. Jang, Y. Imanishi, C. Driessen, M. Matsumura, P. S. Nelson and K. Palczewski (2002) Dual-substrate specificity short chain retinol dehydrogenases from the vertebrate retina. *J. Biol. Chem.* **277**, 45537–45546.
162. Jang, G. F., J. P. Van Hooser, V. Kuksa, J. K. McBee, Y. G. He, J. M. Janssen, C. Driessen and K. Palczewski (2001) Characterization of a dehydrogenase activity responsible for oxidation of 11-cis-retinol in the retinal pigment epithelium of mice with a disrupted RDH5 gene—a model for the human hereditary disease fundus albipunctatus. *J. Biol. Chem.* **276**, 32456–32465.
163. Kedishvili, N. Y., O. V. Chumakova, S. V. Chetyrkin, O. V. Belyaeva, E. A. Lapshina, D. W. Lin, M. Matsumura and P. S. Nelson (2002) Evidence that the human gene for prostate short-chain dehydrogenase/reductase (PSDR1) encodes a novel retinal reductase (RalR1). *J. Biol. Chem.* **277**, 28909–28915.
164. Cideciyan, A. V., F. Haeseleer, R. N. Fariss, T. S. Aleman, G. F. Jang, C. Verlinde, M. F. Marmor, S. G. Jacobson and K. Palczewski (2000) Rod and cone visual cycle consequences of a null mutation in the 11-cis-retinol dehydrogenase gene in man. *Vis. Neurosci.* **17**, 667–678.

165. Yamamoto, H., A. Simon, U. Eriksson, E. Harris, E. L. Berson and T. P. Dryja (1999) Mutations in the gene encoding 11-cis retinol dehydrogenase cause delayed dark adaptation and fundus albipunctatus. *Nat. Genet.* **22**, 188–191.
166. Nakamura, M., Y. Hotta, A. Tanikawa, H. Terasaki and Y. Miyake (2000) A high association with cone dystrophy in fundus albipunctatus caused by mutations of the RDH5 gene. *Investig. Ophthalmol. Vis. Sci.* **41**, 3925–3932.
167. Nakamura, M., J. Lin and Y. Miyake (2004) Young monozygotic twin sisters with fundus albipunctatus and cone dystrophy. *Arch. Ophthalmol.* **122**, 1203–1207.
168. Bonilha, V. L., S. K. Bhattacharya, K. A. West, J. S. Crabb, J. Sun, M. E. Rayborn, M. Nawrot, J. C. Saari and J. W. Crabb (2004) Support for a proposed retinoid-processing protein complex in apical retinal pigment epithelium. *Exp. Eye Res.* **79**, 419–422.
169. Nawrot, M., K. West, J. Huang, D. E. Possin, A. Bretscher, J. W. Crabb and J. C. Saari (2004) Cellular retinaldehyde-binding protein interacts with ERM-binding phosphoprotein 50 in retinal pigment epithelium. *Investig. Ophthalmol. Vis. Sci.* **45**, 393–401.
170. Mata, N. L., E. T. Villazana and A. T. C. Tsin (1998) Colocalization of 11-cis retinyl esters and retinyl ester hydrolase activity in retinal pigment epithelium plasma membrane. *Investig. Ophthalmol. Vis. Sci.* **39**, 1312–1319.
171. Saari, J. C., A. H. Buntmilam, D. L. Bredberg and G. G. Garwin (1984) Properties and immunocytochemical localization of three retinoid binding proteins from bovine retina. *Vision Res.* **24**, 1595–1603.
172. Bunt-Milam, A. H. and J. C. Saari (1983) Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J. Cell Biol.* **97**, 703–712.
173. Gonzalez-Fernandez, F. (2003) Interphotoreceptor retinoid-binding protein—an old gene for new eyes. *Vision Res.* **43**, 3021–3036.
174. Fedorovich, I. B., E. M. Semenova, K. Grant, C. A. Converse and M. A. Ostrovsky (2000) Photosensitized light-induced damage of IRBP (interphotoreceptor retinoid-binding protein): effects on binding properties. *Curr. Eye Res.* **21**, 975–980.
175. Jacobson, S. G., A. V. Cideciyan, C. M. Kemp, V. C. Sheffield and E. M. Stone (1996) Photoreceptor function in heterozygotes with insertion or deletion mutations in the RDS gene. *Investig. Ophthalmol. Vis. Sci.* **37**, 1662–1674.
176. Kemp, C. M., S. G. Jacobson, A. V. Cideciyan, A. E. Kimura, V. C. Sheffield and E. M. Stone (1994) RDS gene-mutations causing retinitis-pigmentosa or macular degeneration lead to the same abnormality in photoreceptor function. *Investig. Ophthalmol. Vis. Sci.* **35**, 3154–3162.
177. Wroblewski, J. J., J. A. Wells, A. Eckstein, F. Fitzke, C. Jubb, T. J. Keen, C. Inglehearn, S. Bhattacharya, G. B. Arden, M. Jay and A. C. Bird (1994) Macular dystrophy associated with mutations at codon-172 in the human retinal degeneration slow gene. *Ophthalmology* **101**, 12–22.
