Molecular basis of dark adaptation in rod photoreceptors

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ARTICLE

Abstract

Following exposure of the eye to an intense light that 'bleaches' a significant fraction of the rhodopsin, one's visual threshold is initially greatly elevated, and takes tens of minutes to recover to normal. The elevation of visual threshold arises from events occurring within the rod photoreceptors, and the underlying molecular basis of these events and of the rod's recovery is now becoming clearer. Results obtained by exposing isolated toad rods to hydroxylamine solution indicate that, following small bleaches, the primary intermediate causing elevation of visual threshold is metarhodopsin II, in its phosphorylated and arrestin-bound form. This product activates transduction with an efficacy about 100 times greater than that of opsin.

Key words Bleaching, Dark adaptation, Metarhodopsin, Noise, Photoreceptors, Sensitivity

Introduction

The phenomena of light adaptation and dark adaptation

The visual system is able to *adapt* to a very wide range of intensities, from starlight levels to bright sunlight. Under many conditions such adaptation occurs very rapidly (within seconds), whether the background intensity is increasing or decreasing, and this ability of the visual system to rapidly alter its adaptational state is referred to as *light adaptation*.

The term *dark adaptation* (synonymous with *bleaching adaptation*) refers to the special case of recovery of the visual system in darkness following exposure of the eye to intense illumination that *bleaches* an appreciable fraction (say, more than 0.01%) of the photopigment, rhodopsin. Following intense exposures it is found that recovery can be very slow, taking many minutes. Indeed, following a total bleach (of 100% of the rhodopsin) complete recovery can take as long as 50 min.

The original dark adaptation results of Hecht, Haig and Chase[1] obtained more than 60 years ago are illustrated in Fig. 1, which plots visual threshold (logarithmically) against time, following 'bleaching' exposures of different strengths. After an almost total bleach (uppermost trace) the visual threshold recovers along the classical bi-phasic curve: the initial rapid recovery is due to cones, and the second slower component occurs when the rod threshold drops below the cone threshold. (Note that in this old work, the term 'photon' was used for the unit now defined as the troland; x trolands is the illuminance at the retina when a light of 1 cd/m^2 enters a pupil with cross-sectional area x mm^2.)

Questions and observations

The basic question in dark adaptation, which has not been answered convincingly in the six decades since the results of Fig. 1 were obtained, is: *Why* is one not able to see very well during the period following a bleaching exposure? Or, more explicitly: What is the molecular basis for the slow recovery of visual performance during dark adaptation? In considering the answers to these questions, there are three long-standing observations that need to be borne in mind.

(1) Firstly, the desensitisation of the overall visual system is far in excess of the fraction of pigment bleached. For example, with a bleach of just 4% (the second lowest trace in Fig. 1), visual threshold is elevated initially by more than 1000-fold (3 log units), and after 1 min of recovery it remains elevated by more than 100-fold (2 log units). This shows that the raised threshold cannot have been caused by lack of rhodopsin available to absorb light, because in this case the vast bulk of the pigment (the other 96%) was still present. Rather than lack of photopigment, it is the *presence* of a photoproduct that causes the desensitisation.

(2) Secondly, it is important to realise that the slowness of recovery of visual sensitivity can in no way be advantageous to the owner of the eye, and indeed it must be disadvantageous, as discussed by Barlow[2] and Lamb.[3] Thus, if an animal (or, perhaps, a caveman) were to enter a dark cave after having been out on a bright sunny beach, then there could be no survival advantage in being unable to see properly for half-an-hour. In this sense, the term 'adaptation' is very misleading as a description of the period

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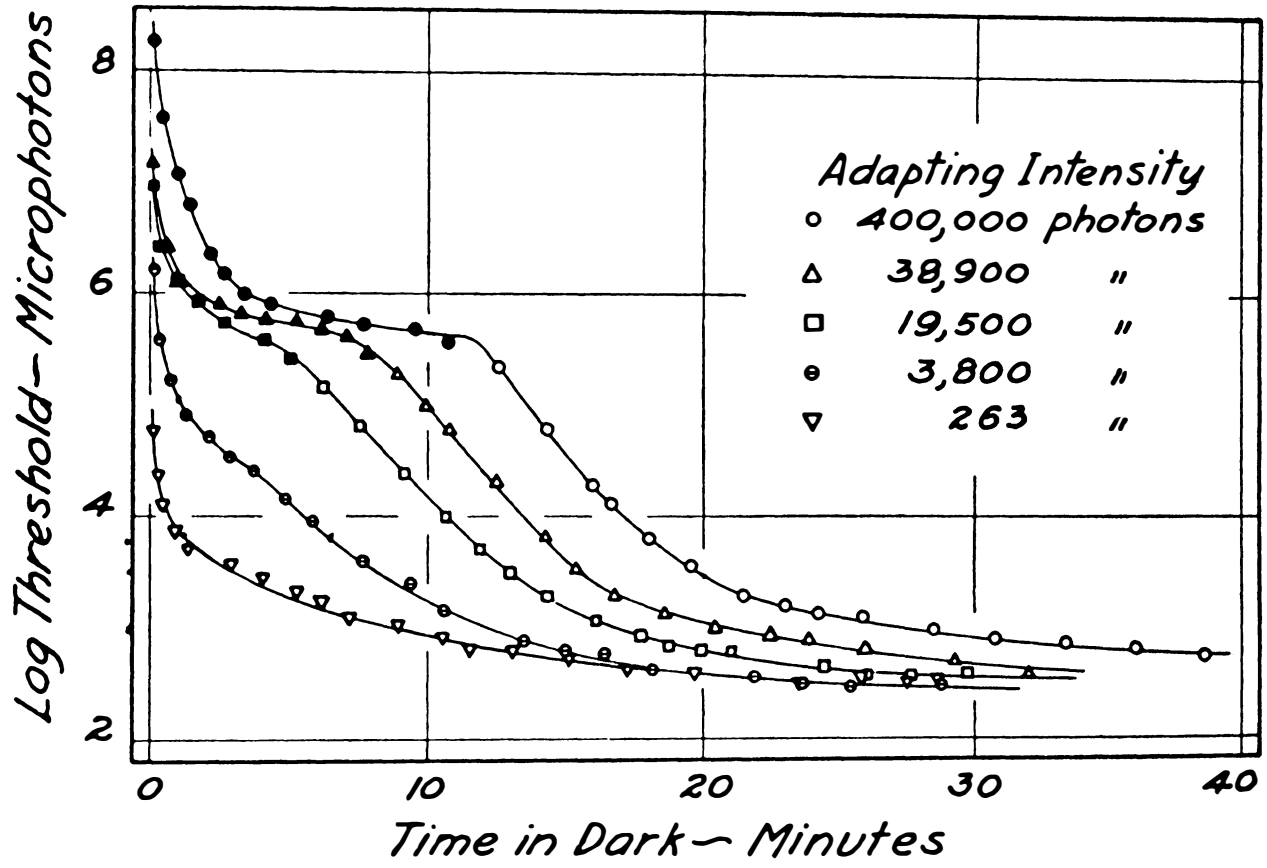

