Thermal and Spectral Sensitivities of Discrete Slow Potentials in *Limulus* Eye

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ABSTRACT The discrete, subthreshold, slow potential fluctuations (SPF's) which can be recorded intracellularly in *Limulus* ommatidia are sensitive to temperature and light wavelength. SPF frequency increases with increasing temperature (Q_{10} about 3.5) and light intensity. The effects are additive. SPF rise and decay time decrease with increasing temperature (Q_{10} between 2 and 3). There is a peak, near 520 nm, in the spectral sensitivity of SPF frequency. This peak may correspond to the wavelength of maximum absorption by rhodopsin in the ommatidia. Hydroxylamine produces a rapid, irreversible reduction of SPF frequency and amplitude perhaps owing to its action on the photopigment. The cornea and crystalline cones fluoresce (peak about 445 nm) when excited by near-ultraviolet energy (380 nm peak) and this fluorescence may influence SPF spectral sensitivity measurements. These findings suggest that the SPF's are the results of photolytic and thermolytic reactions occurring in the ommatidial visual pigments and that they have a role in the mechanisms which transduce light to electrical activity in the visual receptors.

The sequence of physiological and biochemical events between the absorption of light by a photoreceptor and the initiation of nerve impulses in the optic nerve is not completely understood. We know something of the photolytic action of light on the visual pigments (Wald et al., 1963; Hubbard et al., 1965). We suspect that receptor potentials may be associated with ionic currents. In the *Limulus* eye these currents may lead to nerve impulses in the optic nerve (Hartline et al., 1952; Fuortes, 1959 *a*; Tomita et al., 1960); however, the steps between the photolysis of the visual pigment and the receptor potential are unknown.

One sign of an intermediate step between the photolysis of photopigment and the receptor potential may be discrete, subthreshold slow potential fluctuations (SPF's) which have been recorded by intracellular microelectrode in dark-adapted ommatidia of invertebrates. These discrete SPF's have so far been found in *Limulus* (Yeandle, 1958; Fuortes, 1959 *b*; Adolph, 1964; Millecchia et al., 1966), the locust eye (Scholes, 1964), and the leech eye

(Walther, 1966). This paper describes studies on how temperature and the spectral composition of light, two factors which affect the photochemistry of visual pigments (Hubbard, 1958; Wald and Brown, 1950), also affect the SPF's.

METHODS

The lateral eyes of adult *Limulus polyphemus* were used for this series of 20 experiments. Thin (about 1 mm) slices were cut from an eye with a razor blade to expose a layer of ommatidia. For each experiment a slice was pinned to a cork mounting in the bottom of a Plexiglas chamber containing filtered artificial seawater (Instant Ocean, Aquarium Products, Wyckliffe, Ohio) as the saline medium bathing the eye. Drug solutions were applied by a gross perfusion of the chamber while keeping the bath volume nearly constant. It was possible to perfuse the preparation at 1 ml per sec without dislodging the microelectrode.

The temperature of the saline was controlled by a Peltier module which formed the chamber base. A desired temperature or program of temperature changes could be set by a proportional feedback system with a thermistor sensor probe placed near the eye. Bath temperature was monitored with another thermistor probe and bridge circuit. The maximum cooling rate of the system with 5 cc of unstirred saline in the chamber is about 4°C per min. The set temperature is maintained with less than 0.1°C variation.

Micropipette electrodes, filled with 3 M KCl of 6–30 megohms resistance, were used to record membrane potentials. The location of the microelectrode was inferred from the characteristics of the recorded potentials (Purple, 1964; Behrens and Wulff, 1965). Most of the potentials described in this paper were recorded across the membranes of eccentric cells, from their dendrites or cell bodies. The suprathreshold receptor potentials studied in some experiments were probably recorded from the rhabdomeres of retinular cells. Polarizing currents could be passed through the recording microelectrode by a bridge circuit input to the cathode-follower head stage. During an experiment signals were recorded on a multichannel FM tape recorder for subsequent filming and analysis.

A 6 v, 15 w tungsten filament bulb was the light source. The duration of the light stimulus was controlled by an electromechanical shutter; light intensity was adjusted using neutral density filters. Wavelength was selected by narrowband (10 nm) interference filters. A fiber optic system imaged the light stimulus on the retinal slice.

The relative incident energies of the monochromatic stimuli were measured with a photomultiplier photometer. The spectral characteristics of individual interference filters and neutral density filters were determined with a spectrophotometer. Although absolute incident energies of the monochromatic stimuli were too small to be measured by available radiometers, they could be estimated from data like those shown in Fig. 2. The incident energy falling on an area of 10^{-5} sq cm (roughly the area of the rhabdome in cross-section) is 25,000 photons/sec at 500 nm. This figure indicates nothing of the actual number of effective photons. The quantity of photons lost in passage through the saline and cornea, those absorbed by the pigment cells and visual pigment, and the quantum efficiency of any photochemical processes that occur are unknown.

