Effect of Photoregeneration on the Calculation of the Amount of Rhodopsin Bleached by Small Flashes

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ABSTRACT A sample of rhodopsin that is exposed to a series of small light flashes of equal intensity is expected to bleach in successively smaller decrements in proportion to the remaining unbleached rhodopsin. The exponential depletion law describing this effect has been used as a rapid, convenient, and intuitive method for determining the fraction of rhodopsin bleached per flash. This method is commonly assumed to be free of error provided the amount bleached is small, so that there is no significant photoregeneration. We show here, however, that if there is *any* photoregeneration, the bleach fraction calculated in this manner can be in error by a factor of two or more, no matter how little rhodopsin is bleached. This flaw occurs insidiously, without perturbing the expected exponentiality of the bleaching decrements, thereby escaping ready notice. The erroneous bleach values readily propagate as underestimates of metarhodopsin and accompanying G-protein equilibrium and kinetic constants. We derive equations for correcting such errors and illustrate how empirical constants can be obtained from experiments that permit the true fraction bleached to be determined.

INTRODUCTION

When a rod disk membrane suspension or other rhodopsin sample is partially bleached by a brief light flash, analysis of the linked biophysical or biochemical changes generally requires determination of the amount of rhodopsin bleached by the flash. In principle, the fraction bleached can be calculated from the number of photons incident on the sample, the quantum efficiency of bleaching and the overlap integral between the spectral composition of the incident photons and the action spectrum for bleaching of rhodopsin. Alternatively, if rhodopsin or one of its bleaching intermediates is being monitored optically, the fraction bleached can be determined from the initial rhodopsin concentration, the change in optical density upon bleaching, the extinction coefficients for the transitions from rhodopsin to each of the photoproducts that persist and the equilibrium constants between these photoproducts at the particular pH and temperature. A disadvantage of both methods is that they require accurate knowledge of extinction coefficients, quantum efficiencies, equilibrium constants, etc., not all of which are readily determined.

A far simpler approach is to determine the ratio between changes in some measurable quantity like optical density on successive bleaches by flashes of equal intensity. If the measured quantity is proportional to the remaining unbleached rhodopsin, the fraction bleached is just one minus this ratio. For example, if each of a series of responses is 98% as large as the previous one, then it may be concluded that the remaining visual pigment has been reduced 2% by each flash.

A typical experiment of this sort is shown in Fig. 1. A rod disk membrane suspension was partially bleached in a series of 19 flashes of equal intensity separated by 50-s intervals. Formation of metarhodopsin II (MII) was observed at 390 nm. The consecutive changes in optical density plotted against bleach number are well fit by a single exponential decay, as shown in the plot of the logarithm of these changes fitted to a straight line. The apparent fraction bleached is one minus the exponential decay constant.

An obvious advantage of determining the fraction bleached in this way is that it does not depend on any empirical constants. The method is especially useful if a particular coupled reaction (such as binding of G-protein to the activated receptor) is to be titrated by a series of bleaches, because one can readily determine the amount of rhodopsin bleached by each succeeding flash.

In applying this method, however, it is important to be certain that the quantity measured is indeed proportional to the amount of unbleached rhodopsin alone. If there are no long-lived photoproducts that can be reconverted to rhodopsin by subsequent flashes, e.g., when hydroxylamine is used at high enough concentration and temperature to fully convert bleaching intermediates to retinal oxime before the next flash, then this constraint is met and the bleach is indeed correct.

Even without hydroxylamine, however, it has been assumed that a small total bleach ensures accuracy on the grounds that the even smaller amount of photoregeneration would produce only a trivial error in estimating the fraction bleached per flash. It is further assumed that constancy of the

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Abbreviations used: DTT, dithiothreitol; EDTA, ethylenediaminetetraacedic acid; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); MI, metarhodopsin I; MII, metarhodopsin II; MIII, metarhodopsin III; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NH_2OH, hydroxylamine; RDM, rod disk membrane(s); R, rhodopsin; TRIS, tris(hydroxymethyl)aminomethane.

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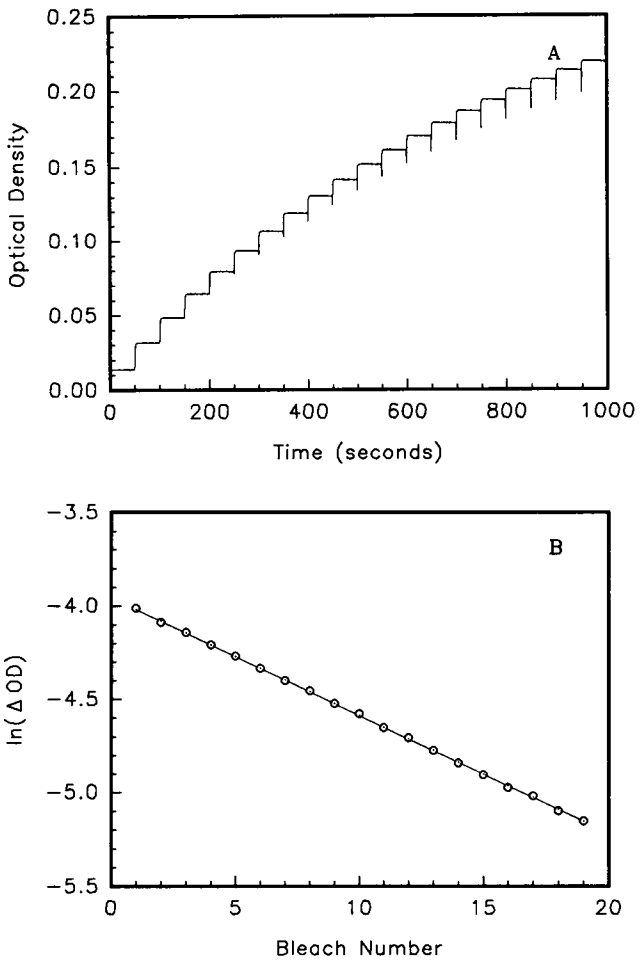

FIGURE 1 (*A*) 1000-s dual-beam (390/426 nm) kinetic trace of the optical density of a visual pigment suspension, showing the successive responses to 19 flashes of equal intensity. Even where there is substantial photoregeneration, the increments in optical density, when plotted versus bleach number, are well fit with a single first order exponential, so that the semilogarithmic plot (*B*) is linear.

observed bleach ratio on successive bleaches is in turn good evidence that no significent photoregeneration *has* occurred.