178. Wells, J., J. Wroblewski, J. Keen, C. Inglehearn, C. Jubb, A. Eckstein, M. Jay, G. Arden, S. Bhattacharya, F. Fitzke and A. Bird (1993) Mutations in the human retinal degeneration slow (Rds) gene can cause either retinitis-pigmentosa or macular dystrophy. *Nat. Genet.* **3**, 213–218.
179. Mata, N. L., R. A. Radu, R. S. Clemmons and G. H. Travis (2002) Isomerization and oxidation of vitamin A in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight. *Neuron* **36**, 69–80.
180. Goldstein, E. B. (1970) Cone pigment regeneration in the isolated frog retina. *Vision Res.* **10**, 1065–1068.
181. Hood, D. C. and P. A. Hock (1973) Recovery of cone receptor activity in the frog's isolated retina. *Vision Res.* **13**, 1943–1951.
182. Saari, J. C., L. Bredberg and G. G. Garwin (1982) Identification of the endogenous retinoids associated with three cellular retinoid binding proteins from bovine retina and retinal pigment epithelium. *J. Biol. Chem.* **257**, 3329–3333.
183. Bok, D., D. E. Ong and F. Chytil (1984) Immunocytochemical localization of cellular retinoid binding protein in the rat retina. *Investig. Ophthalmol. Vis. Sci.* **25**, 877–883.
184. Saari, J. C. and D. L. Bredberg (1987) Photochemistry and stereoselectivity of cellular retinaldehyde-binding protein from bovine retina. *J. Biol. Chem.* **262**, 7618–7622.
185. Das, S. R., N. Bhardwaj, H. Kjeldbye and P. Gouras (1992) Muller cells of chicken retina synthesize 11-cis-retinol. *Biochem. J.* **285**, 907–913.
186. Znoiko, S. L., R. K. Crouch, G. Moiseyev and J. X. Ma (2002) Identification of the RPE65 protein in mammalian cone photoreceptors. *Investig. Ophthalmol. Vis. Sci.* **43**, 1604–1609.
187. Jones, G. J., A. Fein, E. F. Macnichel and M. C. Cornwall (1993) Visual pigment bleaching in isolated salamander retinal cones—microspectrophotometry and light adaptation. *J. Gen. Physiol.* **102**, 483–502.
188. Jin, J., G. J. Jones and M. C. Cornwall (1994) Movement of retinal along cone and rod photoreceptors. *Vis. Neurosci.* **11**, 389–399.
189. Perrault, I., S. Haney, S. Gerber, F. Barbet, D. Ducrocq, H. Dollfus, C. Hamel, J. L. Dufier, A. Munnich, J. Kaplan and J. M. Rozet (2004) Retinal dehydrogenase 12 (RDH12) mutations in Leber congenital amaurosis. *Am. J. Hum. Genet.* **75**, 639–646.
190. Janecke, A. R., D. A. Thompson, G. Utermann, C. Becker, C. A. Hubner, E. Schmid, C. L. McHenry, A. R. Nair, F. Ruschendorf, J. Heckenlively, B. Wissinger, P. Numberg and A. Gal (2004) Mutations in RDH12 encoding a photoreceptor cell retinol dehydrogenase cause childhood-onset severe retinal dystrophy. *Nat. Genet.* **36**, 850–854.
191. Cideciyan, A. V., T. S. Aleman, M. Swider, S. B. Schwartz, J. D. Steinberg, A. J. Brucker, A. M. Maguire, J. Bennett, E. M. Stone and S. G. Jacobson (2004) Mutations in ABCA4 result in accumulation of lipofuscin before slowing of the retinoid cycle: a reappraisal of the human disease sequence. *Hum. Mol. Genet.* **13**, 525–534.
192. Bridges, C. D. B., R. A. Alvarez and S. L. Fong (1982) Vitamin A in human eyes—amount, distribution, and composition. *Investig. Ophthalmol. Vis. Sci.* **22**, 706–714.
193. Radu, R. A., J. Hu, W. N. Moghrabi, N. L. Mata, D. Bok and G. H. Travis (2004) Light-dependent regulation of the visual cycle by non-photoreceptor opsins in the RPE cells. *Investig. Ophthalmol. Vis. Sci.* **45**, E-Abstract 1246.
194. Hao, W. S. and H. K. W. Fong (1999) The endogenous chromophore of retinal G protein-coupled receptor opsin from the pigment epithelium. *J. Biol. Chem.* **274**, 6085–6090.
195. Hao, W. S. and H. K. W. Fong (1996) Blue and ultraviolet light-absorbing opsin from the retinal pigment epithelium. *Biochemistry* **35**, 6251–6256.
196. Chen, P., T. D. Lee and H. K. W. Fong (2001) Interaction of 11-cis-retinol dehydrogenase with the chromophore of retinal G protein-coupled receptor opsin. *J. Biol. Chem.* **276**, 21098–21104.
