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KINETICS OF RHODOPSIN BLEACHING IN THE ISOLATED HUMAN RETINA

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SUMMARY

1. Pieces of human retina were dissected from eyes enucleated because of malignant tumours. The isolated retinas were perfused by an ionic medium (36° C) and investigated spectrophotometrically.

2. Rhodopsin was identified on the basis of its difference spectrum. The maximum absorbance change on bleaching was about 0·1 (λ = 500 nm).

3. The process of bleaching was quantitatively analysed in terms of four slow reactions, viz. (*a*) conversion of metarhodopsin II into metarhodopsin III, (*b*) hydrolysis of metarhodopsin II into retinal and opsin, (*c*) decay of metarhodopsin III to retinal, and (*d*) reduction of retinal to retinol.

4. First-order rate constants for the reactions in 3 were 9×10^{-3} sec^{-1} (*a*), 3×10^{-3} sec^{-1} (*b*), 4×10^{-3} sec^{-1} (*c*), and 3×10^{-2} sec^{-1} (*d*).

5. With two simplified versions of the model summarized in 3 and 4, the description of experimental data was less accurate.

INTRODUCTION

This work is based on experiments with isolated human retinas in which the decomposition of light-exposed rhodopsin was measured spectrophotometrically. The study is confined to the slow steps of bleaching, i.e. to those reactions that start with the decay of metarhodopsin II. The kinetics of the slow decay reactions have recently been studied in the frog retina (Baumann, 1972; Baumann & Reinheimer, 1973). The reaction scheme put forward in these publications was also used to analyse the present measurements. The four reactions involved are: (1) conversion of metarhodopsin II into metarhodopsin III, (2) hydrolysis of metarhodopsin II into retinal and opsin, (3) decay of metarhodopsin III to retinal, and (4) reduction of retinal to retinol. The parameters for the kinetic equations of the scheme were computed from the data by a least squares method.

Two simpler schemes were also tested. The first ignored the direct hydrolysis of metarhodopsin II (cf. Ostroy, Erhardt & Abrahamson, 1966);

the second assumed a direct conversion of metarhodopsin III into retinol without the intermediary appearance of retinal (Cone & Brown, 1969). For both cases, however, the agreement between theory and data was less satisfactory than for the original model.

METHODS

Dissection. The retinas were dissected from eyes enucleated because of malignant tumours. The eyes were dark adapted before the operation which was carried out in dim white light. The anterior segment of the eye was covered with an opaque cap during the operation. The enucleated eyes were put into a light-tight vacuum flask filled with ice cold Ringer solution and transported from the eye clinics to our laboratory. Pieces of retina of approx. 60 mm^2 were dissected (from 1 to 4 hr after enucleation) under dim red light and inserted in the perfusion chamber. All the preparations came from those parts of the peripheral retina definitely not in contact with the tumour.

Perfusion. The preparations were perfused with an ionic medium containing NaCl (140 mM), KCl (2 mM), $CaCl_2$ (0·1 mM), $MgSO_4$ (0·1 mM), phosphate buffer (12 mM; pH = 7·4), and glucose (10 mM). Most of the experiments were carried out at temperatures near 36° C. The temperature was measured inside the perfusion chamber by means of a thermistor.

Spectrophotometry. The spectrophotometer was similar to that described in Baumann (1970), modified to allow stimulation of the retina by monochromatic light of variable intensity and duration. This light was used to bleach the preparation. A radiant flux detector (Hewlett-Packard 8334 A) was used for calibration.

RESULTS

Rhodopsin content of the preparations

The amount of rhodopsin present in the preparations was estimated on the basis of spectra like those in Fig. 1. Though scattering distorts the absorbance spectra, they clearly indicate the presence of two pigments, viz. haemoglobin and rhodopsin. The former can be identified by its Soret band, i.e. the pronounced absorption between 400 and 450 nm. This band does not disappear on bleaching.