RESULTS

Temperature Effects

The average frequency of spontaneous SPF's is remarkably temperature sensitive. Frequency increases with increasing temperature and appears to saturate at temperatures greater than 18° to 20°C (Fig. 1). The Q_{10} of frequency between 12° and 22°C is about 3.5. This Q_{10} may indicate that SPF frequency is related to the rate of an underlying chemical process (Hecht, 1921). Activation energy corresponding to rates at the lower temperatures is

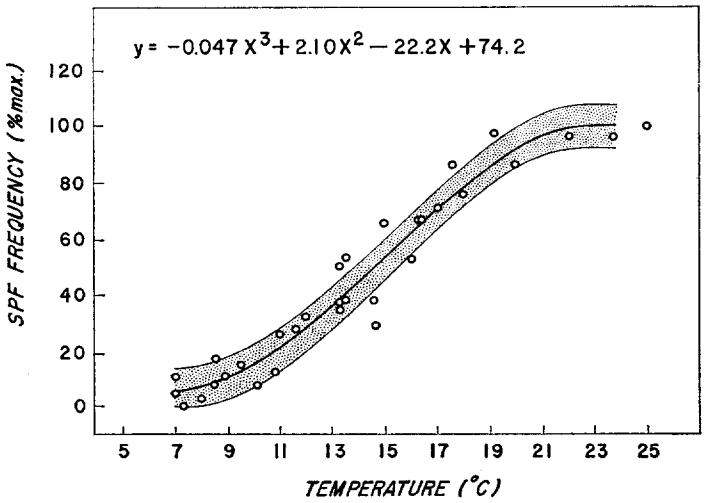

FIGURE 1. Spontaneous miniature potential (SPF) mean frequency as a function o temperature. The heavy middle curve, and its equation (upper left), are the third-order least squares fit to the data. The shaded area indicates 1 SE on either side of the least squares curve.

approximately 40 kcal; that corresponding to rates at the higher temperatures is 20–25 kcal. The absolute frequencies which correspond to the 100% level ranged from 1 per 2 sec to 16 per sec for the pooled data of five experiments shown in Fig. 1.

The effect of light is to increase the frequency of SPF's over the frequency of spontaneous occurrence in the dark (Fig. 2). At a constant temperature, SPF frequency increases as a function of light intensity (Fig. 3). An increase in temperature results in a higher frequency of spontaneous SPF's and a corresponding increase in response to light stimuli.

The time course or shape of an SPF is also sensitive to temperature. The rise time from base line to peak and the decay time from peak to 37% of the peak amplitude are decreasing functions of temperature (Fig. 4). The Q_{10} falls within the range of 2 to 3, somewhat less than the Q_{10} for frequency vs. tem-

perature. The times indicated in Fig. 4 are mean values for distributions of these times at a given temperature in a specific cell. There is also a distribution of peak amplitudes of the SPF's (Adolph, 1964). The data of Fig. 4 are taken from "slow type" SPF's (Adolph, 1964) not "fast," regenerative-like SPF's.

Since the SPF's result from permeability changes in a cell membrane, the

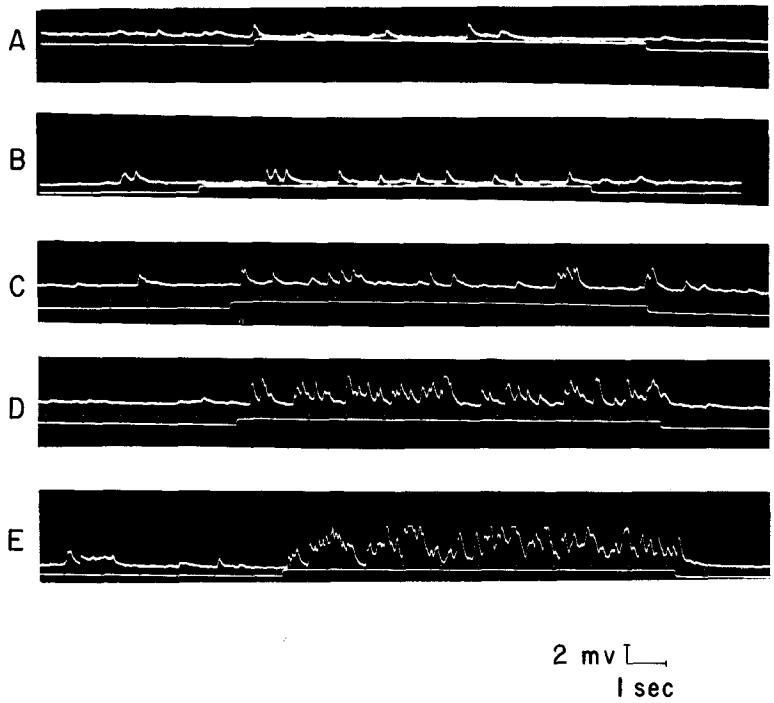

FIGURE 2. Effect of light intensity on SPF frequency. Top trace, intracellular membrane potential. Bottom trace, stimulus signal. The relative light intensity of the stimulus for A, B, C, D, and E was 1, 3.16, 10, 31.6, and 100, respectively. The approximate light flux density in A was 10 nw/cm^2, based on thermopile measurements of the unattenuated incident light.

effects of temperature on membrane permeability might partially explain the striking changes in SPF time constants. As a factor related to permeability, resting membrane resistance was found to decrease as temperature was increased. The Q_{10} between 8° and 18°C is about 1.6, only two-thirds that of the SPF rise and decay times.

