Neural Photoreception in a Lamellibranch Mollusc

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ABSTRACT The pallial nerves of *Spisula solidissima* each contain a single afferent nerve fiber which responds directly to illumination of the nerve, and apparently mediates the "shadow" response of siphon retraction. These units show constant-frequency spontaneous activity in the dark; illumination abruptly inhibits this discharge, and cessation of the light stimulus then evokes a prolonged burst of impulses at high frequency (the off-response). Impulses are initiated at a point near the visceral ganglion, and propagated unidirectionally toward it. Stimulation with monochromatic light has revealed that more than one photoreceptor pigment is involved, since the discharge patterns evoked are wavelength-specific. Inhibition is relatively prominent at short wavelengths, excitation at long wavelengths. Following selective adaptation with blue light, "on" responses can be produced with red stimuli, demonstrating the unmasking of an excitatory event which takes place during illumination. The two photoreceptor pigments may be segregated in two or more cells presynaptic to the recorded unit, or,—more likely—may both be contained in the same cell. The spectral sensitivity function for inhibition shows a single maximum at 540 mμ, and is probably dependent upon a carotenoid pigment. No photoreceptor function has been demonstrated for a hemoprotein, apparently identical with cytochrome *h*, which occurs in high concentration in *Spisula* nerve.

INTRODUCTION

It has been known since the early experiments of Nagel (1894) that the siphons of lamellibranch molluscs contain photoreceptors which mediate withdrawal of that organ upon illumination or darkening. In fact, this withdrawal response was employed by Hecht (1920, 1927) in the classic researches on *Mya* and *Pholas* which produced the first photochemical constants for any visual system. Despite this distinguished experimental history, virtually nothing is known about the electrophysiology of the receptor units involved.

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The present communication will describe electrical responses from a photoreceptor in the pallial nerve of the surf clam, *Spisula solidissima*. The experiments were undertaken not only from a desire to find neural correlates for a classic behavioral response, but also because of a growing interest in the comparative physiology of "simple" photoreceptor systems. It has become clear that some invertebrates possess photosensory cells which are relatively unmodified neural elements, located within the nervous system proper; examples are the caudal photoreceptor in the sixth ganglion of the crayfish (Prosser (1934); Kennedy (1958*b*)), and the radial nerves of certain echinoderms (Yoshida and Millott (1959)). The molluscs seemed a likely group for extension of these comparative studies, especially since many members of that phylum have rich neuronal pigmentation. Moreover, studies by Arvanitaki and Chalazonitis (1949*a*) have demonstrated that the excitability and spontaneous activity of ganglion cells in the nudibranch *Aplysia* can be modified by illumination, and that the effect is mediated through carotenoid and hemoprotein pigments within the neuron somata. Although the absolute sensitivity of these cells to light is not high and their location is unsuitable for a photoreceptor, the existence of the effect provides ample justification for the suspicion that other molluscs may contain functional "neural" photoreceptors. The search for these has been further stimulated by the finding (Arvanitaki and Chalazonitis (1949*b*)) that in some of the *Aplysia* neurons the primary result of illumination is inhibition rather than excitation. Such inhibitory responses have not yet been found in a primary photoreceptor cell.

The receptor unit to be described in this paper shares some of the properties reported by Arvanitaki and Chalazonitis for *Aplysia* neurons, but differs in having much greater sensitivity and a peripheral location. It will be shown that the response to illumination consists of inhibitory and excitatory components, mediated by different photosensitive pigments having different absorption spectra. A preliminary account of some of the experiments has appeared elsewhere (Kennedy (1958*a*)).

METHODS

Living Material Live surf clams (*Spisula solidissima*, referred to mistakenly in a previous communication (Kennedy (1958*a*)) as *Mactra solidissima*) were used in these experiments. They were collected by the Supply Department of the Marine Biological Laboratory and stored in circulated sea water aquaria. Those shipped to Syracuse showed rather low survival, but a sufficient number of healthy animals survived shipment to permit these experiments. Some experiments upon *Mya arenaria* and *Venus mercenaria* are also reported; these molluscs were collected and maintained in the same fashion.

Preparation and Recording In all cases, the left valve of the animal was removed after cutting the adductor muscles, and the mantle and gills of that side were dis-

sected away. The visceral ganglion lies in a mass of connective tissue anteroventral to the posterior adductor muscle. The paired pallial nerves pass ventrally from this ganglion past the siphon, where they each give off branches (the siphonal nerves), and turn anteriorly into the mantle edge. These nerves were freed from connective tissue past the point where the siphonal nerves branch, and their proximal ends cut near the visceral ganglion. They could then be arranged for recording by drawing up the free end, leaving all peripheral endings intact. Alternatively, fairly long segments of the pallial nerve with or without the siphonal branches were dissected entirely free from the animal and placed in a special chamber for recording. In some experiments, fine branches of the siphonal nerves were traced into the siphon and aranged for recording.

Some efforts were made to subdissect the nerves in an attempt to isolate single fibers. All these met with failure; the nerve trunks are surrounded with an extremely tough connective tissue sheath, and the nerves within are fragile and, apparently, easily damaged. This experience duplicated that of other investigators (*e.g.*, Tobias (1952)).

Dissection and recording were performed in sea water. A short segment of nerve was lifted onto a silver or platinum electrode placed just above the bath, with a second lead in the solution. In some cases, a layer of paraffin oil was used to prevent drying of the exposed nerve; usually, however, it was simply allowed to dry. (Under these conditions, the exposed segment of nerve simply acted as an extension of the recording electrode, and resistance did not become high enough to reduce the amplitude of the discharges.) A conventional low-level capacity-coupled preamplifier and oscilloscope were used for displaying action potentials, which were recorded on moving paper with a Grass kymograph camera. The second oscilloscope channel was connected with a photocell which monitored the stimulating light but did not indicate its intensity. Action potentials from the single active photoreceptor fiber in *Spisula* were of fairly low amplitude, usually less than 50 μv., so that producing usable records for analysis depended upon having a favorable orientation of the active fiber in the bundle with respect to the recording electrodes, and upon having a system relatively free from noise. Some preparations yielded action potentials which were of such low amplitude that they could not be reliably used for experimental analysis.

A final note concerns the use of metal electrodes in experiments in which light is employed as a stimulus. It is always possible that photoelectric currents induced between recording electrodes may act as a direct stimulus to the preparation. In these experiments, the capacitative coupling between the recording electrodes makes this extremely unlikely. The responses to light were, furthermore, independent of whether platinum or silver electrodes were used; and experiments with small spots of light directed onto a part of the nerve at some distance from the electrodes showed that such stimuli were effective in inducing activity. The possibility that the responses could represent some kind of photoelectric "artifact" was thus eliminated.

Stimuli In the earlier experiments, white light stimuli were employed. These were produced by a tungsten filament, battery-operated microscope lamp; duration was varied by adjustment of a photographic shutter used to deliver the flashes, and intensity was controlled by Wratten neutral tint filters.

The light source for the experiments in which monochromatic stimuli were used was

a 100-watt zirconium arc lamp. An image of the annular cathode of the source was focused upon the entrance slit of a Bausch and Lomb 500 mm. focal length grating monochromator. This instrument, with a 1200 line/cm. grating, has a linear dispersion of ± 1.6 mμ per mm. In these experiments, the slit widths were 3 mm. or less. Source and monochromator were calibrated with an Eppley thermopile and D. C. amplifier, so that the relative energies produced at the exit slit of the monochromator at each wave length were known. Light from the exit slit then passed through a shutter and was focused on the preparation by means of a short focal length condensing lens, forming a stimulus patch of approximately 3 mm^2.

