Light-Initiated Responses of Retinula and Eccentric Cells in the *Limulus* Lateral Eye

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ABSTRACT The relationship between retinula and eccentric cells in the lateral eye of *Limulus polyphemus* was studied using a double electrode technique which permitted simultaneous recording of light-initiated responses in two sense cells and the labeling of the cells for subsequent histological examination and identification. The following results were obtained: (*a*) light-initiated slow responses with and without superimposed spike potentials were recorded from retinula cells and from eccentric cells (only one eccentric cell yielded responses without superimposed spike potentials); (*b*) spike potentials recorded in different cells within the same ommatidium were always synchronous; (*c*) a complete absence of spike potentials was observed in two experiments in which no eccentric cells could be found in the ommatidia containing the labeled retinula cells; (*d*) the greatest differences in the characteristics of responses recorded simultaneously occurred in those recorded from retinula-eccentric combinations. The results indicate that there is only one source of spike potential activity within an ommatidium (presumably the eccentric cell) and that the light-initiated response of retinula cells may be independent of the eccentric cell response. The suggestion is advanced that the response of the retinula cell may "trigger" the eccentric cell response.

The lateral eye of the horseshoe crab, *Limulus polyphemus*, is organized into subunits called ommatidia, the majority of which contain two types of sense cells (10 to 15 retinula cells and 1 eccentric cell; Demoll, 1914) whose functional relationship is obscure. The results of investigations of lateral eye sense cell function with intracellular electrodes indicate light-initiated slow waves of depolarization which can be categorized as follows: (*a*) slow waves without or with superimposed spike potentials of small magnitude (MacNichol, 1956; Tomita, 1956; Yeandle, 1958; Fuortes, 1958); and (*b*) slow waves with superimposed spike potentials of large magnitude (Hartline, Wagner, and MacNichol, 1952; MacNichol, 1956; Tomita, 1956; Fuortes, 1958). Although the early investigations indicated rather infrequent occurrence of responses of either category upon penetration of an ommatidium with a micropipette

(MacNichol, 1956), subsequent investigations indicated that responses of category (*a*) were obtained much more frequently than responses of category (*b*) (Tomita, 1956; Yeandle, 1958; Fuortes, 1958). These results have been variously interpreted to mean: (*a*) that retinula cells do not respond to illumination and that both the slow potentials and the spike potentials are generated only by the eccentric cells (MacNichol, 1956); (*b*) that slow potentials with small superimposed spikes may be generated by retinula cells (Tomita, 1956; Yeandle, 1958; Fuortes, 1958); and (*c*) that slow potentials with large superimposed spikes are generated only by eccentric cells (Hartline *et al.*, 1952; MacNichol, 1956; Yeandle, 1958; Fuortes, 1958). Slow potentials with large superimposed spikes have been recorded from exposed eccentric cells penetrated under visual control (MacNichol, 1956). Records of electrical activity obtained from the bundle of retinula and eccentric cell axons leaving the proximal end of an ommatidium indicate that spike potentials occur in only one axon in the bundle (Hartline *et al.*, 1952). Similarly, it has been observed (Waterman and Wiersma, 1954) that illumination of ommatidia in the lateral eye of *Limulus* usually elicited conducted spike potentials in only one axon per ommatidium. Exceptions were, however, found; some ommatidia exhibited more than one active axon and some exhibited no spike potential discharge. While most ommatidia contain only one eccentric cell, it is now known that some ommatidia of the lateral eye of *Limulus* contain two eccentric cells and some contain no eccentric cells (Ratliff, Miller, Wolbarsht, and Wagner, 1964). These results indicate that spike potentials originate in the eccentric cell of the ommatidium but they provide no insight into the role of the retinula cell in the lateral eye nor into the functional relationships between the retinula cells and the eccentric cell. In the present experiments, simultaneous recording and cell labeling techniques were employed to study these relationships. A preliminary report of the results of this investigation has appeared (Behrens and Wulff, 1964).