Exposure of the preparations to a strong bleaching light is followed by absorbance changes in both the visible and the ultraviolet region of the spectrum. The time course of these changes will be described in detail below. After some 20 min (at 36° C), there is little further change of absorbance, and difference spectra like the one in Fig. 1*B* can be constructed. These difference spectra are typical rhodopsin spectra, and their height may be considered as a measure for the amount of pigment present in the unbleached preparations. In the peripheral retina of the living eye, Ripps & Weale (1969*a*) measured density changes due to rhodopsin bleaching and found maximum figures at 0·170 and at 0·195. In the retina of Fig. 1, the maximum absorbance change at 500 nm is about 0·1. On the

average, the maximum is (± S.D.) 0·083 ± 0·017 (twenty-seven pieces of retina). Clearly these figures are about half those given by Ripps & Weale, but *in situ* the light used to measure the rhodopsin density is reflected from the *fundus oculi* and passes twice through the retina. Thus the histogram of Fig. 2 suggests that many of the preparations contained the physiological amount of rhodopsin.

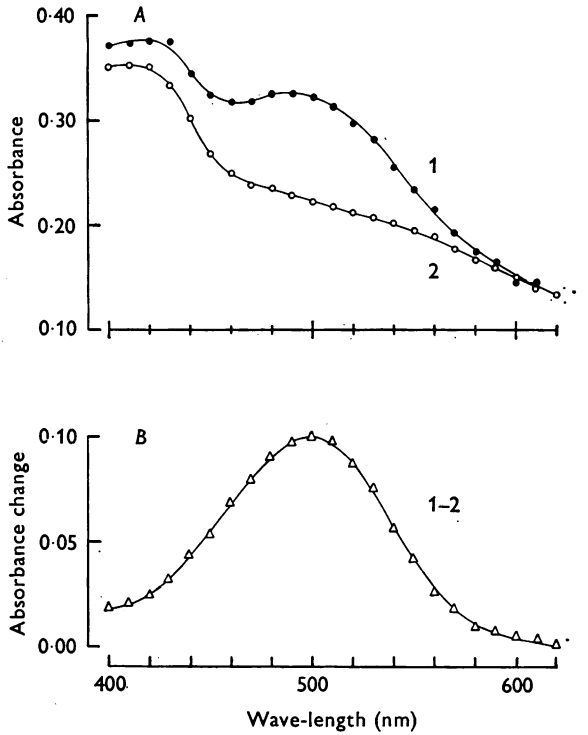

Fig. 1. *A*, absorbance spectra of an isolated human retina before (●, 1) and after 10 min exposure (○, 2) to blue-green light (λ = 502 nm) which bleached virtually all the rhodopsin. *B*, difference spectrum (△, 1–2) calculated from the data of *A*.

Intermediates and final products of bleaching

In the retina, the bleaching ends when all-*trans*-retinal has been reduced to all-*trans*-retinol. The latter has a well-defined absorption band with a maximum at 330 nm. Measurements in this region of the ultraviolet are difficult because the apparent absorbance due to scattering increases markedly with shorter wave-lengths. In addition, the turbidity of the preparations is not always constant but becomes greater towards the end of an experiment. Fortunately in a few experiments, this effect was in-

significant so that the absorbance changes due to the formation of retinol could be measured (Fig. 3). The gain of absorbance at 330 nm is smaller than the loss at 500 nm although the molar absorbance coefficient of retinol (λ_{max} = 328 nm) is known to be larger than that of rhodopsin

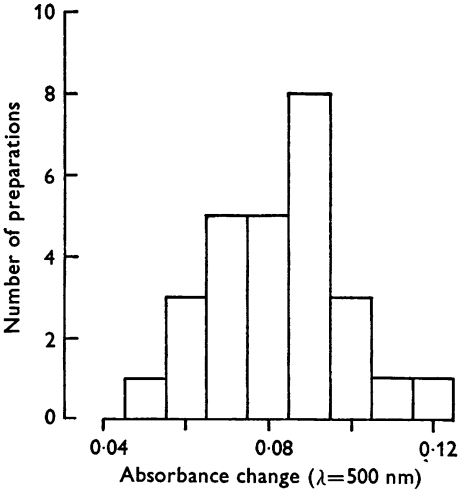

Fig. 2. Frequency histogram of the maximum absorbance changes on bleaching in isolated human retinas; total number of preparations 27.