Intensity at each wavelength was regulated with a series of Wratten neutral tint filters, each calibrated throughout the visible spectrum with a Cary recording spectrophotometer. These filters were available in sufficiently finely graded combinations so that a reasonably accurate threshold for the alteration of discharge frequency could be found. The photoreceptor fiber in *Spisula* shows a "resting" discharge in the dark of about 5 impulses/second. The method for threshold measurement involved finding the intensity, at each wavelength, which would produce either (*a*) a barely perceptible decrease in the firing frequency of the nerve; or (*b*) a total inhibition for some specific length of time, either 5 or 10 seconds. These thresholds could be accurately determined merely by watching the changes in impulse frequency on the oscilloscope face. The reciprocals of the threshold energy values were then plotted against wavelength to yield the spectral sensitivity function. During the course of a single experiment, the sensitivity at one particular wavelength was checked several times to assure that overall sensitivity was not changing during the run. Since the final spectral sensitivity function derived from such intensity values involves the intervention of numerous calibrations and calculations, it was deemed advisable to test the apparatus on a photoreceptor with known properties. The optical system was therefore altered by introduction of a different final lens and a fixation point, and used for psychophysical tests of the scotopic visibility function of the author's own eye. Such measurements, made with a 10° parafoveal rectangular test field subtending approximately 5° in its longer dimension, yielded points which were in good agreement with the average human scotopic luminosity curve (Stiles and Smith (1944)). The calibrations and correction factors for the apparatus may, therefore, be regarded as satisfactory.

Pigment Analysis Solvents and extraction procedures employed in the work dealing with neuronal pigments are described under Results. All spectra of extracted solutions were measured in a Beckman spectrophotometer. Microspectrophotometric measurements were carried out with an apparatus employing a Bausch & Lomb 250 mm. focal length monochromator with a tungsten source. Low power microscope objectives ($\times$ 10) were used with a photomultiplier tube (Photovolt) serving as the detector. This apparatus was lent through the courtesy of Dr. Henry DiStefano of the Anatomy Department, State University of New York, Upstate Medical Center.

Histology Several ganglia, with long segments of both pallial nerves attached, were fixed in 10 per cent neutral formalin. Serial sections of the nerves were cut at 13 μ, beginning at the ganglion and continuing well past the point of branching of the siphonal nerves, and stained with hematoxylin-eosin.

RESULTS

1. *Discharge Patterns of Siphonal Receptors in Various Lamellibranchs*

Initially, attempts were made to record impulse activity in the siphonal nerves of *Mya*, *Venus*, and *Spisula*. It was assumed, partially because of the histological observations of Light (1930) on *Mya*, that photoreceptor elements in these molluscs are scattered through the siphonal wall, and that therefore the siphonal nerve and its branches would contain numerous afferent fibers from them. This expectation was fulfilled in the case of *Mya* and *Venus*. Recordings from the entire siphonal nerve, or from its more peripheral branches, showed that bursts of impulses could be evoked upon illumination of the siphon. The impulses are of very low amplitude, and clearly come from a large number of fibers. They occur both at the onset and the cessation of the stimulus, and there is little sustained activity during prolonged exposures. The fibers mediating this response come from a large number of receptor units, since even the fine peripheral branches of the siphonal nerve show multifiber discharges. Because of the difficulty of recording single fiber discharges (see Methods), no precise measurements of latency or discharge frequency could be made. On the basis of spike amplitudes, the fibers must be much smaller than either the large motor fibers of the same nerve (*cf.* Horridge (1958)) or the numerous tactile afferents which were encountered in these experiments.

In *Spisula*, no multifiber discharges of this sort were recorded from the siphonal nerve or from its branches. When, however, the recordings were made from the central end of the pallial nerve near its point of entry into the visceral ganglion, single fiber activity showing light responses was encountered. The remainder of this communication will deal with this photoreceptor.

2. *Discharge Pattern of the Spisula Photoreceptor*

The response in question is judged to be that of a single afferent neuron because (*a*) the spike amplitudes in any given preparation were constant, and (*b*) the spike intervals during discharge were extremely regular. On no occasion has there been any suggestion that more than one unit is present in each of the paired pallial nerves.

The discharge pattern is shown in Fig. 1. The fiber is active in the dark, discharging at a frequency of about 5 per second. Occasionally, the impulses are not discharged at constant interval, but in "bursts;" in such cases, however, the intervals between bursts and the number of impulses per burst are relatively constant. Upon illumination, the spontaneous discharge is abruptly inhibited. The cessation of illumination is followed by a discharge of higher frequency which gradually declines to the resting or "dark" level. The fre-

quency, latency, and duration of the off-discharge are related in a complex way to the intensity, duration, and wavelength of the stimulus.

A. THE INHIBITORY EVENT The spontaneous activity of the fibers in the dark provides a background against which inhibition can be readily observed. Near threshold, the inhibitory effect consists of a slight decrease in firing frequency during the light exposure; the inhibition builds up to a peak

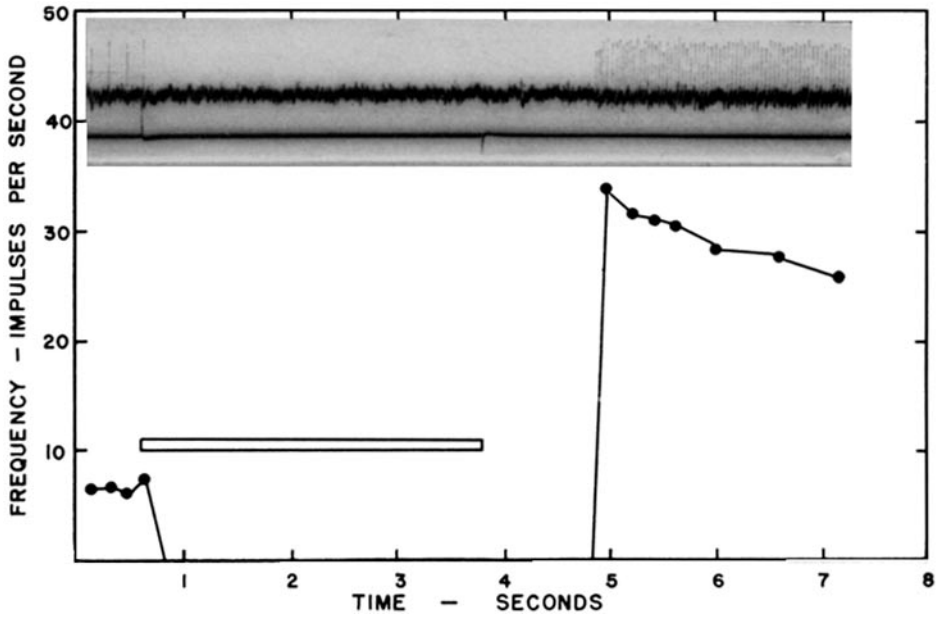

FIGURE 1. Discharge pattern of the *Spisula* photoreceptor fiber in response to a stimulus of about 3 seconds' duration (signalled by artifacts on lower beam of record and by bar on graph). Stimulus wavelength 550 mμ; intensity 6×10^{-3} watts/cm.^{2} Frequencies of off-discharge averaged over one-fifth second periods.

within a second after the onset of illumination, and then shows some tendency towards adaptation ("escape"). At higher intensities, firing during illumination is completely blocked.

The latency of inhibition (time between onset of flash and occurrence of last spike before block) decreases as intensity is increased. By inducing off-discharges and then interrupting them by reillumination, this relationship may be viewed against a stronger background of excitation. Fig. 2 shows such an experiment; it is a plot of impulse interval against time for two 1 second exposures, one near threshold and the other ten times as bright. From such data, an estimate of 40 msec. for the minimum latency of inhibition can be made; but since the method is limited by the frequency of the discharge being inhibited, the real latency minimum may be lower.