MATERIALS AND METHODS

The lateral eye of the horseshoe crab, *Limulus polyphemus*, was excised, sectioned horizontally and perpendicularly to the cornea with a sharp razor blade, and mounted on a wax block in a lucite chamber containing sea water at 10 ± 2°C. A silver chloride–coated silver wire placed in the sea water served as the indifferent electrode and the micropipettes, with inserted silver chloride–coated silver wires, served as the active electrodes. All electrodes were connected to negative capacitance preamplifiers, the outputs of which led to a cathode ray oscilloscope with a multitrace preamplifier and to an ink writer. The deflections of the cathode ray beams were recorded on moving film. The stimulating light source consisted of a 6 watt microscope lamp which, together with lenses, photographic shutter, and filter holder, was mounted on a coarse manipulator that permitted the beam of light to be positioned and focused on the corneal surface. A portion of the stimulating light beam activated a photocell, the output of which was led to the oscilloscope.

A preliminary series of experiments was performed with single micropipette electrodes to evaluate the effectiveness of various cell-labeling techniques. The demonstration by Tomita and Miller (1962) that colored substances could be seen in retinula cells led to the development of the labeling techniques described here. In the majority of the single electrode experiments, a cobalt ferrocyanide marking technique was employed. The electrode contained a 7 per cent solution of potassium ferrocyanide in 1 M potassium chloride, and during the ionophoretic injection of the ferrocyanide ions, 40 per cent cobaltous chloride was applied to the cut surface of the eye. Upon termination of the injection, the eye was placed in a 10 per cent solution of $CoCl_2$ for 5 minutes and then processed as described below.

Most of the experiments were performed with two micropipette electrodes and, since in these experiments it was important to identify each of the impaled cells, a two-color labeling technique was substituted for the marking technique described above. The two micropipette electrodes were held in a jig which permitted adjustment of the interelectrode tip distance and the alignment of the tips, after which they were securely fastened in position. A metal shield was positioned between the pipette barrels to decrease the capacity between the electrodes and the jig was firmly attached to a conventional micromanipulator. One electrode of each pair was filled with a 2.5 per cent aniline blue (water-soluble) solution in 0.5 M KCl. The second electrode was filled with 0.5 M KCl containing 25 gm fast green FCF and 25 gm orange G per liter. The resistances of most electrodes measured between 30 and 100 megohms (on rare occasions, electrodes of resistances above 100 megohms were used). After the desired responses were recorded from impaled sense cells, the electrodes were disconnected from the amplifiers and were connected to a current source for the ionophoretic injection of the dyes. Adequate amounts of dye were injected with a current of 0.4 microampere applied for 20 minutes. After the injection of the dyes, the lateral eye was removed from the electrode chamber and frozen in a block of albumen by liquid nitrogen–chilled isopentane (after Rahman and Lutrell, 1962). The eyes were cut at $-20°C$ into 6 μ sections which were mounted on albumen-coated slides, and examined without further processing. Photomicrographs of sections showing labeled cells were taken in 40 experiments, using Ektachrome X color film and suitable filters.

RESULTS

1. *Identification of Cell Types*

The techniques used in these experiments permitted the simultaneous intracellular recording of two light-initiated responses, the deposition of a green and blue dye by ionophoretic injection, and the subsequent identification of the cells containing the dyes in 69 experiments. The intensity of the color and the distribution of the dye within the cells are indicated in Figs. 1 and 2.

The 69 successful double electrode experiments yielded the following identified cell combinations: (*a*) 49 retinula–retinula cell combinations within the same ommatidium (Figs. 1 and 2, Table II, Fig. 4); (*b*) 10 retinula–eccentric cell combinations within the same ommatidium (Figs. 1 and 2, Table II, Fig. 5); (*c*) 5 cases of two sense cells in adjacent ommatidia (Table II, Fig.