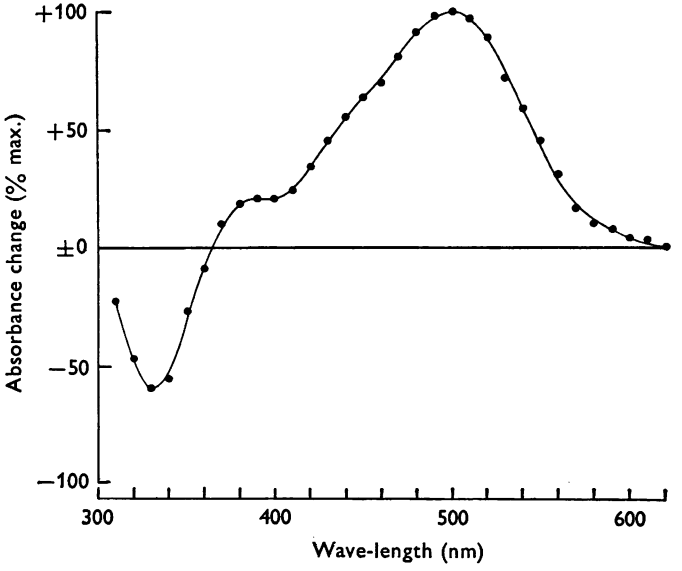

Fig. 3. Bleaching difference spectrum. Average data from two preparations normalized with respect to absorbance change at 500 nm. Absorbance losses shown as positive.

(λ_{max} = 500 nm). Apparently, the chromophores of these two pigments are differently orientated in the receptor cell. Rhodopsin molecules absorb light maximally if the electrical vector of the light is perpendicular to the rod outer segment axis. This is a result of the transverse orientation of the

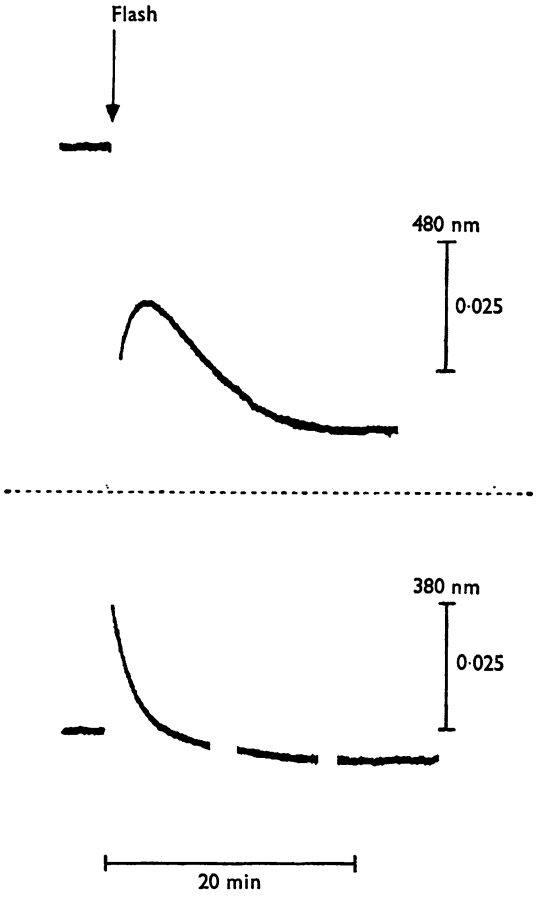

Fig. 4. Time course of absorbance changes in isolated human retinas after 10 sec exposure to the monochromatic bleaching light (595 μW/cm^2; λ = 502 nm). Figures at vertical bars denote measuring wave-length and absorbance, respectively. Interruptions of lower recording were caused by extraneous artifacts. Note that the recordings are from two different retinas differing in initial rhodopsin concentrations.