Fig. 1. *Classical dark adaptation curves for a normal observer. Threshold for detection of a test flash is plotted on a logarithmic scale, against time after bleaching, for bleaches of five levels, each of 2 min duration. Note that the unit of retinal illuminance, the troland, was in those days termed the 'photon'. Reproduced with permission from Hecht* et al.[1]

following a bleach, as it suggests that the visual system has been adjusted in this way so as to improve its performance. Instead, the performance of the visual system has been seriously degraded, and during the period of 'dark adaptation' the system slowly recovers back to the fully sensitive state that it exhibited before the bleach.

(3) Thirdly, in considering the underlying basis of dark adaptation, it is important to bear in mind the classical observations of Stiles and Crawford,[4] who showed in 1932 that during the course of dark adaptation the visual system behaves as though the world is being viewed through a 'veiling light' that gradually fades away. In other words Stiles and Crawford found that, following a bleach, the visual system behaves as though the eye is experiencing something equivalent to light (which they termed the 'equivalent background intensity') and that during the course of dark adaptation the intensity of this equivalent background slowly fades away.

A primary aim of dark adaptation research is to account in molecular terms for the occurrence of this bleach-induced equivalent background. Experiments on isolated rod photoreceptors have demonstrated the existence of equivalent light at the single-cell level, and have indicated the likely molecular basis of the phenomenon.

Post-bleach desensitisation and noise in toad rods

Recordings from rod photoreceptors have shown that, following small bleaches, the rods behave as though they are experiencing light – they exhibit both desensitisation and noise, closely similar to the effects of real light. All the subsequent results in this paper were obtained from rod photoreceptors isolated from the retina of the toad, *Bufo marinus*, and recorded using the suction pipette technique.[5] In considering results from amphibian rods, one needs to bear in mind that responses obtained at room temperature (*c.* 22 °C) are about a factor of 5× slower than responses obtained at mammalian body temperature, and that adaptational changes are similarly slowed down.

Desensitisation

It has long been known that the rod pathway in the retina becomes desensitised following a bleach,[6,7] and that the recovery of sensitivity proceeds steadily and spontaneously. Dowling[6] proposed that the early rapid component of recovery represented a fast neural adaptation, while the slower recovery (taking tens of minutes to hours) was photochemical and depended on the regeneration of rhodopsin. Donner and Reuter,[7] on the other hand, suggested that most of the adaptation was related to processes in the rods themselves, and they

presented evidence indicating that the relatively rapid recovery of sensitivity after small bleaches (3–6%) depended on the decay of the photochemical intermediate metarhodopsin II.

The results in Fig. 2 (from Leibrock *et al.*[8]) typify the desensitisation and recovery that are seen in an isolated rod following very small bleaches (of just under 1% in this case). The traces plot responses to constant dim test flashes, presented either before the bleach (top trace) or at the indicated times (in minutes) after the bleach. Immediately after the bleach, the response was greatly desensitised and considerably accelerated, but as time progressed the response to the fixed test flash grew steadily larger, and its peak moved later, until after about an hour of dark adaptation the response had returned almost to its pre-bleach level.

Under physiological conditions, complete recovery (of the kind illustrated in Fig. 2) must be obtained eventually. However, in experiments on rods isolated from the retinal pigment epithelium (RPE), complete recovery is obtained only in the case of small bleaches, of less than about 5% of the rhodopsin. Delivery of large bleaches in the absence of the RPE leads to a sustained plateau of desensitisation, and full recovery of sensitivity occurs only when 11-*cis* retinal is supplied exogenously.[9,10] This happens because without the RPE, re-isomerisation of retinoid from the bleached all-*trans* form to the native 11-*cis* does not take place. Therefore full recovery in isolated cells is possible only after exposures that bleach less rhodopsin than the endogenous amount of free 11-*cis* retinal present in the photoreceptors, which amounts to less than 10% relative to rhodopsin.[11–13]

This family of responses to flashes following a bleach is indistinguishable from the family of responses that one obtains for a fully dark-adapted rod presented with background illumination (see for example Fig. 12 in Lamb[14]). As indicated by the dashed arrow in Fig. 2, backgrounds of increasing intensity similarly cause progressive decreases in flash sensitivity and acceleration of the response. Hence, following a bleach, the rod is desensitised in exactly the same way as though it were experiencing a background light that slowly faded away. In a subsequent figure (Fig. 4) we plot the 'desensitisation-equivalent intensity' as a function of time after the bleach.