Changes in the membrane resistance produce changes of the resting membrane potential. For instance, an increase in temperature from 8° to 18°C may result in an increased polarization of the membrane by as much as 10 mv. A cell with a resting potential of 40 mv at 8°C has a 50 mv resting potential at 18°C. If nerve impulse threshold is within the range of membrane potentials

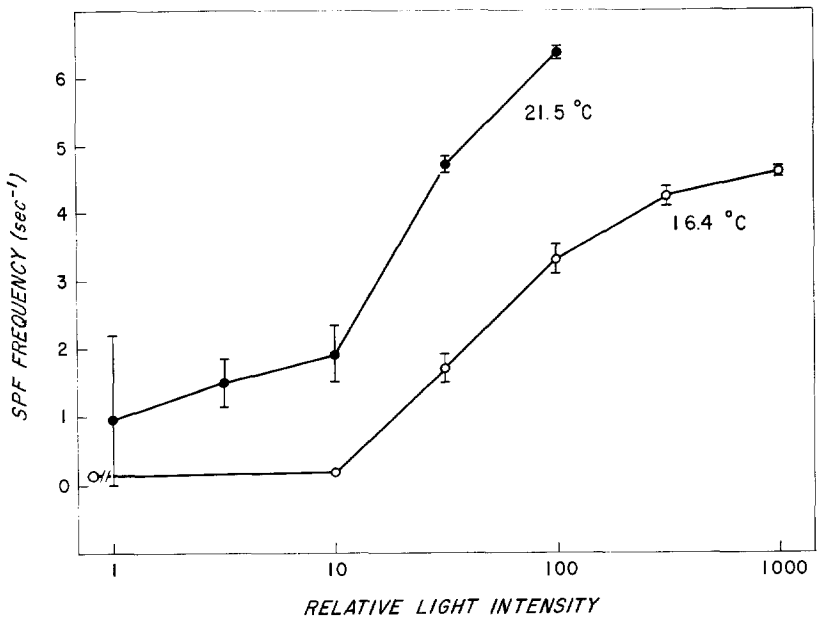

FIGURE 3. Mean SPF frequency as a function of relative light intensity and temperature. Limits indicate 1 SD on either side of the mean. Data for each temperature from separate experiments.

which results from varying the temperature, spontaneous firing will occur as a quiet cell at a warm temperature is cooled.

The equilibrium potentials for the SPF process and for the suprathreshold receptor potential are at a depolarized level compared to resting potential

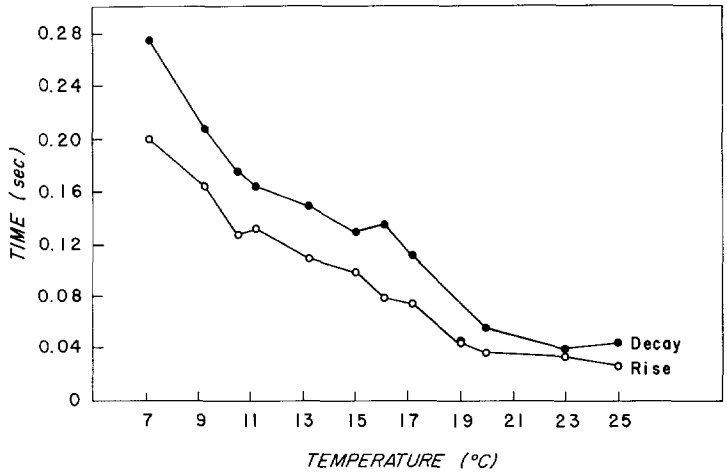

FIGURE 4. SPF time constants as a function of temperature.

level (Adolph, 1964; Benolken, 1961). Therefore, SPF and receptor potential amplitudes decrease as temperature decreases.[1]

Spectral Measurements

The spectral sensitivities of SPF frequency in response to dim, monochromatic light stimuli are not equal (Fig. 5). The spectral sensitivity is defined as the reciprocal of the stimulus energy required to elicit a constant SPF frequency (criterion level) throughout the spectrum. There is a peak in the spectral

