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THE THERMAL ORIGIN OF SPONTANEOUS ACTIVITY IN THE *LIMULUS* PHOTORECEPTOR

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SUMMARY

1. Discrete depolarizations of the photoreceptor cell membrane called discrete waves occur spontaneously and in response to illumination in the eye of the horseshoe crab, *Limulus*. Each light induced discrete wave is caused by the absorption of a single photon.

2. The frequencies of spontaneous and light induced discrete waves were studied at different temperatures from 0 to 25° C using a new method of counting them to avoid errors due to their temporal overlap.

3. The frequency of spontaneous discrete waves followed the Arrhenius relationship with activation energy equal to 48·6 kcal.

4. The frequency of the discrete waves caused by a fixed level of steady illumination was not significantly changed when the temperature of the cell was changed.

5. The relationship of the frequency of spontaneous discrete waves to temperature was compared to a prediction based on the relationship of the quantum relative spectral sensitivity of the *Limulus* eye to the temperature of the eye. The prediction was in good agreement with observation and suggests that spontaneous discrete waves result from thermally induced cis to trans isomerizations of visual pigment molecules.

INTRODUCTION

In the dark-adapted photoreceptor of the lateral eye of the horseshoe crab, *Limulus*, discrete depolarizations of the cell membrane called discrete waves occur spontaneously and in response to illumination (Yeandle, 1958; Adolph, 1964; Fuortes & Yeandle, 1964). Each discrete wave is caused by the absorption of a single photon (for a review of the evidence for this see Wolbarsht & Yeandle, 1967 and Srebro & Yeandle, 1970). Spontaneous and light induced discrete waves have similar properties and are statistically independent of each other (Adolph, 1964; Fuortes &

Yeandle, 1964; Srebro & Yeandle, 1970), and the spontaneous discrete wave frequency depends on the temperature of the photoreceptor (Adolph, 1968). The simplest hypothesis compatible with these observations is that a spontaneous discrete wave is caused by a thermally induced chemical change of a visual pigment molecule identical to that caused by the absorption of a photon (*cis* to *trans* isomerization). If the hypothesis is correct, the physical chemical properties of the visual pigment play a significant role in determining the frequency of spontaneous discrete waves. The absorption spectrum is an important physical chemical property of visual pigment, and it largely determines the relative sensitivity of the photoreceptor to lights of different wave-lengths. In the *Limulus* lateral eye, the relative sensitivity depends on the temperature of the cell as well as the wave-length of the incident light (Srebro, 1966). In this study we show that the shape of the relative sensitivity curve, and the effect of temperature on this curve can be used to predict the relationship of spontaneous discrete wave frequency to temperature. This result provides evidence in support of the hypothesis that spontaneous discrete waves are caused by thermal *cis* to *trans* isomerizations of visual pigment molecules.

THEORY

When a photon collides with a visual pigment molecule, the energy of the molecule is raised from its ground state level E_g to a higher energy level E. The energy of the photon is given by $E_\lambda = hc/\lambda$ in ergs where h is Planck's constant ($6{\cdot}625 \times 10^{-27}$ erg sec), c is the velocity of light ($2{\cdot}988 \times 10^{10}$ cm sec^{-1}) and λ is the wave-length of the light (cm). The ground state energy may be described as a random variable that can have value E_g, for $g = 1, 2, 3, \ldots$ with probability $p(E_g)$. Then E is a function of the random variable E_g given by $E = E_g + E_\lambda$. Let $r(E)$ be the probability that a molecule raised to energy level E absorbs the photon. Then the probability of an absorption $\alpha(\lambda)$, is given by

$$\alpha(\lambda) = \sum_{E_\lambda}^{\infty} r(E)p(E - E_\lambda), \tag{1}$$

where the sum in eqn. (1) is taken over all possible energy levels that equal or exceed E_λ. We make two simplifying assumptions which closely follow the analysis suggested by Stiles (1948).

