Photoreversal of Rhodopsin Bleaching

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ABSTRACT   A mechanistic scheme, showing certain steps of rhodopsin bleaching, provides two ways of viewing the bleaching process: (a) The rate of bleaching depends upon the net rate of accumulation of labile species; and (b) the number of labile molecules which accumulates in a certain period is the number which has absorbed an odd number of quanta by the end of that period. Both views, based on the photoreversibility of bleaching, lend themselves to concise mathematical formulation. The expected amounts of bleaching at various intensities, calculated according to these formulae, give very close fits to the experimental data. The relevance of these results to other experiments is pointed out and emphasis is placed on the explanation of observed quantum efficiencies which obtain at both low and high intensities.

INTRODUCTION

If a molecule of the photopigment, rhodopsin, absorbs a quantum of light, it undergoes a series of chemical changes which result in the eventual dissociation of the molecule. The initial photochemical act is the production of an electronically excited state of the chromophore, 11-*cis* retinal. From this excited state the 11-*cis* isomer emerges in the all-*trans* configuration which apparently does not fit appropriately with the protein, opsin. This lack of fit renders the native protein structure unstable and, by stages, it thermally denatures. This process is usually called "bleaching." The various stages of bleaching have been studied by low temperature photochemical as well as flash photolytic means. As a result of some of these experiments it has been suggested that certain of the bleaching stages are photochemically reversible (1–3). Fig. 1 shows a bleaching scheme proposed by Grellmann, Livingston, and Pratt (1). This diagram was devised in order to explain the results of their flash photolysis experiments. Although other schemes, involving greater detail, have been proposed (2, 3), this one will be shown to be sufficient for the present work. Here, R is rhodopsin, P is prelumirhodopsin, L is lumirhodopsin, and M is metarhodopsin. PRODUCTS refers to all species arising from the decay of M and its related decomposition products. Wavy lines indicate photochemical processes and straight lines, thermal processes. Note that while

679

quantal absorption by R is necessary to start the bleaching process, the process is reversed if P, L, or M absorbs a second quantum, thereby restoring R. P, L, and M have the all-*trans* chromophore and are therefore destined to bleach unless quantal absorption reisomerizes the *trans* to *cis*. However, the absorption must occur before M decays. Therefore, there exists a critical time, t_c, during which photoreversal of bleaching is possible and after which no number of absorptions can prevent the bleaching. This critical time is determined by the lifetimes of the P, L, and M species. Hagins' results would indicate that t_c is about 1 millisecond at, or near, room temperature (4).

If a flash whose duration is less than t_c is delivered to a solution of rhodopsin, no bleaching, as such, has occurred by the end of the flash. However, the
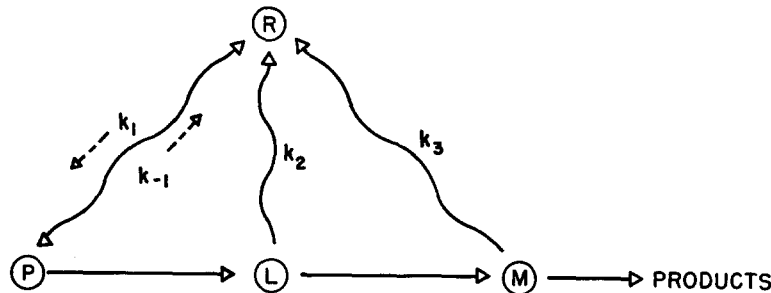

FIGURE 1. Mechanistic scheme for rhodopsin reactions. R is rhodopsin, P is prelumirhodopsin, L is lumirhodopsin, and M is metarhodopsin. PRODUCTS refers to all steps subsequent to the thermal decay of M. Wavy shafted arrows indicate photochemical processes, straight shafted arrows, thermal processes. The k's are specific rates.

number of molecules which are destined to bleach has been strictly determined and is, in fact, the number in the P, L, and M stages of bleaching. The rate at which these labile species accumulate during the flash is the algebraic sum of the rate of production (from R) and the rate of removal (to restore R). Both of these rates depend upon the intensity. Therefore, it should be possible to set up an expression for the net rate of production of these species, integrate it over the flash duration, and find the accumulation of P, L, and M. This is the amount of rhodopsin which, according to the Grellmann scheme, should bleach.