FIGURE 1

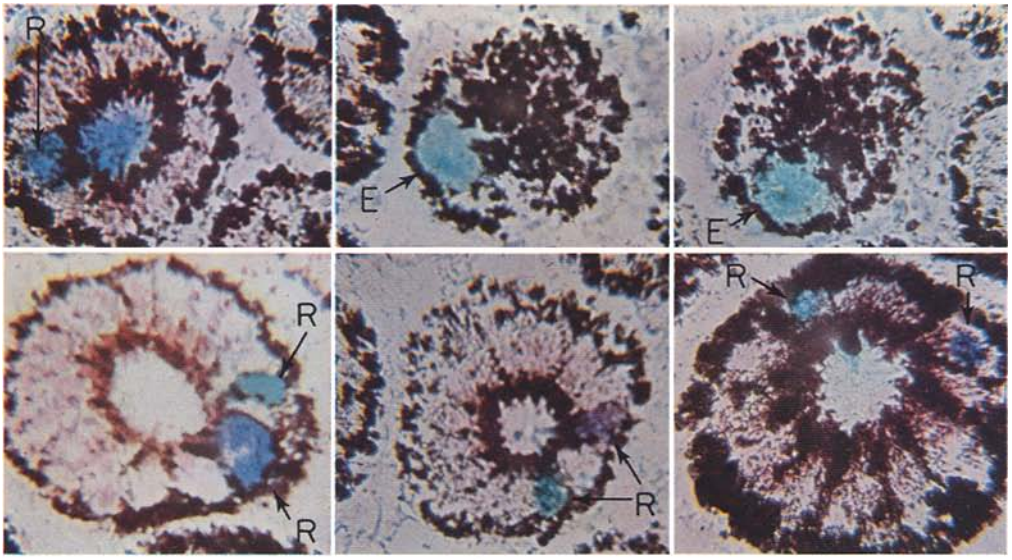
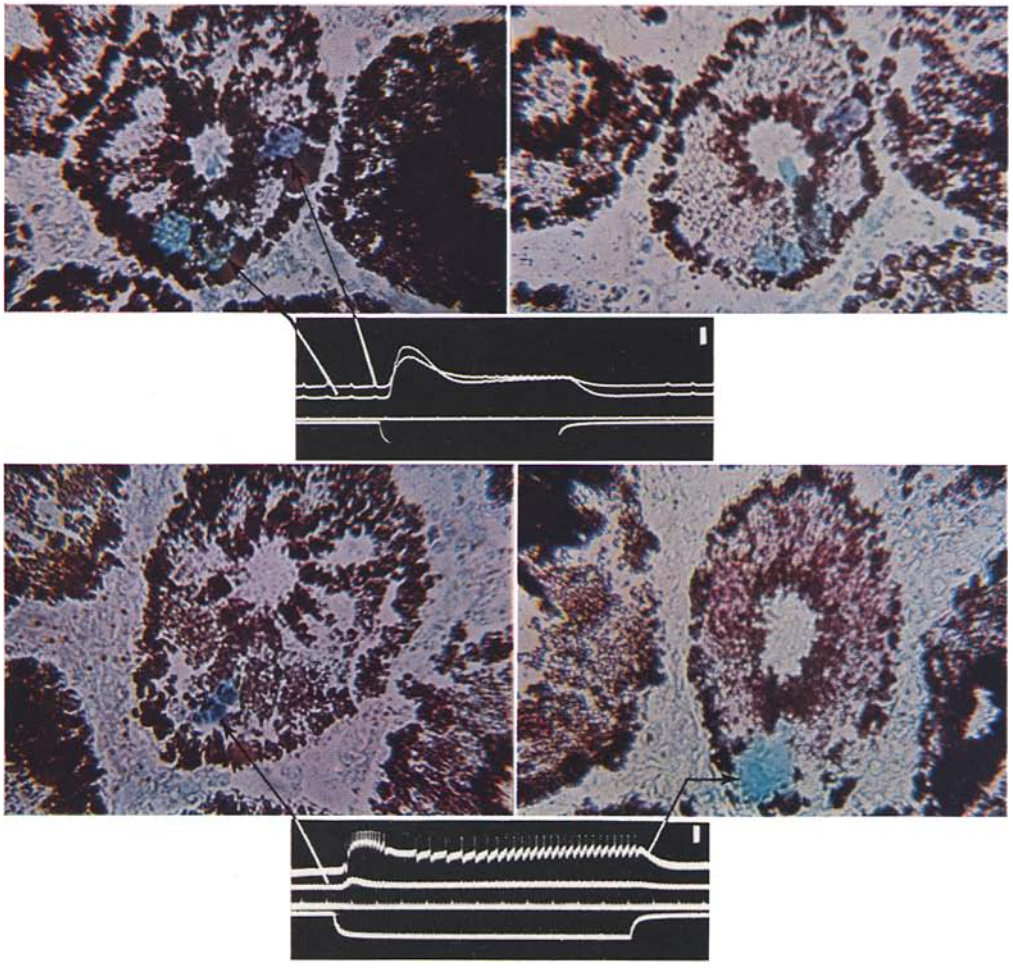


FIGURE 2



4); and (*d*) 5 cases in which both colored dyes were seen in the same retinula cell (Table II, Fig. 4). The results reported here are from the 69 double electrode experiments and, where indicated, from single electrode experiments.