(1) The possible energy levels of the visual pigment molecules are so numerous that E_g may be considered a continuous random variable, E may be considered a function of a continuous random variable, and $r(E)$ and $p(E_g)$ may be considered probability density functions. Under this assumption eqn. (1) may be written:

$$\alpha(\lambda) = \int_{E_\lambda}^{\infty} r(E)p(E - E_\lambda)\mathrm{d}E. \tag{2}$$

(2) The function $r(E)$ is defined by

$$\left.\begin{aligned} r(E) &= 1 \quad (E \geqslant E_0), \\ r(E) &= 0 \quad (E < E_0), \end{aligned}\right\}$$

where E_0 is a critical energy required for the absorption of the photon, and it follows from assumption 2, and eqn. 2 that

$$\left.\begin{aligned} \alpha(\lambda) &= 1 && (E_\lambda \geqslant E_0), \\ \alpha(\lambda) &= \int_{E_0-E}^{E_3} p(E-E_\lambda)\mathrm{d}E && (E_\lambda < E_0). \end{aligned}\right\} \tag{4}$$

The upper limit of the integration in eqn. (4) is taken as E_0 because a visual pigment molecule with energy equal to or greater than E_0 could not exist (by assumption 2).

The simplest assumption about the probability density function $p(E_g)$ is that it is given by Boltzmann's law,

$$p(E_g) = (1/kT)\exp(-E_g/kT), \tag{5}$$

where k is Boltzmann's constant ($1{\cdot}380 \times 10^{-16}$ erg deg^{-1}) and T is the absolute temperature. It follows from eqns. (4) and (5), and the relationship $E = E_g + E_\lambda$ that,

$$\alpha(\lambda) = \int_{E_0-E}^{E_0} (1/kT)\exp(-E_g/kT)\mathrm{d}E_g \quad (E_\lambda < E_0). \tag{6}$$

For $E_\lambda \ll E_0$ the upper limit of the integration in eqn. (6) can be taken as infinity with only a small error and the following approximation holds:

$$\left.\begin{aligned} \alpha(\lambda) &= 1 && (E_\lambda \geqslant E_0), \\ \alpha(\lambda) &= \exp(-(E_0-E_\lambda))/kT && (E_\lambda < E_0). \end{aligned}\right\} \tag{7}$$

Two physical measurements closely related to $\alpha(\lambda)$ are the quantum absorption difference spectrum $a(\lambda)$, and the quantum relative spectral sensitivity $S(\lambda)$. Estimates of $a(\lambda)$ are obtained from measurements of extracted visual pigment and give the fraction of incident photons absorbed by the visual pigment solution as a function of the wave-length of the incident light. $S(\lambda)$ is defined as the reciprocal of the number of photons required to cause a standard response from the photoreceptor containing the visual pigment. It follows from the definition that,

$$a(\lambda) = K\alpha(\lambda), \tag{8}$$

where K is a constant independent of wave-length. From its definition, and noting that two light stimuli at different wave-lengths produce the standard response only if the number of photons absorbed from each stimulus is the same,

$$S(\lambda) = K'a(\lambda)\mu(\lambda), \tag{9}$$

where $\mu(\lambda)$ is the probability that an absorbed photon causes an elementary response (which is a discrete wave in the *Limulus* photoreceptor), and K' is a constant independent of the wave-length. The value of K in eqn. (8) is determined by the concentration of visual pigment in the solution and the geometry of the chamber that contains it. The value of K' in eqn. (9) is determined by the choice of a standard response. Thus K and K' are not of fundamental importance and it is usual to set $a(\lambda)$ and $S(\lambda)$ equal to one at an arbitrary wave-length and scale the values of $a(\lambda)$ and $S(\lambda)$ at all other wave-lengths.

Fig. 1 (open circles) show the values of $a(\lambda)$ for the extracted photopigment of the lateral eye of *Limulus* calculated from the data of Hubbard & Wald (1960), and curve A shows $S(\lambda)$ for the lateral eye of *Limulus* (Srebro, 1966). There is excellent agreement between the values of a (λ) and $S(\lambda)$, and this suggests that $\mu(\lambda) = \mu$, a constant with respect to wave-length. It follows from eqn. (7) to eqn. (9) that

$$\left.\begin{aligned} \log_e S(\lambda) &= \log_e(K'K\mu) && (E_\lambda \geqslant E_0), \\ \log_e S(\lambda) &= \log_e(K'K\mu) - E_0/kT + E_\lambda/kT && (E_\lambda < E_0). \end{aligned}\right\} \tag{10}$$

Since $E_\lambda = hc/\lambda$, and $E_0 = hc/\lambda_0$ where λ_0 is a critical wave-length eqn. (10) can also be written

$$\left.\begin{aligned} \log_e S(\lambda) &= \log_e(K'K\mu) && (\lambda \leqslant \lambda_0), \\ \log_e S(\lambda) &= \log_e(K'K\mu) - hc/kT\lambda_0 + hc/kT\lambda && (\lambda > \lambda_0). \end{aligned}\right\} \qquad (10a)$$