The Grellmann mechanism provides still another way of describing the accumulation of the unstable P, L, and M species during a flash. This is that only those molecules which have absorbed an odd number (1, 3, 5, etc.) of quanta by the end of the flash result in P, L, or M species. Even numbers of quanta absorbed result in a restoration of R. Therefore, a statistical calculation of the number of molecules absorbing an odd number of quanta is in reality a calculation of the number of molecules in the P, L, and M stages and, hence, the number which must bleach.

This paper makes use of both the rate and statistical (odd-even) concepts of bleaching in the interpretation of experimental results obtained by photolyzing rhodopsin solutions with flashes of various intensities. The flash, produced electronically, had an effective duration less than Hagins' t_c, yet it was intense enough to bleach large fractions of the available rhodopsin. Since such large numbers of molecules absorbed quanta, it is a virtual certainty that some of them absorbed more than 1 quantum. Moreover, because the flash duration was less than t_c, the solutions represented the closed system in which no actual bleaching had occurred by the end of the flash. Therefore, the observed amounts of bleaching depend only upon the amounts of P, L, and M which have accumulated during the flash. The sum of the concentrations of these three species was calculated from the rate and statistical considerations described above. The observed amounts of bleaching were compared with these calculations.

METHODS AND MATERIALS

Rhodopsin solutions were digitonin (2 per cent w/v in $M/15$ phosphate buffer, pH 6.5) extracts of rod outer segments obtained from frog (*Rana pipiens*) retinas. One pair of retinas usually yielded enough rhodopsin for easy measurement in 0.6 ml of solution with a 1.0 cm path length. Small volumes of a concentrated NH_2OH solution were added to the rhodopsin stock solutions and the final NH_2OH concentration was made 0.1 M.

The apparatus used is shown schematically in Fig. 2. A cuvette containing the sample, S, was placed in an aluminum block through which water at a constant temperature circulated. The temperature of the sample was allowed to equilibrate with that of the aluminum block and was measured with a copper-constantan thermocouple. A beam of selected monochromatic light from the monochromator was passed through the sample and was partly absorbed therein. The transmitted portion was monitored with the photomultiplier tube, PMT (RCA IP28). The output of the PMT was read on a Hewlett-Packard microvolt meter (425A) and was found to be directly proportional to the intensity of the monochromatic beam. A flash from the strobe source (General Radio, "strobolume") was triggered and was (crudely) focussed upon the sample with the lenses, L. Measurements of the flash duration showed that 90 per cent (or more) of the light was dissipated in about 800 microseconds. The intensity of the flash was controlled with neutral density filters, F, imposed between the strobe and the sample. The spectral distribution of the radiation from the xenon-filled strobe lamp was found to correspond closely to "white" light from a tungsten source.

The amount of bleaching caused by a single flash was calculated from the change of intensity of the monitoring beam reaching the PMT. The original rhodopsin concentrations were obtained by completely bleaching the samples with repetitive flashes and measuring the final transmission. The Beer-Lambert law and a molar absorption coefficient of 40,600 for rhodopsin at 500 $m\mu$ were assumed. The results

do not critically depend upon the accuracy of this value for the absorption coefficient since the results are expressed as the fraction bleached.

Although changes in optical density were measured at several wave lengths, only the results at 500 mμ, the maximum of the difference spectrum as determined in these experiments, will be presented. The temperature of the samples was 20.5 $\pm$ 0.5°C except as otherwise indicated.