It is the purpose of this paper to show that these assumptions are incorrect and to develop the mathematical formalism required to calculate the actual fraction bleached in the presence of photoregeneration. This analysis suggests an experimental procedure to determine the empirical constants that can be used to obtain the true bleach. We demonstrate the use of this procedure and show how it accounts for several otherwise inexplicable experimental results.

MATHEMATICAL ANALYSIS

For discrete bleaches in the absence of photoregeneration (the equations are derived in Appendix I), the amount of rhodopsin remaining after n bleaches, where each flash bleaches a fraction, f, of the remaining rhodopsin is

$$R_n = R_0(1 - f)^n \tag{1}$$

Bleaching by a number of small discrete flashes is sometimes approximated as a continuous process, so that the rhodopsin concentration as a function of number of bleaches is written as

$$R(n) = R_0 e^{-f'n}$$

where f' is the fraction of the rhodopsin molecules absorbing a quantum per bleach, multiplied by the quantum efficiency of bleaching.

Although this exponential formalism may be more familiar than the power law of Eq. 1, the reason that the power law is preferred can be seen from the following. If $R(n)$ is to equal R_n, then $e^{-f'n}$ must equal $(1 - f)^n$. If so, f' equals $-\ln(1 - f)$, so that if f were 0.02, f' would be approximately 0.0202027, reflecting the fact that the most efficient way to bleach rhodopsin with a given number of quanta of light is to expose the rhodopsin to all of the quanta at once. Although the error in using the exponential function is not large for small bleaches (1% of the bleach in the above example), it is comparable with the perturbation caused by photoregeneration on the size of the true fraction bleached. (As will be shown, the effect on the size of the apparent fraction bleached is much larger.) It is therefore important to use the exact function $(1 - f)^n$. For simplicity of notation, the factor, R_0, will be dropped henceforth, so that all functions will represent the change in the fraction rather than in the amount of rhodopsin.

Still assuming no photoregeneration, the fraction of the initial rhodopsin concentration that is bleached by the nth flash is

$$b_n = f \cdot (1 - f)^{n-1} \tag{2}$$

If a quantity proportional to b_n, such as a change in optical density, is measured on consecutive flashes, the ratio of these measurements is

$$\frac{k \cdot b_n}{k \cdot b_{n-1}} = \frac{k \cdot f \cdot (1 - f)^{n-1}}{k \cdot f \cdot (1 - f)^{n-2}} = 1 - f$$

and is independent of the proportionality constant k. Here, without photoregeneration, the apparent fraction bleached, defined as $f_{app} = 1 - b_n/b_{n-1}$, is the same as f, the true fraction bleached. Thus, f can be determined by simply calculating the ratio of the amount of bleaching product formed on consecutive flashes as measured by the change in optical density, as long as the fraction of bleached rhodopsin converted to that product remains constant.

On the other hand, if photoproducts are formed which can photoregenerate on subsequent bleaches, and the mixture is allowed to reach thermodynamic equilibrium between bleaches, the ratio of product formed on consecutive bleaches is still constant (Appendix I). It is possible to calculate from this ratio an *apparent* fraction bleached by each flash, but the *true* fraction bleached, defined below, is no longer equal to the apparent fraction bleached, and it is necessary to take into account the relative efficiency of

photoregeneration of rhodopsin and isorhodopsin from those photoproducts still present on subsequent flashes.

Although the actual amount of photoregeneration may be quite small, failure to include it in the calculation produces an error that is substantial, immediate, paradoxical, undetectable, and independent of bleach size. No matter how small the bleaches are, the apparent bleach may differ from the actual bleach by as much as a factor of two or more and the error will be present from the very first pair of bleaches. The result is paradoxical in that the actual bleach is smaller than if there were no photoregeneration but the apparent bleach is larger. No evidence of the flaw arises because the ratio calculated from successive bleaches is virtually unchanged over a large number of bleaches. The error will be just as great if only a very small total amount of rhodopsin is bleached.

The true fraction bleached when there is photoregeneration can be defined as the net fraction bleached by a single flash of a previously unbleached visual pigment sample after any photoregeneration of its own bleaching products has occurred. Thus, a flash that produces a net 2% bleach must initially bleach more than 2% of the rhodopsin present to compensate for the photoregeneration that takes place from the early intermediates formed during the lifetime of the flash.

Photoregeneration converts some fraction of the bleaching intermediates to isorhodopsin rather than to rhodopsin (Collins and Morton, 1950), so that, in the photostationary state, a portion of the remaining unbleached visual pigment is present in the form of isorhodopsin. Isorhodopsin has an absorption spectrum that is slightly blue-shifted from that of rhodopsin, a somewhat larger molar absorbance (Yoshizawa, 1972; Hubbard, 1956), and rather smaller quantum efficiencies for bleaching and photoregeneration (Kropf and Hubbard, 1970). Mathematical analysis is rendered simpler if we assume rhodopsin and isorhodopsin to have identical quantum efficiencies, as it is only under this condition that the photoequilibrium equations can be solved in analytic form. However, numerical solution of the equations including the relevant quantum efficiencies is only slightly perturbed by introduction of separate bleaching and photoregeneration parameters for rhodopsin and isorhodopsin. Application of these equations and determination of the true quantum efficiencies of bleaching and photoregeneration will be considered in a subsequent paper.