Eqn. (10*a*) correctly predicts several features of $S(\lambda)$ that are in qualitative agreement with curve *A* of Fig. 1. First, it predicts that there is a linear relationship between $\log_e S(\lambda)$ and wave number $(1/\lambda)$ for $\lambda \gg \lambda_0$ and that is similar to the be-

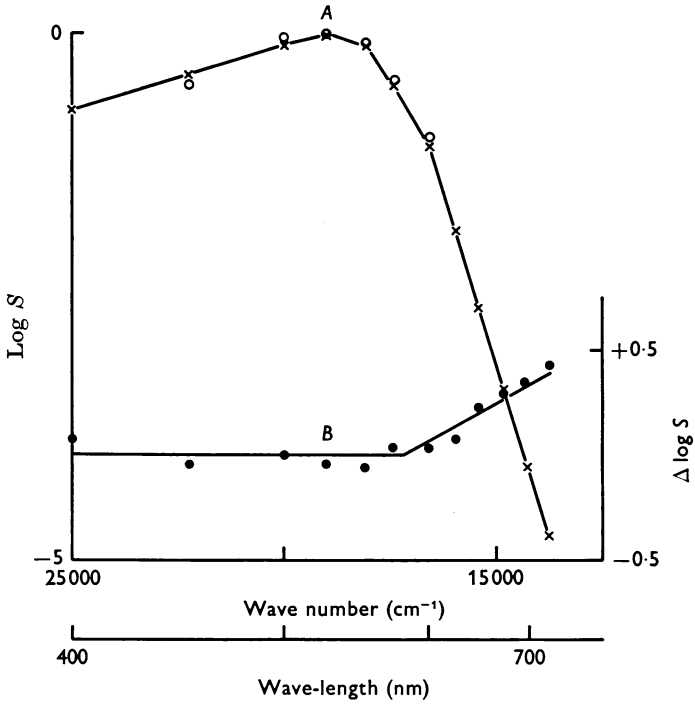

Fig. 1. Quantum relative spectral sensitivity of *Limulus*. Abscissa, wave number (cm^{-1}). The corresponding wave-lengths (nm) are shown below the wave number scale. Left ordinate scale, $\log_{10}$ quantum relative sensitivity. Right ordinate scale, change of $\log_{10}$ quantum relative sensitivity. Curve *A*, crosses and continuous line, quantum relative spectral sensitivity of *Limulus* lateral eye at 27° C, from Srebro, 1966. Open circles, quantum relative absorption difference spectrum for extracted photopigment of *Limulus* lateral eye, from Hubbard & Wald, 1960. Curve *B*, filled circles and continuous line, change of quantum relative spectral sensitivity when the temperature was changed from 7 to 27° C, from Srebro (1966).

haviour of $S(\lambda)$ at wave-lengths greater than 600 nm as shown in Fig. 1 (note the reversal of the wave-number axis in this Figure). Secondly, eqn. (10) predicts that $S(\lambda)$ is constant for $\lambda < \lambda_0$ and that is similar to the behaviour of $S(\lambda)$ in Fig. 1 for wave-lengths less than 500 nm. Finally, eqn. (10) predicts that $S(\lambda)$ is a function of the temperature of the cell for $\lambda > \lambda_0$ but not for $\lambda < \lambda_0$. Curve *B* of Fig. 1 shows

the change in $S(\lambda)$ when the temperature of the eye was changed from 7 to 27° C (calculated from the data of Srebro, 1966). At wave-lengths shorter than about 600 nm there is no significant effect of temperature on $S(\lambda)$, but there is an increasingly strong effect of temperature as λ increases from 600 nm. However, there is poor quantitative agreement between the predictions of eqn. (10*a*) and the observations represented by Fig. 1. For example, the slope of the linear decline of $S(\lambda)$ for long wave-lengths is $1{\cdot}34 \times 10^{-3}$ $\log_{10}$ units cm at 27° C while eqn. (10) predicts that the slope should be $2{\cdot}08 \times 10^{-3}$ $\log_{10}$ units cm.

