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THE DENSITY AND PHOTOSENSITIVITY OF HUMAN RHODOPSIN IN THE LIVING RETINA

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SUMMARY

1. The visual pigment in a 5° circular patch of the living human retina 18° temporal from the fovea was studied with the Rushton retinal densitometer. The measuring light (570 nm) was selected to obviate artifacts from colour photoproducts.

2. The action spectrum of a 10% bleach agrees well with the action spectrum at absolute threshold for the same patch of retina. The quantized C.I.E. scotopic spectral sensitivity curve is a good description of both spectra. Therefore, the visual pigment studied must be human rhodopsin.

3. Its density has been estimated in five different ways. The results are in reasonable agreement. The optical density of human rhodopsin *in vivo* is about 0·35 (common logarithmic units) at its λ_{max}.

4. The photosensitivity of human rhodopsin *in vivo* was determined by studying its rate of bleaching in response to steps of monochromatic light exposed to the dark adapted eye, by measuring the amount bleached in the steady state by monochromatic lights as well as the amount bleached by 10 sec flashes of white light.

5. The results obtained by the different methods are in good agreement with each other and with previous estimates made by others using white light.

6. The photosensitivity of human rhodopsin *in vivo* [$\epsilon\gamma_{max}$ = 62,000 to 120,000 l./cm mole] is much higher than expected from *in vitro* measurements.

INTRODUCTION

Human rod vision begins with the absorption of light by rhodopsin. The speed of this (or any other) photochemical event depends upon the quantum flux density of the incident light, the fraction of the total complement of photosensitive pigment present, its optical density and finally the velocity constant of the reaction, i.e. the photosensitivity of the pigment. This last is defined as the product of the quantum efficiency of the

bleach (γ, the number of chromophores changed per quantum absorbed) and the chromophoric extinction coefficient ($\alpha(\lambda)$, itself a product of the effective area of the chromophore and the probability that a quantum of wave-length (λ) falling within that area will be absorbed).

Knowledge of the photosensitivity of human rhodopsin *in vivo* is essential for understanding a variety of human rod functions. Rushton (1956*a*) was the first to provide direct information. His results have since been confirmed in a number of independent investigations (Rushton, 1961; Weale, 1962*a*; Ripps & Weale, 1969*a*; Alpern, 1971; Rushton & Powell, 1972).

These measurements were made on living subjects with 'white' light bleaching. To obtain an estimate of $\gamma\alpha(\lambda)$ using a light with a broad spectral distribution requires a calculation involving several assumptions (Appendix I). The large photosensitivity value calculated for human rhodopsin from these data has been attributed by Dartnall (1972) to the uncertain nature of these assumptions.

In this paper, the validity of these assumptions, and the possible error introduced by making them, are examined for human rhodopsin *in vivo*, bleaching with both monochromatic and 'white' light. In the first part it is shown that the method studies rhodopsin, in the second the optical density of rhodopsin in living human rods is estimated and in the last part its photosensitivity determined.

METHODS

The apparatus is the Florida model of the Rushton densitometer (Hood & Rushton, 1971) slightly modified in a way already described (Alpern, Maaseidvaag & Ohba, 1971; Alpern, 1971). Briefly, two beams from a single source, one filtered so that it transmitted only deep red light (little absorbed by rhodopsin) the other filtered to transmit a narrow wave-band more strongly absorbed by rhodopsin, were orthogonally polarized and brought together, presented in alternation by a common rotating polaroid and reflected into the eye. The fraction of this light emerging from the fundus through the widely dilated pupil was gathered by a lens and focussed on to a photomultiplier tube. The resulting sine wave photomultiplier tube signal (oscillating at double the frequency of polaroid rotation) was amplified and then reduced to zero by adjusting the relative intensities of the two beams with Inconel filters and a Wratten no. 96 wedge in the red beam. The wedge transmittance required to null this signal constitutes the datum.

A third beam was used for bleaching; it could be made 'monochromatic' by interference filters. A rotating sectored disk in a light-trapped housing alternately exposed either the bleaching beam or the photomultiplier tube at a frequency of 250 Hz in such a way that none of the bleaching light reached the photomultiplier tube.

The bleaching light could be attenuated sufficiently with neutral filters and a calibrated and balanced Wratten no. 96 wedge so that absolute threshold measurements were made on the same apparatus on the same part of the retina studied with the densitometer, and on the same subject. In this way, the action spectrum of

bleaching was compared with the action spectrum for rod vision at threshold. For these psychophysical measurements, the speed of rotation of the sectored disc was reduced so that the subject was presented with a 350 msec flash once every second.

The measurements were carried out on the 18° temporal retina of the subject's left eye. The densitometer measuring field was 5°, the bleaching – and psychophysical threshold testing – field was about twice as large. The subject's pupil was dilated with one drop 1 % Cyclogyl (Alcon Laboratories, Fort Worth, Texas).

Calibration. The spectral interference filters (Baird-Atomic type B-1 with 10 nm half band width, blocked to infinity on each side of the spectrum) and the neutral (Bausch & Lomb Inconel) filters were calibrated from 900 to 300 nm with a Beckman Acta II recording spectrophotometer. The luminance of the bleaching light was determined periodically with an S.E.I. photometer by measuring its reflexion from a white standard test plate ($r = 0{\cdot}82$) placed at 20 cm from the filament image plane normally occupied by the subject's pupil. The angle of observation was always less than 20°. The quantum flux of the unattenuated 'monochromatic' bleaching and measuring lights provided by the interference filters was measured with a calibrated silicon photodiode (United Detector Technology, Santa Monica, California). To convert to quanta sec^{-1} cm^{-2} incident upon the retina, it was necessary to estimate the light losses in the ocular media in the living eye. For this purpose, the results from that one subject of Alpern, Thompson & Lee (1965) whose age (21 yr) was closest to those of the present subjects, were utilized. The data are shown as filled circles in Fig. 4 of that paper. This eye is somewhat more transparent than the Ludvigh & McCarthy (1938), and somewhat less so than the Boettner & Wolter (1962) *in vitro* measurements.

Analysis. The measurements are made by balancing the output of the photomultiplier tube to light of wave-length λ, against its output to deep red light. This is achieved by attenuating the red beam by a wedge calibrated in terms of the fraction (T) of incident red light that it transmits. The measuring light (λ) received by the photomultiplier tube is made up of two parts, signal and stray. The signal has been twice through the rods, the stray light has not been through at all. Light which has only once been through the rods, or partly through, is supposed to contribute in some proportion to signal and in some proportion to stray. Hence the directly measured density is a minimum estimate, diluted by that fraction of λ which is stray light (cf. eqn. (1) below).

Let

T = the red wedge transmittance,
I = the flux density of the λ beam incident upon the eye at the cornea (in quanta sec^{-1} cm^{-2} of retina),
ρ = the product of the reflexion coefficient of the fundus and the square of the ocular media transmittance,
m = fraction of measuring light reflected from the fundus which goes through fully bleached rods,
a = fraction of measuring light reflected from the fundus which does not go through the rods,
b = fraction of measuring light reflected from the superficial parts of the eye,
$\alpha(\lambda)$ = chromophoric extinction coefficient of rhodopsin in cm^2/chromophore,
l = length of the light path in the outer segment in cm,
c_D = concentration of rhodopsin in the rods in full dark adaptation in chromophores/cm^3,
c = concentration of rhodopsin in any other state,
$p = c/c_D$,

$\beta(\lambda)$ = $\alpha(\lambda)lc_D$ = density of rhodopsin in single passage (Napierian logarithmic units) at λ in full dark adaptation ($p = 1{\cdot}0$),
T_0 = T after full bleaching ($p = 0$),
T_D = T after full dark adaptation ($p = 1{\cdot}0$).

Rushton (1956*a*, *b*) and Weale (1962*b*) showed that the contribution of cones to densitometer measurements in the 18° peripheral retina is so small that it can safely be neglected. Tentatively accepting their conclusion (to be verified below in Part I of this paper) then

$$T = KI\{\rho[1-b]\,[me^{-2\beta(\lambda)p}+a]+b\}.$$

In this equation K is a constant which depends upon the wave-length of the measuring beam, the density of neutral filters in the λ and red beams, the relative sensitivity of the photomultiplier tube, the relative spectral reflexion and absorbance of the optical components of the apparatus and the spectral emmittance of the light source in those wave-lengths comprising the red beam. For any given measuring wave-length, these factors all remain unchanged so that

$$\frac{T_0-T_D}{T_0} = [1-s]\,[1-e^{-2\beta(\lambda)}]. \tag{1}$$

s is the fraction of emergent light which is stray light, i.e. does not go through rhodopsin, $\{s = [b+\rho(1-b)\,(a)]/[b+\rho(1-b)\,(m+a)]\}$.
Similarly

$$[T_0-T]/[T_0-T_D] = [1-e^{-2\beta(\lambda)p}]/[1-e^{-2\beta(\lambda)}]. \tag{2}$$

An important simplification of eqn. (2) is the special case in which $\beta(\lambda)$ is so small that in the series expansion

$$1-e^{-2\beta(\lambda)} = 2\beta(\lambda)-[2\beta(\lambda)]^2/2!+[2\beta(\lambda)]^3/3!\ldots$$

the higher order terms can be neglected. Eqn. (2) can then be written

$$(T_0-T)/(T_0-T_D) = p, \tag{2a}$$

and the fraction of pigment unbleached is obtained directly from the wedge settings T. While the evaluation of p from the wedge setting in this way is simpler, eqn. (2) can always be solved for p provided $\beta(\lambda)$ is known. Unfortunately, $\beta(\lambda)$ is unknown and one purpose of this paper is to make precisely this determination. To obviate this problem, a measuring wave-length λ was selected so far from the wave-length of peak extinction that, even granting a reasonable density at the peak, the use of the approximation, eqn. (2*a*) would be valid at the measuring wave-length employed. Unless specified otherwise, this was always 570 nm. For a Dartnall (1953) nomogram pigment with the λ_{max} of human rhodopsin (493 nm, Wald & Brown, 1958; Crescitelli, Pearlman & Dartnall, 1974) $\alpha_{570}/\alpha_{493} = 0{\cdot}114$. It can be shown that if the peak Napierian density of rhodopsin is as large as 1·75, the calculation of p with eqn. (2*a*) from measurements made at 570 nm will mistakenly underestimate the fraction bleached by no more than 0·05 of the total pigment complement. This is about the precision of the measurement at 570 nm. Hence, eqn. (2*a*) has been used in what follows as a first approximation, with the reservation that if the value of $\beta(\lambda_{max})$ [estimated in this way] is very large, the entire calculation may have to be redone with the more complex eqn. (2). As it turned out, this was never necessary.