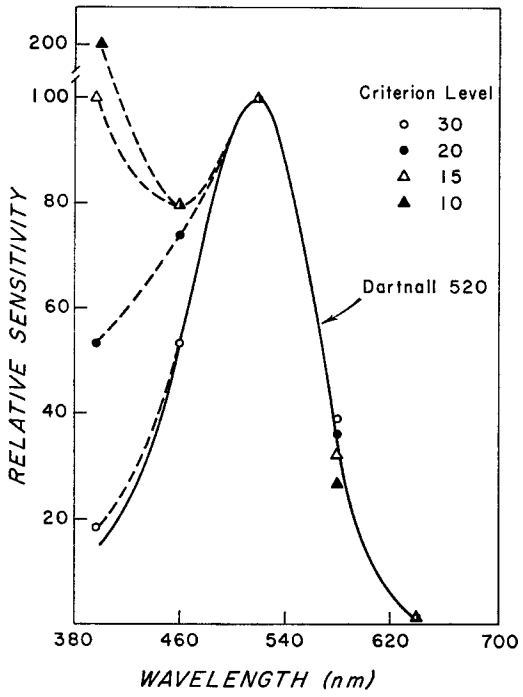

FIGURE 5. SPF spectral sensitivity. Symbols, identified in inset, correspond to different criterion levels (levels of constant SPF frequency) used to determine spectral sensitivity. The four points are superimposed at 520 and 640 nm. Solid line, Dartnall absorption curve with maximum at 520 nm.

sensitivity in the 520–530 nm region of the spectrum. The spectral characteristics illustrated by Fig. 5 were obtained by measuring transfer functions (absolute SPF frequency vs. relative light intensity) at each wavelength of interest (Fig. 6) and noting the relative intensities required for a constant level of response. The spectral characteristics shown in Figs. 5 and 6 illustrate the

[1] In experiments dealing with the effects of temperature on suprathreshold receptor potentials it was found that decreasing temperature reduces the amplitudes of the transient and steady components of the receptor potential. The rate of decrease is greater for the transient than for the steady component. The rise and decay time of the transient and the decay time of the steady component at light off increase as temperature decreases. Kikuchi et al. (1961) and Fuortes (1965) have reported similar findings.

results of one experiment in a series of five similar ones and are representative of the results obtained in all the experiments.

The spectral sensitivity matches the Dartnall (1962) absorption spectrum, for a photopigment with a maximum around 520 nm, at wavelengths longer than the 520 nm peak. However, only the spectral sensitivity for the highest criterion level chosen (30 SPF/10 sec) matches the Dartnall curve at wavelengths shorter than 520 nm (Fig. 5). The spectral sensitivity deviated further from the Dartnall curve as the constant response criterion was lowered.

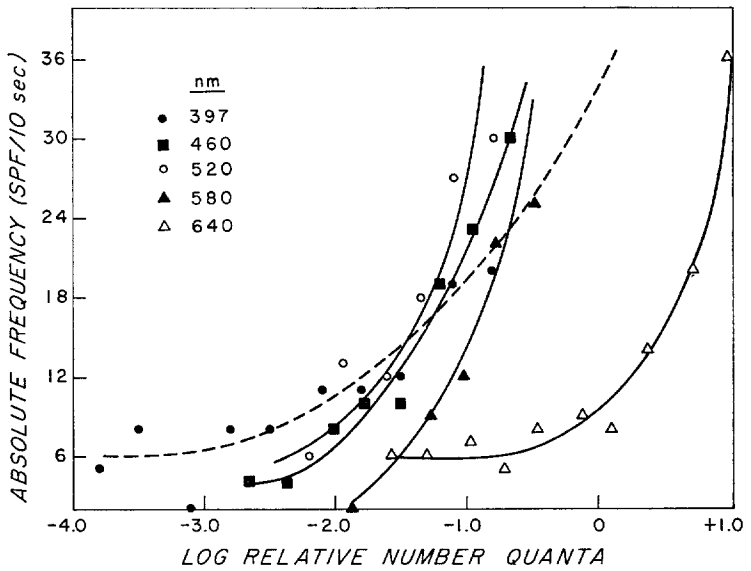

FIGURE 6. SPF spectral transfer functions. Symbols, identified in inset, correspond to absolute SPF frequencies in response to light stimuli of differing intensity at indicated wavelengths. Curves fitted by observation to sets of points for each wavelength.

A series of experiments was performed prior to those mentioned above, in which only one stimulus intensity was used at each of 12 wavelengths across the spectrum. The responses were then adjusted to compensate for the unequal quantum content of the stimuli. The spectral responses in that early series of seven experiments also showed a peak in the 520–540 nm region, in addition to large responses at shorter wavelengths. The unexpected short-wavelength response led to the more extensive spectral measurements reported in this paper.

Not all the spectral measurements, mentioned or reported in detail above, were done at the same temperature, although the temperature during any one experiment was constant. Stimuli lasting 10 sec were used at each of the wavelengths tested in an experiment, and one-half to 1 min. of darkness preceded and followed each stimulus. Wavelengths were scanned at each

intensity in some experiments and intensities scanned at each wavelength, in other experiments. Scanning was usually in one direction, and repeat measurements were done at some wavelengths and/or intensities. Negligible differences were found in comparing SPF frequency for the same wavelength/intensity combination or for the dark condition in a given experiment. This probably indicates a certain stability of conditions during an experiment.