Two plausible corrections have been suggested to resolve the discrepancy. Stiles (1948) suggested that the possible energy levels of the ground state are not uniformly distributed but tend to crowd together at higher levels. Lewis (1955) suggested that several independent vibrational modes contribute to the molecular energy required for absorption. As a result of either assumption the probability density function $p(E_g)$ changes from a simple exponential as in eqn. (5) to a function similar to the gamma density function. Following Lewis' suggestion we assume that there are m independent vibrational modes each following the Boltzmann law, and that the ground state energy is the sum of the m energies. Then

$$p(E_g) = (1/kT)(E_g/kT)^{m-1} \exp(-E_g/kT)/(m-1)! \tag{11}$$

and, substituting eqn. (11) in eqn. (4) and using the same arguments that lead to eqn. (10*a*).

$$\left.\begin{aligned} \log_e S(\lambda) &= \log_e(K'K\mu) && (\lambda < \lambda_0), \\ \log_e S(\lambda) &= \log_e(K'K\mu) \log_e\left(\sum_{j=0}^{m-1} (E_d/kT)^j/j!\right) - E_d/kT && \lambda > \lambda_0, \end{aligned}\right\} \tag{12}$$

where $E_d = E_0 - E_\lambda$. Eqn. (12) was fit to the observed curves of $S(\lambda)$ for the *Limulus* lateral eye at 7 and 27° C given by Srebro (1966) and the values of λ_0 and m were estimated as $\lambda_0 = 575$ nm, and $m = 4$.

By hypothesis a spontaneous discrete wave occurs if $E_g \geqslant E_0$ in eqn. (11), and

$$\log_e F = \text{const.} + \log_e \mu + \log_e\left(\sum_{j=0}^{3} ((E_0/kT)^j/j!)\right) - E_0/kT, \tag{13}$$

where F is the frequency of spontaneous discrete waves, and $E_0 = hc/\lambda_0$ with $\lambda_0 = 575$ nm. For temperature changes from 0 to 25°C, the physiological range for *Limulus*, the summation in eqn. (13) remains nearly constant and the following approximation holds.

$$\log_e F = \text{const.} + \log_e \mu - E_0/kT. \tag{14}$$

The effect of temperature on μ in eqn. (14) can be determined experimentally, and the equation can be used to give an estimate of E_0 from observations of the relationship of the spontaneous discrete wave frequency to temperature. If the hypothesis that spontaneous discrete waves are caused by thermally induced cis to trans isomerizations of visual pigment molecules is correct, the estimate of E_0 should be the same as the estimate made from the curve of $S(\lambda)$ and its change with temperature.

METHODS

Intracellular records were obtained from the *Limulus* lateral eye using standard techniques. The temperature of the eye was kept constant to within 0·1° C by means of a Peltier cooler, and dry nitrogen was blown over the optical surface of the chamber that contained the eye. An optical stimulator produced a 500 nm mono-

chromatic spot of light slightly larger than one ommatidial diameter at the plane of the cornea.

After the cell was penetrated the temperature was set at 15° C and 45 min was allowed for dark adaptation. A 10 min record of spontaneous discrete waves was recorded, d.c. coupled, on analogue magnetic tape. Then a light intensity was selected so that steady illumination caused a just noticeable increase in the discrete wave frequency and another 10 min record was recorded. The procedures were repeated using a light with intensity 1·95 times the first light intensity and again using a light with intensity 3·24 times the first light intensity. The temperature of the cell was changed in 4–8° C steps in an unpatterned way. At each temperature records of spontaneous discrete waves and the responses to exactly the same three intensities of steady illumination were recorded.

The analogue tape records were later amplified using an a.c. coupled amplifier, sampled at 60 samples/sec, and stored in the memory of a computer for further processing.

We report results from eighteen different cells studied at temperatures from 0 to 25° C.

Method of analysis

It was necessary to count discrete waves over a wide range of frequencies. When the discrete wave frequency was of the order of their reciprocal average duration it was not possible to count them accurately by visual inspection. We therefore devised a method of counting discrete waves which did not depend on visual inspection of the records.

It is useful to view discrete waves as a succession of possibly overlapping waves that occur with frequency F and have variable durations. At any time t during an observed period we characterize the system as being in state 0 if no discrete wave covers that instant of time and in state 1 otherwise. The occurrences of discrete waves form a Poisson renewal point process in time, and a device which registers states 0 and 1 is a type II, or paralysable counter, with random dead time (Smith, 1958; Parzen, 1960), with respect to the discrete wave process. Type II counters are used in many physical measurements and the theory of their operation is well developed (Renyi, 1951; Hammersley, 1953; Takacs, 1956; Smith, 1957). It can be shown that

$$F = 1/(T_{11} - T_1) \tag{15}$$

where T_{11} is the average time between consecutive transitions from state 0 to state 1, and T_1 is the average duration of state 1.