rhodopsin chromophore. In frog rods, retinol is aligned axially (Denton, 1959; Liebman, 1969; Kemp, 1973). If this applies also to human rods, then light travelling axially through the receptors (as in the present experiments) would be weakly absorbed by retinol (Fig. 3).

Before retinol is formed as the final product of bleaching, a number

of unstable intermediates appear. The temporary presence of the metarhodopsins II (λ_{max} = 380 nm) and III (λ_{max} = 465 nm) can be inferred from recordings of the type shown in Fig. 4. Exposure of the retinas to a bleaching flash is followed by a sudden drop of absorbance at wave-lengths preferably absorbed by rhodopsin (e.g. 480 nm). This drop is paralleled by an immediate rise of absorbance at 380 nm, reflecting the rapid conversion of rhodopsin into metarhodopsin II. Precursors of metarhodopsin II are not seen because of the time resolution of recording.

Metarhodopsin II decays to metarhodopsin III and presumably also to all-*trans*-retinal. The former substance reaches a maximum concentration after approx. 2 min. Thereafter it decays quite slowly (Fig. 4, top). A very similar time course of formation and decay of metarhodopsin III has been described in the intact human eye by Ripps & Weale (1969*b*) and by Alpern (1971).

The last intermediate to be considered is retinal, which occurs between the metarhodopsins and retinol. Though retinal is known to absorb strongly at 380 nm, the recording at the bottom of Fig. 4 yields little evidence for its presence. The main information about it comes from the mathematical analysis of the bleaching kinetics in the following section.

The kinetics of bleaching

The analysis of the bleaching kinetics makes use of the following reaction scheme

$$R \xrightarrow{k_0} A \xrightarrow{k_1} B \xrightarrow{k_3} C \xrightarrow{k_4} D$$
$$\qquad\qquad\quad \underset{\uparrow}{\llcorner\!\!\lrcorner} \; k_2$$

where R stands for rhodopsin, A for metarhodopsin II, B for metarhodopsin III, C for all-*trans*-retinal, and D for all-*trans*-retinol. The k's are first-order rate constants. k_0 is, in addition, a linear function of the light incident upon the retina. This scheme is essentially the one described by Baumann (1972). In this study, however, rhodopsin was included as one of the reactants, and all data were normalized with respect to its concentration before the bleach. This modification had to be introduced because of the 10 sec exposure to the bleaching light. During this period, noticeable fractions of intermediates decayed away, and the light period had to be considered separately from the subsequent dark period.

The scheme leads to the following set of simultaneous differential equations

$$\frac{dR}{dt} = -k_0 R, \tag{1}$$

$$\frac{dA}{dt} = k_0 R - (k_1 + k_2) A, \tag{2}$$

$$\frac{dB}{dt} = k_1 A - k_3 B, \tag{3}$$

$$\frac{dC}{dt} = k_2 A + k_3 B - k_4 C, \tag{4}$$

$$\frac{dD}{dt} = k_4 C. \tag{5}$$

When the bleaching light is on, k_0 has a finite value. When the bleaching light is off, $k_0 = 0$ and the rhodopsin level remains constant. The solution of (1) is, if the light is on and if $R = 1$ at the time $t = 0$,

$$R = \exp\{-k_0 t\}, \tag{6}$$

where t is the duration of the light exposure, k_0 is the product of the photosensitivity ($\alpha\gamma$) of rhodopsin and of the intensity (I) of the light incident upon the retina. The value of $\alpha\gamma$ for rhodopsin molecules in intact rods is $1{\cdot}5 \times 10^{-16}$ cm^2 (Rushton, 1956; Baumann, 1965). I was calculated from the irradiance at the retina of the monochromatic bleaching light (595 μW/cm^2; $\lambda = 502$ nm) to be $1{\cdot}51 \times 10^{15}$ quanta/sec.cm^2. The constant k_0 was therefore 0·226 sec^{-1}, and during the usual 10 sec exposure time, 90 % of rhodopsin was bleached, a figure confirmed by the spectrophotometric readings.