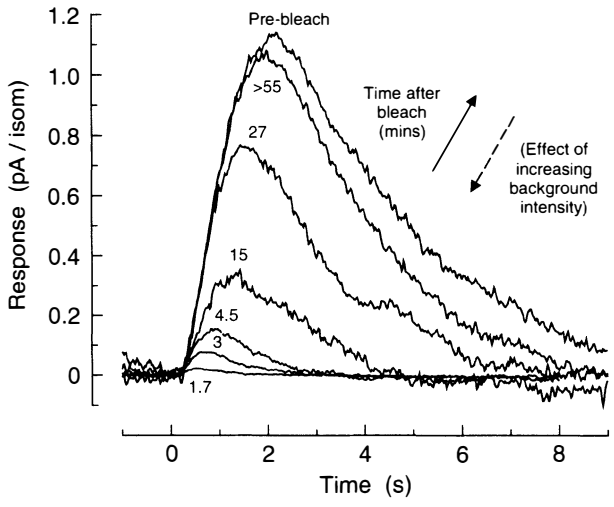

Fig. 2. *Recovery of flash sensitivity and response kinetics in a toad rod, following a bleach of about 0.7%. The responsiveness to a fixed test flash (delivered at time zero in each trace) is plotted, both before ('Pre-bleach') and at the indicated times (in min) following the bleach. Continuous arrow shows the effect of the progression of time after the bleach; dashed arrow shows the effect of applying backgrounds of increasing intensity to a dark-adapted cell. Modified from Leibrock* et al.[8]

Noise

If a photoreceptor were really experiencing something equivalent to light following a bleach, then it would exhibit increased noise as a result of photon fluctuations, i.e. as a consequence of the quantal nature of light. Such bleach-induced fluctuations are indeed observed,[15] as exemplified by the long recording in Fig. 3. In this

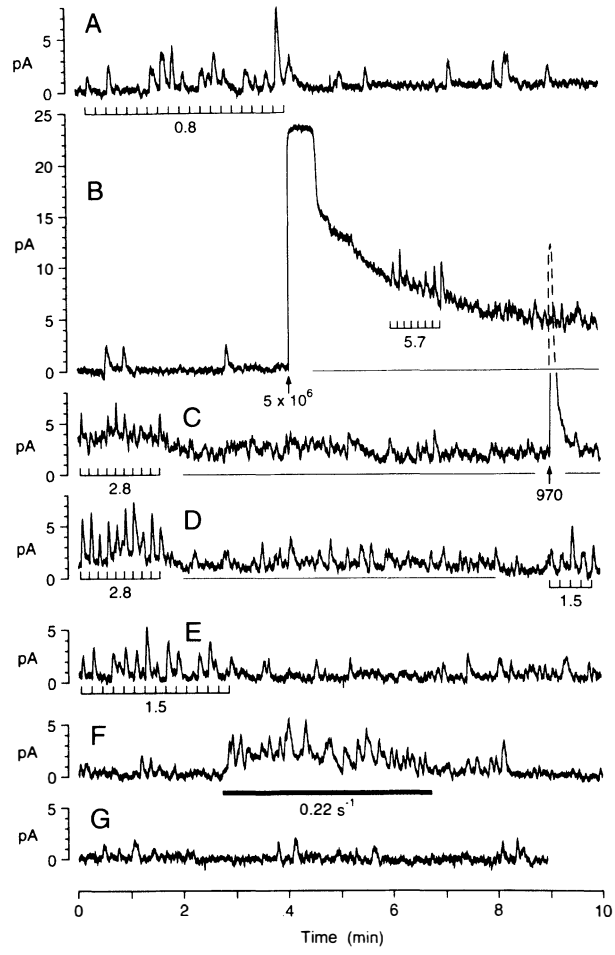

Fig. 3. *Continuous recording of suction pipette photocurrent from a toad rod exposed to a bleach of approximately 0.2%. Each trace is 10 min in duration, and the bleach was delivered 4 min into trace B. The series of small upward markers indicate the timing of delivery of dim flashes (used to estimate flash sensitivity and kinetics, as in Fig. 2), and the numbers adjacent indicate the flash intensities in photoisomerisations. Arrows indicate delivery of brighter flashes, and the bar under trace F indicates delivery of a dim steady light. At all other times the cell was in total darkness. The horizontal lines in traces B, C and D indicate the estimated level of final recovery (0 pA). Reproduced with permission from Leibrock* et al.[8]

experiment, the photocurrent of an isolated toad rod was recorded for more than 5 h, and a stretch of approximately 1 h is presented in seven successive records of 10 min each. The small upward tic-marks monitor the presentation of dim test flashes (to measure sensitivity), and the adjacent numbers give the intensities of those flashes.

In the uppermost record (trace A), and during the initial part of trace B, the cell had been in darkness for a long time; its dark-adapted sensitivity was measured by the dim flashes at the start of trace A, giving an average response generally similar to the pre-bleach trace in Fig. 2 (not shown). Following cessation of the test flashes, a number of discrete 'blips' can be seen in the record. Previously, Baylor *et al.*[16,17] reported that these events are indistinguishable from real photon hits and suggested that they are caused by thermal isomerisation of rhodopsin molecules in complete darkness, i.e. by occasional thermal events that activate a rhodopsin (Rh) molecule from its native form to the activated form, Rh*. During the 10min period of darkness in traces A/B, ten such events are discernible (two of them forming a double), corresponding to a mean rate of approximately 1 event per minute, closely similar to that reported previously under dark-adapted conditions.[16]

Four minutes into trace B an intense light was delivered, which isomerised about 5×10^6 Rh molecules (*c.* 0.2% bleach). The photocurrent was saturated for 40 s, but then began recovering. After 2 min some test flashes were delivered, and in order to achieve a measurable response these were made 8× more intense than the original control test flashes; thus at this time the cell appeared to be desensitised by a factor of roughly 8-fold.