197. Chen, P., W. S. Hao, L. Rife, X. P. Wang, D. W. Shen, J. Chen, T. Ogden, G. B. Van Boemel, L. Y. Wu, M. Yang and H. K. W. Fong (2001) A photic visual cycle of rhodopsin regeneration is dependent on Rgr. *Nat. Genet.* **28**, 256–260.
198. Maeda, T., J. P. Van Hooser, C. Driessen, S. Filipek, J. J. M. Janssen and K. Palczewski (2003) Evaluation of the role of the retinal G protein-coupled receptor (RGR) in the vertebrate retina in vivo. *J. Neurochem.* **85**, 944–956.
199. Van Hooser, J. P., Y. Liang, T. Maeda, V. Kuksa, G. F. Jang, Y. G. He, F. Rieke, H. K. W. Fong, P. B. Detwiler and K. Palczewski (2002) Recovery of visual functions in a mouse model of Leber congenital amaurosis. *J. Biol. Chem.* **277**, 19173–19182.
200. Sun, H., D. J. Gilbert, N. G. Copeland, N. A. Jenkins and J. Nathans (1997) Peropsin, a novel visual pigment-like protein located in the apical microvilli of the retinal pigment epithelium. *Proc. Natl. Acad. Sci. USA* **94**, 9893–9898.
201. Liu, J. H., Y. Itagaki, S. Ben-Shabat, K. Nakanishi and J. R. Sparrow (2000) The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. *J. Biol. Chem.* **275**, 29354–29360.
202. Lamb, L. E. and J. D. Simon (2004) A2E: a component of ocular lipofuscin. *Photochem. Photobiol.* **79**, 127–136.
203. Bazan, H. E. P., N. G. Bazan, L. Feeneyburns and E. R. Berman (1990) Lipids in human lipofuscin-enriched subcellular-fractions of 2 age populations—comparison with rod outer segments and neural retina. *Investig. Ophthalmol. Vis. Sci.* **31**, 1433–1443.
204. Poincelot, R. P., P. G. Millar, R. L. J. Kimbel and E. W. Abrahamson (1970) Determination of the chromophoric binding site in native bovine rhodopsin. *Biochemistry* **9**, 1809–1816.
205. Eldred, G. E. and M. R. Lasky (1993) Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature* **361**, 724–726.

206. Sparrow, J. R., C. A. Parish, M. Hashimoto and K. Nakanishi (1999) A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Investig. Ophthalmol. Vis. Sci.* **40**, 2988–2995.
207. Delori, F. C., C. K. Dorey, G. Staurengi, O. Arend, D. G. Goger and J. J. Weiter (1995) In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. *Investig. Ophthalmol. Vis. Sci.* **36**, 718–729.
208. Delori, F. C., D. G. Goger and C. K. Dorey (2001) Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Investig. Ophthalmol. Vis. Sci.* **42**, 1855–1866.
209. Kim, S. R., N. Fishkin, J. Kong, K. Nakanishi, R. Allikmets and J. R. Sparrow (2004) Rpe65 Leu450Met variant is associated with reduced levels of the retinal pigment epithelium lipofuscin fluorophores A2E and iso-A2E. *Proc. Natl. Acad. Sci. USA* **101**, 11668–11672.
210. Gollapalli, D. R. and R. R. Rando (2004) The specific binding of retinoic acid to RPE65 and approaches to the treatment of macular degeneration. *Proc. Natl. Acad. Sci. USA* **101**, 10030–10035.
211. Law, W. C. and R. R. Rando (1989) The molecular basis of retinoic acid induced night blindness. *Biochem. Biophys. Res. Commun.* **161**, 825–829.
212. Radu, R. A., N. L. Mata, S. Nusinowitz, X. R. Liu, P. A. Sieving and G. H. Travis (2003) Treatment with isotretinoin inhibits lipofuscin accumulation in a mouse model of recessive Stargardt's macular degeneration. *Proc. Natl. Acad. Sci. USA* **100**, 4742–4747.
213. Becker, R. S. (1988) The visual process—photophysics and photoisomerization of model visual pigments and the primary reaction. *Photochem. Photobiol.* **48**, 369–399.
214. Bensasson, R. V., E. J. Land and T. G. Truscott (1983) *Flash Photolysis and Pulse Radiolysis. Contributions to the Chemistry of Biology and Medicine*. Pergamon Press, Oxford.
215. Dillon, J., E. R. Gaillard, P. Bilski, C. F. Chignell and K. J. Reszka (1996) The photochemistry of the retinoids as studied by steady-state and pulsed methods. *Photochem. Photobiol.* **63**, 680–685.
216. Das, P. K. and R. S. Becker (1979) Triplet state photophysical properties and intersystem crossing quantum efficiencies of homologues of retinals in various solvents. *J. Am. Chem. Soc.* **101**, 6348–6353.
217. Truscott, T. G., E. J. Land and A. Sykes (1973) The in vitro photochemistry of biological molecules-III. Absorption spectra, lifetimes and rates of oxygen quenching of the triplet states of  $\beta$ -carotene, retinal and related polyenes. *Photochem. Photobiol.* **17**, 43–51.