Using monochromatic stimuli, an approximation of the absolute threshold for inhibition can be made. The total output of the monochromator at 500 mμ (slit width 3 mm.) is 3.5×10^{-4} watts; this is concentrated by the terminal optical system, after some losses, into a stimulus patch of about 3 mm.², which therefore has an energy flux of 1.2×10^{-2} watts/cm². In the three most sensitive preparations threshold inhibitory responses were obtained with neutral

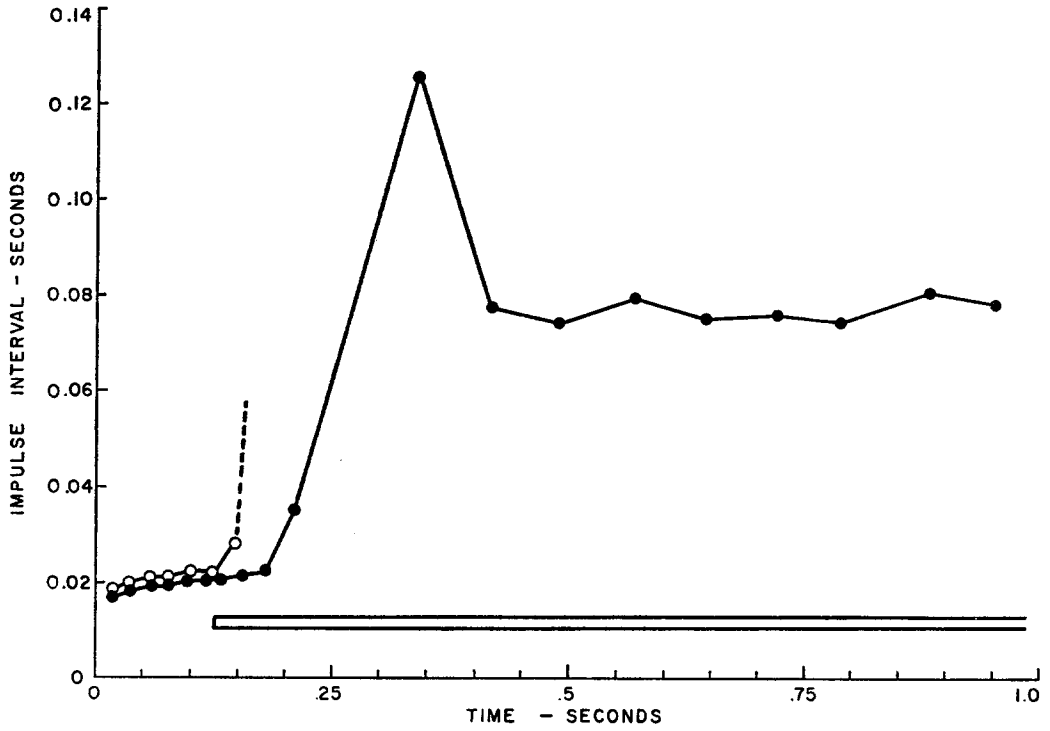

FIGURE 2. Inhibition of off-discharge by reillumination. Initial discharge produced by a 1 minute exposure to white light of 500 foot-candles; the reilluminating flashes, signalled by the bar beneath the graph, had intensities of approximately 150 (open circles) and 15 foot-candles (closed circles). They followed the beginning of the preceding off-discharge by 0.6 sec. Interval preceding each impulse plotted at the time of occrrrence of that impulse.

density filters having an optical density of 6.3 at 500 mμ interposed between monochromator and preparation. The threshold energy flux is thus 6×10^{-2} ergs/sec. × cm.² (field). This clearly represents a maximal estimate of threshold, since some optical system losses are not accounted for and 500 mμ is not quite at the spectral region of greatest sensitivity.

B. THE EXCITATORY EVENT The off-discharge following a brief white light stimulus begins after a latent period which is considerably longer than that for inhibition at "on." At any given intensity, the latent period first in-

creases, then decreases as the stimulus duration is made longer, reaching a minimum of approximately 0.1 sec. in most preparations (Fig. 3). At moderate intensity, such as that used in Fig. 3, frequency shows a graded increase with increase in duration. Firing frequencies may reach over 100 impulses/sec. at the peak of the off-discharge; the frequency often requires over a minute to return to the dark value.

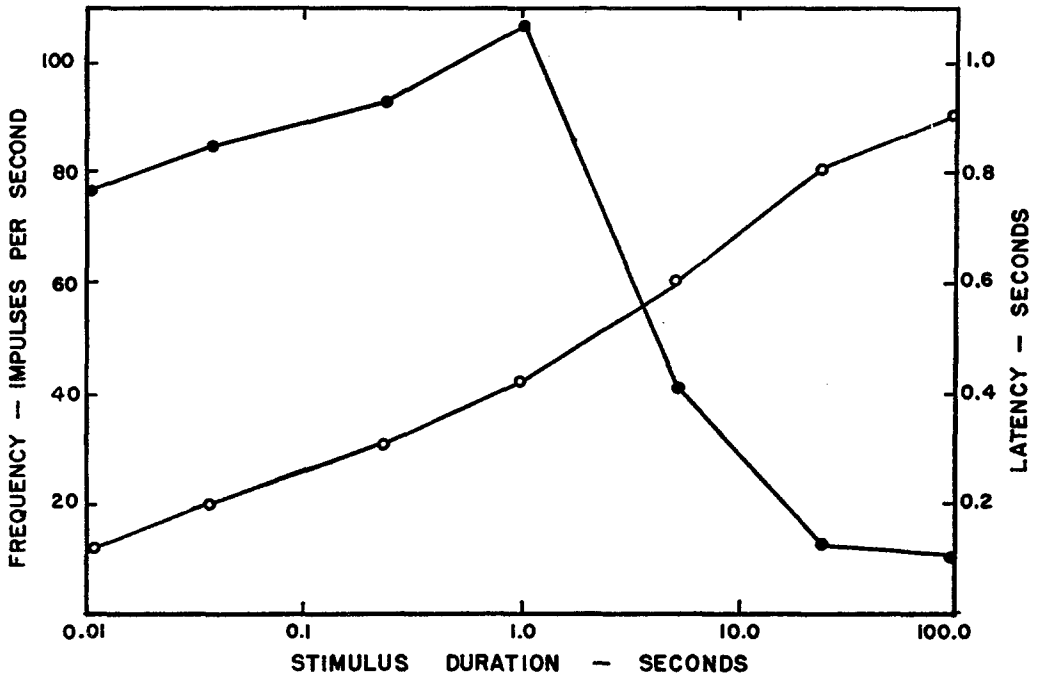

FIGURE 3. Plot of latency (closed circles) and maximum frequency (open circles) of off-discharge as a function of stimulus duration. White light; stimulus intensity approximately 130 foot-candles.

If duration is held constant and intensity varied, the result is also complex. Such an experiment is illustrated in Fig. 4. Impulse frequency (open circles) first increases, then falls as intensity is increased, while the latency (closed circles) increases markedly at high intensity. Although these relationships are somewhat dependent upon the stimulus duration chosen, a frequency maximum at some intermediate intensity is always found.

The off-discharge does not depend upon previous activity in the fiber. When the photoreceptor is under constant illumination, it may be kept inactive for long periods of time. Fractional decreases in the intensity of the stimulus still evoke strong off-discharges, the frequency of which increases as the ratio $\Delta I/I$ becomes larger.