2. *Response Characteristics of Identified Cells*

(A) WAVE FORM AND SPIKE POTENTIALS

Although most of the intracellular responses recorded were characterized by superimposed spike potentials (Figs. 2 and 3 to 5) it is of some interest that spike-free slow potentials were recorded not only from retinula cells but also from one eccentric cell (Fig. 3, top, right). It was also observed that slow potentials recorded simultaneously from different retinula cells within the same ommatidium may be of identical wave form (Fig. 4, middle, left), that slow potentials recorded from two different loci within the same retinula cell may be quite different (Fig. 4, top, right), and that slow potentials recorded from retinula cells in adjacent ommatidia may be very similar in wave form (Fig. 4, bottom, left). Differences in wave form of recorded slow potentials were always greatest in responses recorded from retinula–eccentric cell combinations. In general, the magnitude of the slow potentials recorded from eccentric cells was lower than the magnitude of responses recorded from retinula cells (Table I, Fig. 5).

The slow responses with superimposed spike potentials, recorded from retinula or eccentric cells, exhibited two characteristics of interest: (*a*) the magnitude of the superimposed spike potentials was greatest in responses

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FIGURE 1. *Top.* Photomicrographs of serial cross-sections of a portion of *Limulus* lateral eye showing a retinula cell containing aniline blue (extreme left) and an eccentric cell containing a mixture of fast green FCF and orange G (middle and extreme right). Please refer to text for additional details. × 250.

Bottom. Photomicrographs of cross-sections through three *Limulus* lateral eyes, showing three different ommatidia each showing one retinula cell marked with aniline blue and a second retinula cell containing fast green FCF and orange G. Please refer to text for additional details.

FIGURE 2. *Top.* Photomicrographs of serial sections through a *Limulus* lateral eye containing two labeled retinula cells and, below, photographs of the appropriate light-initiated intracellular responses. The identity of the traces and voltage calibration are given in Fig. 4. The time marks are at 0.1 second intervals. For additional details please refer to the text. × 250.

Bottom. Photomicrographs of serial sections through a *Limulus* lateral eye containing a labeled retinula cell (blue, left) and a labeled eccentric cell (green, right) and, below, photographs of the appropriate light-initiated intracellular responses. The identity of the traces and voltage calibration are given in Fig. 4. The time marks are at 0.1 second intervals. Please refer to text for additional details.