The net fraction bleached by a given flash equals the total fraction bleached minus the fractional photoregeneration from those photoproducts still present from the previous flashes. Defining R_n as the fraction of visual pigment remaining after n flashes, and B_n as the fraction of visual pigment bleached by n flashes, the fraction bleached by the nth flash can be written as

$$b_n = (R_{n-1})(f) - (B_{n-1})(\mathrm{re})(f) \tag{3}$$

where re is defined as the sum of the relative efficiencies of photoregeneration from each bleaching product still present at the time of the next flash, weighted according to their relative concentrations.[1] Thus, the net fraction bleached is the fraction of visual pigment remaining multiplied by the fraction bleached minus the fraction of photoproduct multiplied by the fraction bleached and the relative efficiency of photoregeneration. Because $B_n = 1 - R_n$ this can be written as

$$b_n = f[R_{n-1}(\mathrm{re} + 1) - \mathrm{re}] \tag{4}$$

Using these definitions, the fraction of visual pigment bleached by the nth flash is (Appendix I)

$$b_n = f[1 - (f)(\mathrm{re} + 1)]^{n-1} \tag{5}$$

The total fraction bleached with an infinite number of bleaches (the photostationary state) is

$$B_\infty = \sum_{i=1}^{\infty} b_i = \frac{1}{\mathrm{re} + 1} \tag{6}$$

For example, if re = 1, only one-half of the total rhodopsin can be bleached. The fraction of visual pigment remaining in the photostationary state is

$$R_\infty = 1 - B_\infty = \frac{\mathrm{re}}{\mathrm{re} + 1} \tag{7}$$

For these equations to be useful in practice, two constraints must be met: (*a*) The interval between consecutive flashes must be long enough for bleaching products present in significant amounts to reach thermodynamic equilibrium. If they do not reach equilibrium, then the ratio on successive bleaches will not in general be constant. (*b*) The total time required for a sufficient number of bleaches to reach a photostationary state must be short enough that no significant amount of any other photoproduct such as metarhodopsin III (MIII) or retinal and opsin will have formed. These conditions probably cannot both be realized at temperatures much above 0°.

The effect of photoregeneration can be appreciated by comparing Tables 1 and 2. (We have set re = 1 in Table 2 to simplify the calculations. The actual value of re may be less or greater than one. For example, with a monochromatic bleaching light at 388 nm, at pH 5 the value for re is calculated to be about 1.86. All of the numbers in these tables are exact for the conditions indicated.)

Note that when there is no photoregeneration, the apparent fraction bleached is 0.02, in agreement with the actual fraction bleached. However, when there is photoregeneration, even though the actual fraction regenerated is small (0.02×0.02 for the second bleach), the effect is to double the apparent fraction bleached to 0.04. The effect occurs from the first pair of flashes and continues indefinitely. Thus, although

[1] The relative efficiency of photoregeneration from each bleaching product is the ratio of its quantum efficiency of photoregeneration to the quantum efficiency of bleaching of rhodopsin multiplied by the ratio of the overlap integral between its absorption spectrum and the spectral output of the bleaching flash to the corresponding overlap integral for rhodopsin.

TABLE 1 Effect of bleaching without photoregeneration (f = 0.02, re = 0)

Flash No.	Increment bleached b_n	Rhodopsin remaining R_n	Total bleached B_n	Increment ratio b_n/b_{n-1}	Apparent fraction bleached $1 - b_n/b_{n-1}$
0	0.0	1.0	0.0		
1	0.02	0.98	0.02		
2	0.0196	0.9604	0.0396	0.98	0.02
3	0.019208	0.941192	0.058808	0.98	0.02
4	0.01882384	0.92236816	0.07763168	0.98	0.02
∞	0.0	0.0	1.0	0.98	0.02

TABLE 2 Effect of bleaching with photoregeneration (f = 0.02, re = 1)

Flash No.	Increment bleached b_n	Rhodopsin remaining R_n	Total bleached B_n	Increment ratio b_n/b_{n-1}	Apparent fraction bleached $1 - b_n/b_{n-1}$
0	0.0	1.0	0.0		
1	0.02	0.98	0.02		
2	0.0192	0.9608	0.0392	0.96	0.04
3	0.018432	0.942368	0.057632	0.96	0.04
4	0.01769472	0.92467328	0.07532672	0.96	0.04
∞	0.0	0.5	0.5	0.96	0.04

photoregeneration appears to be a second-order effect, it produces a major error when the fraction bleached is determined by this method.

An operational analog for the effect of photoregeneration is found in the simple rate equation,

$$R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B$$

Assuming $R = 1$ and $B = 0$ at $t = 0$, the time dependence of R can be shown to be

$$R(t) = (k_1 \cdot e^{-k_{obs} \cdot t} + k_{-1})/k_{obs}$$

where the observed rate constant, k_{obs}, equals $k_1 + k_{-1}$. The rate of change of R is given by

$$dR(t)/dt = -k_1 \cdot e^{-k_{obs} \cdot t}$$

At $t = 0$, $dR(t)/dt = -k_1$, regardless of the value for k_{-1}. R falls exponentially to the equilibrium value of k_{-1}/k_{obs} with the rate constant of k_{obs}. If $k_{-1} = 0$ then at equilibrium $R = 0$, and the rate constant is k_1. However, if $k_{-1} = k_1$, $R = 0.5$ at equilibrium, and the rate constant is $k_1 + k_{-1}$, or twice k_1. The point is illustrated graphically in Fig. 2, where the curves are drawn with $k_1 = 1$, and $k_{-1} = 0$ (lower curve) or $k_{-1} = 1$ (upper curve). Although the initial reaction rate determined by the tangent to the depletion curve at time zero is equal to $-k_1$ in either case, the rate constant determined from a fit to the exponential curve will always be k_{obs}, and an estimate of k_1 obtained by equating it to k_{obs} in the case where $k_{-1} = k_1$ (upper curve) will be wrong by a factor of two. Determining the fraction bleached from the ratio of consecutive changes in optical density is analogous to determining the rate constant from a fit to the exponential curve. Note that the two curves are almost indistinguishable until about 15% of the reactant is lost, because with very little product formed, there

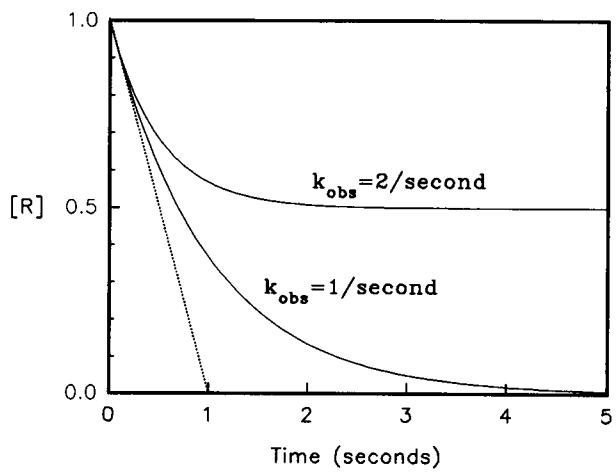

FIGURE 2 Loss of reactant with (k_{obs} = 2/s) and without (k_{obs} = 1/s) a back reaction rate equal to the forward reaction rate. The initial rates of loss are the same, but the exponents, and hence the apparent rates of loss measured by the decrement method, differ by a factor of two.

is initially very little back reaction. Nonetheless the exponents are quite different.