I find that the shape of the SPF does not depend on the wavelength of the stimulus nor does it differ significantly from the shape of a spontaneous SPF. These results were obtained by comparing enlarged versions of relevant SPF responses (Fig. 7).

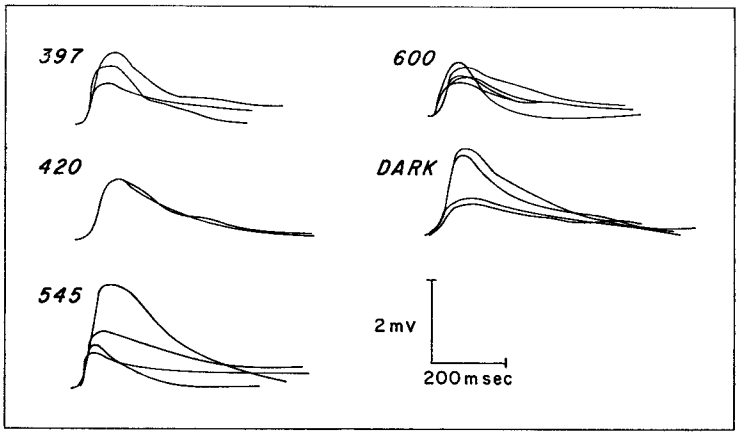

FIGURE 7. SPF waveshapes at different light wavelengths and in the dark. Tracings from filmed records. Numbers at the upper left of each set of tracings indicate light wavelength in nanometers.

Related Experiments

EFFECT OF HYDROXYLAMINE The foregoing experimental evidence indicates that there may be some relationship between the photolysis and thermolysis of visual pigment in an ommatidium and the occurrence of SPF's. One action of the aldehyde-trapping reagent hydroxylamine is to prevent the regeneration of rhodopsin from its photoproducts retinal (vitamin A aldehyde) and opsin (Wald and Brown, 1950). It was therefore of interest to determine whether hydroxylamine has any effect on SPF's.

Three experiments tested the action of hydroxylamine (20–50 mM) on receptor activity. In two experiments, the hydroxylamine appeared to effect a rapid and irreversible reduction in frequency and amplitude of spontaneous SPF's (Fig. 8). The temperature was controlled to within ± 0.1 °C so that temperature effects could be eliminated as the source of frequency change; however, the hydroxylamine solutions were used in a slightly acidic condition

(pH 6.6). The variation in resting potential during the course of hydroxylamine action was no more than 5 mv. A third experiment which tested the action of hydroxylamine on the light response of a retinular cell did not show any significant effects.

DIOPTRIC FACTORS The corneal transmission, as measured spectrophotometrically, is uniform and decreases as the wavelength decreases from 700 to

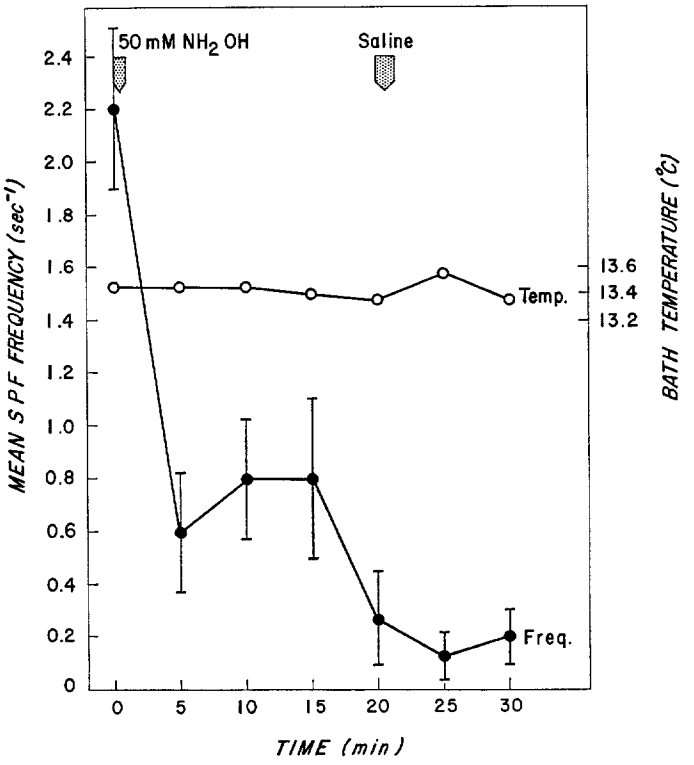

FIGURE 8. Mean frequency of spontaneous SPF's following the application of hydroxylamine. Limits indicate 1 SE above and below mean.