In the subsequent two traces, C and D, at roughly 10 and 20 min after the bleach, it is very clear that the photocurrent fluctuated markedly, while its mean level slowly returned to normal. Such fluctuations are qualitatively very similar to the effects of dim light,[18] despite the fact that they are occurring in total darkness. For comparison, a very dim background light was turned on during trace F, and elicited fluctuations similar to those in darkness in trace D. As time progressed, the magnitude of the noise slowly declined. Note that during traces C-G the fluctuations have the appearance of upward-going blips from the baseline. Such behaviour is as expected if the photoreceptor were experiencing events indistinguishable from photon hits,[18] at a rate that slowly declined with time after the bleach. Eventually, in trace G, the events appear to be occurring at a rate similar to the original dark-adapted rate in traces A/B.

'Equivalence' to real light

Thus, the post-bleach current in the rod appears equivalent to the effects of a slowly fading light in a number of ways: (i) There is a steady level of response that slowly declines. (ii) The cell is desensitised, and its kinetics are accelerated, in the same manner as occurs during steady illumination. (iii) Fluctuations occur that resemble those in steady light (and have the same power spectrum as those in light[8]). (iv) When individual events can be discerned at later times after the bleach they look just like photon-induced events.[18]

Given these similarities, we can extract the 'equivalent intensity' – that is, the intensity of real light to which the phenomena are equivalent. In fact, this equivalence can be determined in at least two ways: (1) we can monitor the desensitisation of the rod, and calculate a 'desensitisation-equivalent intensity'; and (2) we can monitor both the fluctuations and the sensitivity, and calculate a 'noise-equivalent intensity'. For details of these measurements, see Leibrock *et al.*[8] If the molecular events that occur in the rod after a bleach were *identical* to those that occur during steady illumination of an unbleached rod, then these two measures of equivalent intensity would be equal.

For the experiment in Fig. 3, the 'equivalent intensities' calculated by these two procedures are plotted in Fig. 4. Interestingly, the desensitisation-equivalent intensity is higher at all times, by a factor of at least 20×, than the noise-equivalent intensity. What this means is that, at any time after the bleach, there is more desensitisation than one would expect from the observed amount of noise. For example, at 5 min after the bleach the observed fluctuations are equivalent to those that would be produced by a real light of about 1 isomerisation per second, whereas to elicit the observed desensitisation would require a real light of about 20 isomerisations per second. Nevertheless, as shown by the continuous curves, both 'equivalent intensities' begin declining with a common time constant of about 10 min.

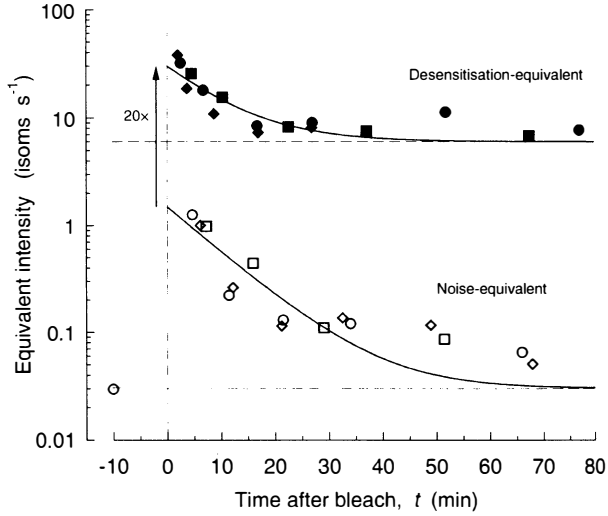

Fig. 4. *Desensitisation-equivalent and noise-equivalent intensities, determined for the cell of Fig. 3 following three bleaches of 0.2%, each represented by a different symbol. The desensitisation-equivalent intensity is determined from the reciprocal of the flash sensitivity, of the kind plotted in Fig. 2, while the noise-equivalent intensity is determined from measurements of both noise and sensitivity. Curves plot exponential recovery of equivalent intensity, in both cases with a time constant of $\tau = 10$ min. Reproduced with permission from Leibrock* et al.[8]

Molecular scheme

These results clearly show that, although a bleach elicits phenomena in the rod that are qualitatively similar to real light, the effects are *not* identical to light. Indeed there appear to be two separate effects, each mimicking real light in its own way. But what molecular mechanisms might underlie these phenomena?

To account for the dark-adaptation behaviour of the overall human visual system, Lamb[19] proposed, in 1981, a scheme in which 'equivalent light' was produced within the rod photoreceptors by the presence of photoproducts of pigment bleaching. The Rh molecule was activated by light to the form Rh*, which then decayed through a series of intermediate forms, each capable (to a different extent) of mimicking light. In producing the equivalent background, these intermediates were proposed either to act by re-forming Rh* (via reverse reactions), or alternatively to act directly on the phototransduction mechanism. In the light of more recent knowledge (discussed below), that model can now be specified in greater precision in the molecular scheme of Fig. 5.

Inactive rhodopsin, Rh, can be activated to Rh* either by photon absorption (lightning bolt) or as a result of spontaneous thermal isomerisation. Rh*, now identified as virgin metarhodopsin II, MII, activates the G-protein cascade by catalysing the conversion of inactive G to its active form, G*; this catalysis by Rh* (arched arrow) is extremely effective. Rh* is rapidly inactivated to the form MII-P-Arr (shown as red), by the combination of phosphorylation and arrestin binding[20] (see Hofmann *et al.*[21] for a recent review).

We can now identify MII-P-Arr as the main photoproduct generating the equivalent light, and the evidence[22] for this identification will be presented subsequently. We propose that MII-P-Arr acts via two pathways, thereby generating the two forms of 'equivalent light' distinguished above in Fig. 4.