Correct determination of the fraction bleached by a given flash using the decrement or exhaustion method thus requires an initial determination of re for the particular bleaching conditions. This can be done by using Eq. 7 to calculate re from the fraction of visual pigment remaining in the photostationary state for a given pH, temperature, and flash spectrum. It is important to note that anything that significantly changes the relative amounts of MI and MII at equilibrium or the overlap integral between the output of the flash and the absorption spectra will give a different value for re. The relative amount of MII is known to increase with increasing temperature and decreasing pH (Matthews et al., 1963; Parkes and Liebman, 1984). The intensity of the flash can be

increased without changing the value of re, provided the color of the flash does not significantly change. The procedure for determining re is discussed in more detail under Materials and Methods.

Simply determining re for a single pH, however, fails to show the functional relationship between re and pH. If in the interval between flashes all of the bleached visual pigment is converted to MI or MII (i.e., no lumirhodopsin remaining, no MIII formed), then re can be written as

$$re = re\,1 \cdot \frac{MI}{MI + MII} + re\,2 \cdot \frac{MII}{MI + MII} \qquad (8)$$

where re 1 and re 2 are the relative efficiencies of photoregeneration of MI and MII, as defined in Footnote 1, above. This gives the functional dependence of re on the relative fractions of MI and MII, and implicitly on the pH of the sample.

DETERMINATION OF RE 1 AND RE 2

If conditions could be chosen so that only MI or only MII were present, then re would correspondingly equal either re 1 or re 2. This is almost possible for MI (pH $\simeq$ 9; T $\simeq$ 0), but not for MII. At too low a pH, the rhodopsin denatures, and with large amounts of Meta II, even at 0°, there appears to be slow formation of Meta III.

Alternatively one could use an orange filter in the bleaching flash so that photoregeneration could only occur from MI. In this case re = re 1 × MI/(MI + MII). Similarly, a near-UV flash would photoregenerate only from MII (Williams, 1968) and re = re 2 × MII/(MI + MII). This method, however, requires accurate knowledge of the relative amounts of MI and MII, which in turn depends on the differential extinction coefficients for the rhodopsin to MI and MI to MII transitions at particular wavelengths. It would be useful to have a general method for determining re 1 and re 2 independently of any empirical coefficients.

PH DEPENDENCE OF PHOTOREGENERATION

Because the proportions of MI and MII are pH-dependent, Eq. 8 predicts that a plot of re versus pH should be a titration curve of the MI – MII equilibrium, with limiting values of re 1 at high pH (all MI) and re 2 at low pH (all MII). If re is determined from photostationary state data at a number of pHs within the range of pH 5 to pH 9, a titration curve with adjustable end-points, midpoint, and slope can be fitted to this data and the best-fitting values of re 1 and re 2 determined. The equation for the titration curve

$$re = \frac{re\,1 + re\,2 \cdot K_{eq} \cdot (H)^n}{1 + K_{eq} \cdot (H)^n} \qquad (9)$$

is derived in Appendix II. As will be shown in the results, the data are well fit by this function. A further advantage of determining re at a number of pHs is that the best-fitting titration curve smooths out small errors in determining individual re values and makes it obvious when a value of re is significantly in error.

MATERIALS AND METHODS

Rod disk membranes (RDM) were prepared by standard sucrose floatation methods from fresh bovine retinas dissected under infrared light. These were suspended in medium consisting of 40 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 100 μM EDTA. RDM suspensions were sonicated on ice before use with three 2-s bursts separated by 30-s intervals using a Heat Systems-Ultrasonic Inc. microprobe with model W-200F power supply adjusted to 3 on a scale of 10. Just before beginning each experiment, a sample of stock RDM, typically 150–200 μM in rhodopsin, was diluted into buffer at the desired pH to give a rhodopsin concentration of 10 to 12 μM. Succinate buffer was used for pHs below 6, MOPS for pHs between 6 and 8.2, and TRIS for pHs above 8.2. 500 μM GTP-γ-S was added to prevent accumulation of MII-G-protein complex, appearing as extra MII. Sonication helped insure that the GTP-γ-S had access to all of the G-protein. Samples were cooled to 0°C in the thermally jacketed cuvette holder of a scanning dual wavelength spectrophotometer (SLM DW2000) and the initial rhodopsin concentration was determined from the absorption spectrum using the Dartnall correction for light scattering.[2]

RDM suspensions were initially bleached by 19 flashes at 50-s intervals using an EG & G xenon flash unit (FX-199 tube with PS-302 power supply) operated at 1000 volts, while continuously recording the optical density in dual wavelength kinetic mode at 390 minus 426 nm. The flash was attenuated by an orange interference filter that removed most of the light below 450 nm. The interval between flashes was made long enough to allow bleaching intermediates to reach equilibrium before the next flash, except at very low pHs where MIII forms slowly from MII. From these data, the apparent fraction bleached could be determined by the method of successive decrements. The suspension was then bleached by 100 additional flashes at 10-s intervals. Each flash produced a true bleach of about 5% of the remaining rhodopsin, so that 100 flashes bleached more than 99% of the rhodopsin above the amount remaining in the photostationary state. Thirty further light flashes were used as a test that photoequilibrium was reached. The fact that these produced no further spectral change was taken as proof that the system had indeed become photostationery. 10 mM hydroxylamine was then added and the suspension warmed to 37°C for 10 min to allow time for complete conversion of the bleaching products to retinal oxime.