350 nm. Some part of that decrease in transmission may be due to Rayleigh scattering. Spectrofluorimetric measurements of the cornea produced a surprising result. The cornea fluoresces in the visible region (445 nm peak) when excited with near-ultraviolet energy (peak about 380 nm). This is illustrated by Fig. 9. Spectrophotometric measurements of light transmission through the screening pigments show that it is uniform over a broad spectrum (300–1500 nm) and is tilted so that short wavelengths are more attenuated than long wavelengths.

Transmission measurements were made with a dual beam scanning spec-

trophotometer and a manually scanned spectrophotometer. Fluorescence spectra were obtained with a scanning spectrophotofluorimeter.

DISCUSSION

In the Introduction, I suggested that the discrete SPF's are one sign of an intermediate step between the photolysis of photopigment and the receptor potential. The work of a number of authors (Fuortes, 1959 *b*; Yeandle, 1958; Adolph, 1964; Millecchia et al., 1966) led to this suggestion. Their findings are:

The SPF's occur spontaneously in the dark, and their frequency increases in response to illumination of the eye.

The SPF's are the result of a stochastic process, and their mean frequency depends upon temperature and history of light adaptation as well as light intensity.

The SPF's and the membrane resistance changes which produce them have similar time courses. In this, they differ from synaptic potentials which are passive membrane responses to short current pulses.

Temporal summation of SPF's occurs. In the dark or with dim lights, the low SPF frequencies produce widely fluctuating summated potentials. Intense lights result in high SPF frequencies and smoother and larger summated potentials.

The SPF's have so far been found to originate only in cells possessing a microvillous structure similar to the rhabdomere of the retinular cells in compound eyes. At present the microvillous structures are thought to be the loci of the visual pigments (Wolken, 1966).

I feel that my experimental findings add further support to the suggestion that the SPF's are one sign of an intermediate step between the breakdown of photopigment and the receptor potential. The findings are in some ways unexpected, and I will therefore discuss them, and their relation to previous knowledge, in some detail.

The temperature sensitivity of spontaneous and light-induced SPF's (Q_{10} of 3 and greater) indicates that any rate-determining reaction in the genesis of the SPF's is probably not a simple photochemical transformation (Q_{10} of 1) or diffusion process (Q_{10} of 1 to 2) (Hecht, 1921). The occurrence of spontaneous SPF's in the dark and their susceptibility to temperature changes also suggest that energy in the specific form of photons is not necessary for SPF production. This is not to say that light does not act on the SPF's; its effectiveness has been demonstrated many times in the past.

Interactions between light and thermal energies were noted by Srebro (1966) for the ERG in *Limulus*, and by Hagins and McGaughy (1967) for "fast photovoltages" in squid retina. Srebro found that the minimum activa-

tion energy required for the ERG response could be supplied by photon quantum energies (of wavelengths shorter than about 600 nm) or by combination of thermal and photon energies. Hagins and McGaughy showed that the fast photovoltages had components due to the visual pigment and various stable photoproducts in addition to a thermal component produced by the incident light.

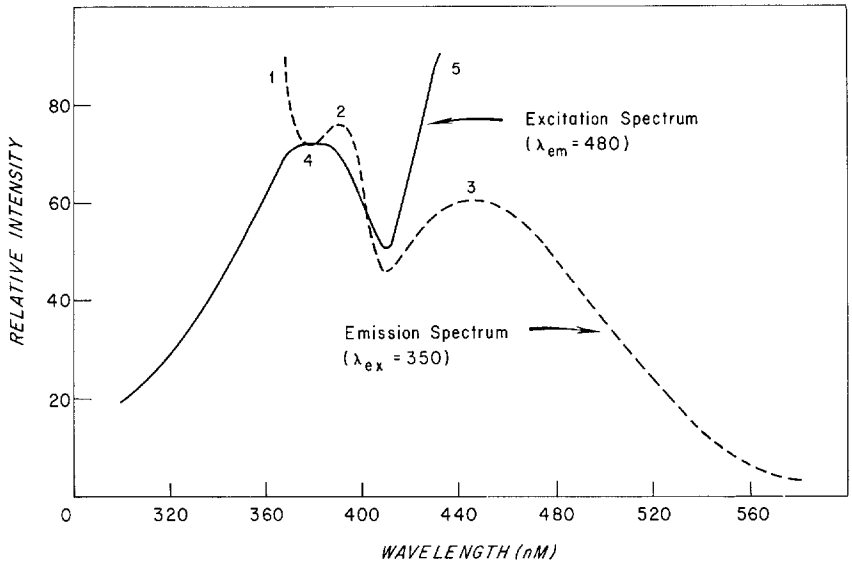

FIGURE 9 *a*. Emission and excitation spectra of corneal fluorescence. Broken line, emission spectrum for a 350 nm excitation wavelength: (1) Rayleigh scattering peak, (2) Raman peak, (3) fluorescence peak. Solid line, excitation spectrum for a 480 nm emission wavelength: (4) excitation peak, (5) scattering peak. Uncorrected spectra from an Aminco-Bowman spectrophotofluorimeter. Correcting for spectral characteristics of light source, gratings, and detector broadens slightly the long-wavelength shoulder of the emission spectrum and sharpens and increases the relative intensity of the excitation peak (4) of the excitation spectrum.