Pathway (1). Firstly, the overall reaction inactivating Rh* to MII-P-Arr will necessarily exhibit some tiny degree of reversibility, so that very occasionally a molecule of MII-P-Arr will revert to Rh* (reverse arrow ①). This mechanism will generate events identical to real photon hits (because the cell will be unable to distinguish the source of Rh* molecules, once formed), and in particular it will generate 'photon-like fluctuations'. As discussed by Lamb[19] and Leibrock, Reuter and Lamb,[8] the degree of reversibility required to explain the observed fluctuations is extremely small: on average an individual molecule of photoproduct will need to spend a waiting time in excess of 10^6 s before reverting to Rh* – so in practice the vast majority of such photoproduct molecules will have been removed from the cell long before any reversion occurs.

Pathway (2). Secondly, we propose that the ability of MII to activate the G-protein cascade is not totally eliminated by phosphorylation and arrestin binding, and that instead MII-P-Arr retains a slight ability to act directly on the phototransduction mechanism (dotted red line ②); see also Okada, Nakai and Ikai[23]). In this way MII-P-Arr will generate steady activation of the G-protein cascade. Unlike pathway (1), pathway (2) will cause negligible fluctuations, because G* will be activated in the form of individual molecules, rather than in bursts of molecules as occurs during the active life of an Rh*. Hence the effects of pathway (2) will generally resemble light, in leading to steady activation of the cascade, but will differ from real light in failing to cause 'photon-like fluctuations'.

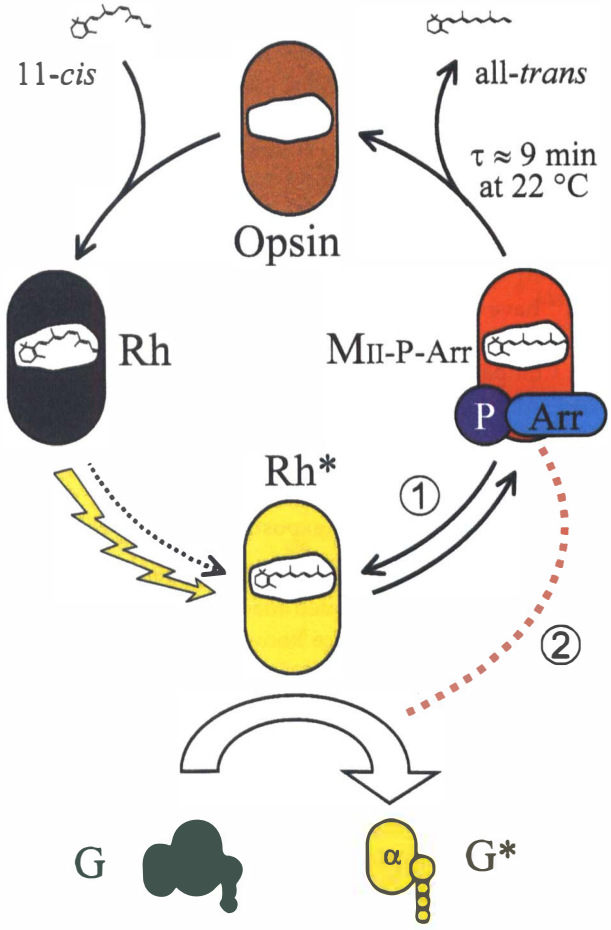

Fig. 5. *Molecular scheme used to explain the results. Rhodopsin (Rh) is activated to Rh* (yellow) either by photon absorption (lightning bolt) or occasionally by thermal activation (dotted arrow). Rh* is extremely effective at catalysing (arched arrow) the activation of the G-protein to G*, which leads subsequently to the formation of PDE* and generation of the light response. Rh*, now identified as virgin metarhodopsin II (MII), is rapidly inactivated, by phosphorylation and arrestin-binding, to MII-P-Arr (red). That reaction is not, however, entirely irreversible, but instead exhibits a small degree of reversibility (reverse arrow, ①); as a result, molecules of MII-P-Arr will very occasionally revert to Rh*. MII-P-Arr also generates activity via a second pathway. The inactivation of Rh* caused by phosphorylation and arrestin-binding is not total, and MII-P-Arr retains a slight ability to activate the G-protien to G* (dotted red line, ②); the efficacy of this activation is* c. 10^{-5} *that of Rh*. Eventually, though, with a time constant of the order of* $\tau \approx 9$ *min at room temperature, the all-*trans *retinoid is hydrolysed off, leaving the apoprotein, opsin (brown). Opsin has also been shown to be able very weakly to activate the G-protein, with an efficacy of* c. 10^{-7} *that of Rh*[24] (not indicated explicitly in the figure). Finally, opsin recombines with 11-*cis *retinal, to re-form rhodopsin, which is completely 'cold' (dark grey). Based on fig. 5 of Lamb[19] and fig. 9 of Leibrock* et al.,*[8] and on the experiments of Leibrock and Lamb.[22,31]*

On this molecular scheme, pathway (1) will generate the 'noise-equivalent intensity', while the summed activity of pathways (1) and (2) will generate the 'desensitisation-equivalent intensity'. Leibrock *et al.*[8] calculated that pathway (1) causes reversions to the state Rh* to occur at a rate of about 2×10^{-7} Rh* s^{-1} per molecule of photoproduct (MII-P-Arr), and from their data we calculate that pathway (2) has an efficacy of about 10^{-5} that of Rh* (see Discussion). Cornwall and Fain[24] have shown that opsin is also able to activate transduction, but with an efficacy only about 10^{-7} that of Rh*. Comparison of our result with theirs indicates that MII-P-Arr is about 100× more effective than opsin in activating transduction.