The selection of the measuring λ = 570 nm has the advantage that in this part of the spectrum the contamination of the measurements by short-lived blue absorbing photoproducts, which otherwise interfere (Rushton, 1956*a*; Ripps & Weale, 1969*b*; Alpern, 1971), is obviated.

There are two disadvantages to using a 570 nm measuring light: The first is that the value of $(T_0 - T_D)/T_0$ is so small that the random measuring error (5–10 % depending on the subject) is higher than when rhodopsin is measured at shorter wave-lengths. The second disadvantage is the uncertainty that what is actually measured at this long wave-length is, in fact, rhodopsin. Though Campbell & Rushton's (1955) evidence argues compellingly that what *they* measured was rhodopsin, working with a 570 nm measuring wave-band is sufficiently different from their method that the validity of generalizing to the present procedure from their results can be questioned. In particular, there is the possibility that (with a measuring wave-band whose peak is almost exactly that of the cone pigment erythrolabe and much closer to that of the other cone pigment chlorolabe than it is to rhodopsin) the measurements are contaminated by a considerable contribution from cone visual pigments. To demonstrate that this method validly studies rhodopsin and it alone, we used it to measure the action spectrum of bleaching.

PART I. THE ACTION SPECTRUM OF BLEACHING

If the measurements at 570 nm contain appreciable contributions from cones, then the measured amount of pigment bleached by a red light will be much larger than the measured amount bleached by a scotopically equated blue light. It is now shown, on the contrary, that lights of all wave-lengths yield identical measured amounts bleached once their quantum content has been equated for rods.

At first thought, studying a 50 % bleach criterion would seem advisable, since densitometer measurements are most sensitive to changes in light intensity at this level. However, if rhodopsin is in the rods in appreciable density its action spectrum of bleaching will vary with the bleaching level. In fact, this proves to be the case (cf. below). Moreover, we wish to compare the action spectrum for rhodopsin bleaching with the action spectrum for rod vision measured under comparable conditions. Since rod vision can never be evaluated psychophysically at light levels where 50 % rhodopsin is bleached at equilibrium (Aguilar & Stiles, 1954) this level is much too high for the present purpose. Instead the lowest level at which reasonable estimates can be made (i.e. about 10 %) was used.

Procedure

The fully dark adapted eye was first exposed to a 10 sec 'white' light of an intensity estimated to bleach 5 % of the rhodopsin. At the end of the 10 sec, a monochromatic interference filter and a neutral filter were introduced. The filter combination had been selected so that the new level would maintain 5 % of the rhodopsin bleached at equilibrium. This was verified by measuring, in five successive determinations, the actual setting of the wedge required. If the estimation were such that the pigment was not at equilibrium, successive measurements showed either a successively larger or successively smaller series of settings. In either event, the measurements were continued until equilibrium was reached as evidenced by a series of successive measurements which fluctuated at random around an equilibrium value. When this was achieved a second interference-neutral filter combination, also estimated to give a 5 % bleach at equilibrium, was introduced and this process was repeated. In this way, a series of eight or nine narrow wave-band bleaches covering the visible spectrum were studied at this same level. At the end of the spectral traverse, the subject relaxed in the dark until full regeneration occurred. The entire process was then repeated twice more, once for an estimated 10 % bleaching level, the second for an estimated 15 % bleach. (The initial estimations, of course, proved to be only approximate.)

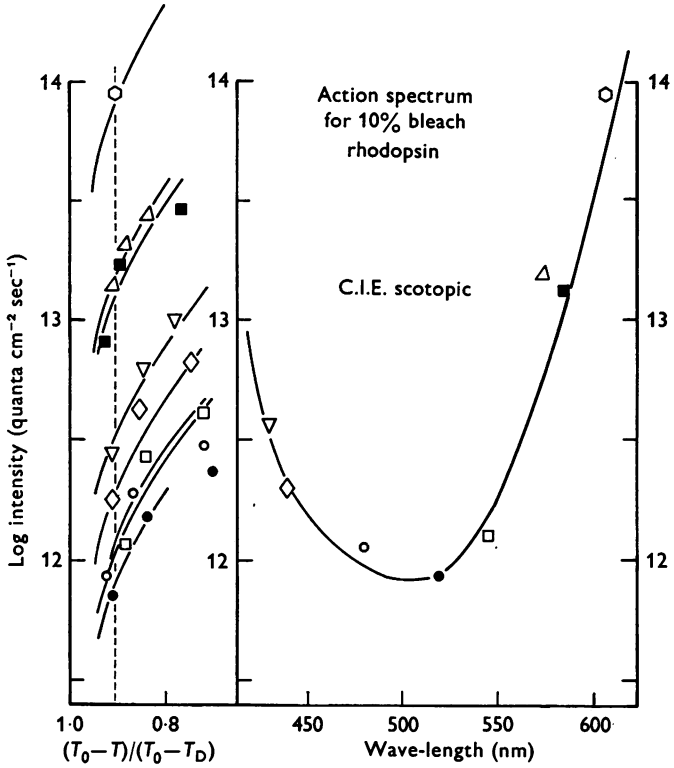

Fig. 1. Action spectrum of 10% bleach. The ordinate scale shows the common logarithm of the quanta cm^{-2} (of retina) sec^{-1} of monochromatic light incident at the cornea. The abscissa scale to the left shows the steady-state value of $(T_0-T)/(T_0-T_D)$ measured at 570 nm; the abscissa scale to the right shows the wave-length of the monochromatic bleaching in nanometres (i.e. metres $\times 10^{-9}$). Each set of symbols represents a different spectral bleaching wave-band, the wave-length of which is indicated on the right hand abscissa scale. The continuous curve through each monochromatic set on the left has the eqn. $(T_0-T)/(T_0-T_D) = I_0/(I+I_0)$. The interrupted line at $(T_0-T)/(T_0-T_D) = 0{\cdot}9$ intersects these successive curves at points which define the ordinates of the action spectrum on the right. The continuous curve through the latter is the reciprocal of the quantized C.I.E. scotopic spectral sensitivity curve vertically shifted for optimum fit. Subject B.

RESULTS

Fig. 1 illustrates the results of a single experimental run. Plotted on the ordinate in this Figure is the $\log_{10}$ of the number of quanta sec^{-1} cm^{-2} of retina incident on the cornea from the bleaching field. The abscissa scale to the left of this Figure shows the value of $(T_0-T)/(T_0-T_D)$ at equilibrium. Different symbols depict the results obtained with different mono-

chromatic bleaching wave-bands. For the three different bleaching levels at each wave band, a continuous curve [whose equation is: $(T_0-T)/(T_0-T_D) = I_0/(I+I_0)$] has been fitted to the results. To this end, a curve with this equation was drawn on transparent paper and slid vertically until the results fell on the curve with the smallest deviation (as judged by eye). A criterion $[(T_0-T)/(T_0-T_D) = 0{\cdot}9]$ was arbitrarily selected. For each wave-length the ordinate at which the continuous curve shows the value of I needed to bleach to this level at equilibrium was read directly from the graph. This defines the action spectrum of a 10 % bleach.

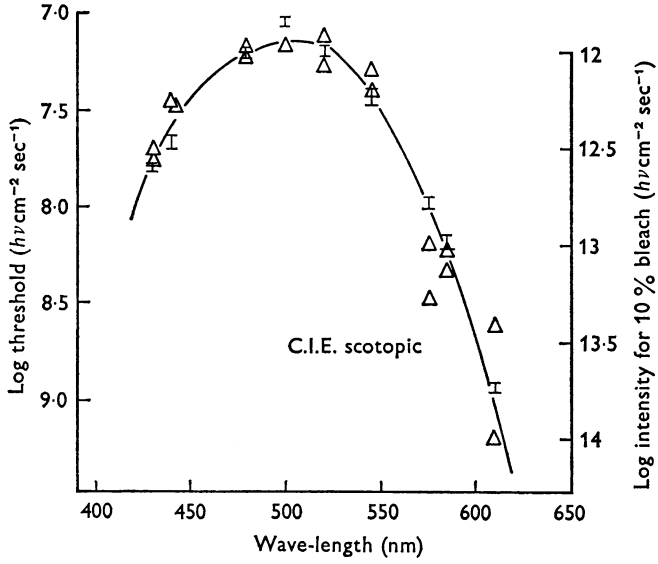

Fig. 2. Action spectrum of 10 % bleach (ordinate scale to the right) and quantized threshold spectral sensitivity in the dark (ordinate scale to the left) for the same retina area. The triangles show the results similar to those on the right half of Fig. 1. The brackets define the geometric mean ± 1 S.E. of mean of fifteen determinations of the absolute threshold in a single run. The smooth curve is the quantized C.I.E. scotopic spectral sensitivity curve. Subject B.

The right half of Fig. 1 shows these intensities plotted as a function of their respective dominant wave-lengths. Each symbol used on the right identifies its partners on the left half of the Figure and hence the wave-length of each respective left-hand set. The curve drawn through the action spectrum on the right is the reciprocal of the quantized C.I.E. scotopic spectral sensitivity curve. This curve is in reasonable agreement with the measurements in keeping with the expectation that the visual pigment which underlies the C.I.E. curve is the one whose action spectrum has been measured.

To confirm directly that the pigment measured is the one this subject uses for night vision in that part of his retina, the action spectrum for the absolute threshold in full dark adaptation was determined. The bracketed lines in Fig. 2 whose limits define the geometrical mean $\pm$ 1 S.E. of mean of fifteen threshold measurements obtained by the method of adjustment in a single spectral traverse in a single session are the results of this determination.