The thermal sensitivity I found in my experiments showed through analysis of Arrhenius plots, that activation energy was not constant. This may indicate that several rate-determining chemical processes are involved in the generation of SPF's. These processes could be: (*a*) enzyme activation or autocatalysis at the higher temperature or (*b*) the activities of two types of cells which contain the visual pigment and which are differentially sensitive to temperature.

A number of investigators have studied the spectral sensitivity of the ERG response in *Limulus* lateral eye (Wald and Krainin, 1963; Srebro, 1966; Chapman and Lall, 1967) and found only one peak in spectral sensitivity at approximately 525 nm. More intense lights than those used in my study are

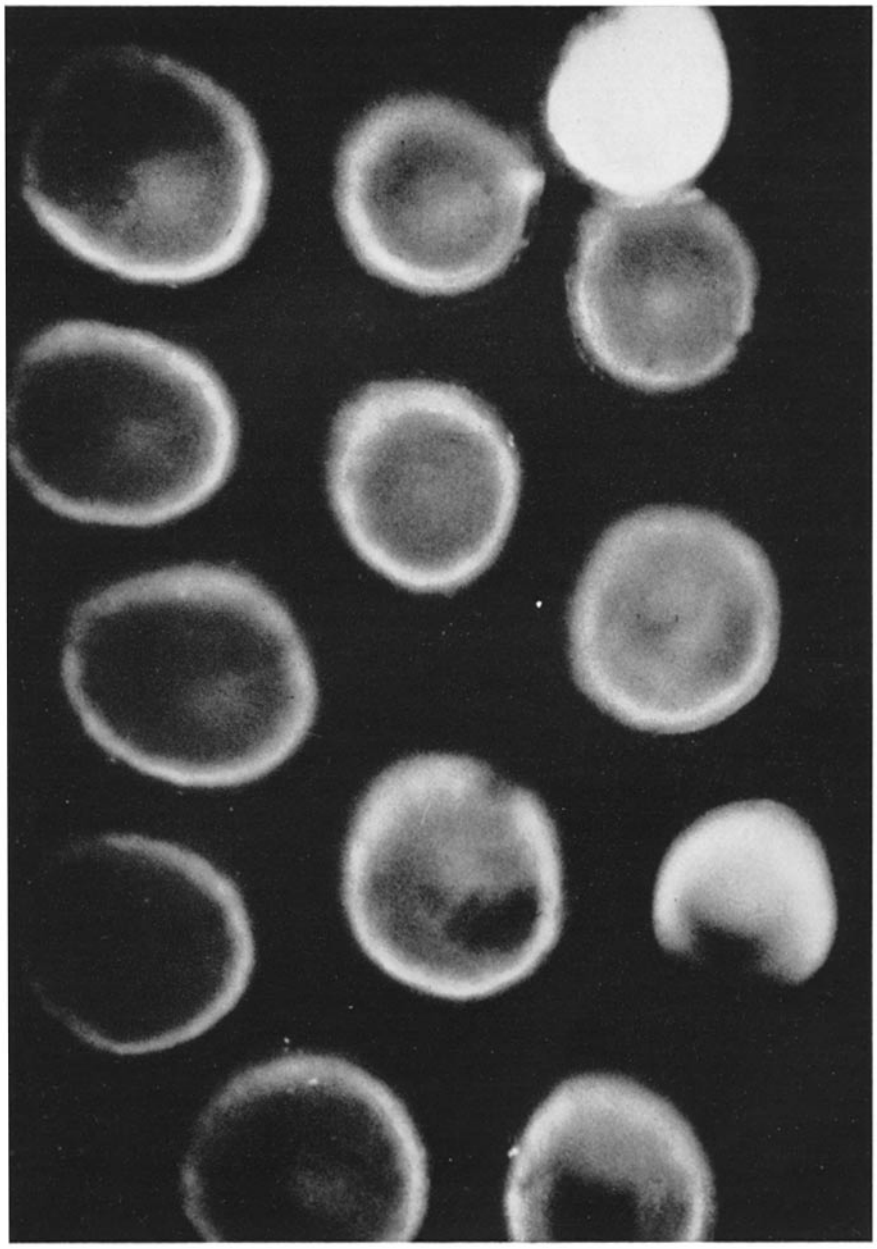

FIGURE 9 *b*. Fluorescence micrograph of a section of cornea through the crystalline cones. Dark-field view of unstained, unfixed (frozen) section in Leitz fluorescence microscope. Excitation wavelength peak about 370 nm. Visible fluorescence is a bright blue (420 to 460 nm range). Variations of the fluorescence seen in different crystalline cones are due to differences of the axial angle of the cones relative to the plane of the section. A fluorescent, lamellar fine structure cannot be seen in this reproduction of the original color micrograph.

required to elicit ERG responses. The difference in light intensity may be a significant factor which caused my findings concerning the short-wavelength characteristics for the lowest criterion levels to differ from the results reported by the others. The SPF spectral characteristics, which I found for the highest criterion levels, are similar to the Dartnall curve and the ERG spectral characteristics reported by the other investigators.