Effects of hydroxylamine exposure

To test the role of metarhodopsin products proposed in the scheme above, we treated toad rods with hydroxylamine, a substance known to destroy metarhodopsins.[25–29] Hydroxylamine acts by attacking the Schiff base bond that links the photoisomerised chromophore (all-*trans* retinal) to the ε-amino group of Lys296 in the protein, thereby leading to formation of retinal oxime plus the apoprotein (opsin). Therefore, treatment with hydroxylamine during the post-bleach period would be expected to remove metarhodopsin products and to produce corresponding amounts of opsin. Very recently, D.A. Cameron, M.C. Cornwall and E.N. Pugh, Jr (personal communication) have found spectrophotometric evidence that hydroxylamine accelerates the removal of metarhodopsin II in isolated salamander rods. They found that normally the dichroism measured at 380 nm decays with a time constant in excess of 4 min, but that in the presence of 50 mM hydroxylamine the decay time constant accelerates to *c.* 5*s*.

Hydroxylamine is a very aggressive substance, and we therefore chose to deliver short exposures (10–30 s) at high concentration (110 mM), similar to those employed recently by Nikonov *et al.*[30] In addition, to maximise entry of the cationic form NH_3OH^+ via the cyclic-nucleotide-gated channels, we reduced the calcium concentration from 1 mM to 0.25 mM. Despite the short exposures only about half the cells we tested survived hydroxylamine treatment for the 30 min to 2 h necessary to monitor bleaching recovery. For details of the methods, see Leibrock and Lamb.[22]

Hydroxylamine treatment reduces the noise

Fig. 6 shows the effect, on the noise-equivalent intensity, of hydroxylamine treatment presented either during recovery from (panel A), or before (panel B), bleaches of 0.2% of the rhodopsin. In both panels the crosses and the continuous curve plot control data: the crosses plot the photon-like event rate measured in control cells that were presented with bleaches of 0.2% but were *not* treated with hydroxylamine, and the continuous curve

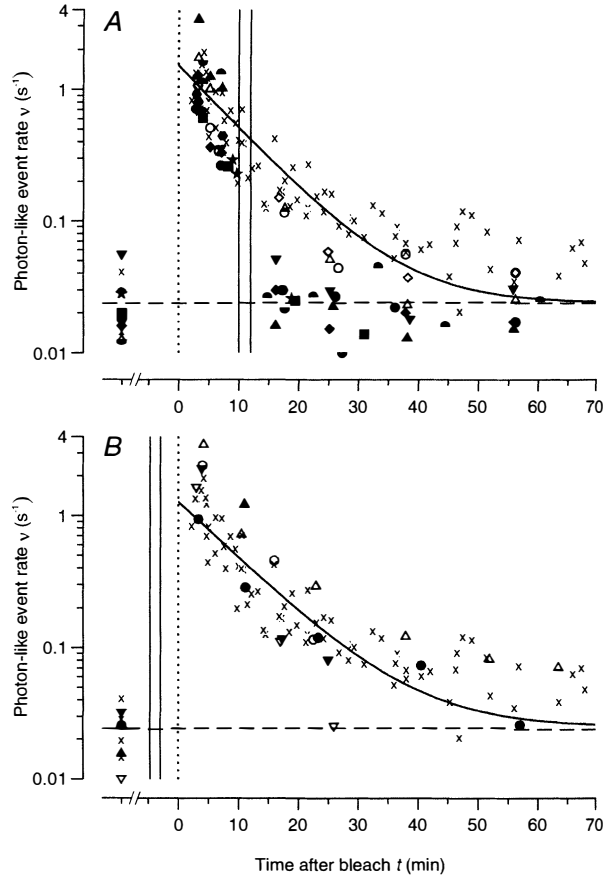

Fig. 6. *Effect of hydroxylamine on the rate of photon-like events, i.e. on the noise-equivalent intensity. The rate of occurrence of events, determined[8] from the measured noise and sensitivity, is plotted as a function of time relative to delivery of bleaches of 0.2%. In (A) the hydroxylamine treatment was given after the bleach, whereas in (B) it was given before the bleach. In both panels the crosses plot measurements from control cells that were not treated with hydroxylamine, and the continuous curve plots the exponential decay time-course reported previously[8] with* $\tau = 9$ *min.* (A) *The filled symbols denote cells that were treated with 110 mM hydroxylamine and 0.25 mM calcium, for approximately 10 s at a time within the window shown by the pair of vertical lines at 10–12 min after the bleach. The open symbols plot three of these cells that were exposed to an additional bleach, and then treated with Ringer solution in which the calcium concentration had been reduced to 0.25 mM. Following treatment, there was a sudden drop in photon-like event rate in the case of the filled symbols, but not in the case of the open symbols.* (B) *Similar experiments, except that the treatment was given before the bleaches, within the window indicated by the pair of vertical lines at −5 to −3 min. Here the filled symbols denote treatment with hydroxylamine, while the open symbols are controls on the same cells in the absence of treatment. In these experiments hydroxylamine treatment had no effect. Reproduced with permission from Leibrock and Lamb.*[22]

plots the exponential decay of equivalent light reported[8] to fit under these conditions, with a time constant of $\tau = 9$ min.

In Fig. 6A the filled symbols are measurements from cells that were treated with hydroxylamine for a period of about 10 s, beginning 10–12 min after the bleach. (The timing of presentation varied slightly from cell to cell, but was always within the window indicated by the pair of vertical lines.) This panel clearly shows that hydroxylamine treatment during bleaching recovery led to a rapid and substantial decline in noise-equivalent

intensity, possibly to the pre-bleach level (indicated by the broken horizontal line). Similar results were also obtained with bleaches 16× larger (*c.* 3%); not shown.

The open symbols in Fig. 6A show control experiments in which cells were treated with a Ringer solution containing reduced calcium concentration but without hydroxylamine; these symbols do not show the sudden reduction in event rate that was elicited by hydroxylamine treatment. As a further control, Fig. 6B shows the results of hydroxylamine treatment *prior* to the bleaching exposures; in this case treatment had no effect. Our interpretation of the lack of effect is that the hydroxylamine that entered the cell during the brief (10 s) exposure either exited the cell, or else reacted (perhaps with 11-*cis* retinal), in the period of 3–5 min before the bleach was delivered at time zero.

