Blue Light's Effects on Rhodopsin: Photoreversal of Bleaching in Living Rat Eyes

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Purpose. To determine whether blue light induces photoreversal of rhodopsin bleaching in vivo.

Methods. Eyes of anesthetized albino rats were exposed to either green (550 nm) or deep blue (403 nm) light, and the time course of rhodopsin bleaching was determined. Rhodopsin was isolated from whole retinas by detergent extraction and measured photometrically. To inhibit photoreversal of bleaching, rats were perfused with 70 mM hydroxylamine (NH_2OH), a known inhibitor of photoreversal. To determine whether blue-absorbing, photoreversible photoproducts were formed, rhodopsin was bleached to near completion with green light and then exposed to blue light. Finally, experimental results were simulated on a computer by means of a simple, three-component model involving a long-lived photoreversible photoproduct.

Results. Photoreversal of bleaching in blue light occurs in vivo as evidenced by the following: In the absence of NH_2OH, bleaching of rhodopsin by blue light was slow and complex. In the presence of NH_2OH, however, blue light bleached rhodopsin very fast with a simple, pseudo–first-order kinetic. A long-lived bleaching intermediate produced by green light exposure was photoreversed to rhodopsin by exposure to blue light. The three-component computer model, invoking a blue-absorbing, photoreversible, long-lived intermediate accurately described the data.

Conclusions. Because of the instantaneous, nonmetabolic regeneration of rhodopsin by the process of photoreversal of bleaching, blue light exposure permits the absorption of large numbers of photons by rhodopsin and by a photoreversible intermediate of bleaching in vivo. These data may have an important impact on resolving mechanisms of blue light–mediated damage to the retina. (*Invest Ophthalmol Vis Sci.* 2000;41:3984–3990)

The chromophore(s) for blue light–induced retinal damage are still not completely elucidated. For exposure to excessive white light, elegant experiments by Noell et al.[1] and by Williams and Howell[2] pointed to rhodopsin as the chromophore for light damage, and a recent study unequivocally shows that the assumptions of these early works are true.[3] Could rhodopsin also be crucial for blue light–induced retinal injury?

The visual pigment rhodopsin consists of 11-*cis*-retinal and opsin, the apoprotein of the pigment. Photon absorption causes bleaching of rhodopsin, a process that is initiated by the photoconversion of 11-*cis*-retinal to all-*trans*-retinal. Subsequently, rhodopsin undergoes a series of dark reactions that culminate in the dissociation of retinal, thus completing the process of bleaching. However, before this dissociation occurs, an intermediate of the bleaching process, metarhodopsin II (MII),[4,5] interacts with the G-protein transducin. This interaction initiates a cyclic nucleotide cascade that ultimately converts the absorbed light into an electrical response of the rod cell. Quenching of G-protein activation occurs by binding of arrestin to MII.[6] Because the absorption maximum of the bleaching intermediate MII is at 380 nm,[7] MII is spectrophotometrically virtually indistinguishable from dissociated, free all-*trans*-retinal, a truly bleached product.[7–9] However, a significant difference between free retinal and MII is that, in MII, the all-*trans*-retinal continues to occupy its chromophoric site in opsin, whereas free retinal is not attached to opsin.[8] Furthermore, the all-*trans*-retinal transiently binds to phosphatidyl-ethanolamine and, after reduction to all-*trans*-retinol, is transported to the pigmented epithelium.[10,11]

Hubbard and Kropf[12] discovered that normal photobleaching could be prevented if additional light was absorbed by bleaching intermediates. This prevention of bleaching is effected by a *trans*-to-*cis* photograph reisomerization of the retinal chromophore after absorption of additional photons, and the process was thus called "photoreversal" of bleaching.[13] That photoregeneration created functional rhodopsin from bleaching intermediates was also suggested by Cone, who reported that a complete bleaching of rhodopsin abolishes early receptor potentials in excised rat eyes, but that they can be recorded again after blue light is flashed onto the eye. Thus, rhodopsin must have been restored by the absorption of the additional light of short wavelength.[14] More light absorbed caused less bleaching. Indeed, working at low temperatures to extend the lifetime of successive intermediates, Hubbard and

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Kropf[12] showed that photograph steady states between a given intermediate and a mixture of rhodopsin and isorhodopsin (9-*cis*-chromophore) were possible and were indefinitely stable as long as the temperature was maintained. That is, the system was "closed." They further showed that the quantum efficiencies for the *cis-trans* and *trans*-to-*cis* reactions were virtually identical (0.7) for the earliest intermediates. A critical stage of bleaching arose when MII appeared during irradiation. Suddenly, less rhodopsin could be photoregenerated, and the maximum amount of rhodopsin bleached by a flash increased dramatically. Although the system became "leaky," it was nevertheless concluded that the quantum efficiency for photoconverting MII to stable, light-sensitive pigments was lower than that of the earlier intermediates, but that it was not zero.[4,15–17]

It can be deduced from the work of Hubbard and Kropf[12] that three conditions are necessary for maintaining photograph steady states between rhodopsin and any given intermediate of bleaching: The intermediate absorbing the additional light must retain retinal at the chromophoric site, the incoming photons must be of the proper wavelength to be absorbed by that intermediate, and the intermediate absorbing the light must have a lifetime that is longer than or at least commensurate with the rate at which photons are coming in (i.e., intensity) and being absorbed.