The triangles in the same Figure show the action spectrum for a 10 % bleach (ordinate scale to the right) obtained as in Fig. 1 (including those results and the results of a second experimental repetition). The agreement in Fig. 2 between triangles and brackets is a strong indication that the pigment, whose bleaching characteristics were measured under these conditions is the one this subject uses for his night vision in this part of his retina, and the agreement of both sets of results with the quantized C.I.E. scotopic curve is good evidence that this pigment is, in fact, normal human rhodopsin.

PART II. THE DENSITY OF HUMAN RHODOPSIN IN THE RODS

The density of rhodopsin in the rods has been estimated in a number of different ways. Each requires certain assumptions. However, the assumptions are different for each method, so that the extent to which the various methods yield convergent estimates suggest the degree to which these estimates are independent of the assumptions.

A. Transmissivity difference. If s, the fraction of the measuring light, reaching the photomultiplier tube which does not go through the rods were known, then β_{570} could be obtained directly using eqn. (1). Evidently assuming $s = 0$, eqn. (1) provides a minimum estimate of $\beta(\lambda)$. (Rushton, 1965, with a densitometer similar to the present instrument has found the superficial stray light b to be very small, but it is difficult, if not impossible directly to measure a, the fraction of light traversing the retina which has not gone through rods. Hagins's (1957) evidence is that this fraction is not likely to be large, under his measuring conditions at least.)

Thirty-three consecutive measurements under identical conditions at 570 nm yielded a value of $(T_0 - T_D)/T_0 = 0{\cdot}165 \pm 0{\cdot}004$ (mean $\pm$ 1 S.E. of mean). Substituting directly in eqn. (1), assuming $s = 0$, gives a value of $\beta_{570} = 0{\cdot}0901 \pm 0{\cdot}003$. If there is no stray light and if all the measuring light goes through the rhodopsin twice, then $\beta_{500} = 0{\cdot}77 \pm 0{\cdot}02$ ($\alpha_{570}/\alpha_{500} = 0{\cdot}117$ in an A_1 visual pigment nomogram with $\lambda_{max} = 493$ nm). Less numerous measurements have been obtained at this wave-length on two other subjects. The mean of nine repetitions on one of them gave a value of $\beta_{500} = 1{\cdot}00 \pm 0{\cdot}061$, while a single measurement on the other led

to $\beta_{500} = 0{\cdot}79$. The mean $\pm$ 1 S.E. of mean of single measurements at $\lambda = 575$ nm on five other subjects yielded $\beta_{500} = 0{\cdot}816 \pm 0{\cdot}038$.

B. Initial bleaching rate. This method is based on the familiar fact that visual pigments in appreciable concentration exhibit 'self-screening'. It depends upon bleaching with two monochromatic wave-bands, one ($\lambda_1 = 500$ nm) near the peak, the other ($\lambda_2 = 585$ nm) far off on the long wave 'tail', of the absorption spectrum. Let $J(\lambda)$ represent the number of quanta from the bleaching beam absorbed $\sec^{-1}$ cm^{-2} of retina. The rate chromophores are destroyed is given by

$$-\mathrm{d}c/\mathrm{d}t = \gamma J(\lambda)/l, \tag{3}$$

where γ is the quantum efficiency of bleaching. According to Beer's and Lambert's law,

$$J(\lambda) = I(\lambda)\tau(\lambda)[1-\mathrm{e}^{-\beta(\lambda)p}],$$

where $\tau(\lambda)$ is the transmissivity of the ocular media at λ and $I(\lambda)$ is now the number of quanta $\sec^{-1}$ cm^{-2} of retina in the bleaching beam incident upon the cornea.

$$-\mathrm{d}p/\mathrm{d}t = \gamma I(\lambda)\tau(\lambda)[1-\mathrm{e}^{-\beta(\lambda)p}]/c_{\mathrm{D}}l, \tag{4}$$

assuming that γ is wave-length independent (Goodeve, Lythgoe & Schneider, 1942).

The rate of bleaching is obtained at $t = 0$ when a monochromatic bleaching light of fixed intensity I is suddenly exposed to the dark adapted eye ($p = 1{\cdot}0$). The experiment is done twice in succession with two different monochromatic lights of wave-lengths λ_1, λ_2 of respective intensities I_1, I_2. It follows from eqn. (4) that

$$\frac{X_2 I_2 \tau_2}{X_1 I_1 \tau_1} = \frac{(1-\mathrm{e}^{-\beta_1})}{(1-\mathrm{e}^{-k\beta_1})}, \tag{5}$$

where X_2^{-1}, X_2^{-1} are the respective empirically determined bleaching rates and $k = \alpha_2/\alpha_1$ is given by the Vitamin A_1 visual pigment nomogram ($\lambda_{\max} = 493$ nm).

The conclusion from this experiment is unaffected by the assumption that the density of rhodopsin at the measuring wave-length is small. If eqn. (2) is differentiated and evaluated at $t = 0$, it is seen that $\mathrm{d}[(T_0-T)/(T_0-T_{\mathrm{D}})]/\mathrm{d}t = C\,\mathrm{d}p/\mathrm{d}t$ in which C depends on the density of rhodopsin at the measuring wave-length and $\mathrm{d}p/\mathrm{d}t$ depends on the bleaching wave-lengths. Since the measuring wave-length is fixed and eqn. (5) involves the ratio of two initial bleaching rates, C does not appear in it.

Procedure. Starting after full dark adaptation, the wedge settings were obtained as a function of time (t) after the onset of a monochromatic bleaching light for some 10 min. For any given experiment (usually, but not invariably completed in the same session) the process was repeated twice: once at $\lambda_1 = 500$ nm, again at $\lambda_2 = 585$ nm. When both were done in one session, sufficient time in the dark (30 min) was interspersed to allow complete recovery and successive sessions were done in reverse order to obviate any systematic errors introduced by the procedure. Ten

measurements of (T_D) the wedge setting in full dark adaptation and at least ten during a complete bleach (T_0) were also obtained. Frequently, the latter were obtained after the initial partial bleach and, if so, the recovery of rhodopsin in the dark was also measured. When the two wave-lengths were studied in separate sessions, T_0 and T_D had to be determined for each session independently.

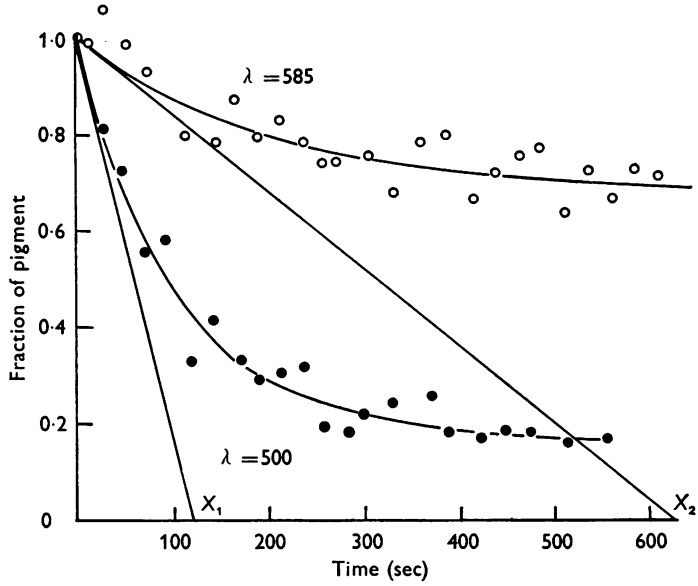

Fig. 3. Rhodopsin fraction (p) as a function of time after the onset of a steady monochromatic light of wave-length 500 nm (filled circles) and 585 nm (open circles) of respective intensities 13·7235 and 13·9815 log quanta sec^{-1} cm^{-2} of retina at the cornea. The continuous curve drawn through the results has been fit by computer to minimize the sum of squared deviations and has the eqn. $p = P+(1-P)e^{-t/Pt_0}$. The slope of these curves at $t = 0$ ($p = 1{\cdot}0$) is shown by the straight line drawn to intersect the abscissa axis at X_1 and X_2 respectively. Subject B.

The results of a typical experiment are shown in Fig. 3 in which fraction of pigment present p, is plotted as a function at time in the light. The open circles are the results for bleaching at $\lambda_2 = 585$ nm, the filled circles those for $\lambda_1 = 500$ nm. A continuous curve has been drawn through each set of points and the tangent to the curve obtained at $t = 0$. The extension of this line strikes the abscissa at the point X, the reciprocal of the initial bleaching rate.

There is a necessarily arbitrary decision about the form of the curve drawn through the experimental points, but the value of X so obtained is not appreciably influenced by this decision provided the resulting fit is equally good (cf. Appendix II for an evaluation of this problem). Alpern (1971) found that a curve of the form $p = P+(1-P)e^{-t/Pt_0}$, where P is

the asymptotic value of p (when $t = \infty$) and t_0 is a constant, provided a good fit to such data. The present results confirm this. The computer was programmed to find the value for P and t_0 which minimized the sum of squared deviations from a curve with this equation. One then calculates

$$X = t_0 P/(1-P).$$

Table 1 shows the parameters in eqn. (5) which were determined for the results in Fig. 3. Substituting these values into eqn. (5) yields a value of $\beta_{500} = 0{\cdot}86$.