Overall, these experiments show that treatment with hydroxylamine rapidly and substantially reduces the noise-equivalent intensity elicited in toad rods by small bleaches, provided that the hydroxylamine is present at the same time as the bleaching products.

Hydroxylamine treatment leads to faster recovery of sensitivity

In the experiments reported above, we thought it important to obtain control measurements of post-bleach noise and sensitivity in each cell, before exposing the cell to hydroxylamine, and in practice this prevented delivery of hydroxylamine until about 10 min after the bleach. By that time the cell's sensitivity had substantially recovered (see Fig. 4), and we could not therefore determine whether treatment with hydroxylamine had any significant effect on sensitivity. In this section we extend those experiments, by presenting hydroxylamine at earlier times (*c.* 5 min) after delivery of bleaches 16× larger.

Fig. 7 plots the sensitivity and time-to-peak of the dim flash response, measured both before and after bleaches of 3%. The open symbols plot control measurements from six cells, while the filled symbols plot measurements from two of these cells, following treatment with hydroxylamine at 5 min into recovery. Immediately after the bleach, each cell was desensitised by about 80–100×, and its time-to-peak was accelerated by 3–4×. At 7 min after the bleach the control cells remained desensitised by about 10×, and their time-to-peak remained accelerated by at least 2×. Hydroxylamine treatment at 5 min brought about a rapid recovery of both sensitivity and kinetics (filled symbols). At the corresponding time, of 7 min post-bleach (2 min post-treatment), the desensitisation had dropped to about 2× and the time-to-peak was barely distinguishable from fully recovered.

Collected results from nine cells treated with hydroxylamine confirmed that the desensitisation was greatly reduced, and that the time-to-peak was returned almost to normal, for exposures presented approximately 5 min after bleaches of 3%. Hence these results show that, as in the case of the noise-equivalent intensity, the desensitisation-equivalent intensity that characterises the post-bleach period is rapidly and significantly reduced by treatment with hydroxylamine.

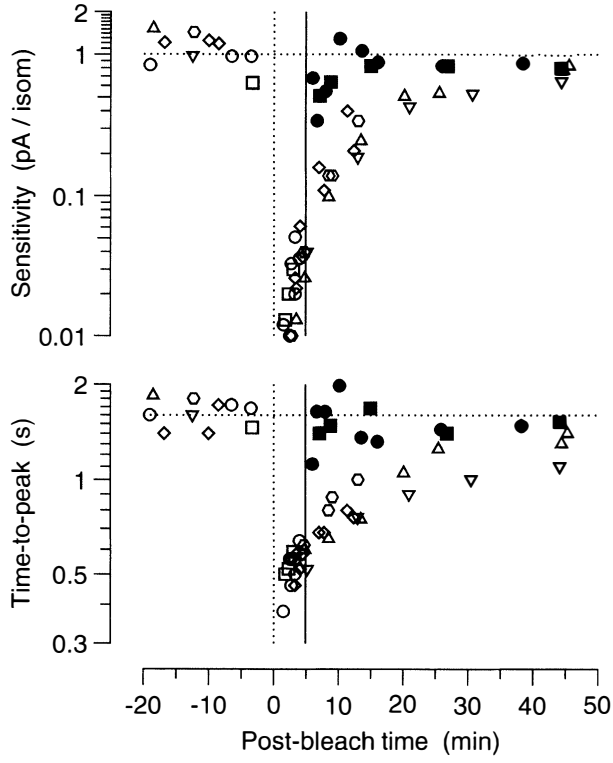

Fig. 7. *Effect of hydroxylamine on flash sensitivity and time-to-peak. The sensitivity and time-to-peak of responses to dim test flashes were extracted, in six cells, from measurements of the kind illustrated in Fig. 2, following bleaches of 3% (i.e. 16× greater than in Figs. 3, 4 and 6). Two of the cells (□, ○), were treated with hydroxylamine, for 30 s at about 5 min after the bleach, and the subsequent measurements (■, ●) showed a rapid and substantial return towards the original pre-bleach levels of sensitivity and time-to-peak. Note that the sensitivity measurements in the upper panel are raw; i.e. they have not been normalised with respect to circulating current. Hence the rapid recovery within the first minute or two reflects both the recovery of the cells' circulating current and the recovery of the transduction steps linking light absorption to channel closure.*

Discussion

The experiments described here have shown that, during darkness following bleaches of around 1% of the rhodopsin, individual rod photoreceptors isolated from the toad retina exhibit phenomena similar to those elicited by light. There is a steady component of response that slowly decays away and that is accompanied both by desensitisation and acceleration of the cell's flash response, and by fluctuations that have the same power spectral properties as those produced by dim light. Consequently, the post-bleach period can be characterised by two 'equivalent intensities' of real light, which would reproduce respectively the desensitisation and the noise. However, as shown in Fig. 4, these two equivalent intensities differ at any time, with the desensitisation-equivalent intensity being a factor of about 20× greater than the noise-equivalent intensity.

The existence of two separate phenomena, each in some respects resembling light and decaying with similar kinetics (Fig. 4), led to a molecular scheme[8] (slightly modified from an earlier one[19]) in which an unidentified photoproduct was proposed to elicit both forms of equivalent background, by activating phototransduction in two ways: (1) by reverse reaction to Rh*, and also (2) by direct activation of the cascade.

Basis of the molecular scheme

In Fig. 5 we have put that scheme on a molecular basis, using information gained from more recent experiments.[22,31] We have identified the molecular species underlying the equivalent light in toad rods as the phosphorylated and arrestin-bound form of meta-rhodopsin II, MII-P-Arr. The basis for that identification, as well as for other features of the molecular scheme, is as follows:

(1) The involvement of a chemical intermediate is indicated by the observed linearity of formation, and the observed first-order kinetics of decay. For the equivalent light both in psychophysical experiments and in experiments on toad rods, the initial magnitude of the equivalent intensity is directly proportional to the size of the bleach, as would be expected for the production of a photoproduct. Furthermore, the decay of the equivalent background follows first-order kinetics for small bleaches, again as expected for the removal of a chemical species.