Except for the flash photolysis study by Hagins[18] and the observations of Cone,[14] all the work referenced thus far was performed in vitro. In the present study we show by direct measurements of the course of bleaching of rhodopsin that blue light photoregenerated a relatively long-lived, blue-absorbing intermediate in vivo. Based on our results and the identification of rhodopsin as chromophore for light damage,[1–3] we show that in experiments of blue light damage,[19–24] photoreversal of bleaching may have occurred, leading to the absorption of high numbers of photons per unit of time and rendering blue light highly effective in the mediation of light damage.

METHODS

Animals

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male, Sprague–Dawley rats, 10 to 12 weeks old, were maintained for 3 to 7 weeks in 3-lux cyclic light (light/dark, 12 hours/12 hours). Rats were dark adapted overnight (16 hours) and anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (23 mg/kg).

Light Exposure

Left eyes of anesthetized rats (ketamine 75 mg/kg and xylazine 23 mg/kg) were kept moist with Methocel (CibaVision, Hergiswil, Switzerland) and exposed either to deep blue light (403 ± 10 nm) or to green light (550 ± 10 nm) for up to 30 minutes. The light exposure system consisted of a xenon short-arc reflector lamp (230 V, 50 Hz, 120 W; Intralux MDR 100; Volpi, Schlieren, Switzerland) with interference filters to eliminate UV and infrared radiation and a liquid fiberoptic light guide (8 mm in diameter) to the animal's eye. The optical system included a switch holder for blue (403 ± 10 nm, bandwidth) or green (550 ± 10 nm bandwidth) interference filters.

Intensities of exposure were at 300 $\mu W/cm^2$ for 403-nm blue light and 409 $\mu W/cm^2$ for 550-nm green light. These intensities gave equal photon fluxes at the cornea of 6×10^{14}/sec per square centimeter.

NH_2OH Studies: Inhibition of Photoreversal

NH_2OH actively removes the retinal from the chromophoric site of late-bleaching intermediates including MII,[25,26] without affecting rhodopsin itself (data not shown).[27–29] Although inhibition of photoreversal of bleaching by NH_2OH is very efficient, it may nevertheless be incomplete: some intermediates may still be able to absorb enough photons to be photoreversed before NH_2OH can remove retinal from the chromophoric site in opsin, which will, perforce, prevent photoreversal of bleaching. To apply NH_2OH, deeply anesthetized animals were cardially perfused with NH_2OH solution (70 mM in 0.9% NaCl [pH 7.2], 100 ml) in dim red light immediately before irradiation of the eye with blue light, as described earlier.

Rhodopsin Measurements and Calculation of the First-Order Rate Constants

Retinas were extruded through slits across the corneas and immersed in 1 ml distilled water for 30 seconds. After centrifugation at 14,000*g* for 3 minutes at room temperature, supernatants were discarded, and 0.7 ml cetyltrimethylammonium bromide (CTAB, 1% in H_2O) was added to each retina pellet. Retinas were homogenized with a polytron for 20 seconds, and the homogenates were centrifuged at 14,000*g* for 3 minutes (room temperature). Supernatants were drawn off quantitatively and scanned on a rapid-recording spectrophotometer (Carry 50; Varian, Basel, Switzerland). After scanning, rhodopsin was bleached completely by exposing the retinal extract to 20,000 lux of white light for 1 minute. Extracts were scanned again, and the difference in absorbancy of rhodopsin at 500 nm was determined. This value was used to calculate from the Beer-Lambert law (molar absorbance coefficient = 42,000 M/cm) the concentration of rhodopsin in the extract, as described recently.[30]

Because the intensity was held constant during a run, the rate of bleaching (K_b) is a first-order rate constant and has units of (time^{-1}). Thus, $K_b = \ln (R_t/R_0)/t$; where (R_t/R_0) is the fraction of rhodopsin remaining after exposure during a time (t).

Computer Simulation of Results

The experimental results were simulated on a computer (Excel; Microsoft, Redmond, WA). The authors will supply details of this simulation on request. The model simulated was

$$R \underset{K_{pr}}{\overset{K_b}{\longleftrightarrow}} B \xrightarrow{K_l} P$$

where R is rhodopsin and B is an as yet unidentified blue-absorbing, photoreversible intermediate that decays slowly to P (products). The rate constants are K_b for bleaching, K_{pr} for photoreversal of bleaching, and K_l for loss of photoreversibility (leak). Numerical values of these rate constants, either measured (K_b) or estimated (K_{pr} and K_l) from the results, were put into the differential rate equations that describe the model. The set of equations was then solved simultaneously and fit to the data by iteration.[31] An important constraint on the iteration was that K_b, the bleaching rate constant in blue light (at 300