The primary and invariant peak in the spectral characteristics which I find in the region of 520–540 nm most probably reflects the spectral absorption properties of the ommatidial visual pigment. According to Hubbard and Wald (1960), this visual pigment is a rhodopsin with an absorption peak at 520 nm. My finding that under certain conditions there may be a significant sensitivity at short wavelengths, is difficult to explain. Peculiarities of the dioptric mechanisms may be one possible explanation. Since the light stimulus was directed obliquely upon a thin slice of eye in these experiments, there were two major pathways by which the light could reach the rhabdomeres. One pathway was primarily transcorneal; the other pathway was directly on to the ommatidium and through the screening pigment into the receptor interior.

The spectrophotometric measurements show that light transmission through the cornea and screening pigments is uniform (no marked peaks or dips) and decreases as wavelength decreases. There is certainly not any increase in transmission at the shorter wavelengths; an increase would be required in order to explain the unusual SPF sensitivity on the basis of light transmission spectrum. Corneal fluorescence may produce responses to short-wavelength stimuli which appear inordinately large compared to the expected responses based on the spectral absorption characteristics of the 520 nm visual pigment.

A second photopigment, absorbing maximally and broadly in the short-wavelength region, is another possibility to explain the unusual aspects of the spectral characteristics. It is necessary to speculate that the absorption properties of the second pigment are highly intensity dependent compared to properties of the primary photopigment. Relatively dim lights would adapt, or suppress, the sensitivity of the second pigment to a much greater extent than they would adapt the primary pigment sensitivity. There would be little difference between the two pigments in the adaptation effects of the very lowest intensity lights. Preliminary results of selective adaptation experiments do not support this hypothesis nor do the findings of other investigators, mentioned earlier in this paper.

A third possible basis for the unusual short-wavelength spectral sensitivity is variability of the data, especially at the SPF frequency levels corresponding to spontaneous activity. This variability will certainly affect the shape of the transfer function curves drawn through the data points. It is difficult to see why such variability would only be effective in selectively distorting the short-wavelength transfer functions so that the resulting spectral sensitivity matches

the pigment absorption characteristics at the highest criterion level and departs from those characteristics in a unidirectional way at lower criterion levels. Spectral sensitivity variations above and below the pigment absorption characteristics and variations at all wavelengths might be expected to result from uncertainty in the transfer functions.

With the information available at present, the speculation about these short-wavelength spectral characteristics seems unproductive. About all that can be said is that they exist and they offer an interesting challenge for a reasonable explanation. The important result of these SPF spectral measurements, it seems to me, is that there is a correlation between the absorption characteristics of a known visual pigment and the invariant peak of the SPF spectral sensitivity. This suggests that the rate of occurrence of the SPF's is at least correlated to the absorption of light by the visual pigment, and that the SPF's may be a direct result of the action of light on the visual pigment.

A brief comment concerning the blocking action of hydroxylamine on rhodopsin regeneration and on the occurrence of SPF's is pertinent. Hydroxylamine is a reagent which, in addition to its well-known retinaldehyde-trapping properties, has various antimetabolic actions (Fruton and Simmonds, 1958; Baxter and Roberts, 1960) and may even cause pigment decomposition (Dartnall, 1962). These antimetabolic and denaturing actions could be the significant effect in my experiments. There are several arguments against an antimetabolite hypothesis but I will not present them here since they are, at present, supported by a limited number of experiments.

My results and previous findings on the nature of SPF's may be interpreted as evidence for the following hypothesis. The SPF's are the electrical signs of one step in the series of events which occurs between the absorption of photons by the visual pigment and the photoreceptor potential. Their immediate cause is a transient and local conductance increase in the rhabdome membrane. There is temporal and spatial summation of the SPF's to produce the receptor potential. The receptor potential is electrically coupled to the eccentric cell dendrite (Smith et al., 1965) and ultimately becomes the generator potential for spikes in the eccentric cell axon.

The events leading up to the single, discrete conductance change are perhaps those outlined by Wald et al. (1963). The critical step for visual excitation in this scheme is the opening up of opsin after the isomerization of the chromophore. Thermal energy may be effective in the opening up. But photoisomerization is necessary before the thermal effects can occur at this stage. My results show that thermal energy is also effective on spontaneous SPF's. Since thermal bleaching of isolated rhodopsin yields 70% unisomerized retinal (Hubbard, 1958), there may still be sufficient isomerization by heat of the chromophore in vivo to account for the effects of thermal energy on spontaneous SPF's.

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