3. *Location of the Photoreceptor Element(s)*

The first observations on the photoactivation of the discharges described above were made with intact preparations, using a large stimulus which illuminated the entire posterior region of the animal, including the siphonal nerve itself. It was soon discovered that illumination of the siphon alone was ineffective and that, in fact, only illumination of the pallial nerve itself distal

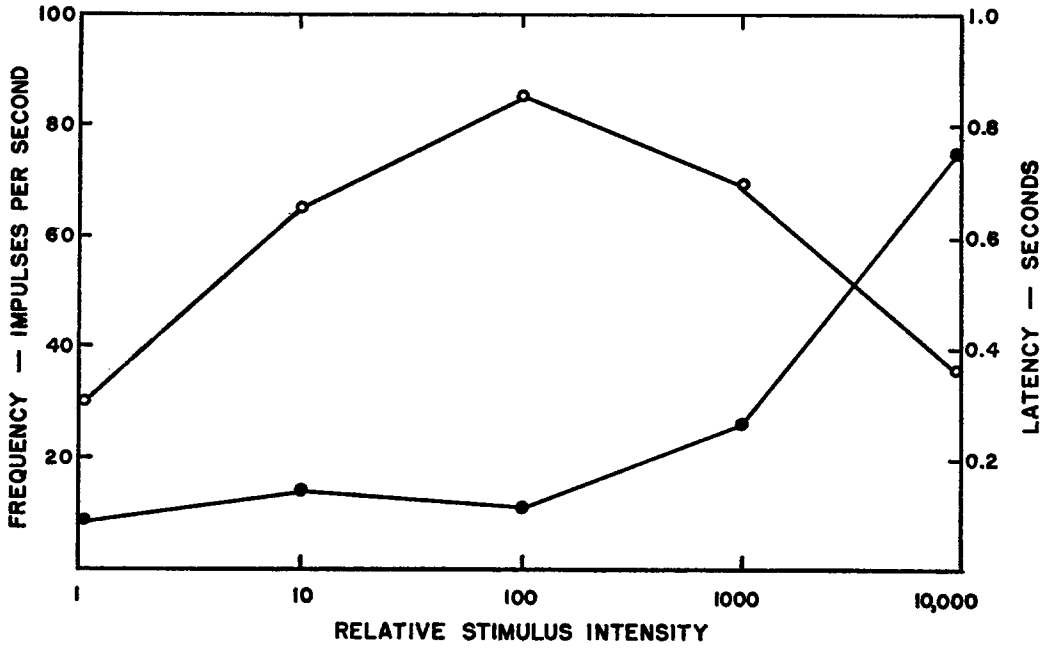

FIGURE 4. Plot of latency (closed circles) and maximum frequency (open circles) of off-discharge as a function of stimulus intensity. White light; stimulus duration 5 seconds, intensity of 1 corresponds to approximately 0.15 foot-candle.

to the visceral ganglion could elicit the response. Subsequently, experiments were performed on excised segments of pallial nerve about 1.5 cm. in length, free of major branches.

The impulses are apparently propagated only towards the visceral ganglion and must originate in an area within 1 cm. of the ganglion. Experiments in which the distal portion of the nerve was arranged for recording and the ganglion region illuminated failed in every case to produce any discernible discharges; in fact, no spontaneous activity having the appropriate dark discharge rate could be recorded under such conditions. When small spots of light were used for stimulation and the recording electrodes placed on the proximal end of the nerve, definite regional variations in threshold were en-

countered. The most sensitive spot appears to be near the point where the siphonal nerve branch occurs.

The suggestion that the photoreceptor element is localized in a definite region of the pallial nerve and that it initiates impulses which propagate centripetally is supported by the changes in action potential waveform which occur under different recording conditions. These are illustrated in Fig. 5. The proximal end of the nerve is slung over one recording electrode (No. 1); the second lead (No. 2) is immersed in the sea water bath in which the remainder of the nerve lies. The potential difference is thus recorded between the air–sea water interface and the proximal end of the pallial nerve, across the relatively large resistance of the exposed segment of nerve. When only a

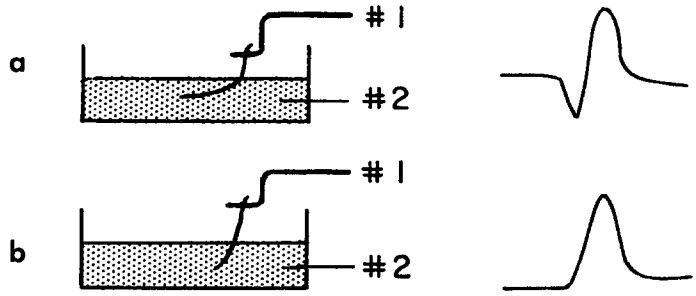

FIGURE 5. Waveform changes of impulses from the photoreceptor fiber under different conditions of recording from isolated segment of pallial nerve. For details, see text.

short segment of the nerve projects from the sea water onto electrode 1, a diphasic potential is recorded as in Fig. 5*a*. But when electrode 1 is raised, drawing the nerve up so that a longer length extends beyond the interface, the discharges become monophasic (Fig. 5*b*). The best explanation is that the impulses are originating at a point fairly close to the proximal end and propagating towards electrode 1; when the initiating region is below the interface, first electrode 2 and then 1 become negative, producing a diphasic wave. When the initiating region is above the interface, the solution electrode can only behave as an inactive lead, and the response is monophasic.

4. *Responses to Monochromatic Stimuli; Spectral Sensitivity*

In order to obtain information about what kind of photosensitive pigments might be involved in the mediation of this response, monochromatic stimuli were employed to measure spectral sensitivity. The routine procedure was to expose the preparation to constant-duration flashes over a broad intensity range at each of a number of wavelengths. For each wavelength tested, a plot was then made of stimulus intensity against any of the response parameters, for example, frequency or latency (*cf.* Graham and Hartline (1935)).

A series of such experiments was carried out upon the pallial nerve photoreceptor in *Spisula*, and produced a somewhat surprising result. When plots were made at several wavelengths relating the intensity of monochromatic stimuli to several response parameters—in particular, (*a*) the duration of the "inhibitory interval" produced by brief flashes and (*b*) the maximum impulse frequency of the off-discharge—the resulting curves in each case showed