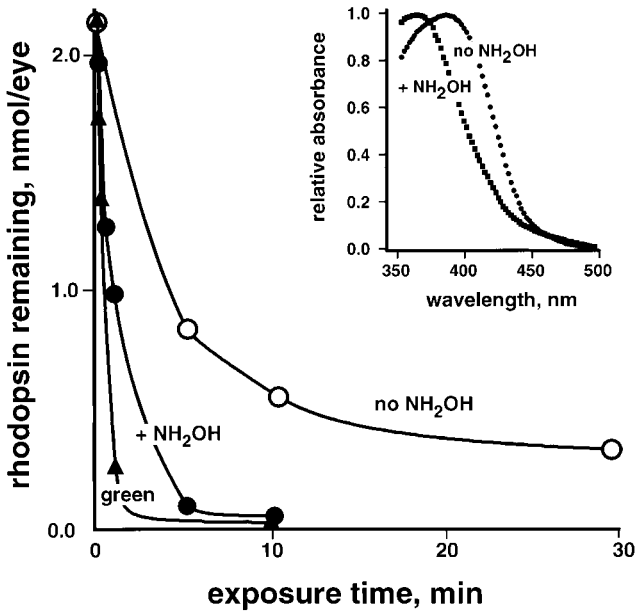

FIGURE 1. Bleaching of rhodopsin in vivo by blue and green light. Eyes of anesthetized rats were exposed to green light (▲; 550 nm; 409 $\mu W/cm^2$), to blue light (○; 403 nm; 300 $\mu W/cm^2$), or to blue light in the presence of NH_2OH to inhibit potential photoreversal of bleaching (●; 403 nm; 300 $\mu W/cm^2$). Rhodopsin disappeared rapidly in green light and in blue light with NH_2OH, conditions with minimized photoreversal of bleaching. However, in blue light without NH_2OH, rhodopsin disappeared slowly, despite irradiation with the same photon fluxes as in the other two conditions. Each data point represents mean values of one to three independent experiments. *Inset*: Spectra of photoproducts formed after bleaching of rhodopsin in eyes of animals that were (+NH_2OH) or that were not (no NH_2OH) cardially perfused with 70 mM NH_2OH. The wavelength of maximal absorption in the absence of NH_2OH is at approximately 380 nm, the correct position for normal rhodopsin-bleaching products. However, the maximal absorption shifted to approximately 365 nm in NH_2OH-perfused animals indicating that NH_2OH reached the photoreceptors and formed the retinal oxime.

$\mu W/cm^2$) was not allowed to change because its absolute value had been measured (see Fig. 3). Thus, all other rates were relative to this anchor, requiring that only two parameters be fit.

RESULTS

Blue Light–Mediated Photoreversal of Bleaching In Vivo

Bleaching of rhodopsin with green light (550 nm) caused the rapid disappearance of the visual pigment leaving less than 5% of rhodopsin after 5 or 10 minutes of exposure. When blue light (403 nm) was used instead, rhodopsin disappeared slowly, with approximately 20% of the dark value left, even after 30 minutes of exposure. However, when the same exposure was tested in an animal perfused with NH_2OH (70 mM), rhodopsin disappeared fast, almost comparable to green light bleaching (Fig. 1). In contrast, when animals were exposed to green light, disappearance of rhodopsin was not influenced by NH_2OH (12% ± 3.8% rhodopsin present after 1 minute of green light exposure in the absence of NH_2OH, $n = 3$; 13% ± 3.9%, rhodopsin present in the presence of NH_2OH, $n = 3$). Therefore, long-wave green light (550 nm) did not cause photoreversal of bleaching as has been suggested by others.[32,33] NH_2OH has no effect on rhodopsin per se (data not shown) but strongly reacts with the retinal chromophore in late intermediates of the bleaching process.[27–29] The interaction results in the removal of the retinal from its chromophoric site and forms a retinal oxime with a maximal absorption at 365 nm, well out of the range of rhodopsin-visible absorption.[27–29] In these experiments, preventing potential photoreversal with NH_2OH greatly enhanced the rate at which rhodopsin disappeared in a blue light exposure.

That NH_2OH, delivered by cardiac perfusion, penetrated the blood–retina barrier and reached the bleaching intermediates in photoreceptors was shown by the different spectra of pigments extracted from retinas of animals that had or had not been perfused with NH_2OH (Fig. 1; insert). Whereas the absorption maximum of the final bleached products in retinas without NH_2OH was at 380 nm, the correct wavelength for all-*trans*-retinal to absorb,[7] perfusion with NH_2OH caused a shift of the absorption maximum to 365 nm, the wavelength of retinal oxime absorption. We conclude that the NH_2OH arrived at the photoreceptors, reacted with late intermediates of bleaching, and efficiently inhibited photoregeneration. However, because NH_2OH prevents photoreversal of bleaching only if removal of retinal from the bleaching intermediate is faster than the absorption of additional photons, we cannot exclude that some few intermediates could be photoreversed, even in the presence of NH_2OH. Nevertheless, the rate of blue light bleaching in the presence of NH_2OH proceeded in an almost straight line (Fig. 3) indicating a very efficient inhibition of photoreversal in our experiments.

Although long-wave green light does not cause photoreversal of bleaching (see above),[32,33] rhodopsin was not bleached to completion. When rhodopsin was bleached using a 5-minute green flash (550 nm; 409 $\mu W/cm^2$), rhodopsin was metabolically regenerated to 13% of the dark value during 15 minutes in darkness (data not shown). Therefore, the metabolic regeneration of rhodopsin in the visual cycle may have restored enough rhodopsin during exposure that bleaching and regeneration reached an equilibrium at very low levels (less than 5%).

Photoregeneration of Rhodopsin by Blue Light after Green Light Bleaching

Green light bleaches rhodopsin without causing photoreversal (see above).[32,33] After 5 minutes of exposure to green light, less than 5% of rhodopsin was left (Fig. 2). Continuation of the green light exposure decreased this value even further. However, when the green light (after 5 minutes) was rapidly switched to blue light (for 1 minute), rhodopsin was regenerated to 28% on average (Fig. 2). Obviously, some intermediates of the green light–induced bleaching process were long lived and capable of reversal to rhodopsin after absorption of additional blue light. More light appeared to cause less bleaching, a hallmark of photoreversal of bleaching.