TABLE 1. Constants needed to evaluate β_{500} from the results in Fig. 3

	λ (nm)	log $\tau(\lambda)$	log I ($h\nu$ sec^{-1} cm^{-2})	$\alpha(\lambda)/\alpha(\lambda_{max})$	X (sec)
1	500	−0·262	13·7235	0·978	121·6
2	585	−0·106	13·9815	0·05	627·1

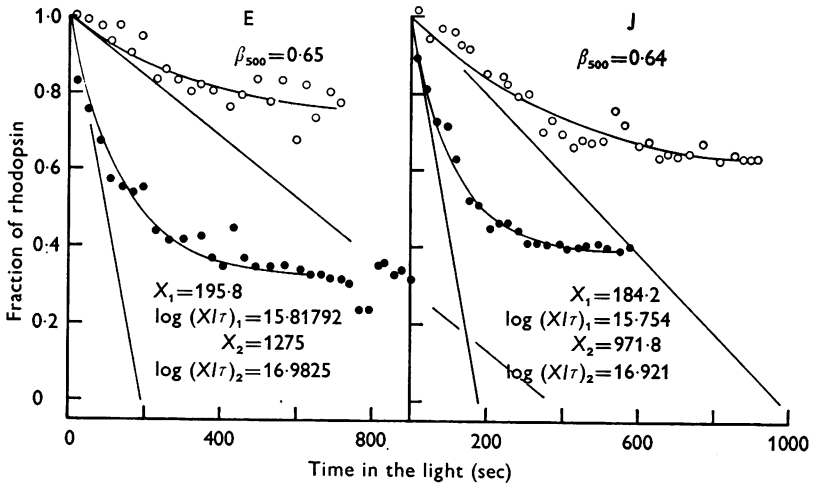

Fig. 4. The results of the same experiments, the results of which are shown in Fig. 3 on two other subjects. For subject J the measuring wave-length was 555 nm not 570 nm; for subject E and all other experiments reported in the paper the measuring wave-length was 570 nm.

This same procedure has been repeated fifteen times for this subject, the geometric mean ± 1 S.E. of mean of these measurements was $\beta_{500} = 0{\cdot}846 \pm 0{\cdot}246$. The agreement with the minimum estimate obtained from the transmissivity difference measurements is satisfactory.

Preliminary observations of this kind at these two wave-lengths have been made on two other subjects. Results on them, comparable to those shown in Fig. 3 are illustrated in Fig. 4. For subject E, the values from this run yield $\beta_{500} = 0{\cdot}65$, but the mean of five repetitions (at $\lambda_1 = 500$,

but only four at $\lambda_2 = 585$ nm) gave a value of $\beta_{500} = 0{\cdot}838$. Subject J was studied with 555 nm measuring light on only one occasion. The estimate of β_{500} from Fig. 4 for her is 0·64.

C. Steady-state bleaching. Alpern (1971) showed that rhodopsin bleaching and regeneration in the eye proceeded independently and that over a range of conditions regeneration can be described by the kinetics of a simple monomolecular reaction. The differential equation which describes this is,

$$-\frac{dp}{dt} = \frac{\gamma I(\lambda)\tau(\lambda)[1-e^{-\beta(\lambda)p}]}{lc_D} - \frac{1-p}{t_0}, \tag{6}$$

in which t_0 is the time constant of regeneration (sec). In the steady state $dp/dt = 0$ and eqn. (6) reduces to

$$\frac{\alpha(\lambda)\gamma I(\lambda)\tau(\lambda)[1-e^{-\beta(\lambda)P}]}{\beta(\lambda)} = \frac{1-P}{t_0}. \tag{7}$$

In order to determine rhodopsin density in the rods, the relation between the fraction of pigment present in the steady state (P) and the intensity of the steady light may be worked out with two different monochromatic bleaching lights of wave-length λ_1, λ_2. Eqn. (7) then simplifies to

$$\frac{(I\tau)_2}{(I\tau)_1} = \frac{(1-e^{-\beta_1 P})}{(1-e^{-k\beta_1 P})}, \tag{8}$$

which can be used to obtain β_1. One needs to determine only the value of I_1 and I_2 at the two wave-lengths necessary to bleach to some fixed criterion level in the steady-state, say $P = 0{\cdot}5$.

Procedure. The dark adapted subject was exposed to a 10 sec 'white' flash the intensity of which had (in preliminary experiments) been selected to bleach to a predetermined level. At the end of the 10 sec an interference-neutral filter combination, estimated to hold bleaching fixed at this level, was introduced and wedge settings were made and recorded until they fluctuated randomly around a steady-state value. The bleaching light was then turned off and full regeneration allowed to proceed in the dark. The same procedure was followed with the same wave-length bleach but a different bleaching level, i.e. 10 sec white flash at a new, predetermined level, followed by a new filter combination and measurements until a steady state was reached. The bleaching light was again turned off; after regeneration in the dark the entire process was repeated still a third time. In any given session, only one bleaching wave-length was studied in this way at eight or so different levels. For λ_1 (500 nm) the light was sufficient to study levels which left only 25 % unbleached, but for λ_2 (575 nm) levels which left 50 % unbleached were never quite achieved. Because we wanted to cover the entire measurable range in each experimental session and subject perseverance limited the number of levels which could be studied in any session, the size of the intervals between different bleaching levels studied was different for the two wave-lengths. Each experiment was repeated six times for each of the two wave-lengths for a total of twelve separate sessions each on a separate day.

Fig. 5 shows the results of these experiments. The plotted points are individual measurements. In this Figure, the ordinate scale shows P as a function of the log of the quanta sec^{-1} cm^{-2} of retina at the cornea. For the two monochromatic wave-bands, this abscissa scale is self-explanatory, but for the 10 sec 'white' flashes the values in effective quanta at 493 nm have been calculated in a way to be discussed presently. The continuous curve drawn through the two monochromatic steady-state bleaches have been fitted by computer to eqn. (7) where (τ = 1·0) and β and ($\alpha\gamma t_0$) were adjusted to minimize the sum of the squared deviations of the measurements from the curve.

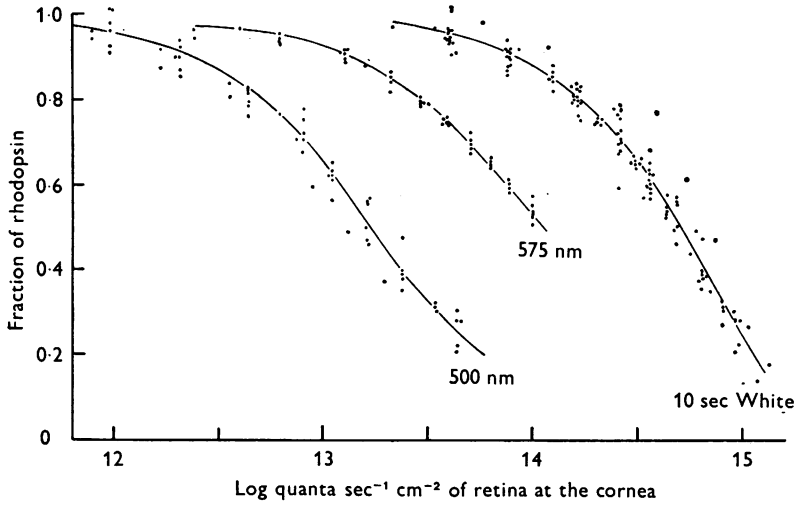

Fig. 5. The two left-hand curves describe the amount of rhodopsin present under steady-state bleaching by monochromatic lights of different intensities and of respective wave-lengths 500 and 575 nm. The continuous curve through the 500 nm results was generated by computer to minimize the sum of squared deviations and is described by eqn. (7) with $-\log(\alpha\gamma t_0)$ = 13·1358 and β_{500} = 0·795. The smooth curve through the 575 nm results is similarly obtained with β_{575} = 0·088 and $-\log(\alpha\gamma t_0)$ = 14·0595. The right-hand curve shows the results obtained by 10 sec white bleach and the curve through the points is the solution to eqn. (9) which minimizes the sum of squared deviations with $-\log(\alpha\gamma)_{max}$ = 15·7466 and $\beta(\lambda_{max})$ = 0·8. Subject B.

Fig. 5 plots results at the cornea. At λ_1 = 500 nm, the estimate of $\log_{10}\tau$ = −0·161, at λ_2 = 575 nm, it is −0·117. Reading from the curves the value for I_1 and I_2 that give P = 0·5, it is found that $(I\tau)_2/(I\tau)_1$ = 9·665. The 493 nm λ_{max} rhodopsin nomogram yields a value for α_{575} = 0·0876. This leads to an estimate of β_{500} = 0·653 in fair agreement with the results obtained by the other methods.

D. Curve fitting. The monochromatic steady-state bleaching results in Fig. 5 can be used individually to estimate the value of $\beta(\lambda)$, assuming that eqn. (7) describes the bleaching and regeneration of human rhodopsin *in vivo*. This assumption is more important in this context than it was in the estimate from eqn. (8) because in the derivation of that equation the terms on the right side of eqn. (7) [i.e. the regeneration terms] cancel. The curves drawn through the results in Fig. 5 were fitted by the computer to eqn. (7) selecting the value of $(\alpha\gamma t_0)$ and $\beta(\lambda)$ which minimized the sum of the squared deviations. These values are $-\log_{10}(\alpha\gamma t_0)_{500} = 13{\cdot}1358$ and $\beta_{500} = 0{\cdot}795$. At $\lambda = 575$ nm the best fit was achieved with 14·0595 and 0·088 respectively.

E. White light 10 sec bleaches. In a 10 sec exposure to the fully dark adapted eye, it has been shown (Rushton, 1956*a*; Ripps & Weale, 1969*b*; Alpern, 1971) that no rhodopsin regeneration occurs. For monochromatic lights the kinetics under these circumstances are described by eqn. (4), but if the light has a broad spectral distribution then the equation is

$$-\frac{dp}{dt} = \frac{\gamma}{c_D l}\int_0^\infty I(\lambda)\tau(\lambda)[1-e^{-k(\lambda)\beta(\lambda_{max})p}]d\lambda. \qquad (9)$$

Eqn. (9) has no formal solution. The spectral distribution of the bleaching light has been measured at fifteen wave bands in the spectrum, so that eqn. (9) could be solved by the Runge–Kutta method.

The abscissa scale in Fig. 5 for the 10 sec white light bleaches is in units of the logarithm of the number of rhodopsin effective quanta $cm^{-2}\,sec^{-1}$ at the cornea, for the fully dark adapted eye ($p = 1{\cdot}0$) $\lambda_{max} = 493$ nm.