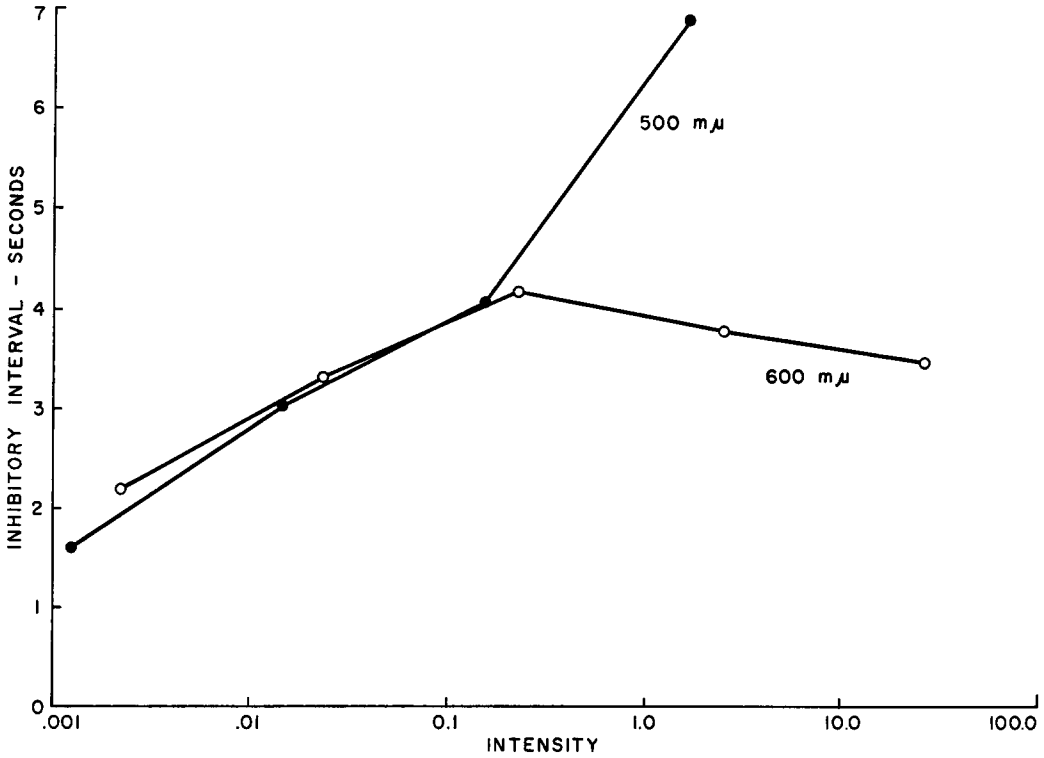

FIGURE 6. Inhibitory interval (elapsed time between last impulse before inhibition of dark discharge and first impulse of off-discharge) as a function of intensity for two wavelengths: 500 mμ (closed circles) and 600 mμ (open circles). Intensity of 1 is approximately 2.6×10^{-4} watts/cm^2.

marked differences which depended upon the wavelength used. An example of this dependence is shown in Fig. 6, which illustrates the relationship between inhibitory interval (the time between the last impulse preceding the flash and the first impulse of the off-discharge) and intensity at two wavelengths: 500 and 600 mμ. At low intensities, the curves are nearly parallel; but at higher values, the latency in response to the 600 mμ stimulus decreases, while that at 500 mμ becomes very long. At high intensities at wavelengths below about 525 mμ a 2 second exposure can actually produce an inhibition which lasts for 2 minutes or more, whereas stimuli of wavelength 600 mμ or

more never give this result. Curves relating frequency of off-discharge to intensity also showed marked differences in form which were wavelength-dependent. Fig. 7 shows examples of actual records from an experiment similar to that illustrated in Fig. 6. The discharge patterns at low intensity at the

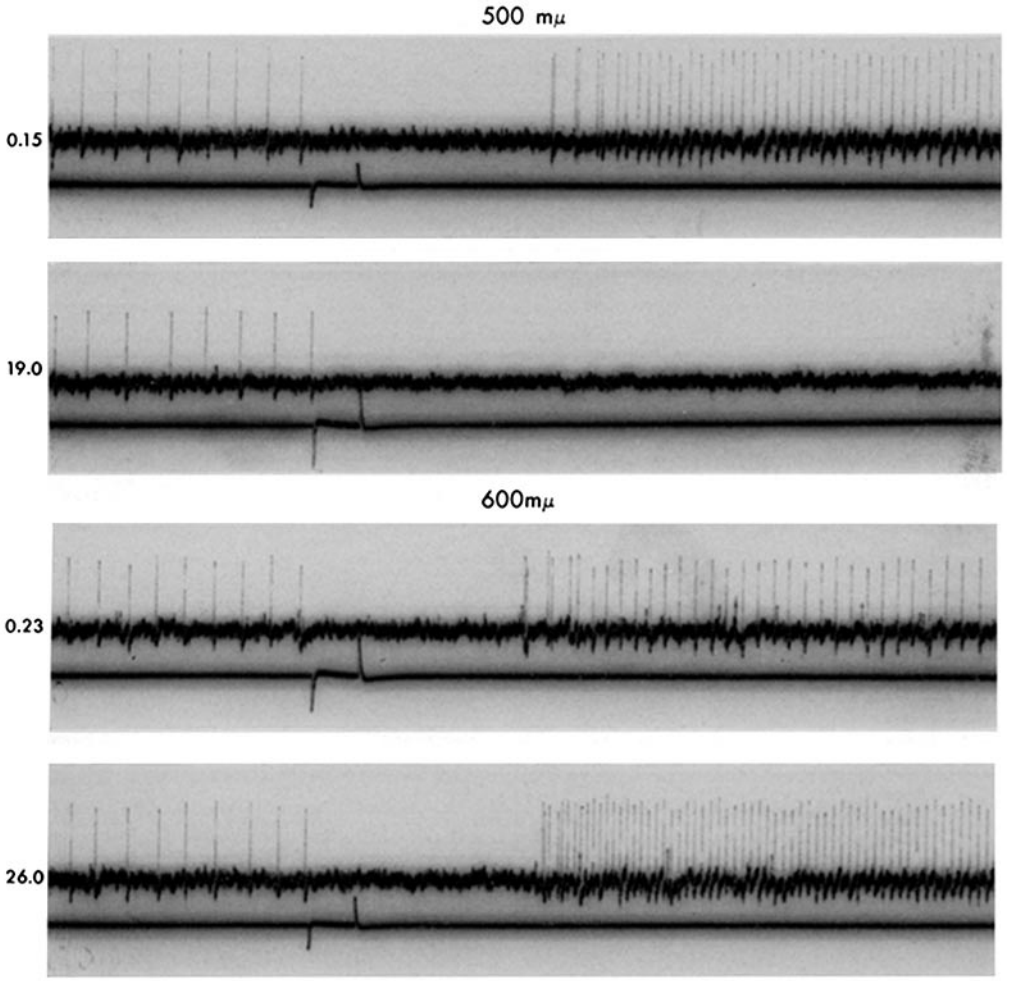

FIGURE 7. Records of responses to monochromatic stimuli showing wavelength dependence of discharge pattern. Numbers to left of records indicate relative intensity; an intensity of 1 is approximately 2.4×10^{-4} watts/cm^2. Deflections of lower trace indicate stimulus duration of approximately 0.2 second.

two wavelengths 500 and 600 mμ are fairly similar, though the frequency of off-discharge at the longer wavelength is somewhat lower. At high intensity, the 600 mμ stimulus produces a high-frequency off-discharge at short latency, whereas the 500 mμ stimulus results in a block which outlasts the record by at

least a minute. Responses to long wavelength stimuli thus show a high frequency of off-discharge associated with a short latency; whereas those to short wavelength stimuli show lower off-discharge frequencies at high intensity and have very much longer latencies for any given value of frequency.