Correction for changes in light scattering

Because the light scattering by the rhodopsin suspension was found to vary with temperature, it was necessary to recool the suspension before recording the spectrum again. To confirm the completeness of the conversion to the oxime, the suspension was rewarmed for 10 min and recooled, and another spectrum was recorded. Finally the visual pigment was fully bleached with white light, warmed for 20 min, and recooled, and the spectrum was recorded. To confirm the completeness of the final bleach, the suspension was again bleached, warmed, and recooled, and a final spectrum was recorded. The pH was measured on a separate aliquot at ambient temperature and corrected for the difference in temperature using the buffer thermal coefficient supplied by the manufacturer.

Fig. 3 shows a typical set of spectra. It is immediately apparent from the downward shift in optical density beyond 700 nm where there is no absorption by rhodopsin or its protoproducts, that a significant change in light scattering has occurred in the course of the experiment. In order to evaluate specific changes in absorbance, it was first necessary to correct for these changes in light scattering. Correction required that some assumption be made about the wavelength dependence of the differential scattering. The fact that the optical density spectra are nearly parallel over 150 nm of the spectrum where pigment absorbance is not changing suggests that there is

[2] $[R] = (1.10\ OD_{500} - 0.77\ OD_{600} - 0.33\ OD_{400})/40{,}000$.

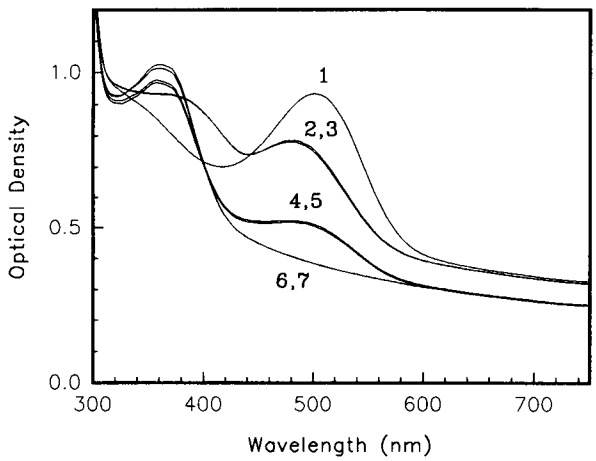

FIGURE 3 Absorbance spectra from a single photostationary state experiment at pH 6.62, 0.0°C. 1) initial rhodopsin spectrum; 2) spectrum following 100 5% bleaches; 3) spectrum after 30 additional 5% bleaches; 4) spectrum after 10 mM NH_2OH addition, warming to 37°C for 20 min, and recooling; 5) spectrum after additional warming for 20 min and recooling; 6) spectrum after 5-min white bleach, warming to 37°C for 20 min, and recooling; 7) spectrum after additional 5-min white bleach, warming, and recooling.

little change in wavelength dependence with bleaching. One way of correcting the data therefore is to assume no wavelength dependence at all and to simply offset the data to the same average value in the region beyond 700 nm.

A second method of correcting for the light scattering requires experimental determination of the pure scattering curve. To this end, a spectrum of RDM was obtained and 1% Ammonyx LO was added, and the spectrum of the detergent-solubilized rhodopsin, corrected for dilution, was subtracted from that of the suspension. A scaled first derivative of the rhodopsin detergent spectrum was subtracted to compensate for a small apparent contribution from rhodopsin anomalous dispersion that remained, thus obtaining a pure scattering spectrum that increased smoothly with decreasing wavelength. Assuming that the light scattering after bleaching had the same wavelength dependence as that before, the pure absorbance spectra could thus be obtained.

A third method combined the first two. The spectra were all offset to zero in the region beyond 700 nm and the scattering curve was then scaled in amplitude to match the spectral data curve in the region below 310 nm on the assumption that there was no absorbance change in this region. This method will approximately correct for a differential scattering that has a different wavelength dependence than does the total scattering from the prebleach spectrum.

Although the three methods of correction for scattering produced significantly different absorption spectra, it was found that the values of re for a given pH, calculated from a ratio of differences in these spectra, rarely differed by more than 0.02 from each other. It is probable, given the degree of accuracy to which the apparent bleach can be determined, that any of the methods of correcting for scattering would be satisfactory. Certainly the offset method is the easiest.

Use of corrected spectra to determine re

A final photoequilibrium difference spectrum was obtained by subtracting the fully bleached hydroxylamine spectrum from the final photoequilibrium spectrum in hydroxylamine, each spectrum in turn having been corrected for light scattering by one of the above methods. The photoequilibrium difference spectra so obtained showed increasing amplitude in the 500-nm region with increasing pH but had λ_{max} values distinctly shorter than 500 nm consistent with the presence of photoregenerated isorhodopsin with the rhodopsin. The amounts of photoregenerated rhodopsin and isorhodopsin remaining were determined from the best fitting mixture of rhodopsin and isorhodopsin basis spectra using a Nelder-Mead Simplex fitting algorithm. The rhodopsin basis spectrum was obtained from an unbleached sample, corrected for light scattering and from which a hydroxylamine bleach spectrum was subtracted as above. The isorhodopsin basis spectrum was similarly derived except that the starting prebleach spectrum was obtained after regenerating a bleached sample with a threefold molar excess of 9-cis retinal. Molar amounts of the two photoregeneration products were then determined using an extinction coefficient of 40,000 liters $mole^{-1}$ cm^{-1} for rhodopsin (@500 nm) and 44,000 for isorhodopsin (@493 nm) (Hubbard, 1956). The sum of the molar concentrations remaining was divided by the original rhodopsin concentration to give the fraction, R_∞, of original pigment remaining. This made it possible to calculate re according to Eq. 7.

Similar experiments were conducted at 13 different pHs, in the range from 4.9 to 9.1. Finally, the overall experiment was done five times, under somewhat different conditions. In one data set, where the suspension was not recooled after addition of hydroxylamine and warming, the values calculated for re were found to be significantly lower than the others, no matter which correction for scattering was used. This data set was not included in the calculation of re. The other data sets were combined and another Simplex fitting routine was used to determine the values of re 1 and re 2 that gave the best fit for Eq. 9 to 111 pairs of data for pH and re.