218. Rozanowska, M., J. Wessels, M. Boulton, J. M. Burke, M. A. J. Rodgers, T. G. Truscott and T. Sarna (1998) Blue light-induced singlet oxygen generation by retinal lipofuscin in non-polar media. *Free Radic. Biol. Med.* **24**, 1107–1112.
219. Harper, W. S. and E. R. Gaillard (2001) Studies of all-trans-retinal as a photooxidizing agent. *Photochem. Photobiol.* **73**, 71–76.
220. Pawlak, A., M. Wrona, M. Rozanowska, M. Zareba, L. E. Lamb, J. Roberts, J. D. Simon and T. Sarna (2003) Comparison of the aerobic photoreactivity of A2E with its precursor retinal. *Photochem. Photobiol.* **77**, 253–258.
221. Fisher, M. M. and K. Weiss (1974) Laser photolysis of retinal and its protonated and unprotonated N-butylamine schiff base. *Photochem. Photobiol.* **20**, 423–432.
222. Harper, W. S. and E. R. Gaillard (2003) A photochemical study of (E,E,E)-2-(9-(2-hydroxyethyl)imino)-3,7-dimethyl-1,3,5,7-decatrien-1-yl-1,3,3-trimethylcyclohexene, a derivative of all-trans-retinal and ethanolamine. *Photochem. Photobiol.* **78**, 298–305.
223. Kim, J. E., M. J. Tauber and R. A. Mathies (2001) Wavelength dependent cis-trans isomerization in vision. *Biochemistry* **40**, 13774–13778.
224. Rozanowska, M. B., B. Rozanowski, A. Pawlak, M. Zareba, M. E. Boulton and T. Sarna (2003) Phosphatidylethanolamine prevents retinal photodamage. In *10th Congress of the European Society for Photobiology Programme and Book of Abstracts* (Edited by H. Honingsmann), p. 48 Vienna, Austria.
225. Crabb, J. W., Z. Q. Nie, Y. Chen, J. D. Hulmes, K. A. West, J. T. Kapron, S. E. Ruuska, N. Noy and J. C. Saari (1998) Cellular retinaldehyde-binding protein ligand interactions—GLN-210 and LYS-221 are in the retinoid binding pocket. *J. Biol. Chem.* **273**, 20712–20720.
226. Pogozheva, I. D., I. B. Fedorovich, M. A. Ostrovsky and N. M. Emanuel (1981) Photodamage of rhodopsin molecule—oxidation of SH-group. *Biofizika* **26**, 398–403.
227. Sun, H. and J. Nathans (2001) ABCR, the ATP-binding cassette transporter responsible for Stargardt macular dystrophy, is an efficient target of all-trans-retinal-mediated photooxidative damage in vitro—implications for retinal disease. *J. Biol. Chem.* **276**, 11766–11774.
228. Ablonczy, Z., D. R. Knapp, R. Darrow, D. T. Organisciak and R. K. Crouch (2000) Mass spectrometric analysis of rhodopsin from light damaged rats. *Mol. Vis.* **6**, 109–115.
229. Murata, M. and S. Kawanishi (2000) Oxidative DNA damage by vitamin A and its derivative via superoxide generation. *J. Biol. Chem.* **275**, 2003–2008.
230. Rozanowski, B., M. B. Rozanowska and M. E. Boulton (2003) Toxicity of all-trans retinal to the retinal pigment epithelium. *Investig. Ophthalmol. Vis. Sci.* **44**, 1643.
231. Delatour, T., J. Favrot, M. H. Baron, J. Belloc and C. De Loze (1982) Photochemical evolution of trans-retinal and trans-n-retinylidene n-butylamine under laser beam irradiation. *Chem. Phys. Lett.* **87**, 379–383.
232. Rozanowska, M. B., B. Rozanowski, A. Pawlak, T. Sarna and J. D. Simon (2003) Peroxidized docosahexaenoic fatty acid as a photo-generator of reactive oxygen species in the retina. *Investig. Ophthalmol. Vis. Sci.* **44**, E-Abstract 396.
233. Rozanowski, B., M. E. Boulton and M. B. Rozanowska (2004) Action spectra of photo-oxidation for bleached and dark-adapted photo-receptor outer segments. *Investig. Ophthalmol. Vis. Sci.* **44**, E-Abstract 717.
234. Organisciak, D. T., A. Xie, H. M. Wang, Y. L. Jiang, R. M. Darrow and L. A. Donoso (1991) Adaptive-changes in visual cell transduction protein-levels—effect of light. *Exp. Eye Res.* **53**, 773–779.
235. Williams, T. P., A. Squitieri, R. P. Henderson and J. P. P. Webbers (1999) Reciprocity between light intensity and rhodopsin concentration across the rat retina. *J. Physiol.* **516**, 869–874.
236. Kaldi, I., R. E. Martin, H. Huang, R. S. Brush, K. A. Morrison and R. E. Anderson (2003) Bright cyclic rearing protects albino mouse retina against acute light-induced apoptosis. *Mol. Vis.* **9**, 337–344.
237. Bush, R. A., C. E. Reme and A. Malnoe (1991) Light damage in the rat retina—the effect of dietary deprivation of n-3 fatty-acids on acute structural alterations. *Exp. Eye Res.* **53**, 741–752.