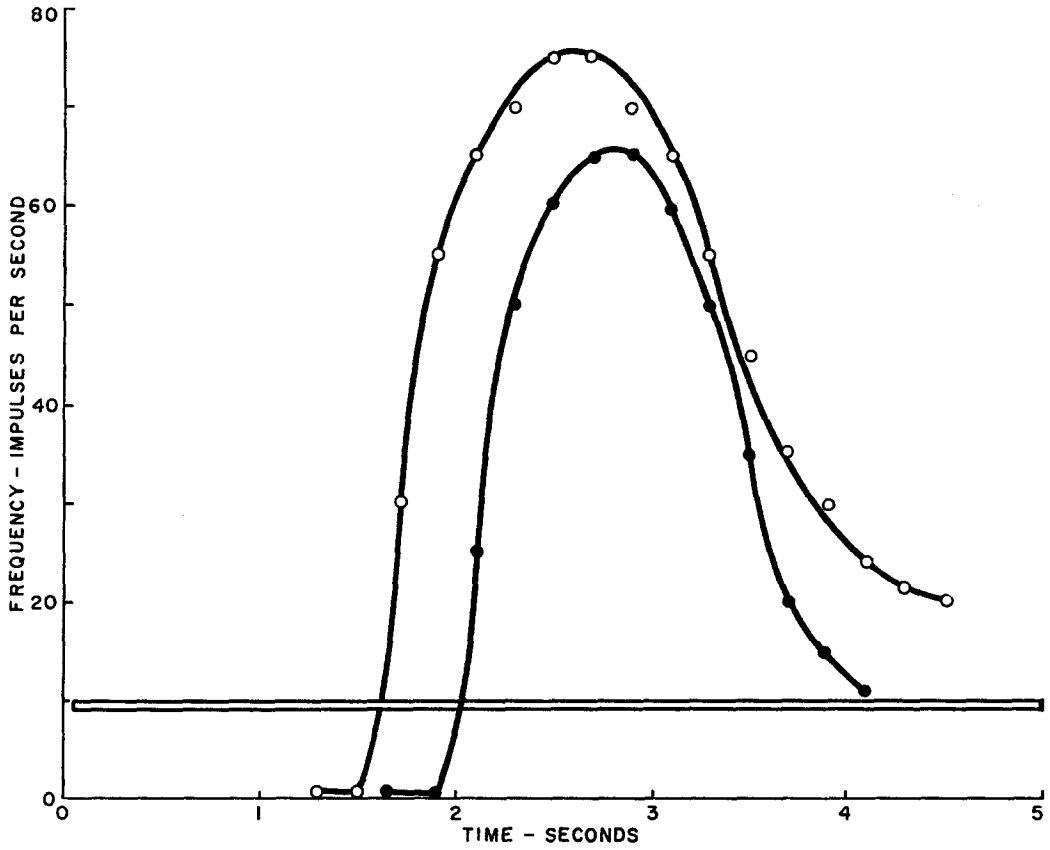

FIGURE 8. Frequency plots of on-discharges following selective adaptation to light of 475 mμ. Stimulus, indicated by bar, is at 630 mμ and has an intensity of approximately 7×10^3 watts/cm.^{2}; it begins 10 seconds after the adapting light is turned off. Intensity of adapting light 4×10^{-3} watts/cm.^{2}; duration 10 seconds for response plotted with filled circles, 30 seconds for response plotted with open circles.

Since these results suggested that more than one pigment was involved in the act of photoreception (see Discussion), selective monochromatic adaptations were performed to obtain supporting data. A test flash of 3 to 10 seconds' duration was given at 630 mμ; it resulted in a total inhibition of the dark discharge during the exposure, and was followed by the usual off-discharge. Then the preparation was adapted to light of 475 mμ for a period of from 5 to 30 seconds. This adapting exposure suppressed impulse activity for a period of

at least a minute in the dark. When, however, the adapting light was followed, after an interval of 20 to 45 seconds, by another light stimulus of 630 mμ, impulse discharge occurred both at the onset and the cessation of illumination. The "on"-discharge increased in frequency and number of impulses and decreased in latency as the duration of the previous 475 mμ adapting exposure was increased A sample experiment is illustrated in Fig. 8. On-discharges

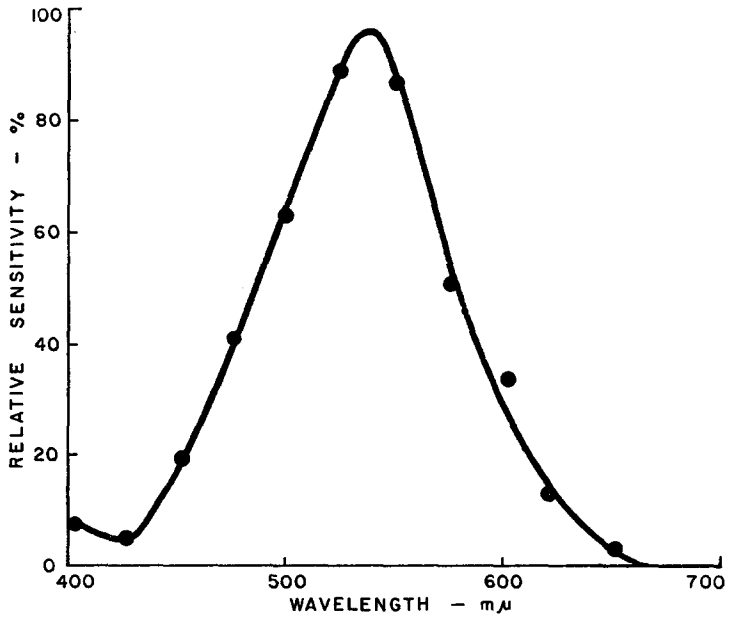

FIGURE 9. Averaged spectral sensitivity function for the inhibitory process. Sensitivity for inhibition plotted as a function of wavelength; each point is the average of seven determinations of four different preparations.

have been observed in response to long wavelength stimuli following blue light adaptation in all experiments, and occur only under these conditions.

Since the comparatively low threshold for inhibition of dark discharge can be accurately determined, a spectral sensitivity curve was obtained for this event. The experiments were performed on four different preparations. In those preparations with a very regular dark discharge, the sweep of the oscilloscope could be locked in phase with the occurrence of impulses on the screen, and the intensity found at each wavelength which would just produce a visible frequency decrease. In others, the discharge was too irregular to permit this, and it was necessary to define as the "threshold" the intensity (for continuous exposure) necessary to produce a predetermined period of "block" (usually 5 to 10 seconds). The two methods yielded data with no apparent differences,

and the results have been pooled in Fig. 9, which is the average of determinations made from seven runs on the four preparations.

5. *Neuronal Pigments*

It has been known for some years that molluscan ganglia are rich in carotenoid pigments; and some apparently contain hemoproteins as well (Arvanitaki and

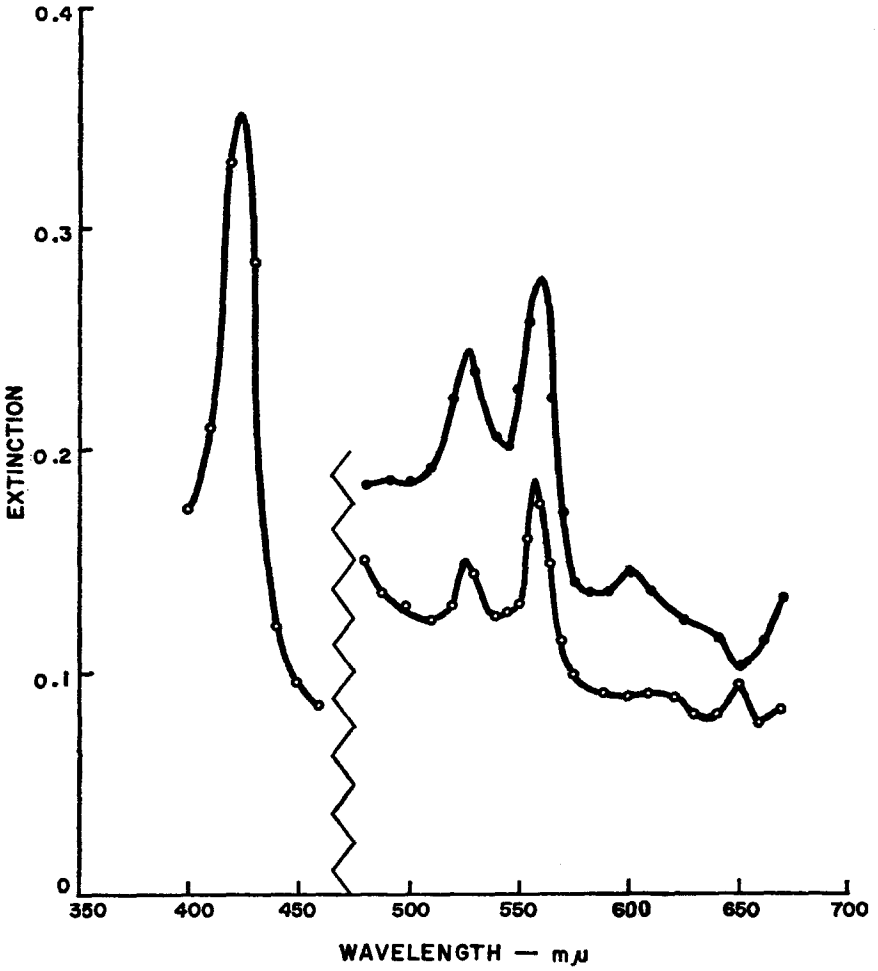

FIGURE 10. Absorption spectra of *Spisula* nerve determined *in vivo* by microspectrophotometry. Filled circles, ganglion cell layer in visceral ganglion; open circles, peripheral nerve trunk. Soret band of latter spectrum (to left of broken line) plotted at one-half extinction.