RESULTS

The Rate Concept

The rate expression for the accumulation of the P, L, and M species during a flash follows from the mechanism in Fig. 1. Let $[R]_0$ be the preflash concentra-
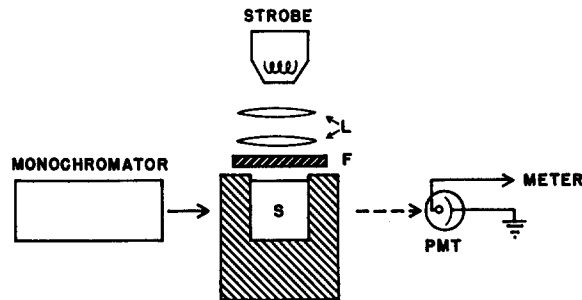

FIGURE 2. Schematic diagram of apparatus. The intensity of the monochromatic light passing through sample, S, is monitored by the photomultiplier tube, PMT. A flash from the strobe source is focussed by lenses, L, and attenuated by the neutral density filter, F, before it falls on the sample. The sample is held in an aluminum block, through which water at a given temperature circulates. The temperature of the sample is measured directly with a thermocouple (not shown).

tion of rhodopsin, I the intensity of the flash, and $[X]$ the sum, $[P] + [L] + [M]$.
Then

$$d[X]/dt = k_1 I ([R]_0 - [X]) - k_{-1} I [P] - k_2 I [L] - k_3 I [M]. \tag{1}$$

The values of the rate constants in this equation are not known. They are all specific rates of geometrical isomerization processes, some of which, perhaps, are complicated by thermal processes (3). For the present, it is assumed that all the k's are equal. (This assumption will be discussed later.) If one substitutes k_i for each, Equation 1 becomes

$$d[X]/dt = k_i I [R]_0 - 2k_i I[X]. \tag{2}$$

Integration over the flash duration, t_f, gives

$$(1/2k_i t_f) \ln \{[R]_0/([R]_0 - 2[X])\} = I, \tag{3}$$

where k_i (at a given temperature) and t_f are constants. $[R]_0$ can be taken as unity and $[X]$, the fraction in the labile states, can be calculated as a function of the relative intensity. Even though t_f is known to be about 800 microseconds, the product $k_i t_f$, is simply a proportionality constant whose value is not required for the calculation of intensity on a relative scale. The calculated dependence of $[X]$ on log I is the solid curve in Fig. 3. Three sets of measured fractions of rhodopsin bleached are shown for comparison. Each set of points has been slid along the abscissa to correspond with the curve and they all
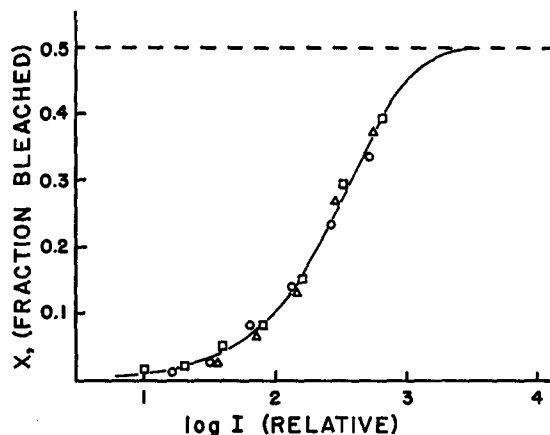

FIGURE 3. Comparison of experimental bleaching results and calculated curve. The curve is derived from rate theory applied to the reaction mechanism. The squares, triangles, and circles are three separate sets of results which have been slid along the abscissa to correspond with curve on the relative intensity scale. Note the direct proportionality between X, the fraction bleached, and intensity at low intensities. Also, note the calculated upper limit on bleaching is 0.50.

provide a close fit. It is evident that no systematic deviations from the calculated curve obtain. This lends support to the simple mechanism given in Fig. 1 and indicates that no important aspects of the process have been omitted.

Two features of Fig. 3 warrant some discussion at this point. First, at low intensities, the fraction bleached is directly proportional to the intensity. According to the mechanism, the quantum efficiency, γ, was assumed to be unity for the step: $R + h\nu \rightarrow P$. That is, each R absorbing reacts to produce P. This agrees with the suggestion of Dartnall, Goodeve, and Lythgoe (5) that the quantum efficiency in their experiments was unity (or not much less). Their bleaching experiments were performed with relatively low intensities when the rate-limiting step was likely to have been the initial absorption of a quantum and hence photoreversal was unimportant.