(2) The identification of MII-P-Arr as the source of the equivalent light comes from consideration of our hydroxylamine results. Hydroxylamine is known to attack metarhodopsins, thereby *producing* opsin, yet the equivalent intensity is always *reduced* by hydroxylamine treatment. This shows that the phenomena we observe cannot be produced by opsin, since the equivalent intensity declines when more opsin is being produced. Instead our results indicate that a metarhodopsin product underlies the desensitisation and noise that we observe. But which form of metarhodopsin? Metarhodopsin III is most unlikely, both because of the slow kinetics of its production and because almost none is formed after small bleaches;[11] appreciable quantities are formed only after large bleaches. This leaves the various forms of metarhodopsin II. Virgin MII is ruled out, firstly because it has been identified as Rh*, and secondly because it is removed (by phosphorylation) very quickly. For small bleaches, virtually all the Rh* (MII) should have been phosphorylated and bound by arrestin within the order of seconds following light absorption. These arguments indicate that the substance underlying the component of desensitisation and noise that decays with a time constant of $\tau \approx 9$ min at room temperature is MII-P-Arr. This result therefore confirms the proposal made more than 30 years ago by Donner and Reuter[7] that the post-bleach recovery of rod sensitivity in the retina is mediated by the decay of metarhodopsin II.

Efficacy of the photoproducts in activating transduction

It is interesting to determine the relative efficacy, in activing transduction, of the two bleaching products shown in Fig. 5: MII-P-Arr and opsin. Leibrock *et al.*[8] showed that, after bleaches ranging from 0.02% to 3% of rhodopsin, the rate of photon-like events was directly proportional to the number of bleached molecules, and that reversions to Rh* occurred with a time constant of 5×10^6 s. The component of recovery they studied is the one that we have now identified as being caused by MII-P-Arr, and so their result indicates that reversions to Rh* via pathway (1) occur at a rate of 2×10^{-7} Rh* s^{-1} per molecule of MII-P-Arr. They also showed that pathway (2) was at least 20× more effective than pathway (1), in terms of equivalent background intensity. After a bleach of 5×10^6 Rh* (0.2%), the desensitisation was equivalent initially to a background of about 35 Rh* s^{-1}. Since the effective lifetime of Rh* is $\tau_{Rh^*} \approx 2$ s (Pepperberg *et al.*[32]), the relative efficacy of pathway (2) compared with Rh* is $(35\ Rh^*\ s^{-1} \times 2\ s)/(5 \times 10^6\ Rh^*) = 1.4 \times 10^{-5}$. In other words, there would need to be some 70 000 molecules of MII-P-Arr present in the rod to have the same effect in activating G to G* that a single molecule of Rh* has on average. For comparison, Cornwall and Fain[24] have shown that opsin activates transduction with an efficacy of about $1–2 \times 10^{-7}$ that of Rh* at small bleaches. Hence MII-P-Arr appears to be about 100× more potent than opsin in activating the transduction cascade.

Implications for dark adaptation of the human visual system

We think it likely that the component of recovery that we observe in toad rods, with a time constant of $\tau \approx 9$ min at 22 °C, corresponds to the so-called second component of recovery in human psychophysics; that component is linear and first-order for small bleaches, and exhibits a time constant of just under 2 min at 37 °C (Lamb[19]). A factor of 5× acceleration is about what would be expected for the 15 °C increase in body temperature. Accordingly we propose that the second component of recovery in human dark adaptation similarly arises from decay of MII-P-Arr.

It has previously been shown that bleaching products, including opsin, are able to activate the transduction cascade weakly.[23,24,33] Although the effect of opsin will undoubtedly be important following large bleaches in the absence of the RPE, our results indicate that it cannot be important with very small bleaches. Furthermore, we suggest that in human dark adaptation, under normal physiological conditions when 11-*cis* retinal is readily available from the retinal pigment epithelium, opsin will play a negligible role. Nevertheless, under pathological conditions (e.g. lack of 11-*cis* retinoid) the situation might be very different, and opsin might play a significant role. Indeed, recent dark adaptation results from a patient deficient in vitamin A[34] appear to be explicable by a

model of slow access of retinoid, in which opsin contributes to generating the psychophysical equivalent intensity.[35,36]

Stiles and Crawford[4] reported that, following bleaches, the visual system behaved as though it were experiencing something equivalent to light. What, then, is the meaning and relevance of the existence at the photoreceptor level of two *different* phenomena similar to light? Presumably the occurrence of photon-like events in rods will signal to the overall visual system a phenomenon that is identical to light. And it seems reasonable to think that perhaps the steady activation of transduction (despite its lack of fluctuations) will similarly be interpreted by the visual system as 'real light'. Indeed, it is hard to imagine that any signal from the photoreceptors could be interpreted as anything other than equivalent to light. So, despite the lack of a precise equivalence between the two phenomena in the rods, it seems likely that the visual system will experience 'light'.

Furthermore, even though at the level of the photoreceptor the steady activation is equivalent to a higher intensity than the noise is equivalent to, the same need not necessarily apply at the level of the overall visual system. The scotopic system is specialised for operation in the 'photon counting' regime, and it seems possible that under some circumstances visual sensitivity might be compromised to a larger extent by photon-like events than by a steady level of activity in the rods. For example it is possible that, whereas the steady activation contributed by pathway (2) may dominate in determining visual sensitivity at high scotopic and mesopic levels, the photon-like events contributed by pathway (1) might instead dominate when the system is almost fully dark-adapted.

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