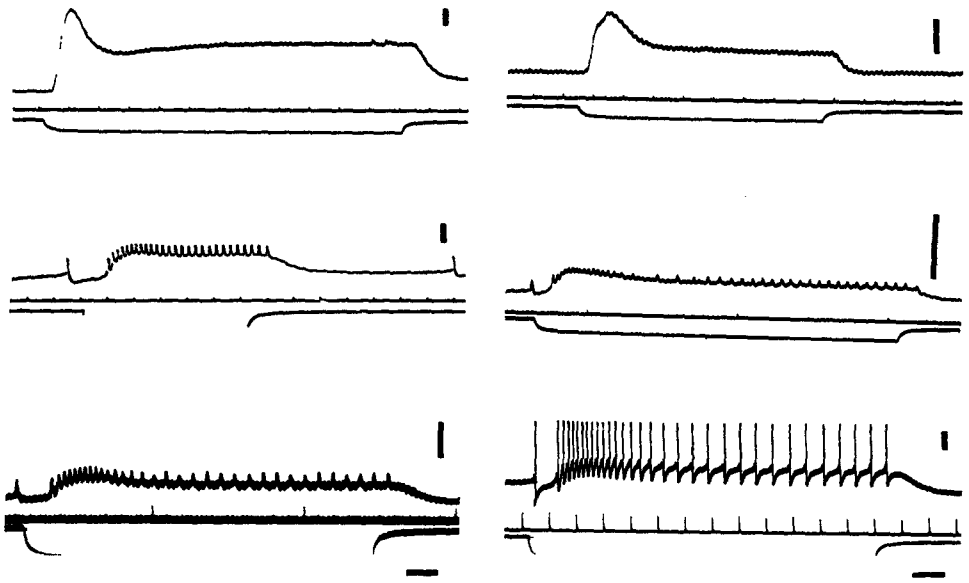

FIGURE 3. Photographs of light-initiated intracellular responses recorded from cells identified as retinula (left column) and eccentric (right column). In all records the upper trace defines the light-initiated response, the lowest trace the onset and duration of the stimulating light, and the second trace from the bottom the elapsed time. The vertical bar at the top right of each record indicates the deflection produced by a 10 mv calibration signal and the horizontal bar indicates 0.1 second of elapsed time. The responses in this figure are from preliminary experiments with single micropipette electrodes.

recorded from eccentric cells (Table I, Fig. 5); and (*b*) the spike potentials present in records obtained from retinula–retinula and retinula–eccentric cell combinations, provided they were in the same ommatidium, were always synchronous (Figs. 2, 4, and 5). Asynchronous spike potentials were recorded in only five cases in which the electrodes penetrated sense cells in different ommatidia (Fig. 4).

In two of the experiments light-initiated slow responses without superimposed spike potentials were recorded from cells identified as retinula in ommatidia which appeared to contain no eccentric cells. This statement regarding the absence of eccentric cells is based upon the failure to find eccentric cell bodies and dendrites. However, the organization of the rhabdome and the dendrite of eccentric cells cannot always be seen in unstained sections. Therefore, definitive proof of the absence of eccentric cells in ommatidia from which light-initiated slow potentials without superimposed spike potentials are recorded from retinula cells must come from preparations in which

TABLE I

The range and average amplitudes of light-initiated slow potentials and spike potentials recorded intracellularly from retinula and eccentric cells are presented. This table includes data from single electrode experiments as well as the double electrode experiments. For additional details, please refer to the text.

Cell type	No.	Response amplitude, *mv* Slow potential Range	Slow potential Average	Spike potential Range	Spike potential Average
Retinula	151	3–54	18.6	0–3	0.8
Eccentric	27	4–22	11.8	0–23	10.3