238. Bush, R. A., A. Malnoe, C. E. Reme and T. P. Williams (1994) Dietary deficiency of n-3 fatty-acids alters rhodopsin content and function in the rat retina. *Investig. Ophthalmol. Vis. Sci.* **35**, 91–100.
239. Penn, J. S. and R. E. Anderson (1987) Effect of light history on rod outer segment membrane composition in the rat. *Exp. Eye Res.* **44**, 767–778.
240. Sokolov, M., A. L. Lyubarsky, K. J. Strissel, A. B. Savchenko, V. I. Govardovskii, E. N. Pugh and V. Y. Arshavsky (2002) Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron* **34**, 95–106.
241. Danciger, M., M. T. Matthes, D. Yasamura, N. B. Akhmedov, T. Rickabaugh, S. Gentleman, T. M. Redmond, M. M. La Vail and D. B. Farber (2000) A QTL on distal Chromosome 3 that influences the severity of light-induced damage to mouse photoreceptors. *Mamm. Genome* **11**, 422–427.
242. Wenzel, A., C. Grimm, M. Samardzija and C. E. Reme (2003) The genetic modifier RPE65 Leu(450): effect on light damage susceptibility in c-Fos-deficient mice. *Investig. Ophthalmol. Vis. Sci.* **44**, 2798–2802.
243. Nusinowitz, S., L. Nguyen, R. Radu, Z. Kashani, D. Farber and M. Danciger (2003) Electoretinographic evidence for altered photo-transduction gain and slowed recovery from photobleaches in albino mice with a MET450 variant in RPE65. *Exp. Eye Res.* **77**, 627–638.
244. Sparrow, J. R. (2003) Therapy for macular degeneration: insights from acne. *Proc. Natl. Acad. Sci. USA* **100**, 4353–4354.
245. Sieving, P. A., P. Chaudhry, M. Kondo, M. Provenzano, D. Wu, T. J. Carlson, R. A. Bush and D. A. Thompson (2001) Inhibition of the visual cycle in vivo by 13-cis retinoic acid protects from light damage and provides a mechanism for night blindness in isotretinoin therapy. *Proc. Natl. Acad. Sci. USA* **98**, 1835–1840.
246. Ishizawa, Y., R. Pidikiti, P. A. Liebman and R. G. Eckenhoff (2002) G protein-coupled receptors as direct targets of inhaled anesthetics. *Mol. Pharmacol.* **61**, 945–952.
247. Keller, C., C. Grimm, A. Wenzel, F. Hafezi and C. E. Reme (2001) Protective effect of halothane anesthesia on retinal light damage:

- inhibition of metabolic rhodopsin regeneration. *Investig. Ophthalmol. Vis. Sci.* **42**, 476–480.
248. Rapp, L. M. and A. J. Ghalayini (1999) Influence of UVA light stress on photoreceptor cell metabolism: decreased rates of rhodopsin regeneration and opsin synthesis. *Exp. Eye Res.* **68**, 757–764.
  249. Noorwez, S. M., V. Kuksa, Y. Imanishi, L. Zhu, S. Filipek, K. Palczewski and S. Kaushal (2003) Pharmacological chaperone-mediated in vivo folding and stabilization of the P23H-opsin mutant associated with autosomal dominant retinitis pigmentosa. *J. Biol. Chem.* **278**, 14442–14450.
  250. Katz, M. L., C. L. Gao and L. M. Rice (1999) Long-term variations in cyclic light intensity and dietary vitamin A intake modulate lipofuscin content of the retinal pigment epithelium. *J. Neurosci. Res.* **57**, 106–116.
  251. Fite, K. V., L. Bengston and B. Donaghey (1993) Experimental light damage increases lipofuscin in the retinal pigment epithelium of Japanese quail (*Coturnix coturnix Japonica*). *Exp. Eye Res.* **57**, 449–460.
  252. Schutt, F., B. Ueberle, M. Schnolzer, F. G. Holz and J. Kopitz (2002) Proteome analysis of lipofuscin in human retinal pigment epithelial cells. *FEBS Lett.* **528**, 217–221.
  253. Eldred, G. E. and M. L. Katz (1988) Fluorophores of the human retinal pigment epithelium—separation and spectral characterization. *Exp. Eye Res.* **47**, 71–86.
  254. Parish, C. A., M. Hashimoto, K. Nakanishi, J. Dillon and J. Sparrow (1998) Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc. Natl. Acad. Sci. USA* **95**, 14609–14613.
  255. Eldred, G. E. (1998) Lipofuscin and other lysosomal storage deposits in the retinal pigment epithelium. In *The Retinal Pigment Epithelium Function and Disease* (Edited by M. F. Marmor and T. J. Wolfensberger), pp. 651–668. Oxford University Press, New York.
  256. Delaey, J. J. and C. Verougstraete (1995) Hyperlipofuscinosis and subretinal fibrosis in Stargardt's disease. *Retin.-J. Retin. Vit. Dis.* **15**, 399–406.