Immediately after the end of the light exposure, most of the bleached rhodopsin is present as metarhodopsin II but a fraction of this has already been converted into metarhodopsin III which in turn has partially decayed to retinal. In terms of the scheme this means that A will be smaller than 0·9 (the fraction of rhodopsin bleached) and that B and C (and probably also D) will be larger than 0. The exact proportions can be calculated after solving eqns. (2) to (5). However, the correct values for k_1, k_2, k_3, and k_4 cannot be obtained at this stage from the absorbance changes in the dark, since the exact mathematical description of the dark reactions themselves require well defined initial conditions.

Because of this dilemma, a preliminary analysis of the data was attempted by ignoring the finite duration of the exposure period. It was assumed that no decay of A (metarhodopsin II) occurs during the bleach and that B, C, and D are 0 at the beginning of the dark period. As $k_0 = 0$ in the dark, eqn. (2) reduces to

$$\frac{dA}{dt} = -(k_1 + k_2) A. \tag{2a}$$

If $A = A_0$ and $B = 0$ at time $t = 0$, the solution of eqn. (3) in connexion with eqn. (2a) becomes

$$B_{\text{approx.}} = A_0 \frac{k_1}{k_3 - (k_1 + k_2)} (\exp\{-(k_1 + k_2)t\} - \exp\{-k_3 t\}), \tag{7}$$

where the subscript approx. denotes the approximate nature of this expression. Equation (7) was used for a tentative computation of k_1, k_2 and k_3. Mathematically, this is done with a least-squares method, i.e. the constants are varied until the error mean square becomes minimum and the function (eqn. 7) fits the data (Fig. 4, top) in an optimum way. The fourth constant, k_4, can be found when, by an analogous procedure, recordings at 380 nm are analysed (for further details see Baumann, 1972). The numerical figures of the preliminary constants are: $k_1 = 8 \times 10^{-3}\,\text{sec}^{-1}$, $k_2 = 3{\cdot}5 \times 10^{-3}\,\text{sec}^{-1}$, $k_3 = 3 \times 10^{-3}\,\text{sec}^{-1}$, and $k_4 = 4 \times 10^{-2}\,\text{sec}^{-1}$.

The next step is the integration of the five simultaneous differential eqns. (1)–(5) with the initial conditions

$$R = 1; \quad A = B = C = D = 0 \quad \text{at time} \quad t = 0.$$

The result is five equations that describe the change of R, A, B, C, and D with time *during* the light exposure. Then by using k_0 and the preliminary values of k_1, k_2, k_3 and k_4 it can be calculated that at the end of a bleaching period of 10 sec the relative concentrations of the five reactants are 0·10 (R), 0·83 (A), 0·05 (B), 0·02 (C), and 0·00 (D), respectively. Since the zero point of the time scale henceforward used in all computations and diagrams refers to the beginning of the *dark* period,

$$A_0 = 0{\cdot}83; \quad B_0 = 0{\cdot}05; \quad C_0 = 0{\cdot}02; \quad \text{and} \quad D_0 = 0{\cdot}00.$$