We specified the conditions for state 1 by defining a critical depolarization V_c such that state 1 corresponded to a depolarization greater than V_c. Instead of setting V_c visually, we determined the relationship of F calculated from eqn. (15) to different values of V_c for each record that we analysed. The exploratory values of V_c started at a level of depolarization equal to the average base line depolarization, which we call $V_c = 0$, and ranged to about 1 mV in 0·1 mV steps.

Fig. 2 graph *a* shows a typical plot of $\log_{10} F$ *vs.* V_c for a single record that contained spontaneous discrete waves. There are two clearly defined linear segments. At levels of V_c near 0 there are many 0–1 transitions which we attribute to random fluctuations of the base line caused largely by electrode noise. At higher levels of V_c there are fewer 0–1 transitions and these are due largely to discrete waves. The two linear segments have markedly different slopes and this reflects the different statistical properties of electrode noise and discrete waves. The intersection of the two linear segments was used to determine F for the discrete wave process.

In order to check the accuracy of the counting procedure, we simulated a run as

follows. A computer program generated a different random number every msec. If the number was greater than a specified value, C, a very short pulse was made to appear on an output channel of the computer. The pulse was used as the input to a two-staged RC-coupled amplifier, whose output was a smoothly rising and falling wave that lasted approximately 150 msec and simulated the time course of a discrete wave. The output of the amplifier to a random sequence of pulses was recorded on analogue tape and was processed exactly as if it were experimental data. By changing the value of C it was possible to vary the frequency of the pulses that the computer

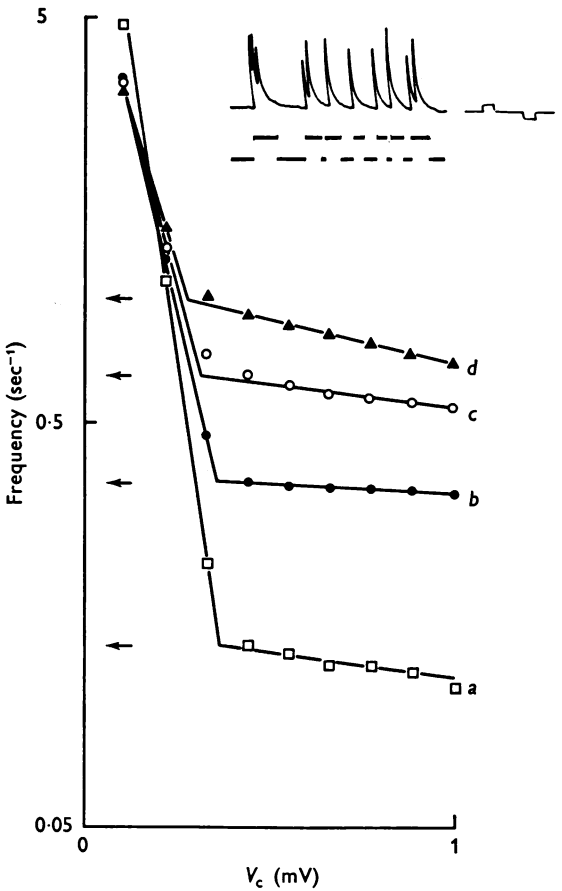

Fig. 2. Detection and counting of discrete waves. Ordinate, $\log_{10}$ frequency (discrete waves/sec). Abscissa, critical voltage (mV). The value of 0 on the abscissa corresponds approximately to the average value of the base line. Straight lines fit by eye. Curve *a*, spontaneous discrete waves. Curves *b* to *d*, progressively increasing levels of steady illumination with relative intensities 1, 1·95, and 3·24 respectively. Insert, illustration of counting procedure. Top trace, segment of d.c. coupled record used to obtain curve *d*. Bottom trace, indicates state 0 when at lower level and state 1 when at higher level. Calibrations, 1 mV pulses separated by 2 sec. Retinula cell, 19·2° C.

generated. We counted the number of pulses and compared this value to the results of the counting procedure already described. In this way we determined that the counting procedure was accurate when the pulse rate was as high as 5 sec^{-1}.