  257. Birnbach, C. D., M. Jarvelainen, D. E. Possin and A. H. Milam (1994) Histopathology and immunocytochemistry of the neurosensory retina in fundus flavimaculatus. *Ophthalmology* **101**, 1211–1219.
  258. Curcio, C. A. (2001) Photoreceptor topography in ageing and age-related maculopathy. *Eye* **15**, 376–383.
  259. Roth, F., A. Bindewald and F. G. Holz (2004) Key pathophysiologic pathways in age-related macular disease. *Graefes Arch. Clin. Exp. Ophthalmol.* **242**, 710–716.
  260. Kliffen, M., T. L. Vanderschaft, C. M. Mooy and P. deJong (1997) Morphologic changes in age-related maculopathy. *Microsc. Res. Tech.* **36**, 106–122.
  261. Lorenz, B. and M. N. Preising (2005) Best's disease. Overview of pathology and its causes. *Ophthalmologie* **102**, 111–115.
  262. Kolb, H. and P. Gouras (1974) Electron microscopic observations of human retinitis pigmentosa, dominantly inherited. *Investig. Ophthalmol.* **13**, 487–498.
  263. Bergsma, D. R., B. N. Wiggert, M. Funahashi, T. Kuwabara and C. J. Chader (1977) Vitamin A receptors in normal and dystrophic human retina. *Nature* **265**, 66–67.
  264. Young, R. W. (1987) Pathophysiology of age-related macular degeneration. *Surv. Ophthalmol.* **31**, 291–306.
  265. Dorey, C. K., G. Wu, D. Ebenstein, A. Garsd and J. J. Weiter (1989) Cell loss in the aging retina—relationship to lipofuscin accumulation and macular degeneration. *Investig. Ophthalmol. Vis. Sci.* **30**, 1691–1699.
  266. Taylor, A., P. F. Jacques and C. K. Dorey (1993) Oxidation and aging—impact on vision. *Toxicol. Ind. Health* **9**, 349–371.
  267. Kennedy, C. J., P. E. Rakoczy and I. J. Constable (1995) Lipofuscin of the retinal pigment epithelium: a review. *Eye* **9**, 763–771.
  268. Klein, R., B. E. K. Klein, S. C. Tomany, S. M. Meuer and G. H. Huang (2002) Ten-year incidence and progression of age-related maculopathy. *Ophthalmology* **109**, 1767–1779.
  269. Katz, M. L. (2002) Potential role of retinal pigment epithelial lipofuscin accumulation in age-related macular degeneration. *Arch. Gerontol. Geriatr.* **34**, 359–370.
  270. Boulton, M., M. Rozanowska, B. Rozanowski and T. Wess (2004) The photoreactivity of ocular lipofuscin. *Photochem. Photobiol. Sci.* **3**, 759–764.
  271. Eagle, R. C. J., A. C. Lucier, V. B. J. Bernardino and M. Yanoff (1980) Retinal pigment epithelial abnormalities in fundus flavimaculatus: a light and electron microscopic study. *Ophthalmology* **87**, 1189–1200.
  272. Winkler, B. S., M. E. Boulton, J. D. Gottsch and P. Sternberg (1999) Oxidative damage and age-related macular degeneration. *Mol. Vis.* **5**, U50–U60.
  273. Beatty, S., H. H. Koh, D. Henson and M. Boulton (2000) The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv. Ophthalmol.* **45**, 115–134.
  274. Rozanowska, M., J. Jarvis-Evans, W. Korytowski, M. E. Boulton, J. M. Burke and T. Sarna (1995) Blue light-induced reactivity of retinal age pigment—in vitro generation of oxygen reactive species. *J. Biol. Chem.* **270**, 18825–18830.
  275. Boulton, M., A. Dontsov, J. Jarvis-Evans, M. Ostrovsky and D. Svistunenko (1993) Lipofuscin is a photoinducible free-radical generator. *J. Photochem. Photobiol. B-Biol.* **19**, 201–204.
  276. Wassell, J., S. Davies, W. Bardsley and M. Boulton (1999) The photoreactivity of the retinal age pigment lipofuscin. *J. Biol. Chem.* **274**, 23828–23832.
  277. Dontsov, A. E., R. D. Glickman and M. A. Ostrovsky (1999) Retinal pigment epithelium pigment granules stimulate the photo-oxidation of unsaturated fatty acids. *Free Radic. Biol. Med.* **26**, 1436–1446.
  278. Rozanowska, M., W. Korytowski, B. Rozanowski, C. Skumatz, M. E. Boulton, J. M. Burke and T. Sarna (2002) Photoreactivity of aged human RPE melanosomes: a comparison with lipofuscin. *Investig. Ophthalmol. Vis. Sci.* **43**, 2088–2096.
  279. Shamsi, F. A. and M. Boulton (2001) Inhibition of RPE lysosomal and antioxidant activity by the age pigment lipofuscin. *Investig. Ophthalmol. Vis. Sci.* **42**, 3041–3046.
  280. Reszka, K., G. E. Eldred, R. H. Wang, C. Chignell and J. Dillon (1995) The photochemistry of human retinal lipofuscin as studied by EPR. *Photochem. Photobiol.* **62**, 1005–1008.