Bleaching in the Absence of Photoreversal

The bleaching functions (Fig. 1) obtained with green light and with blue light in the absence of photoreversal (that is in the presence of NH_2OH), gave the rates of photon absorptions by

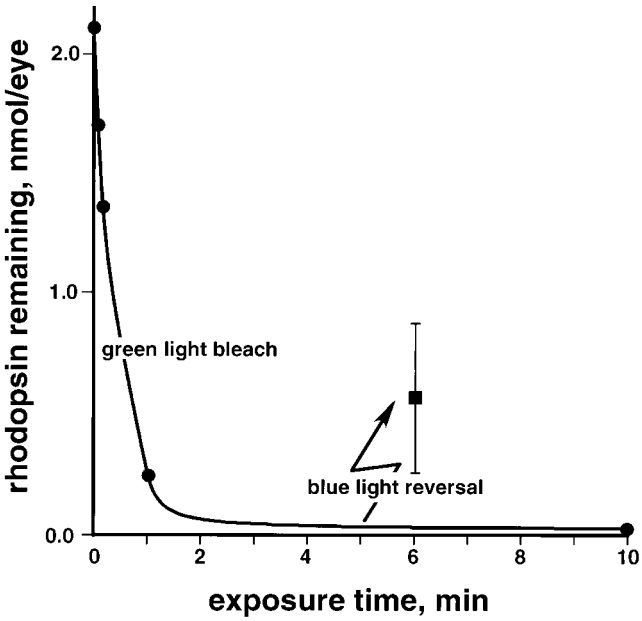

FIGURE 2. Photoregeneration of rhodopsin from intermediates of the bleaching process. Eyes of anesthetized rats were exposed to green light (●; 550 nm; 409 μW/cm^2) for up to 10 minutes. For some animals, the wavelength of the light was rapidly changed to 403 nm (■; blue; 300 μW/cm^2) after 5 minutes of green light exposure and irradiation continued for an additional 1 minute. Rhodopsin bleaching in green light generated photoproducts from which rhodopsin (28% ± 15%) could be photoreversed by the additional blue light exposure. Data points for green light bleach are from Figure 1. Data points for photoregeneration are shown as mean ± SD from eight independent experiments.

rhodopsin at 550 and 403 nm, respectively. To determine the corresponding values of the rate constants for these bleaches, we plotted the bleaching functions as integrated first-order processes and determined their slopes (Fig. 3). The rate constant (slope) for the bleach in blue in the presence of NH_2OH (K_b), was calculated to be 0.61 per minute. This value was used in the model that will be described. The constant in green light was 2.11 per minute. The rates differed by a factor of 3.5, showing that the photosensitivity of rhodopsin was greater at 550 nm than at 403 nm, consistent with the absorption spectrum of rhodopsin combined with the transmissions of the green and blue filters.

Of interest is that the lines were straight over 2 and 3 ln units (Fig. 3; $r^2 > 0.99$ for both). This implies that photoreversal is virtually completely avoided in green light and inhibited by NH_2OH in blue light. If photoreversal were to occur, as in the bleaching process with blue light in the absence of NH_2OH, the lines would be curvilinear—the more photoregeneration, the greater the curvature. This is further supported by direct measurements of the rhodopsin remaining after a 1-minute green light bleaching in the absence or presence of NH_2OH. The value was identical in both conditions (12%–13%) strongly suggesting that photoreversal of bleaching does not occur in long-wave green light (550 nm) or only very little so.

A Model to Determine Bleaching

Development of the model was based largely on the value of K_b and on the shape of the blue no-NH_2OH curve in Figure 1. Inspection of the function without NH_2OH shows that the curve declined very slowly after an initial bleaching burst and that a substantial amount of rhodopsin remained even after 30 minutes of exposure. If bleaching depended only on rhodopsin and a photoreversible intermediate, the system would be closed and the *cis-trans* isomerizations (bleaching and photoregeneration) would come into steady state, the level of which would be determined by the relative photosensitivities of rhodopsin and the blue absorber. However, the slow decline of the rhodopsin level in blue light indicated that the system was not closed; it was leaky. Photoreversibility was gradually lost, and a steady state could not be maintained, even though the light intensity was held constant. This leak in the system was most likely due to the slow decay of the photoreversible intermediates (MII) to products (opsin and free all-*trans*-retinal, which is converted rapidly to all-*trans*-retinol). The products enter the visual cycle to regenerate rhodopsin physiologically through a reisomerization– oxidation step of the all-*trans*-retinol in the pigment epithelium.

The measured value of K_b and estimated values of K_{pr} and K_l were put into a set of differential rate equations as described in the Methods section, and the equations were solved simultaneously. The results of this simulation are given in Figure 4, along with the experimentally determined data points of the