The agreement of the results with the Dartnall *et al.* experiment is, in itself,

not surprising, it was a condition imposed upon the mechanism at the outset. However, the curve in Fig. 3 clearly shows that the fraction bleached does not remain proportional to the intensity but, in fact, becomes proportional to log I over a certain range and finally yields a maximum bleaching of 50 per cent. Although these data do not verify it, this upper limit on bleaching has been observed experimentally by Hagins (4). Thus, one continuous function agrees not only with the low intensity result that γ is unity but also correctly sets an upper limit on bleaching at 50 per cent. It achieves this generality, of course, by utilizing the concept of photoreversal of bleaching. Hubbard and Kropf (2), discussing photoreversal, anticipated this result when they pointed out that γ cannot remain constant, independent of intensity and temperature.

The Statistical Concept

So far, the rate theory treatment of the bleaching process has been successful at reproducing the data. It remains now to describe the process in terms of the statistical nature of absorption of odd and even numbers of quanta. As mentioned earlier, there must be a distribution of absorbed quanta over the ensemble of molecules in a sample. It is assumed that the Poisson exponential binomial limit describes this distribution. The individual terms of the Poisson function then give the number of molecules, n_q, with q quanta absorbed. Before these terms can be calculated, however, it must be assumed that there is some fixed, average probability, p, of absorbing a quantum by rhodopsin or any of the P, L, or M species. The probability of absorption by any one will be somewhat different from the others, but the assumption is that there exists some average p for all. It is not necessary to know the real value of p, only that it is a constant, independent of intensity.

According to the Poisson distribution, then, the fraction of molecules absorbing at least 1 quantum is

$$\frac{\sum n_q}{N} = \sum_{q=1}^{Q} (a^q\, e^{-a})/q!. \tag{4}$$

Here, N is the total number of photopigment molecules in the sample, Q is the total number of quanta impinging upon the sample, and a is the average number of quanta absorbed per molecule; i.e., $a = pQ/N$. By varying Q, the analog of the experimental intensity, one can calculate the fraction of molecules absorbing at least 1 quantum as a function of Q. Summing the numbers of molecules with only odd numbers of quanta absorbed gives the curve labeled "odd" in Fig. 4, while the even absorptions give the "even" curve. Observed fractional bleaches at various intensities are given for comparison (only one set is shown for clarity). The highest point of this set is tied to the odd-curve at its corresponding level of bleaching and the other points

plotted in accordance with this anchor. The agreement is clearly very good. When compared with the even-curve, the same data (now shown as X's) do not fit. It might be mentioned that the odd-curve in this figure is identical with the curve calculated from rate considerations and shown in Fig. 3. Consequently the odd-curve shows the same high and low intensity limits on bleaching as did the rate curve of Fig. 3. This time, however, these limits can be viewed in a different way: At low intensities, the probability of any molecule absorbing more than 1 quantum before the end of t_c is quite small. One, being an odd number, means the molecule must bleach, and hence the quan-
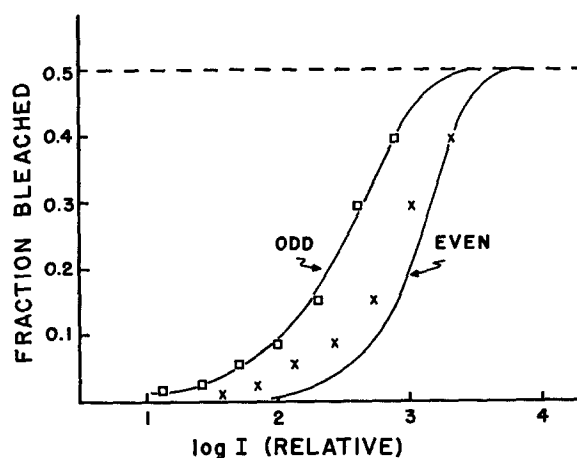

Figure 4. Comparison of experimental bleaching results and calculated curves. The curves are calculated from a statistical formulation of the numbers of molecules absorbing odd and even numbers of quanta during a short flash. A set of data (shown as squares) can be fit to the odd curve but not the even curve (same data are now shown as X's). The close fit to the odd-curve indicates that only those molecules absorbing odd numbers of quanta during the flash bleach.

tum efficiency is unity. As the intensity is increased, this condition holds only until the number of even absorptions becomes important. Since even absorptions cause photoreversal, the quantum efficiency falls. Finally, however, so many quanta have been absorbed that the number of even equals the number of odd absorptions and the 50 per cent limit obtains.