  281. Gaillard, E. R., S. J. Atherton, G. Eldred and J. Dillon (1995) Photophysical studies on human retinal lipofuscin. *Photochem. Photobiol.* **61**, 448–453.
  282. McLean, A. J., D. J. McGarvey, T. G. Truscott, C. R. Lambert and E. J. Land (1990) Effect of oxygen-enhanced intersystem crossing on the observed efficiency of formation of singlet oxygen. *J. Chem. Soc.-Faraday Trans.* **86**, 3075–3080.
  283. Rozanowska, M., A. Pawlak, B. Rozanowski, C. Skumatz, M. Zareba, M. E. Boulton, J. M. Burke, T. Sarna and J. D. Simon (2004) Age-related changes in the photoreactivity of retinal lipofuscin granules: role of chloroform-insoluble components. *Investig. Ophthalmol. Vis. Sci.* **45**, 1052–1060.
  284. Davies, S., M. H. Elliott, E. Floor, T. G. Truscott, M. Zareba, T. Sarna, F. A. Shamsi and M. E. Boulton (2001) Photocytotoxicity of lipofuscin in human retinal pigment epithelial cells. *Free Radic. Biol. Med.* **31**, 256–265.
  285. Feeney-Burns, L., E. S. Hilderbrand and S. Eldridge (1984) Aging human RPE—morphometric analysis of macular, equatorial, and peripheral cells. *Investig. Ophthalmol. Vis. Sci.* **25**, 195–200.
  286. Zinn, K. M. and J. V. Benjamin-Henkind (1979) The retinal pigment epithelium. In *Anatomy of the Human Retinal Pigment Epithelium* (Edited by K. M. Zinn and M. F. Marmor), pp. 3–31. Harvard University Press, Cambridge.
  287. Weiter, J. J., F. C. Delori, G. L. Wing and K. A. Fitch (1986) Retinal pigment epithelial lipofuscin and melanin and choroidal melanin in human eyes. *Investig. Ophthalmol. Vis. Sci.* **27**, 145–152.
  288. Wing, G. L., G. C. Blanchard and J. J. Weiter (1978) The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Investig. Ophthalmol. Vis. Sci.* **17**, 601–607.
  289. Roberts, J. E., B. M. Kukielski, D. N. Hu, D. S. Miller, P. Bilski, R. H. Sik, A. G. Motten and C. F. Chignell (2002) The role of A2E in prevention or enhancement of light damage in human retinal pigment epithelial cells. *Photochem. Photobiol.* **75**, 184–190.
  290. Sparrow, J. R. and M. Boulton (2005) RPE lipofuscin and its role in retinal-pathobiology. *Exp. Eye Res.* **80**, 595–606.
  291. Schutt, F., M. Bergmann, F. G. Holz and J. Kopitz (2002) Isolation of intact lysosomes from human RPE cells and effects of A2-E on the integrity of the lysosomal and other cellular membranes. *Graefes Arch. Clin. Exp. Ophthalmol.* **240**, 983–988.
  292. De, S. and T. P. Sakmar (2002) Interaction of A2E with model membranes. Implications to the pathogenesis of age-related macular degeneration. *J. Gen. Physiol.* **120**, 147–157.

293. Bergmann, M., F. Schutt, F. G. Holz and J. Kopitz (2004) Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. *FASEB J.* **18**, U450–U469.
294. Shaban, H., P. Gazzotti and C. Richter (2001) Cytochrome c oxidase inhibition by N-retinyl-N-retinylidene ethanolamine, a compound suspected to cause age-related macula degeneration. *Arch. Biochem. Biophys.* **394**, 111–116.
295. Suter, M., C. Reme, C. Grimm, A. Wenzel, M. Jaattela, P. Esser, N. Kociok, M. Leist and C. Richter (2000) Age-related macular degeneration—the lipofuscin component N-retinyl-N-retinylidene ethanolamine detaches proapoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells. *J. Biol. Chem.* **275**, 39625–39630.
296. Gao, B., A. Wenzel, C. Grimm, S. R. Vavricka, D. Benke, P. J. Meier and C. E. Reme (2002) Localization of organic anion transport protein 2 in the apical region of rat retinal pigment epithelium. *Investig. Ophthalmol. Vis. Sci.* **43**, 510–514.
297. Sparrow, J. R., K. Nakanishi and C. A. Parish (2000) The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. *Investig. Ophthalmol. Vis. Sci.* **41**, 1981–1989.
298. Sparrow, J. R. and B. L. Cai (2001) Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by bcl-2. *Investig. Ophthalmol. Vis. Sci.* **42**, 1356–1362.
299. Sparrow, J. R., J. L. Zhou and B. L. Cai (2003) DNA is a target of the photodynamic effects elicited in A2E-laden RPE by blue-light illumination. *Investig. Ophthalmol. Vis. Sci.* **44**, 2245–2251.
300. Sparrow, J. R., J. Zhou, S. Ben-Shabat, H. Vollmer, Y. Itagaki and K. Nakanishi (2002) Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE. *Investig. Ophthalmol. Vis. Sci.* **43**, 1222–1227.