RESULTS

Typical sets of spectra for pH 8.61 and 6.17 are shown in Fig. 4, *A* and *B*. The spectrum initially obtained at the photostationary state is drawn by a solid line; the second spectrum, after additional bleaches, is drawn with a dashed line. The data shown here were corrected for light scattering by the offset method described above. It is clear that high pH favors larger amounts of remaining rhodopsin and isorhodopsin as predicted from the larger spectral overlap of MI with the flash spectrum. The value of re can be obtained by visual inspection of these data: If the loss of absorbance at 500 nm upon bleaching to the photostationary state (spectrum 1 minus spectrum 4) is scaled to equal one, then the remaining absorbance at 500 nm equals re. (This neglects the difference in extinction coefficient between rhodopsin and isorhodopsin.)

Fig. 5 shows the fit of Eq. 9 to the 111 data sets using the Simplex algorithm which returned the following values for the free parameters: re 1 = 0.737, re 2 = 0.0298, $K_{eq} = 3.798 \times 10^7$, and $n = 1.134$. Note that K_{eq} is not the midpoint of the titration curve when n has a value other than one. The relatively low value for re 2 is explained by the presence of the short wavelength cutoff filter in our bleaching apparatus. From these parameters the value of re for any pH can be calculated. It should, of course, be noted that these values for re 1 and re 2 are not applicable to any bleaching apparatus other than this one because they depend on the type and age of the flash lamp, the voltage applied, the filters used, etc.

DISCUSSION

A mathematical analysis of decrement or exhaustion curves obtained from a rod outer segment suspension in response to a series of equal bleaches was described by Emeis and Hofmann, (1981). No attempt was made to treat the effect of photoregeneration in this analysis, yet under the experimental conditions described, some photoregeneration would

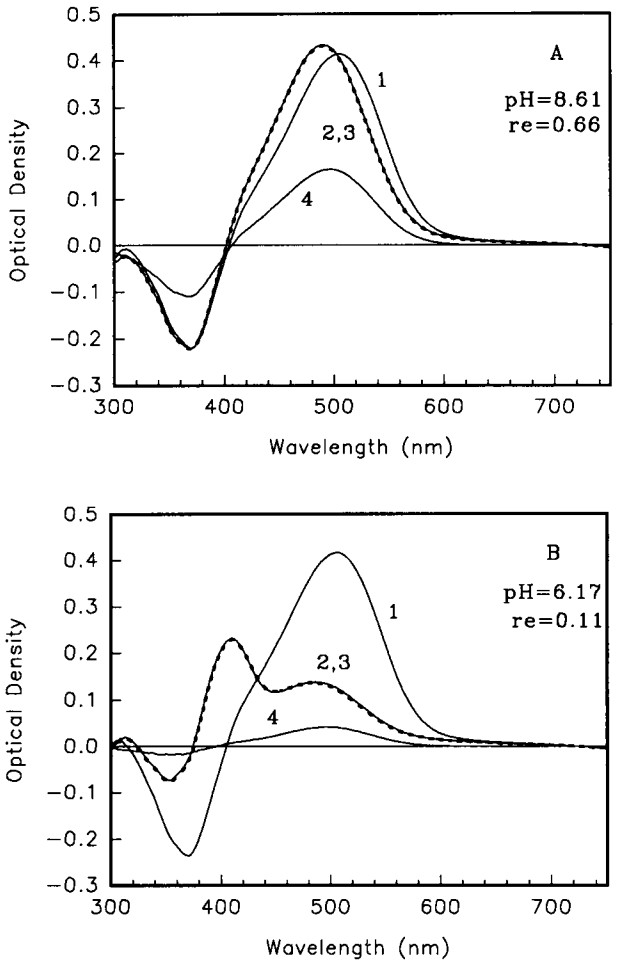

FIGURE 4 Difference spectra from two experiments, at pH 8.61 (A) and 6.17 (B), corrected for changes in scattering by the offset method discussed in the text, and normalized to 10 μM rhodopsin. The final postbleach spectrum has been subtracted from each. 1) initial rhodopsin spectrum; 2) (solid line) spectrum after 100 5% bleaches; 3) (dashed line) spectrum after 30 additional 5% bleaches; 4) spectrum after 10 mM NH_2OH addition, warming to 37°C for 20 min, and recooling.

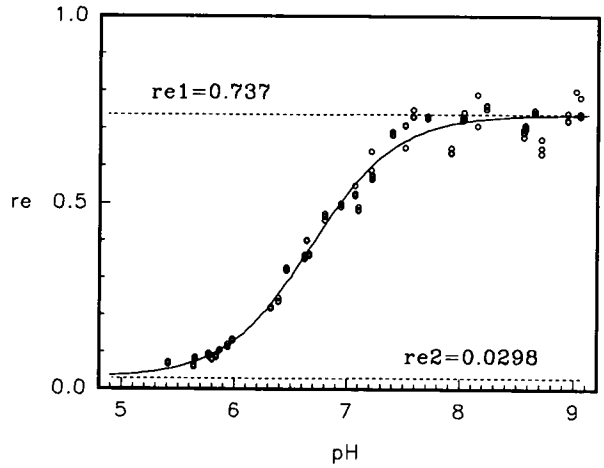

FIGURE 5 Least-squares fit of a titration function to 111 pairs of data for pH and re, showing the best-fit values for the relative efficiencies of photoregeneration from MI and MII (re 1 and re 2), determined from the asymtotes to this function.

have occurred. This method has been used explicitly in a number of papers and others may have used this method to calculate the fraction bleached without specifying how the result was obtained.

In some cases an error in estimating bleach size is not important. But if the amount of bleached rhodopsin is used to measure some other quantity, such as a rate constant (for example, Parkes and Liebman, 1984) or the amount of G-protein bound to rhodopsin, then the error is significant. Much of the uncertainty in determining the ratio of rhodopsin to G-protein in visual receptors by this method, variously estimated to be from 10:1 to 20:1, may be due to this error.

We observed some years ago (Parkes and Liebman, unpublished observations) that the apparent bleach size obtained by the decrement method did not agree with that calculated from the loss of optical density at 500 nm unless the rhodopsin suspension contained detergent and hydroxylamine. This was thought to be due to the failure to completely correct either for the formation of metarhodopsin I or for changes in light scattering. The real reason for the disagreement is now apparent.