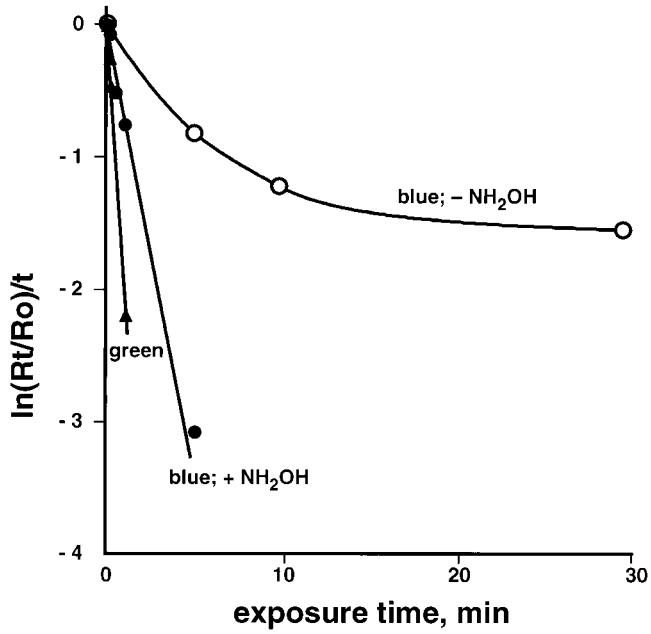

FIGURE 3. Determination of the kinetics of rhodopsin bleaching. Data from Figure 1 were plotted as integrated, first-order processes. R_0 describes the dark value of rhodopsin, R_t stands for the amount of rhodopsin at time (t) during bleaching. The lines of bleaching in green light (▲; 550 nm; 409 μW/cm^2) and of bleaching in blue light (●; 403 nm; 300 μW/cm^2) in the presence of NH_2OH were straight over several half-lives, indicating that very little or no photoreversal of bleaching occurred in green light or in blue light with NH_2OH present. In contrast, the line of rhodopsin bleaching in blue light (○; 403 nm; 300 μW/cm^2) without NH_2OH present was curvy, indicating that bleaching in this condition is not a simple, first-order process. The rate constants for rhodopsin bleaching in vivo were determined from the slopes of the straight lines. The values are K_b = 0.61 per minute (bleaching with blue light in the presence of NH_2OH) and K_g = 2.1 per minute (bleaching with green light).

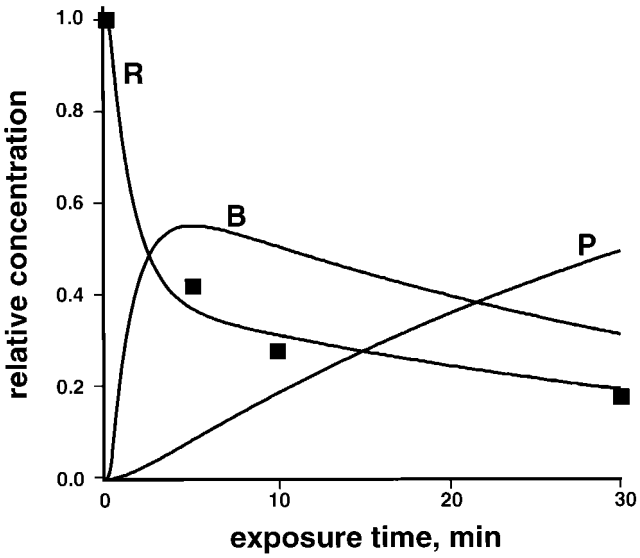

FIGURE 4. Computer simulation of the three-state model for bleaching of rhodopsin in blue light. The model involves rhodopsin (R), a photoreversible blue absorber (B), and an irreversible product (P). (■) Experimentally determined data of rhodopsin bleaching in blue light (403 nm; 300 $\mu W/cm^2$) without NH_2OH present (see Fig. 1). Because the data points are fit very well by the simulation, the result implies that a simple three-component model is sufficient to describe the bleaching of rhodopsin by blue light.

blue bleach (in the absence of NH_2OH, Fig. 1). With K_b fixed at the measured value of 0.61 per minute, the best fit values of K_{pr} and K_l were 0.35 per minute and 0.075 per minute, respectively. This suggests that *trans-cis* (photoreversal) absorptions at 403 nm occur approximately half as often as do *cis-trans* (bleaching) absorptions (K_{pr}/K_b = 0.35/0.61 = 0.57). Also, the calculated leak rate was approximately one fourth the photoreversal rate (K_l/K_{pr} = 0.075/0.35 = 0.21), large enough to prevent a true photograph steady state between rhodopsin and the blue absorber. The good fit of the measured data points to the calculated curve indicates that the model with only two photoconvertible states and one leak step is sufficient to describe the data.

Number of Photons Absorbed by Rhodopsin

The results in Figure 1 can be used to calculate the number of photons absorbed by rhodopsin and by the photoreversible blue absorber. The calculation begins with the measured rate of rhodopsin bleaching in blue light in the absence of photoreversal (NH_2OH curve, Fig. 1; also, Fig. 3). Whether the rod outer segments are dark adapted or partially bleached, rhodopsin (as distinct from the bleaching intermediate that absorbs blue light) in those outer segments absorbs photons at a rate given by $K_b R_t/\Gamma$, where K_b is the rate constant for rhodopsin bleaching with NH_2OH in blue light of 300 $\mu W/cm^2$—namely, 0.61 per minute. R_t is the rhodopsin present at any time (t), and Γ is the quantum efficiency with which rhodopsin is bleached, independent of the wavelength (0.7 molecules bleached/absorbed photon).[12]

In Table 1 we summarize the results of the calculations to determine the numbers of photons absorbed by rhodopsin in the presence and absence of NH_2OH. We also present the numbers of photons absorbed by the blue absorber, assuming its time course is as given by the model (Fig. 4) and that it is MII. To obtain these numbers we integrated the curves graphically (and, for confirmation, by numerical means as well; data not shown) from t = 0 to t = 5, 10, and 30 minutes. The total number of absorptions by the average molecule (combining absorptions while in the rhodopsin state with those while in the MII state) during 30 minutes of blue light exposure (403 nm at 300 $\mu W/cm^2$) was approximately 55. This is a surprisingly large amount of light absorbed by individual molecules.

Because rhodopsin bleaching has a quantum efficiency of Γ = 0.7, 1.4 (1/0.7 = 1.4) photons must be absorbed, on average, to bleach one molecule. Similarly, the photoreversal of MII has a quantum efficiency of 0.22 for producing one rhodopsin[34]; therefore, 4.5 (1/0.22 = 4.5) photons, on average, must be absorbed by MII to produce one rhodopsin.