Chalazonitis (1949*b*)). In *Spisula*, however, (and in many gastropods as well) the nerve trunks and connectives as well as the ganglia contain a reddish pigment. This situation has been described briefly by Tobias (1952), who ob-

served that the pigment appears to be contained within axons but did not explore its chemical nature.

Since the pigment is present in the nerves of *Spisula* but not of the other lamellibranchs studied, it was naturally supposed that it might be the mediator of the photoreceptor process in the pallial nerve. Accordingly, some observations were made on its nature.

Microspectrophotometric measurements were performed on freshly isolated tissue, either nerves or ganglia, with low-power objectives; unpigmented regions of the tissue were used as "blanks." Typical absorption spectra obtained in this way are shown in Fig. 10; they indicate that the red pigment of *Spisula* neurons is a hemoprotein. It is spectrally different from the more familiar vertebrate cytochromes, but does resemble cytochrome *h*, a hemoprotein previously isolated from several molluscan tissues (Keilin (1956)). The extinction ratio of the Soret (γ) band to the green (α and β) bands suggests that the pigment is primarily in the reduced state *in vivo*. The similarity of the two spectra in Fig. 10 shows that ganglia and nerves both contain the same pigment; within the ganglia, it occurs in greatest concentration in the peripherally located neuron somata.

Extractions of the pigment were attempted using several different methods, including some of the standard procedures for cytochromes (*e.g.*, digitonin); the most successful method was aqueous extraction in M/15 phosphate buffer followed by centrifugation at high speed. The clear supernatant contains the pigment, but its absorption spectrum differs from that in Fig. 10 in (*a*) having a much higher Soret extinction compared to that of the α and β bands, and (*b*) exhibiting altered relative extinction and position of the α and β bands. These changes suggest that the extracted pigment is in the oxidized condition, though attempts at reversible oxidation and reduction of the pigment *in vitro* were not successful.

The ganglia contain, in addition to the hemoprotein, a yellow pigment or pigments; they thus have an orange-red appearance. The yellow pigment is soluble in acetone, petroleum ether, and other organic solvents, and is presumably a carotenoid. Acetone extracts of peripheral nerve yield none of this pigment.

6. *Histological Examination*

Serial sections were cut at 13 μ, beginning at the visceral ganglion and extending through the pallial nerves to a point well past the siphonal branch. Preparations made in this way of six pallial nerves, stained with hematoxylin and eosin, revealed that in general the structure is similar to that described for other molluscan peripheral nerves; *e.g.*, those of *Helix* (Schlote (1955)). The nerve fibers are small in diameter and usually stellate in cross-section; and, as

with other molluscs, ganglion cell bodies occasionally occur along the nerve trunk. In the pallial nerve of *Spisula*, however, these are quite rare; in four of the six preparations, one or two cell bodies were found at the junction of the pallial and siphonal nerves, and in two others single cell bodies occurred elsewhere along the nerve. But in no case did groups of more than two such cells appear within the same area; and the maximum number of somata seen in any one series of sections from a nerve (involving a linear distance of more than 0.5 cm.) was three.

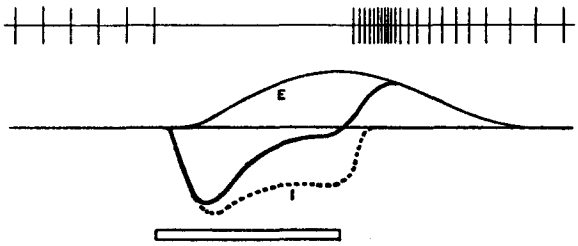

FIGURE 11. Postulated scheme for the contributions of inhibitory (*I*) and excitatory (*E*) processes to the discharge pattern of the receptor unit. See text.

DISCUSSION

Excitatory and Inhibitory Processes

The experiments described above show that the response of the *Spisula* photoreceptor is composed of an excitatory and inhibitory component. The inhibitory process has the lower threshold, since it dominates during the period of illumination; but upon cessation of the stimulus the excitatory event is revealed, presumably because it has a longer time course. A scheme for the interaction of these two components is shown in Fig. 11, where the baseline represents a "resting" level of excitability so poised that the cell fires at a frequency of 5 impulses per second. The response of the cell is determined by the algebraic sum of the excitatory and inhibitory events (labeled *E* and *I*), indicated by the bold curve. The scheme accounts for the long time course of the off-discharge, and is consistent with the peculiar relationships which its latent period shows to intensity and duration of the stimulus. The only alternative would be to view the off-response as a simple post-inhibitory rebound phenomenon (*cf.* "anode-break" excitation). Its duration, however, is so long that this seems unlikely; and the "unmasking" of excitatory events *during illumination* by selective preadaptation reveals that both events actually do coexist during the stimulus.

That these two components are mediated by different photosensitive pigments is demonstrated by the finding that various parameters of the response to illumination are wavelength-specific—that is, for example, that a response

to blue light cannot be matched by the use of red stimuli at any intensity. Such wavelength specificity is not shown by systems in which a single photosensitive pigment is involved.

Since the inhibitory component of the response is especially prominent at short wavelengths (*e.g.*, Fig. 7), it seemed reasonable to suppose that one pigment—having a relatively prominent absorption at short wavelengths—is involved in the mediation of inhibition, and another—absorbing more strongly at long wavelengths—mediates excitation. It is, of course, necessary to assume that their absorption spectra show considerable overlap, since inhibition occurs at all wavelengths. The results of the selective adaptation experiments, through which a further excitatory action can be unmasked through preexposure to blue light, provide proof that this view is essentially correct. No decision can be made from such data, however, as to whether the two pigments are intermingled in the responding cell or occur in separate presynaptic cells.

Nature of the Photoreceptor Elements

Although the *Spisula* photoreceptor is evidently a neural element, the data do not permit definite conclusions as to whether the primary receptor structure is a cell body or an axon—or whether, in fact, the unit from which the impulses are recorded is first order or not. The latter question is of particular interest, since the experiments show that separate pigments mediate excitatory and inhibitory responses of the cell. If the recorded unit is indeed first order, then its inhibitory response represents a new type of activity in primary photoreceptor cells. This problem is reminiscent of the one faced by Hartline (1938) in his interpretation of responses from the mantle eye of another mollusc, *Pecten*. There, the retina is divided into distal and proximal portions; fibers originating in the distal layer yield only off-discharges. Hartline clearly laid out the two possibilities, and concluded that the distal layer could be synaptic and the off-response thus of secondary origin. The subsequent elucidation (Ratliff, Miller, and Hartline (1958)) of the role of lateral inhibition in giving rise to complex discharge patterns in the *Limulus* eye has strengthened the view that off-responses in photoreceptors often arise secondarily, occurring as the result of inhibitory synaptic action.