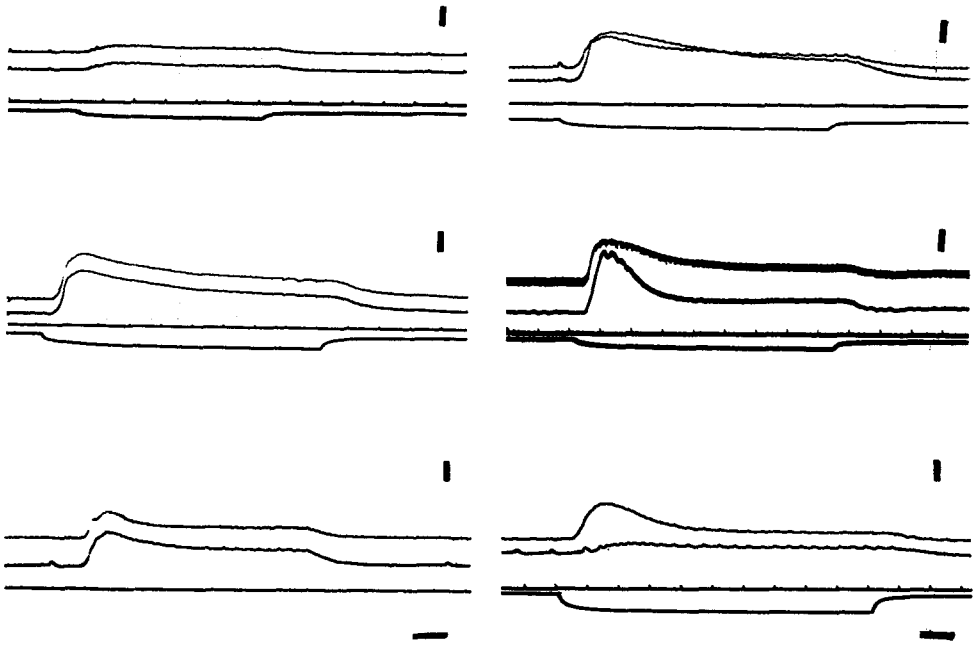

FIGURE 4. Photographs of simultaneously recorded light-initiated intracellular responses recorded as follows: (*a*) upper records, simultaneous responses from different loci in the same retinula cell; (*b*) middle records, simultaneous responses from different retinula cells in the same ommatidium; and (*c*) lower records, simultaneous responses from retinula cells in adjacent ommatidia. In all records the upper two traces define the responses, the third trace defines the elapsed time, and the lowest trace the onset and duration of the stimulating light. (The light stimulus trace is absent in bottom record, left column.) The vertical bar at upper right indicates the deflection produced by a 10 mv calibration signal, and the horizontal bar indicates 0.1 second of elapsed time. Please refer to text for additional details.

the organization of the rhabdome clearly shows the absence of a dendrite and serial sections show the absence of an eccentric cell body.

(B) LATENCY AND RATE OF POTENTIAL CHANGE

The simultaneous recording of light-initiated responses from two sense cells within a single ommatidium of the *Limulus* lateral eye permitted a comparison

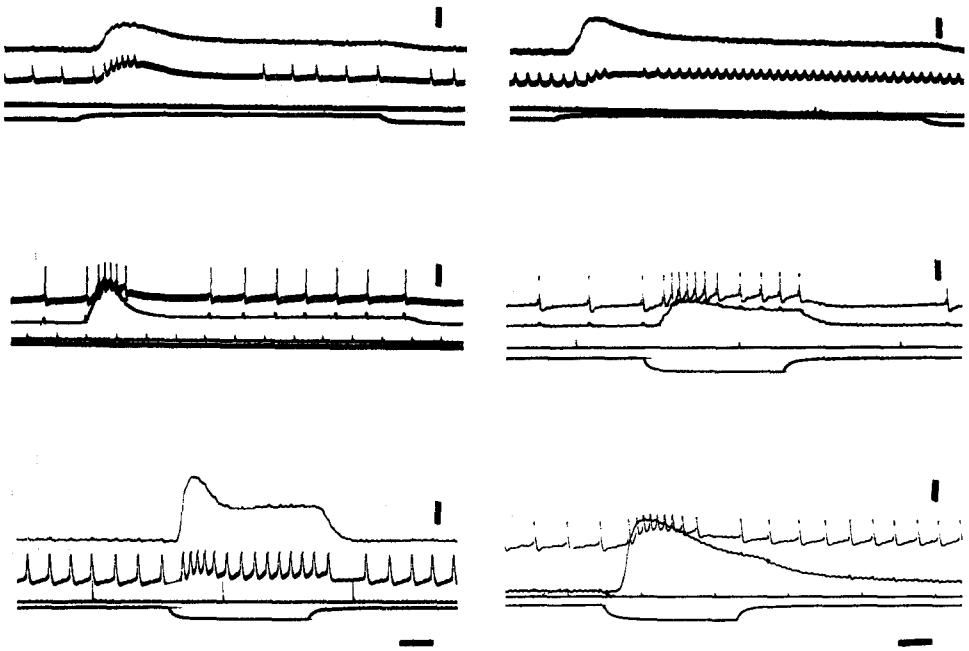

FIGURE 5. Photographs of simultaneously recorded light-initiated intracellular responses obtained from cells within the same ommatidium identified as retinula and eccentric. The upper two records were obtained from the same cell combination, the left response soon after penetration and the right response sometime later. Each of the remaining four records are from different retinula–eccentric cell combinations. Traces and calibrations as in Fig. 4, except that the light stimulus signal is absent in the middle record, left column. In each record the response characterized by spike potentials of larger magnitude is that of the eccentric cell. Please refer to text for additional details.

of the latent periods and of the slopes of the rising phases of the recorded slow potentials. Measurable latency differences (6 to 30 msec.) occurred in 1 of the 49 retinula–retinula cell combinations and in 6 of the 10 retinula–eccentric cell combinations, the retinula cell response leading the eccentric cell response in all cases (Table II). Since the determination of the onset of a slow response is somewhat uncertain, the slopes of the linear portions of the rising phase of the slow potentials recorded from retinula–eccentric cell combinations were determined. In five of the six cases in which a latency difference was meas-

ured, the slope of the rising phase of the slow potential change recorded from the retinula cell was greater than that recorded from the eccentric cell; in the sixth case the slopes were equal. In the four retinula–eccentric cell combinations in which latency differences were not detected, the slope of the rising phase of the slow potential change of the retinula cell response was greater than that of the eccentric cell in one case, less than that of the eccentric cell in two cases, and equal to that of the eccentric cell in one case.