301. Shaban, H., C. Borrás, J. Vina and C. Richter (2002) Phosphatidylglycerol potentially protects human retinal pigment epithelial cells against apoptosis induced by A2E, a compound suspected to cause age-related macula degeneration. *Exp. Eye Res.* **75**, 99–108.
302. Holz, F. G., F. Schutt, J. Kopitz, G. E. Eldred, F. E. Kruse, H. E. Volcker and M. Cantz (1999) Inhibition of lysosomal degradative functions in RPE cells by a retinoid component of lipofuscin. *Investig. Ophthalmol. Vis. Sci.* **40**, 737–743.
303. Schutt, F., S. Davies, J. Kopitz, F. G. Holz and M. E. Boulton (2000) Photodamage to human RPE cells by A2-E, a retinoid component of lipofuscin. *Investig. Ophthalmol. Vis. Sci.* **41**, 2303–2308.
304. Finnemann, S. C., L. W. Leung and E. Rodriguez-Boulan (2002) The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. *Proc. Natl. Acad. Sci. USA* **99**, 3842–3847.
305. Bermann, M., F. Schutt, F. G. Holz and J. Kopitz (2001) Does A2E, a retinoid component of lipofuscin and inhibitor of lysosomal degradative functions, directly affect the activity of lysosomal hydrolases? *Exp. Eye Res.* **72**, 191–195.
306. Lamb, L. E., T. Ye, N. M. Haralampus-Grynaviski, T. R. Williams, A. Pawlak, T. Sarna and J. D. Simon (2001) Primary photophysical properties of A2E in solution. *J. Phys. Chem. B.* **105**, 11507–11512.
307. Kanofsky, J. R., P. D. Sima and C. Richter (2003) Singlet-oxygen generation from A2E. *Photochem. Photobiol.* **77**, 235–242.
308. Pawlak, A., M. Rozanowska, M. Zareba, L. E. Lamb, J. D. Simon and T. Sarna (2002) Action spectra for the photoconsumption of oxygen by human ocular lipofuscin and lipofuscin extracts. *Arch. Biochem. Biophys.* **403**, 59–62.
309. Haralampus-Grynaviski, N. M., L. E. Lamb, C. M. R. Clancy, C. Skumatz, J. M. Burke, T. Sarna and J. D. Simon (2003) Spectroscopic and morphological studies of human retinal lipofuscin granules. *Proc. Natl. Acad. Sci. USA* **100**, 3179–3184.
310. Cantrell, A., D. J. McGarvey, J. Roberts, T. Sarna and T. G. Truscott (2001) Photochemical studies of A2-E. *J. Photochem. Photobiol. B-Biol.* **64**, 162–165.
311. Ragauskaite, L., R. C. Heckathorn and E. R. Gaillard (2001) Environmental effects on the photochemistry of A2-E, a component of human retinal lipofuscin. *Photochem. Photobiol.* **74**, 483–488.
312. Ben-Shabat, S., Y. Itagaki, S. Jockusch, J. R. Sparrow, N. J. Turro and K. Nakanishi (2002) Formation of a nonaoxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. *Angew. Chem. Int. Ed.* **41**, 814–817.
313. Gaillard, E. R., L. B. Avallé, L. M. M. Keller, Z. Wang, K. J. Reszka and J. P. Dillon (2004) A mechanistic study of the photooxidation of A2E, a component of human retinal lipofuscin. *Exp. Eye Res.* **79**, 313–319.
314. Dillon, J., Z. Wang, L. B. Avallé and E. R. Gaillard (2004) The photochemical oxidation of A2E results in the formation of a 5,8,5',8'-bis-furanoid oxide. *Exp. Eye Res.* **79**, 537–542.
315. Sparrow, J. R., H. R. Vollmer-Snarr, J. L. Zhou, Y. P. Jang, S. Jockusch, Y. Itagaki and K. Nakanishi (2003) A2E-epoxides damage DNA in retinal pigment epithelial cells—vitamin E and other antioxidants inhibit A2E-epoxide formation. *J. Biol. Chem.* **278**, 18207–18213.
316. Avallé, L. B., Z. Wang, J. P. Dillon and E. R. Gaillard (2004) Observation of A2E oxidation products in human retinal lipofuscin. *Exp. Eye Res.* **78**, 895–898.
317. Radu, R. A., N. L. Mata, A. Bagla and G. H. Travis (2004) Light exposure stimulates formation of A2E oxiranes in a mouse model of Stargardt's macular degeneration. *Proc. Natl. Acad. Sci. USA* **101**, 5928–5933.
318. Mainster, M. A., W. T. Ham and F. C. Delori (1983) Potential retinal hazards—instrument and environmental light sources. *Ophthalmology* **90**, 927–932.
319. Darrow, R. A., R. M. Darrow and D. T. Organisciak (1997) Biochemical characterization of cell specific enzymes in light-exposed rat retinas: oxidative loss of all-trans retinol dehydrogenase activity. *Curr. Eye Res.* **16**, 144–151.