Figure 5 shows an abbreviated scheme for conceptualizing bleaching and photoreversal in 403-nm light. To complete one bleaching and regeneration cycle, a rhodopsin molecule (and the reversible intermediate) must therefore absorb approximately six photons of blue light at the given wavelength and intensity. In the 30 minutes during which 55 absorptions occur, a single rhodopsin molecule completes on average approximately nine cycles of bleaching and regeneration.

DISCUSSION

These results show that photoreversal of a blue-absorbing, long-lived intermediate of rhodopsin bleaching could occur in vivo. This conclusion is drawn from four observations: (1) When photoreversal is inhibited by NH_2OH, bleaching with a steady blue light is rapid; (2) in such conditions the bleaching is simple first order, as befits pseudo–first-order photochemical processes; (3) when no NH_2OH is used, the bleaching is slow and when plotted as first order, the function is curved; and (4) when most of the rhodopsin in an eye is bleached with long-wave green, nonphotoreversing light, and the products of that bleach are exposed to blue light, substantial amounts of rhodopsin are regenerated by the blue exposure.

Therefore, we conclude that the apparent slowness of rhodopsin bleaching by blue light, in the absence of NH_2OH, is not due to a failure of rhodopsin to absorb that light. Rather, the slowness is a result of the rapid regeneration of the rhodopsin by the photograph reisomerization of the all-*trans*-retinal that resides in the chromophoric site of a long-lived photoproduct. Before bleached rhodopsin can absorb another photon, it usually must be regenerated in the visual cycle, a slow process (15 minutes in darkness regenerated rhodopsin

TABLE 1. Numbers of Photons Absorbed by the Average Molecule in 403-nm Light

	Exposure Time (min)		
	5	10	30
Rhodopsin: NH_2OH present	1.34	1.37	1.39‡
Rhodopsin: NH_2OH absent*	3.6	5.4	10.0
Blue absorber: NH_2OH absent†	15.6	24.3	45.0
Totals (*) + (†)	19.2	29.7	55.0

‡ When bleaching is complete, this value is 1.4, as required by the known photosensitivity of rhodopsin in nonphotoreversing light.

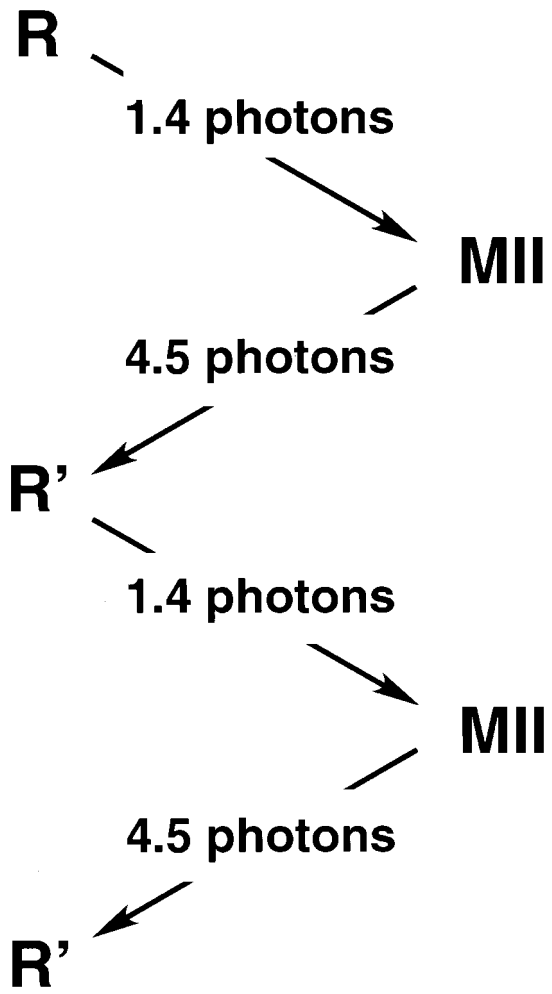

FIGURE 5. Scheme for calculating the total number of photons absorbed by rhodopsin (R) and the photoreversible intermediate, assuming it is MII. Photoregenerated rhodopsin is shown as R′. The numbers on the *arrows* indicate the average numbers of photons needed to cause the reactions and reflect the fact that the quantum efficiencies of bleaching and photoreversal are not unity.

to 13% of dark levels after a 5-minute green light bleaching; data not shown) that involves shuttling of the all-*trans*-retinol to the pigment epithelium, enzymatic reisomerization to 11-*cis*-retinol, and the transport back to the photoreceptors. In blue light conditions, regeneration can occur very fast through the process of photoreversal, and a single rhodopsin molecule can therefore absorb many photons and can enter the bleaching pathway several times in a short period.

Evidence suggests that the long-lived intermediate that is photoreversed by blue light may be MII: (1) The retinal is still attached to the chromophoric site in MII. Thus, any reisomerization of the MII retinal to 11- or 9-*cis* occurs in the chromophoric pocket of opsin and thus efficiently restores rhodopsin. (2) MII, similar to retinal, has an absorption maximum of 380 nm,[35] which is sufficiently close to the 403 nm used in this study. MII absorbs light at 403 nm approximately 70% to 80% as efficiently as rhodopsin does at 500 nm.[36] Furthermore, Williams[34] found that in vitro 1 of 4.5 absorptions converts MII to rhodopsin, and therefore the quantum efficiency of this process (0.22) is high enough to photoconvert reasonable amounts of the all-*trans* to 11- or 9-*cis*, even at 403 nm. (3) MII is a long-lived bleaching intermediate with a lifetime measured in seconds.[37] Our light source projects a large number of photons every second onto the cornea ($\sim 3 \times 10^{15}$ photons/sec). Even though approximately 20% of these photons (at 403 nm wavelength) are absorbed by the rat lens and therefore do not reach the retina[38] and a large fraction of the photons is not absorbed by rhodopsin,[31] such intensities are sufficient to "hit" substantial numbers of MII molecules before they decay irreversibly.