TABLE II

Tabulation of the results of a comparison of the onset of light-initiated responses recorded simultaneously from two sense cells in the *Limulus* lateral eye. Differences of 6 msec. or more were scored as latency differences. Please refer to the text for additional details.

Cell combination	Total No.	No. of observations of:		
		Difference	No Difference	Uncertain
Retinula-retinula	49	1	37	11
Retinula-eccentric	10	6*	2	2
Same retinula cell	5	0	5	0
Sense cells in different ommatidia‡	5	0	1	4

* Retinula cell leads in all cases.

‡ Identified as retinula-retinula in three cases.

DISCUSSION

1. *Dual Electrode Penetrations and Labeling of Cells*

The results of this investigation show that two micropipettes, properly positioned and rigidly held in a microdrive, may be introduced into exposed ommatidia of the lateral eye of the horseshoe crab and impale two cells whose light-initiated responses may then be recorded simultaneously.[1] Recently, double units have been obtained in all lateral eye preparations which were "good"; *i.e.*, offered minimal resistance to penetration and generated large light-initiated responses. Since most of the intra-ommatidial mass consists of retinula cells, it is not surprising that the yield of retinula-retinula pairs exceeds retinula-eccentric pairs (Table II).

The results of this investigation confirm the demonstration by Tomita and Miller (1962) that sense cells in ommatidia of the *Limulus* lateral eye can be adequately labeled by the ionophoretic injection of substances (Figs. 1 and 2). Recently, successful labeling of double units and visual recovery of the labeled

[1] After this paper was accepted for publication experiments were reported in which a double electrode technique was employed to investigate the effects of currents on the penetrated cells of *Limulus* lateral eye (Smith, Baumann, and Fuortes, 1965).

cells in 6 μ cross-sections of ommatidia have been achieved in about 60 per cent of the experiments.

Throughout this study, the assumption was made that the cell containing the colored label was the cell which was impaled by the micropipette containing that label at the time the light-initiated responses were recorded. It was necessary to make this assumption since it is always possible that the electrode could have moved to a new locus within the ommatidium between the time of the last record and the end of the ionophoretic injection of the dye. To minimize this uncertainty, it would have been necessary to inject some of the dye, record the desired responses, and then inject more of the dye. Such a procedure could not be used since the current required to inject an adequate amount of the dye undoubtedly injured the impaled cells, for in all cases in which light-initiated responses were recorded after ionophoretic injection of the dyes, they were of inverted polarity. Movement of the electrode during ionophoretic injection or leakage of dye out of the impaled cell would result in deposition of a given label in more than one cell. Since the label was either confined to one cell (in most cases) or more densely deposited in one cell than in the adjacent cells (Figs. 1 and 2), we believe that the densely marked cells were the cells from which the responses were recorded.

Not only did the impaled cells sustain injury by the currents flowing during ionophoretic injection of the dyes, but also there was evidence of injury to cells during the recording phases of the experiments, particularly between the impalement of one cell and the capture of a second cell. The influence of such injury on the responses of cells to light could have been quite subtle and may have contributed to some of the response characteristics described below, such as the absence of spike potentials in the responses elicited from an eccentric cell (Fig. 3, top, right).

2. *Response Categories*

The results obtained in this study confirm the suggestion of MacNichol (1956), Tomita (1956), Yeandle (1958), and Fuortes (1958) that intracellular responses recorded from ommatidia in the lateral eye of the horseshoe crab fall into two classes: (*a*) large amplitude slow potentials with small amplitude superimposed spike potentials or without superimposed spike potentials; and (*b*) small amplitude slow potentials with large amplitude superimposed spike potentials. Generally, the responses of category (*a*) were recorded from labeled and identified retinula cells (Fig. 2, Table I, Figs. 3 to 5) and the responses of category (*b*) were recorded from labeled cells identified as eccentric cells (Fig. 2, Table I, Figs. 3 and 5). Although the responses submit to categorization, identification of the cell of origin by the response characteristics is not always possible. Responses recorded from retinula cells were, in many cases, similar to responses recorded from eccentric cells and, in one

experiment, a slow potential without superimposed spike potentials was recorded from an eccentric cell (Fig. 3).