Thus, it has been shown that the results of these experiments as well as certain others are adequately described by rate theory and the statistical nature of multiple absorptions. Both views rest on the principle of photoreversal. A simple test was devised to show the effects of photoreversal in a more direct way and thereby help to confirm its existence and importance in these experiments. The test was based on the idea that there exists an upper limit on bleaching with a short flash ($t_f < t_c$) regardless of its intensity. Hence,

two very bright flashes delivered within t_c should not bleach more than this limiting amount. But if the second flash follows the first by an interval greater than t_c it should be able to contribute to the bleaching. Accordingly, arrangement was made for the production of two equally intense flashes from the strobe source. The interval between the flashes could be varied at will. Unfortunately, the strobe could not produce the twin flashes separated by intervals shorter than 100 milliseconds. Ordinarily this would be far too long an interval since t_c at room temperature is about 1 millisecond. Therefore, the solutions were cooled (to 2.5°C) in order to extend t_c by prolonging the life
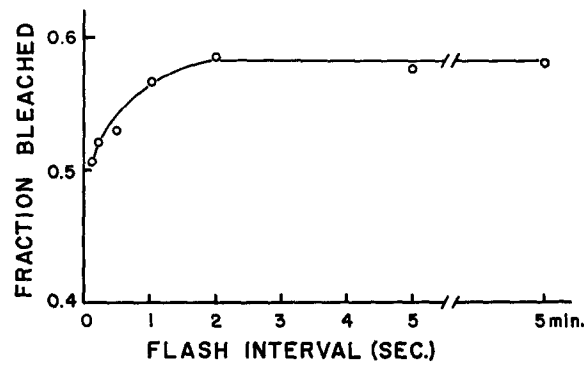

Figure 5. Results of double flash experiment at 2.5°C. Two identical flashes bleach increasingly more rhodopsin as the interval between them is increased. The increase in fraction bleached is made possible by metarhodopsin decay (explained in text). Note that extrapolation to zero interval indicates that no more than 50 per cent of the rhodopsin could have been bleached by the two flashes, presented simultaneously.

of the last photoreversible species which, according to the reaction diagram, is metarhodopsin.

As predicted Fig. 5 shows that increasingly more bleaching is effected by the second flash as the interval between the flashes is increased. It was found that each flash alone was capable of bleaching 36 per cent of the available rhodopsin. (Other experiments showed that the fraction bleached was independent of the rhodopsin concentration.) Hence, the two flashes, separated by long intervals should bleach 59 per cent, which they do within experimental error. Extrapolation to zero interval, condition of simultaneity of the flashes, shows that only about 50 per cent (probably somewhat less) could have been bleached. This suggests that the upper limit is about 0.5 in these experiments.

If the second flash impinges upon the sample within t_c, it finds some R molecules available for bleaching as well as a substantial number of M molecules, produced by the first flash, which can be photoreversed. (Very few P and L species will exist since their lifetimes are so short.) In this case, the

second flash will, of course, cause some bleaching of its own, but it will also prohibit some of the bleaching started by the first flash. However, if the second flash follows by an interval greater than t_c, some M species from the first flash have decayed and cannot be reversed. The net result is increased bleaching with increased intervals. Indeed, the increased bleaching caused by the combination of flashes at any given interval is exactly equal to the amount of decay suffered, during that interval, by the last photoreversible species which the first flash produced. Therefore, the curve in Fig. 5 is a monomolecular decay function. When an integrated, first-order rate plot was made from the data, monomolecularity was confirmed and the specific rate of the decay process was found to be 1.1 sec.$^{-1}$. In separate experiments in this laboratory the specific rate of metarhodopsin decay was found to be 0.8 sec.$^{-1}$ at 2.5 °C. This good agreement confirms that the last photoreversible species is metarhodopsin. Only its decay can prevent photoreversal and hence this double flash experiment demonstrates the importance of photoreversal in these experiments.