The dark reactions can be described in full detail if the system of eqns. (2*a*), (3), (4), and (5) has been solved. For the given initial conditions, the solution is

$$A = A_0 \exp\{-(k_1+k_2)\,t\}, \tag{8}$$

$$B = B_{\text{approx.}} + B_0 \exp\{-k_3 t\}, \tag{9}$$

$$C = K_1 \exp\{-(k_1+k_2)\,t\} + K_2 \exp\{-k_3 t\} + K_3 \exp\{-k_4 t\}, \tag{10}$$

$$D = A_0 + B_0 + C_0 + D_0 - (A+B+C), \tag{11}$$

where

$$K_1 = A_0 \frac{k_1 k_3 + k_2 (k_3 - k_1 - k_2)}{(k_3 - k_1 - k_2)\,(k_4 - k_1 - k_2)},$$

$$K_2 = A_0 \frac{k_1 k_3}{(k_1 + k_2 - k_3)\,(k_4 - k_3)} + B_0 \frac{k_3}{k_4 - k_3},$$

$$K_3 = A_0 \frac{k_1 k_3 + k_2 (k_3 - k_4)}{(k_1 + k_2 - k_4)\,(k_3 - k_4)} + B_0 \frac{k_3}{k_4 - k_3} + C_0.$$

Evaluation of the rate constants

The eqns. (8), (9), (10) and (11) will fit the experimental data, viz. the time course of absorbance changes in the dark, provided appropriate values for the various k's have been chosen. As the data recorded at

480 nm (Fig. 4; upper post-flash curve) are directly proportional to B of eqn. (9), this equation can be used to find k_1, k_2 and k_3. The mathematical procedure which is based on the method of least squares was carried out with the programme BMDX 85 (Dixon, 1970). The quality of fit is illustrated in Fig. 5 where a set of normalized experimental data (filled circles) is fitted by eqn. (9). The respective rate constants are shown in Table 1 (Expt no. 2).

TABLE 1. Parameters of eqns. (9) and (12), k_1, k_2, k_3, k_4, determined by the method of least squares. s = residual S.D.

Expt no.	Temp.	Eqn.	$k_1 \times 10^2$	$k_2 \times 10^2$	$k_3 \times 10^2$	$k_4 \times 10^1$	$s \times 10^2$
1	27·2	9	0·35	0·11	0·12	—	1·45
2	35·3	9	0·85	0·32	0·32	—	1·00
3	35·4	9	0·63	0·18	0·59	—	1·10
4	35·6	12	1·12	0·33	0·38	0·14	0·58
5	35·9	12	1·04	0·17	0·31	0·44	0·32
6	35·8	12	1·05	0·67	0·33	0·22	0·60

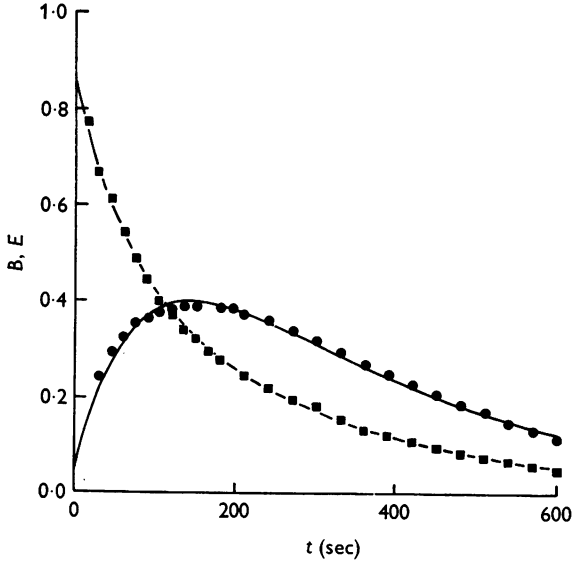

Fig. 5. Data from Expt. no. 2 (circles) fitted by $B(t)$, i.e. eqn. (9), and data from Expt. no. 4 (squares) fitted by $E(t)$, i.e. eqn. (12). Numbering of expts. same as in Table 1.