There is good evidence, however, that this assumption may not be correct for the present case. It is certainly possible that a network of pre- and postsynaptic fibers mediating this response occurs in the pallial nerve. The presence of cell bodies in molluscan peripheral nerve has been demonstrated, and functional reflex connections between sensory and motor fibers in the periphery seem to exist (Horridge (1958)). The histological examinations of the *Spisula* pallial nerve described above, however, have shown the presence of only one or two ganglion cells in the photosensitive region.

The assumption that the fiber recorded in these experiments is post-synaptic requires that *at least* two presynaptic (sensory) cells and one post-synaptic unit be present in a restricted segment of the nerve; and this appears doubtful from the histology.

Moreover, a precedent already exists for believing that photosensitive neural elements in molluscs may display primary inhibition. Arvanitaki and Chalazonitis (1949*a*, *b*, 1958) have shown that the spontaneous discharge of ganglion cells in *Aplysia*, which contain both carotenoid and hemoprotein pigments, may be modified by illumination. These effects may be inhibitory or excitatory, or both, depending at least partially upon the nature of the pigmentation; so that "on", "off", and "on-off" cells may be recognized. These cells are well buried in the ganglia of the central nervous system, have high illumination thresholds, and show rather low frequencies of firing even to intense stimuli. They thus are probably not functional sensory cells; but it may be that this basic cell type has been adapted to serve as a photoreceptor unit in other forms.

The problem of the distal sense cell layer in *Pecten* may also be open to a new interpretation. Miller (1958) has published electron photomicrographs which indicate the presence of typical lamellar photoreceptor organelles in this layer, which is the one responsible for the generation of off-responses. Since it appears that this is a true sensory layer, the likelihood is that off-discharges from it arise from primary and not synaptic inhibition.

The whole question of whether primary inhibition exists in molluscan photoreceptors is thus still an open one. To summarize its present status, it appears (*a*) that in the present case of the neural photoreceptor in *Spisula* it seems most likely that the inhibitory response occurs in a primary receptor neuron; (*b*) that in the case of *Pecten* the existence of such responses is suggested by the structure of the distal sensory cells, and (*c*) that in the behavior of non-sensory, integrating ganglion cells of *Aplysia* a strong precedent for the existence of such a mechanism exists. Although the matter must await proof, it seems appropriate to entertain seriously the possibility that molluscan photoreceptors, and perhaps others, have evolved a mode of primary response to light which has not heretofore been associated with any photosensory system.

Behavioral Effects

Lamellibranch molluscs are all known to perform siphon retraction and (often) valve closure in response to photic stimuli. In some species (e.g., *Pecten*), these responses and others are mediated by highly organized and probably image-forming eyes located on the mantle edge; but most lamellibranchs lack such obvious structures. Early workers such as Nagel (1894) and Wenrich (1916) showed that some members of the latter group (*e.g.*, *Mya*)

respond primarily at the onset of illumination, but that others (including some European species of *Spisula*) are "skioptic"; *i.e.*, respond more prominently to "shadowing." The few behavioral experiments performed incidental to the present work suggested that *Spisula solidissima* is considerably more responsive to decreases in intensity than to sudden illumination, though Wenrich (1916) classified it as sensitive to both. The discharge pattern of the photoreceptor system shows that it could function as a detector of sudden illumination following darkness and of shadowing, and so the behavior actually correlates quite well with the properties of the receptor.

The location of the photosensitive region of the nerve is not so inconvenient as it might seem for serving this function. When the clam is in the feeding position, light can impinge on it through the openings of the extended siphon, since the sensitive region of the nerve is near its base and covered only by a thin, translucent layer of connective tissue. Presumably, light passing through the opened valves may also be effective. The sensitivity of the system is high compared with other invertebrate photoreceptors. The approximate threshold value of 6×10^{-2} erg/sec. $\times$ cm.^{2} is, to be sure, some 5 log units higher than that for human rod vision (see Pirenne (1956)); but it appears to be very much lower than, for example, that of the *Limulus* ommatidium for which Hartline shows threshold responses to white light at about 10^3 lux (Hartline (1934)). The sensitivity of the *Spisula* receptor may be more easily appreciated through the fact that threshold inhibitory responses are obtained by illuminating the nerve with a 500 mμ stimulus which the experimenter, viewing the preparation in the dark, cannot see reflected from the nerve. The absolute sensitivity of the receptor system is thus high enough to overcome the deficiencies of internal location, and there seems no reason to doubt its status as the sense organ responsible for "shadow responses"—especially in view of the apparent absence of multiple photosensory end-organs in the siphon.

Photosensitive Pigments

As a consequence of the experiments dealing with wavelength-specific discharge patterns and with selective adaptation, it can be stated with considerable assurance (*a*) that the response of the receptor consists of an excitatory and an inhibitory event, the latter having the lower threshold; (*b*) that these processes are mediated by separate photosensitive pigments, either located in some part of the cell recorded from or segregated in at least two presynaptic cells; and (*c*) that the absorption spectrum of the pigment mediating inhibition has its maximum at shorter wavelengths than that mediating excitation. The problem of correlating these responses with known photosensitive pigments is obviously far from any solution; but a few specifications can be made. The

spectral sensitivity function for the inhibitory process may be assumed to represent fairly well the absorption spectrum of the pigment mediating inhibition, since the process obviously has the greatest sensitivity and can be viewed without interference. This sensitivity function has a single maximum at around 540 mμ, and a general shape similar to the absorption spectra of some carotenoid proteins; it is clearly *not* consistent with those of hemoproteins, and it appears certain that the extractable hemoprotein from *Spisula* nerve is not mediating this process. The probability that the pigment is a carotenoid protein is supported by the ubiquity of such pigments in photosensory processes, and by the findings of Arvanitaki and Chalazonitis (1949*b*) in *Aplysia* neurons. They showed that in the cells of young animals, where both carotenoids and hemoproteins are found, illumination in the region of absorption of the carotenoid produced inhibition of spontaneous activity, whereas the hemoprotein(s) appeared to mediate excitation. As in the present case, the thresholds for inhibition were lower than for excitation. If the inhibitory process in the *Spisula* receptor is, indeed, produced through photoactivation of a carotenoid protein, it is a different one from those found in *Aplysia* cells, which have absorption maxima at 482 and 447 mμ (Arvanitaki and Chalazonitis (1949*b*)), and different also from the rhodopsins of cephalopod molluscs, which have absorption maxima at 478 *m*μ and 492 mμ (Hubbard and St. George (1958); Brown and Brown (1958)).

Little can be said concerning the nature of the pigment mediating excitation, since a true spectral sensitivity function of that process appears impossible to determine against the background of the more sensitive inhibitory event. The most effective wavelengths for the production of on-responses following blue light adaptation lie above 600 mμ; but a sensitivity function derived by such a process would only be in fact a "difference spectrum" between an unknown amount of remaining inhibitory pigment and the excitatory one. One can say only that its greatest absorption occurs at longer wavelengths than that of the inhibitory pigment. It is interesting to speculate that the responsible pigment might be a hemoprotein, possibly the one which occurs in such high concentration in *Spisula* nerve, since this would provide another consistency with the *Aplysia* neuron and would implicate an entirely new category of molecules in photosensory processes. This possibility is not provable, however, and in fact is argued against strongly by the lack of sensitivity of the excitatory process in the spectral region 400 to 420 mμ, occupied by the Soret band of hemoproteins.

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