We also noted a pronounced pH dependence of the apparent bleach of a rhodopsin suspension by flashes of equal intensity, the bleach size decreasing with decreasing pH. This can be seen clearly in our present data (Fig. 6) where the apparent and true bleaches are plotted as a function of pH, and separately fit to straight lines. (Linear regression was chosen for simplicity in this analysis, and not because of any theoretical justification. In particular, if the dependence of the true bleach on pH were linear, the apparent bleach would not be linear, but would depend on the titration curve.)

The apparent bleach declined from about 8.5% at pH 9 to about 5.7% at pH 5. Given the shift in the MI/MII equilibrium toward MII at low pH, together with the relatively weak output of the filtered xenon flash below 450 nm, the explanation is now apparent. With less photoregeneration at pH 6,

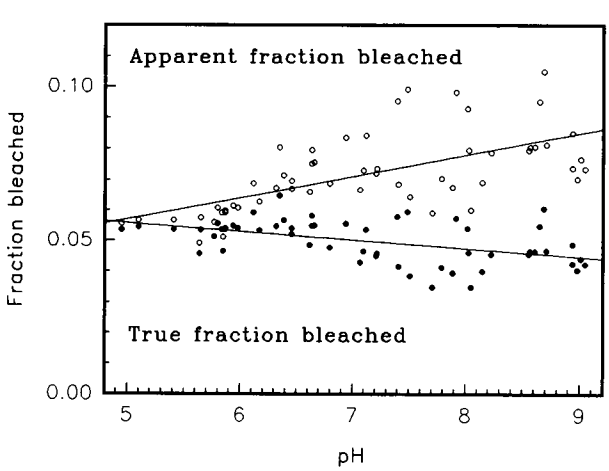

FIGURE 6 Least-squares linear fits to the apparent (open circles) and true (closed circles) fractions bleached in 54 experiments, plotted as a function of pH. The slopes of the lines and their standard deviations are 0.00731 ± 0.00208 for the apparent bleach and −0.00246 ± 0.00120 for the true bleach.

the apparent bleach is smaller, closer to the true bleach. Once the data are corrected for photoregeneration, this decrease disappears. Indeed the true bleach appears to increase slightly with decreasing pH. Most of this increase can be shown to be due to the error made in considering isorhodopsin to be the same as rhodopsin, and to ignoring the effect of screening by MI on the intensity of the bleaching flash.

It should be noted that while the titration curve for re versus pH was obtained on the assumption that only two intermediates (MI and MII) were present, the validity of this assumption is not essential to the usefulness of the parameters re 1, re 2, K_{eq}, and n. It is only necessary that the data be adequately fit by a curve of this shape, so that re can be predicted from the pH of the sample, because re is the only parameter needed to calculate the true bleach from the apparent bleach. If there are more than two intermediates present in equilibrium, then re 1 and re 2 would probably have to be interpreted differently, but the calculated value for re would still be correct to within the accuracy of the photostationary state data.

CONCLUSION

Calculations of the fraction of rhodopsin bleached by a light flash using the successive decrement or exhaustion method need to be corrected for the relative efficiency of photoregeneration. Only one constant, re, is necessary to make this correction, and it can readily be determined for a specific set of bleaching conditions. The pH dependence of re at 0° is seen to fit a generalized titration curve and the parameters describing this curve can be determined.

APPENDIX I: DERIVATION OF BLEACHING FUNCTIONS

It is useful to define three functions:

R_n = fraction of rhodopsin remaining after n flashes
B_n = fraction of rhodopsin bleached by n flashes
b_n = fraction of rhodopsin bleached by the nth flash

Consider first the case in which there is no photoregeneration. Each flash will bleach a given fraction f of the remaining rhodopsin, and will leave unbleached the fraction $1 - f$ of the remaining rhodopsin. The amount remaining after n flashes is

$$R_n = (1 - f)^n \tag{A1}$$

while the amount bleached by n flashes is

$$B_n = 1 - R_n = 1 - (1 - f)^n$$

and the fraction bleached by the nth flash is

$$\begin{aligned} b_n &= R_{n-1} - R_n = (1 - f)^{n-1} - (1 - f)^n \\ &= (1 - f)^{n-1} \cdot \{1 - (1 - f)\} \\ b_n &= f \cdot (1 - f)^{n-1} \end{aligned} \tag{A2}$$

If there is photoregeneration, the net amount of bleaching by a given flash will equal the total amount bleached minus the amount of photoregeneration from those photoproducts still present from the previous flashes. This can be written as

$$b_n = (R_{n-1})(f) - (B_{n-1})(\mathrm{re})(f) \tag{A3}$$

where f is the net amount bleached by a single flash of a previously unbleached visual pigment sample, after any photoregeneration of its own bleaching products has occurred, and re is the sum of the relative efficiencies of photoregeneration from those bleaching products still present at the time of the next flash, weighted according to their relative concentrations. Because $B_n = 1 - R_n$, this can be written as

$$b_n = (f)[R_{n-1}(\mathrm{re} + 1) - \mathrm{re}] \tag{A4}$$

To solve for the ratio of the amount of MII formed on consecutive bleaches, write this as

$$\frac{b_n}{b_{n-1}} = \frac{f \cdot [(R_{n-1})(\mathrm{re} + 1) - \mathrm{re}]}{f \cdot [(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}]}$$

Because $R_n = R_{n-1} - b_n$, this can be rewritten as

$$\frac{b_n}{b_{n-1}} = \frac{(R_{n-2} - b_{n-1})(\mathrm{re} + 1) - \mathrm{re}}{(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}}$$

$$= \frac{(R_{n-2})(\mathrm{re} + 1) - \mathrm{re} - (b_{n-1})(\mathrm{re} + 1)}{(R_{m-2})(\mathrm{re} + 1) - \mathrm{re}} = 1 - \frac{(b_{n-1})(\mathrm{re} + 1)}{(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}}$$