DISCUSSION

The reaction scheme given in the Introduction was declared to be sufficiently detailed for the present purposes and the high degree to which it fits the data provides strong support for this declaration. Greater detail would have involved the inclusion of isorhodopsin, tautomeric forms of metarhodopsin, and the possible photoproduction of all-*trans* as well as *monocis* isomers (other than 9- or 11-*cis*) from P, L, and M (3). Tests, based on $\lambda_{(max)}$ shifts in once flashed solutions, disclosed no conclusive evidence that isorhodopsin was present. Hence it was not considered. The other species were omitted only because their inclusion represented a complexity which was not necessary for the mathematical description of the data.

The data have been described by means of two different calculations of the dependence of bleaching on intensity. It was necessary to make two major assumptions in order to carry out these calculations: (a) All isomerization rate constants are equal; and (b) an average probability of absorbing by R, P, L, and M exists and is independent of intensity.

There exists some experimental evidence in favor of the first assumption. The evidence derives from certain experiments done at low temperatures where the bleaching process is arrested at one of the P, L, or M stages (3, 6, 7). (Note: some of these experiments have complicating features due to the presence of isorhodopsin and/or the use of certain wave lengths of monochromatic radiation. The latter will be discussed below.) In these studies it has been shown that long term irradiation can result in a steady state mixture of about 50 per cent labile and 50 per cent stable products. Using the rate equation (2), let $[X']$ be the concentration of any one of the species, P, L,

or M and k'_i the appropriate specific rate of isomerization. At steady state, $d[X']/dt = 0$, whence

$$([R]_0 - [X'])/[X'] = 0.5/0.5 = k'_i/k_i .$$

This shows that the 50 per cent labile fraction obtains only if $k'_i = k_i$. Since the labile fraction can be P, L, or M, the equality of all k's is indicated. On the other hand, Yoshizawa and Wald have indicated that the reversal steps of L and M have thermal components. This seems reasonable since thermal energy was required to reach the L and M stages. Even if this is the case, the low temperature results would indicate that the photoisomerization part of the process is rate-limiting and therefore, the assumption may still be valid.

Arguments, pro and con, could similarly be advanced regarding the assumption of a p value for absorption. In the final analysis, however, the most persuasive reason for accepting the two assumptions is that they not only fit the present data but also explain other results as well. This sort of reasoning, while standard procedure in the field of reaction kinetics, is admittedly dangerous and often misleading.

It should be stressed that the 50 per cent limit on bleaching is far from a unique solution of the general rate equation (1). This result obtains because the rate constants were assumed equal. This assumption, in turn, implies that the radiation is absorbed with equal probabilities by all species. This can only occur if white light is used and the oscillator strengths of all the species are equal. It simply happens that these conditions are closely approximated in these, and apparently Hagins' experiments. Theoretically, an infinitely intense flash of monochromatic light, if absorbed only by rhodopsin, should bleach all of the available rhodopsin. Of course, the appropriate wave length for this purpose is hypothetical since the absorption band of rhodopsin is overlapped and bound on both sides by those of the photoreversible species. However, one of these species, prelumi, is enough different from rhodopsin that it is possible to convert all R into P (3) at $-195°C$. In ordinary room temperature experiments such as the ones reported here this would be difficult, at best, to do.

It still remains to be shown that photoreversal of bleaching is of physiological importance. The intensities required for achieving multiple absorptions by one molecule are quite high and thus photoreversal obtains only under special conditions of vision. Even then, the neural components of adaptation may well mask the phenomenon. Nevertheless, it is of interest to note that the photopigment molecules, themselves, have the built-in capability of making inefficient, indeed, contradictory, use of stimuli.

Finally, both the data and the calculated curves show that there is a range of intensities wherein the amount of bleaching is directly proportional to log I. Rushton (8) has postulated that such a relationship probably exists and per-

haps the data and mechanisms given here confirm and explain his postulate. Again, the high intensities required to produce this relationship suggest that this conclusion is only tentatively acceptable.

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