RESULTS

Fig. 2 shows a typical set of measurements for a retinula cell at 19·2° C. Each of the graphs marked *a* through *d* shows the relationship between the frequency calculated from eqn. (15) and the critical level of depolarization, V_c. Each of the arrows indicates the frequency corresponding to the intersection of the two line segments in each graph which was taken as the estimate of the frequency of discrete waves. Graph *a* corresponds to a record of spontaneous discrete waves, and graphs *b*, *c* and *d* correspond, respectively to relative light intensities 1, 1·95, and 3·24. The insert of the Figure shows part of the record of discrete waves used to construct graph *d*. The trace just below it represents the state of the counter when V_c was 0·4 mV (corresponding to the intersection of the two line segments of graph *d*). Counter state 1 is represented by the higher of the two levels.

The average frequency of spontaneous discrete waves for the eighteen cells studied was 0·44 sec^{-1} at 15° C. The average rates of discrete waves for the light stimuli at relative intensities 1, 1·95, and 3·24 at 15° C were, respectively, 0·88, 1·07, and 1·54 sec^{-1}. These values could be represented by a linear relationship between discrete wave frequency and light intensity. However, this was not always the case. At higher intensities there was sometimes a fall of the observed frequency from the linear prediction. We attribute this effect to light adaptation, and it is of no importance to our analysis because we compared responses at identical light intensities.

Eqn. (14) was used to fit the observed values of spontaneous discrete wave frequency to the temperature of the cell, for each cell studied, and E_0 was determined from the slope of the line. We report our results in molar units, that is the energy of Avagadro's number of photons. The values of E_0 ranged from 34·4 kcal to 78·2 kcal with mean value 48·6 kcal and S.E. 11 kcal. The average value of E_0 corresponds to $\lambda_0 = 587$ nm.

Since there was appreciable variation of the frequency of spontaneous discrete waves from cell to cell at 15° C, a composite scatter diagram was prepared by scaling the frequency at each temperature relatively to the frequency at 15° C for each cell that we studied. The composite scatter diagram is shown in Fig. 3 and the line corresponds to $E_0 = 48·6$ kcal.

For each of the light intensities that we used the frequency of light induced discrete waves was calculated by subtracting the frequency of spontaneous discrete waves from the total frequency. The relationship of the frequency of light induced discrete waves to the temperature of the

cell was fit by an equation identical to eqn. (14). The fit was done separately for each intensity and each cell. A constant $E\mu$, analogous to E_0 in eqn. (14) was estimated from each set of data. The values of

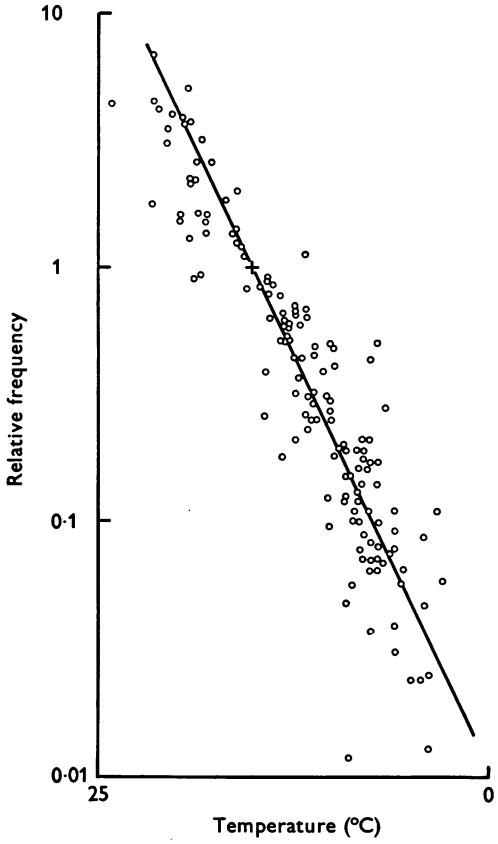

Fig. 3. Relationship of spontaneous discrete wave frequency to temperature. Abscissa, reciprocal temperature (° K) shown as ° C for convenience. Ordinate, relative frequency of spontaneous discrete waves (sec^{-1}). The observed frequency of spontaneous discrete waves was divided by the frequency at 15° C for each cell. Scatter diagram of this Figure is for eighteen cells. Straight line corresponds to E_0 = 48·6 kcal. in eqn. (14), the average slope for all cells studied.