The continuous curve drawn through the results of the 10 sec white light exposures in Fig. 5 is the computer solution to eqn. (9) which minimizes the sum of the squared deviations after correction for losses in the eye media in keeping with the results expressed there in $h\nu$ $cm^{-2}\,sec^{-1}$ at the cornea. This curve has as its two constants $-\log_{10}[\alpha\gamma](\lambda_{max}) = 15{\cdot}7466$, $\beta(\lambda_{max}) = 0{\cdot}8$. This gives $\beta_{500} = 0{\cdot}782$, in good agreement with the other estimates.

Curve fitting the 10 sec 'white' light results as well as the monochromatic steady-state measurements yield values for β_{500} in good agreement with those obtained by eqns. (1), (5) and (8). Unfortunately, there is a fair amount of imprecision in these estimations by curve fitting. The difficulties are examined in detail in Appendix II where it is shown that these methods are inherently insensitive to a relatively wide range of values for β even when, as is here not always the case, the variability in the experimental data is small.

PART III. THE PHOTOSENSITIVITY OF RHODOPSIN *IN VIVO*

The final part of this paper is devoted to the determination of the velocity constant of bleaching human rhodopsin, i.e. its *in vivo* photosensitivity. *In vitro* estimates of the photosensitivity of cattle rhodopsin give convergent results in the range $\log_{10}[\alpha(\lambda_{max})\gamma] = -15{\cdot}9978$ to $-15{\cdot}9627$ cm^2/chromophore (Dartnall, 1968). How do such measurements compare with human rhodopsin in the living eye? They are too small by a factor of 2·2–3·5!

The calculation of photosensitivity from the experimental results described in Part II is straightforward once the density of rhodopsin in the rods is known; almost all the equations have already been derived. The estimation has been made in three different ways.

A. Initial bleaching rate. Upon sudden exposure of the dark adapted retina to a monochromatic light λ, the initial rate of bleaching, X^{-1} is given by eqn. (4) by setting $p = 1{\cdot}0$. It follows that

$$\alpha(\lambda)\gamma = \beta(\lambda)/XI(\lambda)\tau(\lambda)[1-e^{-\beta(\lambda)}], \qquad (4a)$$

in which all terms on the right are known. In the experiment illustrated in Fig. 3, $\log_{10}(\alpha\gamma)_{500}$ can be calculated directly from the information in Table 1 by substitution in eqn. (4*a*) ($\beta_{500} = 0{\cdot}86$). The result is $-15{\cdot}37$. The geometric mean (± 1 S.E. of mean) of the fifteen repetitions of this experiment (using the mean value of $\beta_{500} = 0{\cdot}846$) gives $\log_{10}(\alpha\gamma)_{500} = -15{\cdot}466 \pm 0{\cdot}041$. A second subject repeated this experiment 5 times. The mean β_{500}, 0·838, yields the value of $\log_{10}(\alpha\gamma)_{500} = -15{\cdot}40 \pm 0{\cdot}107$. The third subject was available for only one experiment (Fig. 4); the value obtained was $\log_{10}(\alpha\gamma)_{500} = -15{\cdot}62$ ($\beta_{500} = 0{\cdot}64$).

B. Steady-state bleaching. The equilibrium measurements in Fig. 5 at $\lambda = 500$ nm may also be used to determine $(\alpha\gamma)_{500}$ with the help of eqn. (7). This equation assumes rhodopsin regeneration *in vivo* follows monomolecular kinetics with a time constant t_0. Simple exponential curves fit the measurements of rhodopsin regeneration after long bleaches in this subject as well as they do those of subjects previously described (Alpern, 1971) and this assumption therefore seems reasonable. Thirty-three regeneration curves following long full bleaches gave $t_0 = 374 \pm 13{\cdot}6$ sec (mn ± 1 S.E. of mean). (This is slightly faster than $387 \pm 10{\cdot}8$ sec, the value previously (Alpern, 1971) reported for a different subject.) This value may be used in conjunction with the results in Fig. 5 to compute $(\alpha\gamma)_{500}$, correcting, as before, for losses in the eye media, it is $\log_{10}(\alpha\gamma)_{500} = -15{\cdot}445$. The equilibrium bleaches in Fig. 5 at $\lambda = 575$ nm also give an estimate of $\log_{10}(\alpha\gamma)_{500}$ in a similar way using the nomogram to obtain

$\alpha_{575}/\alpha_{500}$. After correcting for losses in the eye media it is $-15{\cdot}468$. For a second subject two repetitions at $\lambda = 575$ nm give a value $-15{\cdot}360$ (in agreement with $-15{\cdot}40$ obtained for him in the experiment described above).

C. White light bleaches. These photosensitivity estimates are the first made of human rhodopsin *in vivo* with monochromatic lights. The solution of eqn. (9) fit to the white light results in Fig. 5 also provides an estimate of $\log_{10}(\alpha\gamma)_{500}$. It is $-15{\cdot}558$, a result somewhat less than the estimates made with monochromatic bleaching lights, but perhaps not unreasonably so given the differences in the assumptions and the imprecision of the measurements.

DISCUSSION

Generality of the results. The major results of this work concern the visual pigment in a circular patch of retina 5° in diameter, centred 18° from the fovea along the horizontal meridian in the temporal retina of a single subject B. The results indicate that this pigment is human rhodopsin, that it is the visual pigment which serves the night vision of this patch of his retina, that it is present in considerable density [$\beta(\lambda_{max}) = 0{\cdot}8$] and that its peak wave-length photosensitivity in the retina exceeds that of cattle rhodopsin *in vitro* by a factor of 2·2–3·5.

Fewer observations of a similar kind have been made on three other subjects, which suggests that these results are by no means peculiar to subject B. The results of all these experiments have been collected together and are summarized in Table 2.

Up until now in this paper, the density has been expressed in natural logarithmic units and the peak wave-length photosensitivity has been expressed in units of the chromophoric extinction coefficient [cm²/chromophore] because the respective quantities expressed in this way follow more directly from theoretical considerations. Unfortunately, these are not the most frequently employed units and in Table 2 the density values have been converted to common (instead of Napierian) logarithmic units and peak wave-length photosensitivity to units of the molar absorbance coefficient [$\epsilon_{max}\gamma$, l./cm mole] as is more customary. [$\epsilon(\lambda) = 2{\cdot}6165(10)^{20}\alpha(\lambda)$.] The values in Table 2 are given at the retina after correcting for losses in the eye media.

The demonstration that the density of human rhodopsin *in vivo* is about the same as that of chlorolabe in the green cones of the protanope (Rushton, 1963*a*, *b*; Miller, 1972) and of erythrolabe in the red cones of the deuteranope (Miller, 1972; King-Smith, 1973*a*, *b*) is in good accord with the microspectrophotometric observations of Dobelle, Marks & MacNichol (1969). They found that '...the pigment density in primate rods and cones is similar and...comparable to the densities for frog rods and fish cones'. Furthermore, their minimum estimate is 0·3, remarkably close to the results tabulated in Table 2.

TABLE 2. Optical density $\log_{10}(I_i)/(I_t)$ and photosensitivity ($\epsilon_{max}\gamma$) of human rhodopsin *in vivo* at the λ_{max} (493 nm)

Subject	Method	N	$\log_{10}(I_i)/(I_t)$	($\epsilon_{max}\gamma$) l. (cm mole)$^{-1}$	
	Transmissivity difference eqn. (1) $s = 0$	33	0·34 ± 0.001		
	Initial bleaching rate eqn. (5)	15	0·376 ± 0·109	91,640 ± 8310	eqn. 4(*a*) $\lambda = 500$ nm
	Initial bleaching rate eqn. (5)	15		86,801 ± 8748	eqn. 4(*a*) $\lambda = 585$ nm
B	Monochromatic equilibrium 575/500 eqn. (8)	6	0·290		
	Curve fitting				
	$\lambda = 500$ eqn. (7)	6	0·353	96,010	
	$\lambda = 575$ eqn. (7)	6		91,170	
	White light 10 sec bleach curve fitting eqn. (9)	14	0·347	74,080	
	Transmissivity difference eqn. (1) $s = 0$	9	0·444 ± 0·027		
E	Initial bleaching rate eqn. (5)	5	0·372	107,650 ± 23,502	eqn. 4(*a*) $\lambda = 500$ nm
	Initial bleaching rate eqn. (5)	4		104,552 ± 36,617	eqn. 4(*a*) $\lambda = 585$ nm
	Curve fitting $\lambda = 575$ eqn. (7)	2		116,840	
J*	Initial bleaching rate eqn. (5)	1	0·284	64,190	eqn. 4(*a*) $\lambda = 500$ nm
	Initial bleaching rate	1		62,770	eqn. 4(*a*) $\lambda = 585$ nm
D	Transmissivity difference eqn. (1) $s = 0$	1	0·350		

* Measured at $\lambda = 555$ nm

That rhodopsin in the rods, absorbs more than half the 510 nm quanta incident on the retina points up a major error in the calculation of Hecht, Shlaer & Pirenne (1942) who drew the inference that the absorption of a single quantum could excite a human rod, in part, from the estimate that 'at most' only 20% of such incident quanta would be absorbed. That inference drawn from this calculation applied to their own data can no longer be considered strong, but it remains firm when applied to the elegant results of Stiles (1939). His measurements are sufficient to establish this with high probability even if all of the quanta incident on the retina were absorbed as Brindley (1960) has already pointed out.

Of the various estimates of density, the greatest precision was obtained from the results using eqn. (1). However, this estimate provides only a lower limit assuming that the fraction of measuring light which does not go through rhodopsin (s) is negligible. The next most precise estimate for the principle subject B was that using eqn. (5). If the difference between these two values is attributed exclusively to the fraction of stray light, then s at 570 nm can be estimated from eqn. (1). The result is 0·0816. This value is a good deal less than King-Smith's (1973*b*) estimation for stray light in the measurement of foveal erythrolabe with a nearly identical instrument.