Because $b_{n-1} = (f)[(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}]$ from Eq. 1, this equals

$$\frac{b_n}{b_{n-1}} = 1 - \frac{(f)[(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}](\mathrm{re} + 1)}{(R_{n-2})(\mathrm{re} + 1) - \mathrm{re}} = 1 - (f)(\mathrm{re} + 1)$$

so that the fraction bleached per flash decrements with the constant ratio $1 - (f)(\mathrm{re} + 1)$. Thus, because $b_1 = f$,

$$b_n = f[1 - (f)(\mathrm{re} + 1)]^{n-1} \tag{A5}$$

The total fraction bleached with an infinite number of flashes is

$$B_\infty = \sum_{i=1}^{\infty} b_i = \frac{b_1}{1 - \mathrm{ratio}} = \frac{f}{1 - [1 - (f)(\mathrm{re} + 1)]} = \frac{1}{\mathrm{re} + 1} \tag{A6}$$

so that, if re = 1, only one-half of the total rhodopsin can be bleached. The total fraction bleached after n flashes is

$$B_n = \sum_{i=1}^{n} b_i = \sum_{i=1}^{\infty} b_i - \sum_{i=n+1}^{\infty} b_i = \frac{b_1}{(f)(\mathrm{re} + 1)} - \frac{b_{n+1}}{(f)(\mathrm{re} + 1)}$$

$$= \frac{1}{\mathrm{re} + 1} - \frac{[1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1} = \frac{1 - [1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1}$$

Finally, the fraction remaining after n bleaches is $R_n = 1 - B_n$

$$R_n = 1 - \frac{1 - [1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1} = \frac{\mathrm{re} + [1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1}$$

In the photostationary state, the fractional amount of visual pigment remaining is

$$R_\infty = \mathrm{re}/(\mathrm{re} + 1) \tag{A7}$$

It can be readily verified that $b_n = R_{n-1} - R_n$, and that $b_n = B_n - B_{n-1}$.

Note that on setting re = 0, each of these equations reduces to the corresponding expression for no photoregeneration:

$$R_n = \frac{\mathrm{re} + [1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1} \Rightarrow (1 - f)^n$$

$$B_n = \frac{1 - [1 - (f)(\mathrm{re} + 1)]^n}{\mathrm{re} + 1} \Rightarrow 1 - (1 - f)^n$$

$$b_n = (f)[1 - (f)(\mathrm{re} + 1)]^{n-1} \Rightarrow (f)(1 - f)^{n-1}$$

If all of the bleached rhodopsin is converted to MI or MII in the interval between flashes, then re can be written as

$$\mathrm{re} = \frac{\mathrm{re}\ 1 \cdot \mathrm{MI}}{\mathrm{MI} + \mathrm{MII}} + \frac{\mathrm{re}\ 2 \cdot \mathrm{MII}}{\mathrm{MI} + \mathrm{MII}} = \frac{\mathrm{re}\ 1 + \mathrm{re}\ 2 \cdot K_a}{K_a + 1} \tag{A8}$$

where K_a = MII/MI and re 1 is defined as

$$\text{re } 1 = \frac{\text{qe(photoregeneration from MI)}}{\text{qe(bleaching of rhodopsin)}} \times \frac{\int \text{absorption of MI}(\lambda) \cdot \text{flash}(\lambda)\, d\lambda}{\int \text{absorption of rhodopsin}(\lambda) \cdot \text{flash}(\lambda)\, d\lambda}$$

That is, re 1 is the ratio of the quantum efficiency of photoregeneration of MI relative to the quantum efficiency of bleaching for rhodopsin, multiplied by the ratio of the overlap integral between the absorption spectrum of MI and the spectral output of the bleaching flash, compared with the corresponding overlap integral for rhodopsin. Similarly, re 2 is the relative efficiency of photoregeneration for MII. Note that while the relative quantum efficiencies of MI and MII are constant, the overlap integrals, and hence re 1 and re 2, will be constant only for the particular bleaching apparatus used in the experiment. Their weighted average, re, depends additionally through K_a on the pH and temperature of the sample.

It should be noted that because each bleaching product can be photoconverted either to rhodopsin or isorhodopsin, re 1 and re 2 are themselves composites. On the assumption that isorhodopsin is equivalent to rhodopsin, if re 1 = 0.72, re 1→R (photoregeneration of rhodopsin) = re 1→isoR (photoconversion to isorhodopsin) = 0.36. In the more general case, where re 1→R is not equal to re 1→isoR, their sum will be somewhat greater than 0.72.

APPENDIX II: DERIVATION OF TITRATION FUNCTION

To develop the mathematical form for this titration curve we start with the general equation for the MI – MII equilibrium:

$$\text{MI} + nH^+ \rightleftarrows \text{MII}$$

where n is left as a free parameter. From this one can write

$$K_{eq} = \frac{\text{MII}}{\text{MI} \cdot (H)^n} = \frac{K_a}{(H)^n}, \quad \text{so that} \quad K_a = K_{eq} \cdot (H)^n$$

where K_{eq} is the pH-independent association constant, and K_a is the pH-dependent association constant. The equation on the left can be rewritten as

$$K_{eq} \cdot (H)^n = \text{MII/MI}$$

Because pH = $-\log(H)$, and defining pK_{eq} as $\log(K_{eq})$, this can be written as

$$pK_{eq} - n \cdot pH = \log\left(\frac{\text{MII}}{\text{MI}}\right), \quad \text{or} \quad \frac{\text{MII}}{\text{MI}} = 10^{(pK_{eq} - n \cdot \text{pH})}$$

which is the Henderson-Hasselbach equation, generalized to nonunitary values for n. Then

$$\frac{\text{MI}}{\text{MI} + \text{MII}} = \frac{10^{(n \cdot \text{pH} - pK_{eq})}}{1 + 10^{(n \cdot \text{pH} - pK_{eq})}} \quad \text{and} \quad \frac{\text{MII}}{\text{MI} + \text{MII}} = \frac{1}{1 + 10^{(n \cdot \text{pH} - pK_{eq})}},$$

which yields a generalized titration curve when either MI/(MI + MII) or MII/(MI + MII) is plotted against pH.

Substituting for K_a in Eq. A8

$$\text{re} = \frac{\text{re } 1 + \text{re } 2 \cdot K_{eq} \cdot (H)^n}{1 + K_{eq} \cdot (H)^n} \tag{A9}$$

which is the form of the equation used by the Simplex to solve for the best-fit values of re 1 and re 2.

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