Other deep blue–absorbing photoproducts can be ruled out as candidates for the reversible intermediate, even though some of them fulfill some of the necessary criteria. These molecules include retinal, retinol, *N*-retinylidene-*N*-retinylphosphatidyl-ethanolamine, *N*-retinylidene-*N*-retinylethanolamine,[39,40] and *N*-retinylidene phosphatidylethanolamin.[11] The spectra of these are appropriate for absorbing 403-nm light strongly. Of importance, however, is that the retinoid components of these molecules no longer occupy the chromophoric site on opsin, and they are therefore unlikely candidates for photoreversible molecules. Furthermore, after dissociation, retinal and retinol assume orientations in the disc membrane that are perpendicular to the disc. Thus, their absorption dipoles are orthogonal to the **e** vector of incoming light.[41,42] They are therefore extremely weak absorbers of light in vivo. The orientations in vivo of the other three compounds have not been determined.

Another blue-absorbing candidate, metarhodopsin III (MIII, λ_{max} = 480 nm), should be considered separately from the others, because there is discord about its photoreversibility. Williams[34] found that MIII was not photoreversible but Mathews et al.[8] reported that it was weakly and indirectly (through MII) reversible with light. Even if weakly reversible, the time course of MIII's appearance does not fit our observations: Figure 1 shows that photoreversibility in the blue light was present early during the irradiation. This is evidenced by the fact that the two blue curves (with and without NH_2OH) diverged early—i.e., the difference in bleachability appeared soon during the exposures. In addition, the model (Fig. 4) indicates that photoreversibility began to be lost after approximately 1 minute as the product appeared. Taken together, these facts strongly support the notion that the photoreversible intermediate is MII, because MII appears within approximately a millisecond[4,13,15,34] and then begins a slow decay to MIII (or retinal).[8,9] If MIII were the photoreversible blue absorber, photoreversibility would have increased in long time interval as MIII appeared. Although we cannot completely exclude that a minor amount of rhodopsin is regenerated from MIII, we nevertheless conclude, by a process of elimination, that MII is the main reversible blue absorber in the rat retina.

It is not clear what the molecular or cellular effects of so many photon absorptions by one molecule might be. It seems certain, however, that photoregenerated pigment can produce electrophysiological responses and thus constitutes functional rhodopsin.[14,43] Beyond this, however, a number of questions remain unanswered. (1) Is MII photoreversible when either transducin or arrestin is attached, or must these ligands be dissociated? Arnis and Hofmann,[4] using an excess of transducin, found in vitro that the binding to transducin inhibits reversal of the intermediate if no nucleotides are available, but it is not clear what happens in in vivo conditions. (2) What happens to transduction or to response shutoff if absorptions occur while MII is bound to these ligands? (3) Do some of the

deleterious effects of blue light derive from interfering with these processes by *trans-cis* photoisomerization? Answers to such questions could help to elucidate whether damage in blue light is due simply to an excessive number of photon absorptions or to absorptions at certain time points (e.g., during transduction or response shutoff).

In conclusion, we have shown that the apparent slowness of rhodopsin bleaching in blue light is not due to a failure of rhodopsin to absorb that light[44] but is caused by the photoreversal of bleaching. Conclusions about the identity of the chromophore that mediates blue light–induced damage should therefore be reexamined. Further, it is possible from experiments such as these to calculate the numbers of photons absorbed by rhodopsin and by the photoreversible intermediate(s). The calculations indicate that previously unsuspected large numbers of photon absorptions are occurring in blue light, and this insight could be valuable in understanding blue light–induced retinal damage.