This difference is probably due to the rather large contribution to stray light made by specular reflexion at the fovea pit, a contribution absent in the 18° peripheral retina under study here. This is the only obvious source of stray light seen when the photomultiplier tube is replaced by the experimenter's eye in aligning the subject and it must certainly be the major source of stray light in King-Smith's measurements.

To test this interpretation, King-Smith's (1973*b*) method was used in an attempt to obtain the density of rhodopsin and the amount of stray light in the peripheral retina. Unfortunately the experiments were inconclusive. The difficulty is that differences in fundus reflectivity in full dark adaptation and after full bleaching must be obtained in two parts of the spectrum, for one of which the fundus reflexion coefficient is a good deal higher than for the other. For foveal erythrolabe King-Smith used 620 and 560 nm, the respective fundus reflexion coefficients of which are about in the ratio of 3·5 to 1. But for the best wave-length combinations for studying rhodopsin (535 and 585 nm) that ratio is only 1·5 to 1. Furthermore, the presence of coloured bleach products, notably transient orange which absorbs differentially at these two wave-lengths, further confounds the application of King-Smith's method to the measurement of the density of rhodopsin and our attempts to obtain consistent and reliable data were unsuccessful.

The density estimate obtained by eqn. (1) may be erroneously high because that fraction of the measuring light which goes through cones (about 4% of the total number of photoreceptors in this region of the retina, if Østerberg's (1935) measurements apply to these subjects) con-

tributes to the results as if it were traversing rhodopsin and not as if it were stray light. That this is not a serious source of error in the present work is suggested by the very good agreement found between the action spectrum for a 10 % bleach and the action spectrum of night vision shown in Fig. 2.

Neither of these action spectra is well fit by the absorption spectrum of a 493 nomogram pigment assumed to be in density 0·35 in the eye. The difficulty is that it is technically impossible to apply exact corrections for eye media losses for the observer whose night vision is under study. To minimize the problem it is advisable to use rod spectral sensitivity measurements obtained on subjects in whom the eye's lens has been removed and replaced by a lens selected for each wave-length to produce a sharp image on the retina (taking into account the transmission of that lens at that wave-length). The most accurate published data of this kind were made in this laboratory by Tan (1971). The continuous lines between the brackets in Fig. 6 show the mean ± 1 S.E. of mean of his results on ten such subjects. The continuous curve represents the normalized values of the log of the incident quanta for constant absorption as a function of wave-length provided by Dartnall nomogram assumed in (common logarithmic) density 0·35 at the λ_{max} (493 nm) in an aphakic eye in which the residual ocular media has the transmission characteristics found in human (lensless) eyes by Boettner & Wolter (1962). The fit is neither so good as to provide a compelling argument that night vision in these subjects is mediated by a nomogram pigment with $\lambda_{max} = 493$ nm, nor so bad as to exclude that possibility, given the familiar individual differences in eye media transmissivity. The fit can be improved somewhat for wave-lengths smaller than 490 nm by using Crescitelli, Pearlman & Dartnall's (1974) more recent results on human rhodopsin which are narrower in this part of the spectrum than the nomogram. For wave-lengths longer than 490 nm the human results are fit very well by the nomogram, however, and it seems pointless to press the comparison to this degree of refinement given the uncertainty of the transmissivity correction.

There is remarkable agreement between the estimates of the photosensitivity of human rhodopsin *in vivo* given by Rushton (1961), by Ripps & Weale (1969*a*), by Alpern (1971) and by Rushton & Powell (1972) using results from different instruments in different laboratories on different subjects. But these estimates are all much larger than either the *in vitro* (Dartnall, 1972) or the *in situ* (Hagins, 1954, rabbit; Baumann, 1965, frog; Dowling & Ripps, 1971, skate) estimates. The possibility that these differences are due to errors in assumptions made necessary by using 'white' bleaching lights, as suggested by Dartnall (1972), is excluded by the present results (cf. Appendix I) which show, if anything, a lower

sensitivity with white light bleaches than with monochromatic light (Table 2).

There are both classical (Braude, 1950) and quantum mechanical (Kauzmann, 1957) ways of estimating the theoretical upper limit to the *extinction coefficient* of rhodopsin which yield values very close to, if not actually lower than, the *photosensitivity* values of human rhodopsin *in vivo* shown in Table 2. One (not very likely) interpretation is that human rhodopsin *in vivo* has a quantum efficiency of bleaching very close to, if

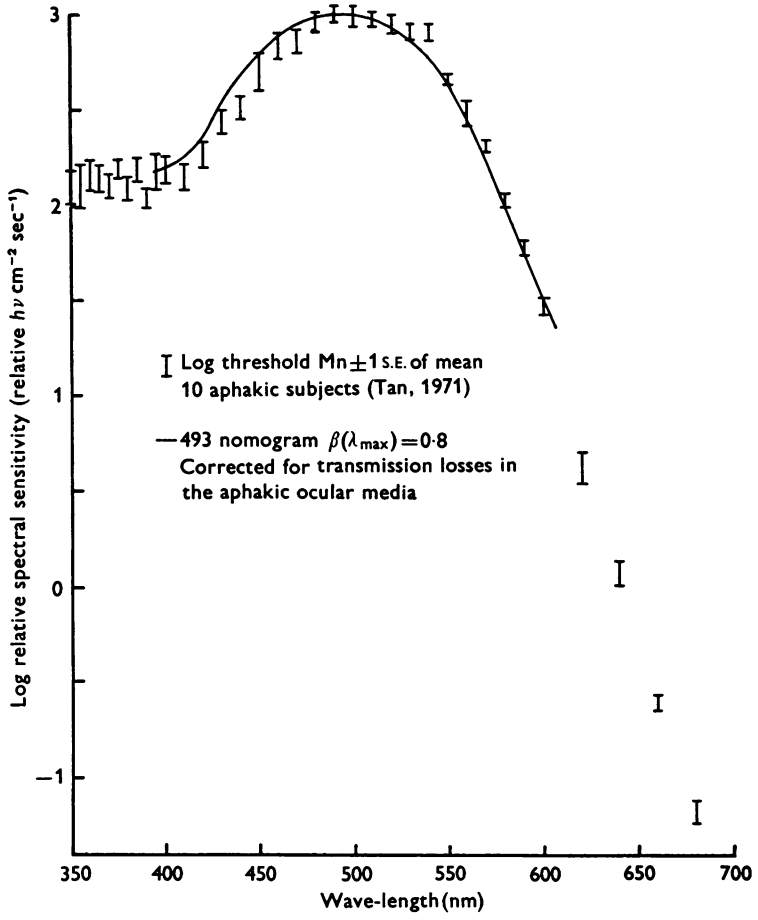

Fig. 6. The rod spectral sensitivity of 10 aphakic subjects measured in the 15° temporal retina on the horizontal meridian with a rectangular test 1 × 3° by Tan (1971). The brackets define the geometric mean ± 1 S.E. of mean. The continuous curve shows the normalized values of the log of the quanta incident at the cornea required for constant absorption in a vitamin A_1 nomogram pigment with λ_{max} = 493 nm assumed to have a density of 0·35 with media losses of the aphakic eye (Boettner & Wolter, 1962).

not larger than, unity. Two other possibilities have been suggested by Rushton (1956*a*, *b*, 1963*b*):

(1) *Orientation of the pigment in the rods.* This should increase the *in situ* estimates by 50% over the unoriented rhodopsin studied by *in vitro* methods. It brings Hagins' (1954), Baumann's (1965) and Dowling & Ripps' (1971) data into good accord with the *in vitro* results presuming that frog rhodopsin is 1·4 times more sensitive than that of rabbit. But all these *in situ* estimates are still 2–3 times too small compared with human rhodopsin *in vivo*.

(2) *Optical funneling of the light by the inner segments in its passage through the retina to reach the rhodopsin in the outer segments.* Because the first kind of Stiles–Crawford Effect in man (and frog) is a property of cones alone (Flamant & Stiles, 1948; Donner & Rushton, 1959) it is sometimes supposed that there is no optical funneling in rods. However, Brindley (1966) provided clear evidence that human rods funnel the incident light, albeit less strongly than cones. He suggests that the relative luminances of the low force deformation phosphene (68/28) seen respectively by rods and by cones (with fully dilated pupil) can be explained by the stronger funneling characteristics of the latter. If so (and if the high *in vivo* photosensitivity of rhodopsin we find is due in part to funneling in the rods), one might expect the photosensitivity of cone pigments *in vivo* to be 0·38 $\log_{10}$ units higher still than that reported here for human rhodopsin. Rushton's (1963*b*) estimate of $\log_{10}(\alpha_{552}\gamma)$ of chlorolabe in the protanope ($-15{\cdot}155$) is in excellent agreement with this expectation.

Whether the differences between the results of the *in situ* and the *in vivo* photosensitivity measurements are to be attributed to differences in optical properties of receptors in eyes removed from the living body or to species differences remains to be shown. Weale's (1964) results certainly support the latter interpretation, but those characteristics of the human retina which would give its rhodopsin so much higher photosensitivity than that of rabbit, frog and skate, are by no means obvious and the question merits a good deal of further study.

APPENDIX I

THE MEASUREMENT OF RHODOPSIN PHOTOSENSITIVITY WITH A BROAD SPECTRAL DISTRIBUTION AND VISUAL PHOTOMETRY

Previous estimates of the photosensitivity of human rhodopsin *in vivo* by Rushton (1956*a*, 1961), Ripps & Weale (1969*a*), Alpern (1971) and by Rushton & Powell (1972) all used bleaching lights of broad spectral distribution (i.e. 'white' light) and visual photometry. In photometry the human eye is used to determine

$$I'\tau' = \int_0^\infty I(\lambda)\tau(\lambda)V'(\lambda)\,\mathrm{d}\lambda,$$

by subjective matching such as establishing the scotopic identity of the broad spectral distribution to a monochromatic light of known quantum content I' and ocular media transmissivity τ'. $V'(\lambda)$ is the quantized C.I.E. scotopic spectral sensitivity curve.