The evaluation of k_4 is somewhat more complicated. This constant governs the conversion of retinal into retinol. However, the progress of this reaction is difficult to monitor. The absorbance of retinal is maximum near 380 nm but that of metarhodopsin II is likewise maximum at this

wave-length. In addition, the absorbance of metarhodopsin III (λ_{max} = 465 nm) cannot be neglected in this region of the spectrum. Any recording of absorbance changes at 380 nm will therefore be influenced by the presence of three reactants, viz. A, B, and C. The contribution of D (retinol) can be ignored as absorbance changes due to the formation of this substance are hardly measurable at 380 nm. If it is assumed that the absorbance coefficients at 380 nm of metarhodopsin II, metarhodopsin III and retinal stand in the ratio 1:0·2:1 (cf. Baumann, 1972), then the absorbance changes at this wave-length are proportional to the following function

$$E = A + 0{\cdot}2B + C, \tag{12}$$

where A, B, and C are given by the eqns. (8)–(10), respectively. Fitting of the experimental data by eqn. (12) is possible (cf. Fig. 5) and yields numerical figures of all the constants including k_4.

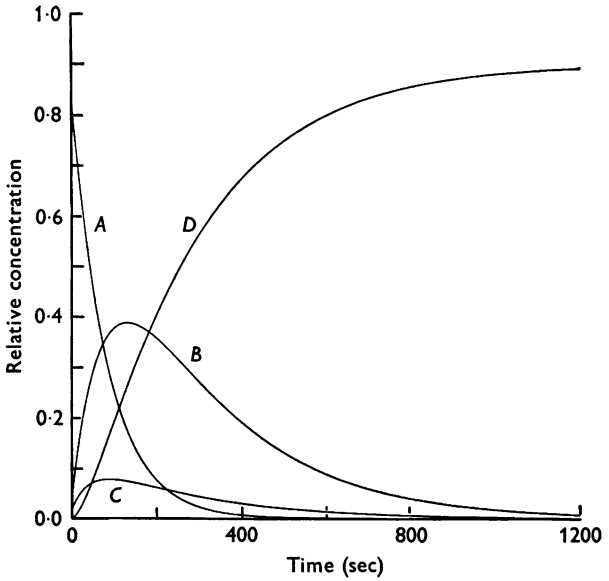

Fig. 6. Kinetics of slow bleaching reactions in the human retina. The relative concentrations of metarhodopsin II (A), metarhodopsin III (B), retinal (C), and retinol (D) are plotted *vs.* time.

The result of six least-squares fittings is shown in Table 1. The first set of constants refers to an experiment carried out at 27° C but in all the other cases the temperature was 35·6 ± 0·3° C and thus not far off the normal temperature in the human eye. The column on the far right gives the residual S.D. as a measure of the goodness of fit. At temperatures near 36° C, the rough mean values of the constants amount to 9×10^{-3} sec^{-1}

(k_1), 3×10^{-3} sec^{-1} (k^2), 4×10^{-3} sec^{-1} (k_3), and 3×10^{-2} sec^{-1} (k_4), respectively. With one exception (k_2 of Expt. no.6), the individual figures of the k's do not deviate more than $\pm 50\%$ from their respective mean value. This amount of scattering is approx. twice as large as it was in an earlier study with isolated frog retinas. However, the present analysis is based upon signals considerably smaller and noisier than in that previous work.

Fig. 6 is a synopsis of the four dark reactions analysed in this study. The curves represent eqns. (8), (9), (10) and (11), respectively. The initial conditions are identical with those calculated above on the basis of preliminary values for the rate constants. They remain almost the same, however, if the final values of the k's are used instead. The diagram shows that in addition to the more obvious appearance of the metarhodopsins II and III, a small amount of retinal is transiently present. The accumulation of retinol (curve *D*) was directly observed in earlier experiments with frog retinas (Baumann, 1972). This required the recording of absorbance changes at 325 nm. In the human retina, the kinetic data obtained at this wave-length all had to be discarded because of a steady rise of absorbance during the whole length of the experiment. This was most probably due to the increasing turbidity of the ageing preparation. Though this made a quantitative analysis impossible, the recordings showed the formation of a u.v. absorbing substance as predicted by curve *D*.