References

1. Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. *Invest Ophthalmol.* 1966;5:450–473.
2. Williams TP, Howell WL. Action spectrum of retinal light-damage in albino rats. *Invest Ophthalmol Vis Sci.* 1983;24:285–287.
3. Grimm C, Wenzel A, Hafezi F, et al. Protection of Rpe65-deficient mice identifies rhodopsin as mediator of light-induced retinal degeneration. *Nat Genet.* 2000;25:63–66.
4. Arnis S, Hofmann KP. Photoregeneration of bovine rhodopsin from its signaling state. *Biochemistry.* 1995;34:9333–9340.
5. Tachibanaki S, Imai H, Mizukami T, et al. Presence of two rhodopsin intermediates responsible for transducin activation. *Biochemistry.* 1997;36:14173–14180.
6. Palczewski K. Is vertebrate phototransduction solved? New insights into the molecular mechanism of phototransduction. *Invest Ophthalmol Vis Sci.* 1994;35:3577–3581.
7. Ebrey TG. The thermal decay of the intermediates of rhodopsin in situ. *Vision Res.* 1968;8:965–982.
8. Mathews R, Hubbard R, Brown PK, Wald G. Tautomeric forms of metarhodopsin. *J Gen Physiol.* 1963;47:215–240.
9. Blazynski C, Ostroy SE. Pathways in the hydrolysis of vertebrate rhodopsin. *Vision Res.* 1984;24:459–470.
10. Saari JC. Biochemistry of visual pigment regeneration: the Friedenwald Lecture. *Invest Ophthalmol Vis Sci.* 2000;41:337–348.
11. Bok D. Photoreceptor: "retinoid pumps" in health and disease. *Neuron.* 1999;23:412–414.
12. Hubbard R, Kropf A. The action of light on rhodopsin. *Proc Natl Acad Sci USA.* 1958;44:130–139.
13. Williams TP. Photoreversal of rhodopsin bleaching. *J Gen Physiol.* 1964;47:679–689.
14. Cone RA. Early receptor potential: photoreversible charge displacement in rhodopsin. *Science.* 1967;155:1128–1131.
15. Williams TP. Upper limits to the bleaching of rhodopsin by high intensity flashes. *Vision Res.* 1974;14:603–607.
16. Williams TP. An isochromic change in the bleaching of rhodopsin. *Vision Res.* 1970;10:525–533.
17. Fesenko EE, Ratner VL, Lyubarsky AL, Bagirov IG. Photoinduced isochromic rearrangement in rhodopsin. *Gen Physiol Biophys.* 1984;3:135–146.
18. Hagins WA. The quantum efficiency of bleaching in situ. *J Physiol (Lond).* 1955;129:22P.
19. Wu J, Chen E, Soderberg PG. Failure of ascorbate to protect against broadband blue light-induced retinal damage in rat. *Graefes Arch Clin Exp Ophthalmol.* 1999;237:855–860.
20. van Norren D, Schellekens P. Blue light hazard in rat. *Vision Res.* 1990;30:1517–1520.
21. Busch EM, Gorgels TG, van Norren D. Temporal sequence of changes in rat retina after UV-A and blue light exposure. *Vision Res.* 1999;39:1233–1247.
22. Gorgels TG, Van Norren D. Two spectral types of retinal light damage occur in albino as well as in pigmented rat: no essential role for melanin. *Exp Eye Res.* 1998;66:155–162.
23. Remé CE, Hafezi F, Munz K, Reinboth J. Light damage to retina and pigement epithelium. In: Wolfensberger TJ, Marmor MF, eds. *The Retinal Pigment Epithelium*: *Current Aspects of Function and Disease*. New York: Oxford University Press; 1996.
24. Grimm C, Wenzel A, Williams TP, et al. Rhodopsin mediated blue light damage to the rat retina: effect of photoreversal of bleaching. *Invest Ophthalmol Vis Sci.* In press.
25. Brin KP, Ripps H. Rhodopsin photoproducts and rod sensitivity in the skate retina. *J Gen Physiol.* 1977;69:97–120.
26. Pepperberg DR, Okajima TI. Hydroxylamine-dependent inhibition of rhodopsin phosphorylation in the isolated retina. *Exp Eye Res.* 1992;54:369–376.
27. Wald G, Brwon PK. The molar extinction of rhodopsin. *J Gen Physiol.* 1953;37:189–200.
28. Bridges CDB. Studies on the flash-photolysis of visual pigments, IV: dark reactions following the flash-irradiation of frog rhodopsin in suspension of isolated photoreceptors. *Vision Res.* 1962;2:215–232.
29. Dartnall HJ. The photosensitivities of visual pigments in the presence of hydroxylamine. *Vision Res.* 1968;8:339–358.
30. Kueng–Hitz N, Grimm C, Lansel N, et al. The retina of c-fos$^{+/+}$ and c-fos$^{-/-}$ mice: electrophysiological, morphological and biochemical aspects. *Invest Ophthalmol Vis Sci.* 2000;41:909–916.
31. Williams TP, Henrich S, Reiser M. Effect of eye closures and openings on photostasis in albino rats. *Invest Ophthalmol Vis Sci.* 1998;39:603–609.
32. Williams TP, Webbers JP. Photometer for measuring intensity and rhodopsin distributions in intact eyes. *Appl Optics.* 1995;34:5720–5724.
33. Williams TP, Squitieri A, Henderson RP, Webbers JP. Reciprocity between light intensity and rhodopsin concentration across the rat retina. *J Physiol* (*Lond*). 1999;516:869–874.
34. Williams TP. Photolysis of metarhodopsin II: rates of production of P470 and rhodopsin. *Vision Res.* 1968;8:1457–1466.
35. Dowling JE. *The Retina*: *An Approachable Part of the Brain*. Cambridge, MA: Belknap Press of Harvard University Press; 1987.
36. Kropf A, Hubbard R, The mechanism of bleaching rhodopsin. *Ann NY Acad Sci.* 1958;74:266–280.
37. Pugh EN, Jr, Duda T, Sitaramayya A, Sharma RK. Photoreceptor guanylate cyclases: a review. *Biosci Rep.* 1997;17:429–473.
38. Gorgels TG, van Norren D. Spectral transmittance of the rat lens. *Vision Res.* 1992;32:1509–1512.
39. Eldred GE, Lasky MR. Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature.* 1993;361:724–726.
40. Weng J, Mata NL, Azarian SM, et al. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell.* 1999;98:13–23.
41. Kaplan MW. Rhodopsin lateral diffusion as a function of rod outer segment disk membrane axial position. *Biophys J.* 1984;45:851–853.
42. Azuma K, Azuma M, Walter AE, Williams TP. Axial diffusion of all-trans retinol in single rods following bleach of rhodopsin. *Zool Sci.* 1991;8:431–436.
43. Huddleston SK, Williams TP. Physiological activity of isorhodopsin in rat rods. *Vision Res.* 1977;17:711–714.
44. Rapp LM, Smith SC. Morphologic comparisons between rhodopsin-mediated and short-wavelength classes of retinal light damage. *Invest Ophthalmol Vis Sci.* 1992;33:3367–3377.