Alternatively the match may be made by the photopic eye with a visual photometer and the retinal illuminance in scotopic trolands (E) calculated by straightforward procedures outlined in Wyszecki & Stiles (1967). $I' = KE$, is then the equivalent effective quanta at the $\lambda_{\max}$ (in visual photometry this is 507 nm) $K = 5{\cdot}25(10)^8$ *quanta* $\mathrm{sec}^{-1}\,\mathrm{cm}^{-2}$ (scotopic td)$^{-1}$ and

$$\tau' = \frac{\int_0^\infty I(\lambda)\tau(\lambda)V'(\lambda)\,\mathrm{d}\lambda}{\int_0^\infty I(\lambda)V'(\lambda)\,\mathrm{d}\lambda},$$

(which for tungsten light of spectral distribution of C.I.E. Illuminant A is 0·227).

If $V'(\lambda)$ were based upon a rhodopsin density in the rods so small that the shape of its absorption spectrum remained essentially unchanged over the range from $p = 1{\cdot}0$ to $p = 0$, the integral in eqn. (9) could be treated as a time independent constant instead of a function $p(t)$. $I'\tau'$ could then be directly substituted for

$$\int_0^\infty I(\lambda)\tau(\lambda)\,[1-\mathrm{e}^{-k(\lambda)\beta(\lambda_{\max})p}]\,\mathrm{d}\lambda,$$

into eqn. (9). For the initial condition $p = 1{\cdot}0$ when $t = 0$ the solution is

$$(\mathrm{e}^{p\beta}-1) = (\mathrm{e}^{\beta}-1)\mathrm{e}^{-\alpha\gamma I'\tau' t}. \qquad (10)$$

In the present paper it was found, however, that in the rods rhodopsin has a measurable density [$\beta(\lambda_{\max}) \simeq 0{\cdot}8$] and the question naturally arises as to how erroneous the estimates of the photosensitivity of human

rhodopsin *in vivo* will prove to be when made from the simplifying assumption implicit in using visual photometry. The assumption is equivalent to assuming that the change in colour of the source due to the narrowing of the absorption spectrum with bleaching is negligibly small.

Rushton (1961), Ripps & Weale (1969*a*), Alpern (1971) and Rushton & Powell (1972) all measured the intensity of white light pulses required to bleach human rhodopsin *in vivo* to e^{-1}. Their respective values at the cornea were 6·94, 6·90, 7·0, and 7·0 log scotopic td sec. If the value of $\beta(\lambda_{max})$ in these cases were such that higher order terms in the series expansion of $e^{-\beta(\lambda_{max})}$ can be neglected, then these data yield estimates of the λ_{max} photosensitivity of human rhodopsin with the above assumption of $3{\cdot}667(10)^{-16}$, $4{\cdot}02(10)^{-16}$, and $3{\cdot}193(10)^{-16}$ (cm^2/chromophore)

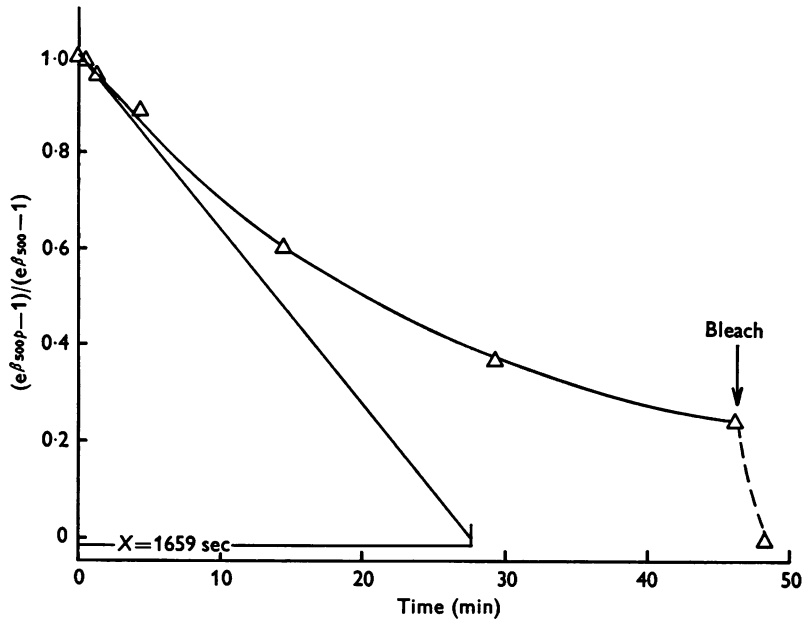

Fig. 7. Calculation of photosensitivity of cattle rhodopsin in hydroxylamine from the measurements of Wald (1954). The ordinate shows $(e^{\beta(500)p}-1)/(e^{\beta(500)}-1)$ as a function of time after the onset of white light of 324 ml. viewed through a 3 mm artificial pupil by a water eye backed by a cuvette of digitonin micelle suspension of cattle rhodopsin in 0·1 M hydroxylamine. The change in extinction at 500 nm was monitored by a separate measuring beam. The curve is a simple exponential function of time. The slope at $t = 0$ was found to be $1/1659$ sec^{-1}.

respectively [or 96,700, 106,000 and 84,000 l./cm mole]. If we assume the rhodopsin densities in these subjects were equivalent to those found in the present work ($\beta_{max} \simeq 0{\cdot}8$) but still use the photometric simplification

introduced in deriving eqn. (10), then these respective estimates become $4{\cdot}67(10)^{-16}$, $5{\cdot}12(10)^{-16}$, and $4{\cdot}07(10)^{-16}$ (cm^2/chromophore) respectively or (123,000, 135,000 and 107,000 (l./cm mole)). In either event, given the observed individual differences these values are in reasonable agreement with those tabulated in Table 2. Evidently the implicit assumption of a constant absorption spectrum used with white light bleaching and visual photometry does not introduce major errors in the estimation of the photosensitivity of human rhodopsin *in vivo*.

This conclusion is substantiated by the results of Dowling & Ripps (1971) on the photosensitivity of skate rhodopsin. Using flashes of 'white' light they measured the photosensitivity of rhodopsin in the open eye cup. They found $\log(\alpha\gamma)_{max} = 7{\cdot}2$ log scotopic td sec. Following the procedure just outlined this can be converted to $(\epsilon\gamma)_{max} = 31{,}500$ l./cm mole, in excellent agreement with Hagins' *in situ* estimate for the rabbit (27,000) and Baumann's *in situ* measurements on frog (38,100).

The usefulness of the photometric assumption and eqn. (10) can be illustrated by employing them to derive the photosensitivity of cattle rhodopsin *in vitro* from the results of a 'white' light bleaching experiment (Wald, 1954). A glass model eye filled with water was placed directly in front of a cuvette of cattle rhodopsin solution (in 0·1 M hydroxylamine). At time $t = 0$ the ensemble was exposed to an opal glass of 324 ml. luminance which the eye viewed through a pupil 3 mm in diameter. Wald followed the change of extinction at $\lambda = 500$ nm for 47 min after which time the remaining rhodopsin was bleached by a bright light. (The hydroxylamine assured that only changes in rhodopsin were studied.) The results are shown in Fig. 7. Wald plotted his results in terms of extinction at 500 nm (at $t = 0$, β_{500} was 0·576), but in Fig. 7, $(e^{\beta(500)p} - 1)/(e^{\beta(500)} - 1)$ is shown as a function of t.

The continuous curve drawn through these results has been fitted by computer to the equation of a single exponential curve by adjusting its time constant to minimize the sum of the squared deviations. By direct analogy with the analysis of the results in Figs. 3 and 4 the slope of this smooth curve at $t = 0$.

$$-\mathrm{d}\,[(e^{\beta p} - 1)/(e^{\beta} - 1)]/\mathrm{d}t = 1/X = \alpha(\lambda)\gamma I'\tau'.$$

Since X can be measured and I' is known, assuming a value of τ' will permit an estimate of photosensitivity of cattle rhodopsin *in vitro*, if the 'white' light assumptions are valid in this context.

324 ml. viewed through an artificial pupil 3·0 mm in diameter is equivalent to $7{\cdot}29(10)^3$ td. The colour temperature of the lamp was 3240° K before absorption by the opal glass (G. Wald, personal communication). The log of the factor needed to convert from photopic to scotopic td is

therefore 0·215 (Wyszecki & Stiles, 1967). This gives $1{\cdot}20(10)^4$ scotopic td. In Fig. 6 $X = 1659$ sec; assuming that 95% of the incident light reached the rhodopsin $\log(\alpha\gamma)_{500} = -15{\cdot}98642$ (after correcting for the fact that I' in eqn. (10) refers to equivalent quanta at 507 nm). Hence $(\epsilon\gamma)_{500} = 27{,}000$ l./cm mole. This value falls precisely in the range of the latest measurements of the peak wave-length photosensitivity of cattle rhodopsin in 'solutions' of hydroxylamine (26,300–28,500 l./cm mole) according to Dartnall (1968). Because of uncertainties in correcting for losses in the eye media this agreement may be partly fortuitous. However, it none the less suffices to illustrate the value of visual photometry and eqn. (10) as a simplification of eqn. (9) when 'white' light is used to estimate rhodopsin photosensitivity.

APPENDIX II

CURVE FITTING

The fitting of curves in this paper has been achieved by minimizing the sum of the squared deviations from a smooth function for three different experimental situations: (i) steady-state bleaching with monochromatic lights of varying quantum content; (ii) bleaching by white light flashes (10 sec duration) of different intensities exposed to the fully dark-adapted eye and (iii) the time change in the fraction of rhodopsin present in response to a step of monochromatic light exposed to the dark adapted eye.

(i) *Steady-state monochromatic bleaching*

Eqn. (7) has two unknown parameters $I_0 = 1/\alpha(\lambda)\gamma t_0$ and $\beta(\lambda)$; corresponding to every pair of such values is a unique curve which shows the relation between P and $[I(\lambda)]$. Although the equation cannot be solved directly for $P[I(\lambda)]$ a simple numerical analysis method can be used to generate the error function $\chi^2[I_0, \beta(\lambda)] = \Sigma_{I(\lambda)}\{P_{obs} - P[I(\lambda)]\}^2$ in which P_{obs} represents the empirical measurements at a given steady-state intensity $I(\lambda)$ and $P[I(\lambda)]$ the steady-state level of P expected from eqn. (7) given a specific parameter pair $[I_0, \beta(\lambda)]$. Reasonably detailed plots of the error functions were made and the global minimum determined by a search routine (Chandler, 1965).