$E\mu$ were $4{\cdot}8 \pm 7{\cdot}7$, $5{\cdot}5 \pm 5{\cdot}6$, and $6{\cdot}4 \pm 6{\cdot}0$ kcal for relative light intensities 1, 1·95 and 3·24 respectively. These values of $E\mu$ are not significantly different from 0.

Fig. 4 shows a composite scatter diagram of the relationship of light induced discrete waves to temperature constructed in a way analogous to

Fig. 3. The scatter diagram is for relative intensity 1. The line corresponds to $E\mu$ = 4·8 kcal. The scatter diagram shown in Fig. 4 is similar to those observed for relative intensities 1·95 and 3·24.

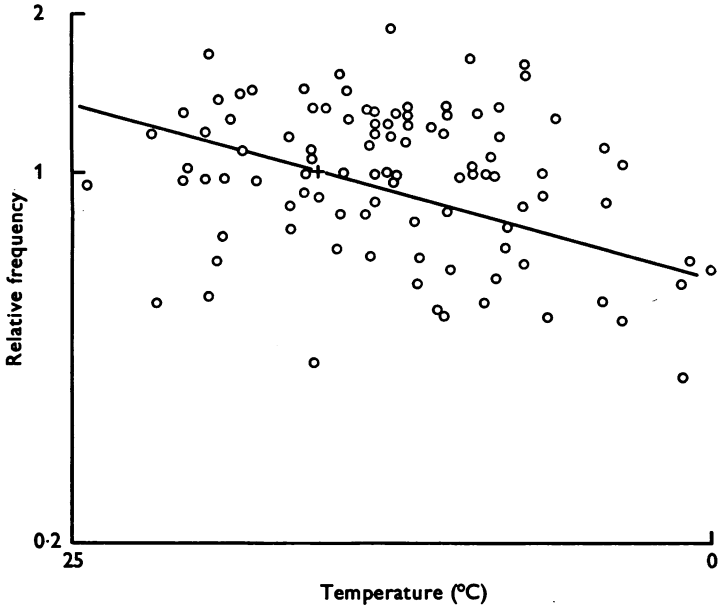

Fig. 4. Relationship of light induced discrete wave frequency to temperature. Abscissa, reciprocal temperature (° K) shown in ° C for convenience. Ordinate, relative frequency of light induced discrete waves (sec^{-1}). The observed frequency of light induced discrete waves (total frequency minus spontaneous discrete wave frequency) was divided by the frequency at 15° C for each cell studied. Scatter diagram of this Figure is for eighteen cells. Steady illumination at relative intensity 1. This intensity was kept constant as the temperature of the cell was changed. An equation analogous to (14) was used to fit the data with E_μ replacing E_0. Straight line corresponds to E_μ = 4·8 kcal the average slope for eighteen cells.

DISCUSSION

Our results show that the frequency of light induced discrete waves caused by a fixed level of steady illumination is not significantly affected by the temperature of the cell. Thus μ in eqn. (14) does not depend on temperature and no correction is needed for the estimate of λ_0 made from fitting the data to this equation. Within the limits of experimental accuracy, equation 14 is a good description of the relationship of the spontaneous discrete wave frequency to the temperature of the cell. The estimate of λ_0 from eqn. (14) is λ_0 = 587 nm, and the estimate of λ_0 from the spectral sensitivity data is λ_0 = 575 nm. These estimates are in reason-

able agreement. This supports the hypothesis that spontaneous discrete waves result from thermal isomerizations of visual pigment molecules.

Our results suggest that spontaneous discrete waves are not caused by cellular processes such as primary random fluctuations of the cell membrane conductance in the dark, or an enzymic reaction in the dark. We note the formal analogy of eqn. (14) to the Arrhenius relationship,

$$k = A\,\mathrm{e}^{-Ea/RT}, \tag{16}$$

where k is the rate constant, A is the frequency factor, E_a is the activation energy, and R is the gas constant. The relationship between k in eqn. (16) and F in eqn. (14) is given by