3. *Unitary Origin of the Ommatidial Spike Discharge*

The results obtained in this investigation confirm the finding (Hartline *et al.*, 1952; Waterman and Wiersma, 1954) that, in most ommatidia, there is only one source of spike potentials and all the evidence implicates the eccentric cell as the source of the spike potential discharge. In the study reported here, spike potentials superimposed on responses recorded from different cells within the same ommatidium were always synchronous; the only observed cases of lack of synchrony in recorded spike potentials were those in which the micropipettes impaled sense cells in different ommatidia. Although most ommatidia of the *Limulus* lateral eye contain only one eccentric cell, some ommatidia have two and some have no eccentric cells (Ratliff *et al.*, 1964). Oscillograms of ommatidial nerve bundles indicating activity of a single axon or of two axons, as well as those exhibiting no spike activity (Waterman and Wiersma, 1954; Tomita, 1957; Tomita, Kikuchi, and Tanaka, 1960) presumably can be attributed to ommatidia containing one, two, or no eccentric cells, respectively. During the investigation reported here, there were two cases of a complete absence of recorded spike activity in which no eccentric cells could be found upon examination of unstained serial sections of the ommatidia containing the labeled cells.

4. *Some Features of Simultaneously Recorded Light-Initiated Responses*

In general, the results of this study indicate that a high degree of synchrony exists within each ommatidium. Responses recorded from two different retinula cells within the same ommatidium can have very similar wave forms (Fig. 4, middle, left) and in most cases appear to begin after identical latent periods (Table II). These observations, together with the previously discussed observation that the spike potentials of the responses of two cells within a single ommatidium were always synchronous, confirm the suggestion by Tomita *et al.* (1960) that electrical coupling (presumably electrotonic) may exist between intra-ommatidial retinula cells and between intra-ommatidial retinula and eccentric cells.

Responses recorded simultaneously from retinula and eccentric cells within the same ommatidium were, in all cases, rather different (Figs. 2 and 5). In six out of the ten retinula–eccentric cell combinations reported, the retinula cell slow potential appeared to begin 6 to 30 msec. earlier than the eccentric cell slow potential, and in five of these six cases, the slope of the linear portion of the rising phase of the slow potential was greater in the retinula cell response than in the eccentric cell response. In two of the retinula–eccentric cell combinations (one of which is presented in Fig. 5, top) the potential of the eccen-

tric cells decreased during the period of observation, while the responses of the retinula cells increased in magnitude. It is likely that the retinula cell responses increased because of dark adaptation (it was not feasible to control the level of light adaptation in these experiments) or because of recovery from the act of impalement. It is entirely possible that the observed reduction in magnitude of the slow potentials recorded from the eccentric cells may have resulted from a movement of the tip of the micropipettes to a different locus and that the *in situ* response of the eccentric cell did not, in fact, change. Although the latter possibility cannot be ruled out, it is suspect, for in both of these cases the frequency of the spontaneous spike discharge increased as the slow potential of the eccentric cell decreased (Fig. 5, top). Such an increase in the frequency of spontaneous spike discharge is usually interpreted as a sign of "deterioration" of the cell. The possibility exists, therefore, that the observed reduction in magnitude of the slow potential recorded from the eccentric cells was real. Since only one eccentric cell was observed in each of the two ommatidia in question, it follows that the response of the retinula cells may be independent of the response of the eccentric cells. This conclusion is supported by the results of two experiments in which light-initiated responses without superimposed spike potentials were recorded from retinula cells located in ommatidia which appeared to contain no eccentric cells.

Although the results of this investigation do not permit a definitive description of the functional relationship between retinula and eccentric cells within the same ommatidium of the *Limulus* lateral eye, they do indicate that: (*a*) light-initiated slow potential changes can be recorded from both retinula and eccentric cells; (*b*) that there is only one source of spike potential activity, presumably the eccentric cell; (*c*) that the retinula cells generate an electrical response upon illumination that may be independent of the response of the eccentric cell; and (*d*) that the response of the retinula cells may "trigger" the response of the eccentric cells.

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