In the determination of the best fit it was found, unfortunately, that the two parameters trade off for one another in a systematic way. The major axis of the ellipses in Fig. 8*A* illustrates this 'tradeoff trough' for the condition $\lambda = 500$ nm. The ellipses themselves show approximate contours for $1{\cdot}5\chi^2_{min}$, $2\chi^2_{min}$ and $5\chi^2_{min}$ when $\log(\alpha\gamma t_0)$ is plotted as a function of $\beta(\lambda)$.

The continuous curve (1) in Fig. 8*B* shows a plot of the r.m.s. deviation

($\sqrt{\chi^2/N}$) as a function of Napierian density $\beta(\lambda)$ along the major axis of the ellipses in Fig. 8*A* between *c* and *b*. Despite the existence of an absolute minimum $\beta = 0{\cdot}8$ the tradeoff is exceedingly tight and the difference between the r.m.s. at $\beta = 0{\cdot}0$, $\log I_0 = 13{\cdot}227$ (r.m.s. = 0·0404) and that at $\beta = 0{\cdot}8$, $\log I_0 = 13{\cdot}136$ (r.m.s. = 0·0385) is very small. It must be concluded that the experimental data do not sharply discriminate between parameters which lie near the trough determined by the error surface over the parameter space.

A similar analysis has been performed on the steady-state results when bleaching with 575 nm. The error function plots obtained are quite similar to those found for $\lambda = 500$ nm and illustrated in Fig. 8*A*. The trade off between the two parameters was very similar to that shown in this Figure, but the results are not reproduced here. Instead only the variation of the r.m.s. as a function of β_{575} are plotted from the trench along the major axis of the ellipses as curve 2 in Fig. 8*B*. Once again no sharp discrimination between parameter pairs near the minimum can be made although the minimum r.m.s. value occurs at $\beta_{575} = 0{\cdot}088$.

Is this indeterminancy in curve fitting due to exceptional variability in these particular sets of data, or to an indeterminancy inherent in the theoretical curves $P[I(\lambda)\,|\,I_0, \beta(\lambda)]$? Almost certainly the indeterminancy is intrinsic, and unlikely to be removed with data of ordinary variability. Consider two *theoretical* curves described by the pairs of parameters which lie along the line *bc* of minimum r.m.s. in Fig. 8*A* [for example, the two equilibrium bleaching curves determined by $(\log I_0, \beta) = (13{\cdot}227, 0{\cdot}0)$ and $(\log I_0, \beta) = 13{\cdot}052, 1{\cdot}6)$]. Using a grid of $\log I$ values spaced evenly at 0·05 log units between $P = 0{\cdot}975$ and $P = 0{\cdot}025$, the r.m.s. difference between the curves,

$$\text{r.m.s.} = \{\Sigma_I[P(I\,|\,13{\cdot}227,\ 0{\cdot}0) - P(I\,|\,13{\cdot}052,\ 1{\cdot}6)]^2\}^{0{\cdot}5}\, N^{-0{\cdot}5},$$

was computed as a measure of their separation; it was found to be only 0·0254! Thus, attempts at distinguishing between the hypotheses $\beta = 0{\cdot}0$ and $\beta = 1{\cdot}6$ on the basis of curve fitting to equilibrium bleaches alone are unlikely to be conclusive. (Other pairs of curves along the trough in Fig. 8*A* yielded equally discouraging results.)

(ii) *White light* 10 *sec bleaches*

The error function plots of the 10 sec white light bleaches using eqn. (9) show the identical indeterminancy already described for the steady-state monochromatic bleaching lights. This is shown by curve 3 in Fig. 8*B* in which it is seen that the r.m.s. minimum is even less conspicuous than those obtained with steady-state monochromatic bleaching. The method makes no crisp distinction between densities over the range tested though

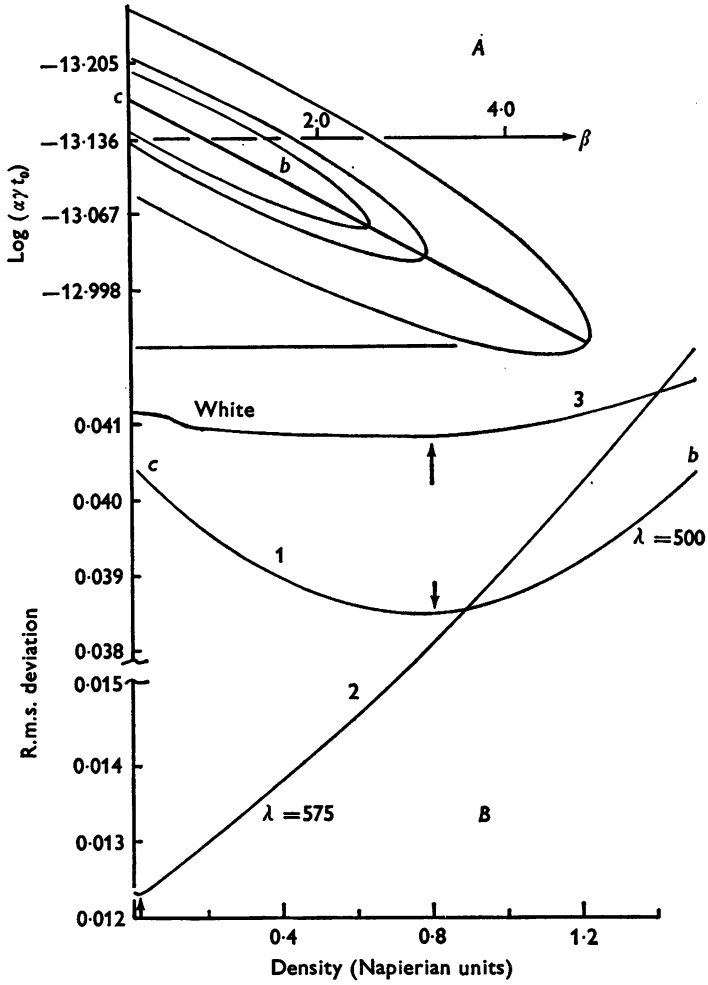

Fig. 8. *A*, the tradeoff between the photosensitivity and density for best fit to eqn. (7) for the steady-state monochromatic (λ = 500 nm) bleaching results in Fig. 5. The ordinate shows log $\alpha\gamma t_0$ while $\beta(\lambda)$ is plotted along the abscissa. The straight line through *bc* is the trough of minimum root mean square deviation and the variation of the r.m.s. deviation with density along the trough is shown by curve (1) in Fig. 8*B* (only over the range *c* to *b*). Note the minimum at β_{500} = 0·795 (arrow). The ellipses in Fig. 8*A* show contours of equal χ^2 around this trough of 1·5 χ^2, 2 χ^2, and 5 χ^2 respectively. Fig. 8*B* also shows the r.m.s. deviation as a function of density along similar troughs for steady-state bleaching at 575 nm as well as for the results of bleaching with 10 sec white light exposures fit to the solution of eqn. (9). The arrows in all three curves in Fig. 8*B* show the density at which the r.m.s. deviation is at a minimum. Subject B.

the minimum r.m.s. does, in fact, occur at $\beta_{500} = 0{\cdot}8$. The results for white light bleaches shown in Fig. 5 have also been well fit with the simplifying assumption introduced by visual photometry (and the resulting eqn. (10)), but in this case the minimum r.m.s. occurs at $\beta = 0{\cdot}4$. In view of the uncertainty of curve fitting no importance has been attached to this discrepancy.

(iii) *Initial rate of bleaching measured from pigment changes in response to a step of monochromatic light*

The routine use of a computer to fit curves of the form $p(t) = P + (1-P)e^{-t/Pt_0}$ [P is the value of p when $t = \infty$] to these data is based purely on the empirical observation that the equation describes the data with precision. On theoretical grounds a more consistent approach would be to solve eqn. (6) for the initial condition $p = 1{\cdot}0$ when $t = 0$. Unfortunately, this equation has no analytical solution and efforts to apply the Runge–Kutta method to these results quickly showed that the unspecified constants $\tau\gamma/c_D l$, t_0 and β could not be uniquely determined in this way. Each set of data does, however, specify a definite value of P and since at equilibrium

$$\frac{\gamma I(\lambda)\tau(\lambda)}{c_D l}\,[1-e^{-\beta(\lambda)P}] = (1-P)/t_0$$

the units of intensity can be defined such that

$$I''(\lambda) = \frac{\gamma I(\lambda)\tau(\lambda)}{c_D l} = \frac{(1-P)/t_0}{1-e^{-\beta(\lambda)P}}.$$

This reduces the number of unknown parameters to two, $\beta(\lambda)$ and t_0. In these units eqn. (6) becomes

$$dp/dt = -I''(\lambda)\,[1-e^{-\beta p}] + (1-p)/t_0.$$

The Runge-Kutta method was used to solve this form of eqn. (6) for six representative bleaches at $\lambda = 500$ nm and for one at 585 nm. (There is, in fact, no inconsistency in using the simpler procedure for the latter case since none of the estimates of rhodopsin density *in vivo* at $\lambda = 585$ nm is sufficiently large to justify the more complex eqn. (6) in this instance.)

This analysis yielded equally satisfactory fits to the data as those obtained with the less complicated procedure. The initial slope was always within measurement precision ($\pm 10\,\%$) of the estimate obtained by using the simpler $p(t) = P + (1-P)e^{-t/Pt_0}$. The parameter values β and t_0 which yielded best fits to individual runs were not unreasonable, but showed great variability from run to run. Data in the individual runs were not sufficiently accurate to use the curve fitting of the solution of eqn. (6) to estimate β with any precision and we therefore used the simpler equation to estimate the initial rate of bleaching.

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