DISCUSSION

The present analysis of slow bleaching reactions makes use of a scheme worked out in previous studies with frog retinas (Baumann, 1972; Baumann and Reinheimer, 1973). The scheme has been confirmed by Reuter (1973) and seems to be suitable for the interpretation of both data from isolated rat retinas (Kemp, 1973) and from human retinas. However, the interpretation of complex reactions is never quite unambiguous, and the data may be consistent with more than one scheme. Therefore, two other schemes were also considered. They are both simplifications of the original one.

In the first case it was assumed that no direct hydrolysis of metarhodopsin II occurs so that this intermediate decays entirely via metarhodopsin III (Ostroy *et al.* 1966). Mathematically this means that k_2 is zero. When the data of Table 1 were calculated, the computer was free to set k_2 equal to zero if this was required for an optimum fit. But the computations always led to finite values of k_2. Consequently, if k_2 was deliberately set equal to zero, the agreement between experimental findings and theoretical curves became nearly twofold worse. The individual figures of the k's showed also a larger amount of scattering. This effect applied particularly

to the values of k_4 which fell in quite different orders of magnitude. On the basis of its poor performance, this model can be dismissed.

Cone & Brown (1969) observed in isolated rat retinas a metarhodopsin III decay to retinol and opsin but not to retinal. If one assumes that this is the case also in the human retina, the above scheme has to be modified so that reactant B decays directly to D, and C has only A as its source. This alters the differential eqns. (4) and (5) which change to

$$\frac{dC}{dt} = k_2 A - k_4 C, \tag{4a}$$

and to

$$\frac{dD}{dt} = k_3 B + k_4 C, \tag{5a}$$

respectively. Equations (8), (9) and (11) remain exactly the same and equation (10) becomes much simpler. Again the quality of fit was not as good as for the original scheme and in addition some more scattering of the parameters k_2 and k_4 was found. It should be noted that the modified scheme is not strikingly inferior to the original one but with the same number of parameters, the original scheme fits the data better. There is thus little reason to prefer this version over the original model.

In two recent papers dealing with rhodopsin kinetics *in vivo*, evidence for the temporary presence of metarhodopsin III in intact retinas has been given (Ripps & Weale, 1969*b*; Alpern, 1971). The time course of formation and decay of this intermediate was described in terms of three constants of which two were the time constants for the decay of the metarhodopsin III precursor and of metarhodopsin III itself. The formula of Ripps & Weale (1969*b*) is analogous to eqn. (7) if one assumes that their time constants T_1 and T_2 equal $1/(k_1+k_2)$ and $1/k_3$, respectively. As the average figure of k_1+k_2 is approx. 0·0125, its reciprocal value equals 80 sec. This agrees fairly well with the figure (60 sec) given by Ripps and Weale for T_1. Separation of the two constants, k_1 and k_2, is not possible on the basis of the *in vivo* data. However, the relatively low maximum concentration of metarhodopsin III (Ripps & Weale, 1969*b*; Alpern, 1971) provides indirect evidence for the shunt, i.e. for a second pathway of metarhodopsin II decay which does not result in the formation of metarhodopsin III (cf. Weale, 1972).

The agreement between T_2, the time constant of metarhodopsin III decay *in vivo*, and $1/k_3$ is less perfect. The data of Ripps & Weale (1969*b*) confirmed by those of Alpern (1971) indicate a faster decay in the living eye than in the isolated retina. Part of the difference may be caused by the somewhat low temperature in our experiments (approx. 1·5° C below the normal level of 37° C). It may be, however, that flash bleaching of

rhodopsin, as carried out in the *in vivo* studies, results in the formation of a more labile metarhodopsin III (cf. Alpern, 1971).

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