$$F = \mu\,\mathrm{dN/d}t = \mu kN, \tag{17}$$

where N is the number of visual pigment molecules per ommatidium. By analogy to eqn. (16), the activation energy of the discrete wave process is 48·6 kcal, and the Q_{10} in the range 10–20° C is 19. This value should be compared to the Q_{10} of the membrane process associated with an action potential in the squid axon, about 3 (Hodgkin & Katz, 1949). The Q_{10} of the latency of discrete waves in the photoreceptor of *Limulus* is about 4 (Srebro & Bahbehani, 1971). Most enzymic reactions have a Q_{10} of the order of 2 (Baldwin, 1948). Thus the Q_{10} for spontaneous discrete waves is unusually high. However, this is exactly what one might expect if the underlying reaction causing a spontaneous discrete wave were a thermal isomerization, because the energy for the reaction would ordinarily be supplied by a photon. The energy of a mole of photons at the wavelength of maximal absorption for the *Limulus* photopigment is approximately 54 kcal. Additional support for this conclusion derives from the result that reducing the calcium ion concentration of the bathing fluid of the ventral photoreceptor of *Limulus* causes spontaneous discrete waves to become larger but does not substantially affect their frequency of occurrence (Millecchia & Mauro, 1969).

Several objections to the model we propose could be raised.

(1) The model predicts $\mathrm{S}(\lambda)$ for wave-lengths longer than the wavelength of maximal sensitivity but does not predict the decline at shorter wave-lengths. A correction could be made by assuming that $r(E)$ is not a step function at $E = E_0$, as we have assumed, but has a slowly increasing value approaching 1 at $E = E_0$ and then a sharp decline for $E < E_0$. The major effect of this correction with regard to our analysis of spontaneous discrete wave frequency is to decrease the slope of $\log F$ *vs.* $(1/T)$ in eqn. (14). The reason for this is that as the temperature increases, slightly higher energy states are expected to become occupied, and these states have a lower probability of reaction. But the effect must be small because the occurrence of a spontaneous discrete wave depends largely on the high energy tail of

$p(E_g)$ which declines exponentially with E_g, and thus the values of E_g just greater than E_0 are the most important.

(2) The assumption of independent vibrational modes in the visual pigment molecule is arbitrary. We justify this assumption on the more general grounds that a molecule as large and complex as a visual pigment molecule is likely to have a greater number of possible high energy states than a simple diatomic molecule. Even if a single vibrational mode were involved, it would exist in the complex electronic potential field of the visual pigment chromophore and would probably act as an anharmonic oscillator for which there is a clustering of available energy states at values higher than the zero level (Davidson, 1962). In the last analysis, the model is justified on empirical grounds because it predicts spectral sensitivity data not only for *Limulus* but for the human rod system as well (Lewis, 1955). We do not think that the value of m in eqn. 8 has the simple meaning that our theoretical section implies.

(3) Fuortes & Yeandle (1964) reported that hyperpolarizing the ommatidium decreased the frequency of spontaneous discrete waves, but they gave no statistical confirmation of the finding. Furthermore, a change in the rate of spontaneous discrete waves could occur if μ is affected by hyperpolarizing current. We have repeated Fuortes' and Yeandle's experiment using hyperpolarizing currents up to 8×10^{-9} A and have not observed a significant change in the rate of spontaneous discrete waves.

(4) Hubbard (1958) reported that the bleaching of cattle rhodopsin by heat resulted in the denaturation of the protein, opsin, and did not cause a *cis* to *trans* isomerization. The measurements were made at temperatures from 58·3 to 65·7° C because at physiological temperature the rate of thermal bleaching was so low that it could not be measured. It is difficult to interpret Hubbard's results because it is possible that thermal denaturation predominates at high temperatures and masks thermal *cis* to *trans* isomerization. To estimate the magnitude of the effect we can use eqns. (16) and (17) and our estimates of E_a and F to determine the expected rate of thermal isomerization at 65·7° C for *Limulus* photopigment. Taking 2×10^9 as an estimate of N in eqn. (16) (Hubbard & Wald, 1960) and 0·25 as an estimate of μ (Millecchia & Mauro, 1969), the percentage loss of visual pigment at 15° C is calculated to be about 10^{-7} %/sec, and the percentage loss at 65·7° C is about 3×10^{-2} %/sec. If cattle rhodopsin had similar kinetics, then the loss of rhosopsin due to thermal isomerization would be about 1·8 % in 3 min at 65·7° C. But Hubbard found that the rhodopsin loss was approximately 13 % in 3 min at 65·7° C. We suggest that there are two competing reactions: thermal denaturation and thermal isomerization. According to Hubbard, thermal denaturation of cattle rhodopsin has an activation energy of 100 kcal. We would expect that

thermal isomerization would have an activation energy not greater than the energy of a mole of photons at the wave-length of maximal absorption, or about 58 kcal. This suggests that thermal denaturation predominates at the temperatures Hubbard used.

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