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Spectral sensitivity of photoreceptors in insect compound eyes: Comparison of species and methods

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Summary. Three different methods were used to determine the spectral sensitivity of retinula cells in the compound eyes of three species of hymenopteran insects (*Apis mellifera, Melipona quadrifasciata, Osmia rufa*). The conventional flash method gives the least reliable results. Sensitivity is extremely sensitive to small fluctuations of the resting potential and long lasting changes induced by preceding test flashes. The ramp method, which speeds up a spectral scan to about 1 min and keeps effective illumination constant at every flash, determines $S(\lambda)$ much more reliably. The best results are obtained with the spectral scan method, which provides the experimenter with a $S(\lambda)$ function of high spectral resolution within 20 s. Using this method we demonstrate that the high observed variability in $S(\lambda)$ of individual receptors is the result of the inadequacy of the flash method, which was the only method used in earlier studies.

Double microelectrode experiments and variations of the stimulus conditions reveal that field potentials and return flow of electric current produced by activated neighboring cells have no effect in the bee eye. We conclude that the model of Shaw (1975, 1981) of current flow in the locust and fly eye does not apply to the bee eye. Very rare recordings (about 1%) of UV receptors with hyperpolarizing responses to long wavelength light are interpreted as having a synaptic inhibitory connection to green receptors.

The improvement of spectral measurements of single receptors allows us for the first time to model the spectral input to a color-coding network with great precision.

Introduction

Accurate measurements of the spectral properties of photoreceptors are needed for several reasons: the spectral absorption of photopigments can be determined, the type of photopigment and the possible existence of antennal or screening pigments can be tested, optical and electrical interactions between neighboring receptors can be studied, the spectral input to a neural color-coding network can be elucidated, and comparative studies between species differing in ecological adaptation and phylogenetic relationship can be made. Ever since the first spectral measurements with intracellular electrodes on insect photoreceptors (Autrum and Burkhardt 1960), such measurements have displayed high variability and unexpected results. They were based on the assumption that individual photoreceptors contain only one type of photopigment and are electrically isolated from one another (see review by Menzel 1974). Burkhardt (1962) and Autrum and von Zwehl (1964) were the first to demonstrate three different spectral receptor types in animal (fly and honeybee) eyes with single-cell recording techniques, and were able to correlate the individual spectral sensitivity functions with the color vision of the bee. They interpreted the great variability of their spectral sensitivity functions and the often significant deviation from a theoretical absorbance spectrum of rhodopsin (enhanced sensitivity outside the main sensitivity band, narrower main sensitivity bands) as indicating artifactual recordings from more than one cell and damage to the cell membrane. This paper will demonstrate that their interpretation is essentially correct, although these deviations could have also been caused by: optical filtering and self-screening in a fused rhabdom which contains three different photopigments (Snyder et al. 1973), competition for Na^+ in the limited extracellular space surrounding the microvilli of the rhabdom (Hamdorf et al. 1973), electrical coupling between receptors of different spectral type (Shaw 1969), mixture of two or more pigments in one retinula cell (Kirsch-

feld et al. 1977; Hardie et al. 1979) return currents forced through the synaptic terminals by an active cell due to the high resistance of the basement membrane (Shaw 1975, 1981), or synaptic interaction on the level of the retinula axons (reviewed by Menzel 1974, 1979). Any one of these factors may actually influence the spectral sensitivity $S(\lambda)$ of insect photoreceptors and has indeed been demonstrated as doing so in certain insect species. The questions posed here are: what factors influence $S(\lambda)$ in the honeybee and other hymenopteran species, which factors are artifactual, and what is an accurate estimate of $S(\lambda)$ of the different spectral receptor types?

Our motivation for studying this question arises from an attempt to determine, as accurately as possible, the spectral input functions of the receptors as determinants of color vision. We are interested in correlating the receptors' $S(\lambda)$ functions with color discrimination in various species of hymenopteran insects and in searching for ecological adaptations. We need to know, therefore, the $S(\lambda)$ function of individual photoreceptors with as little disturbance from intracellular recording techniques as possible.

Methods

Three different kinds of stimulation have been used: (a) the flash method, (b) the ramp method, and (c) the spectral scanning method.

Flash method. This is the conventional technique for determining the spectral sensitivity of photoreceptors. Short (in our case 300 ms) flashes of monochromatic light at various intensities (12 steps over 4 log I units) and 19 wavelengths (321–686 nm, Schott II or DIL interference filters) were delivered to the eye after carefully centering the point light source (0.8° visual angle) to the recorded cell. Intensity-response functions (V/log I functions) were used to calculate $S(\lambda)$. We stored the receptor potentials on tape (Hewlett Packard 3964 A) and measured the potentials either from a paper trace or with the aid of a computer that digitized the responses and determined the peak (highest DC depolarization within 70 ms after flash-ON) and the plateau (DC depolarization 250 ms after flash-ON). A hyperbolic function of the form

$$\frac{V}{V_{max}} = \frac{(R \cdot I)^n}{(R \cdot I)^n + 1} \quad (1)$$

(I is the stimulus intensity in quantal flux; V is the amplitude of the receptor potential, either peak or plateau, in mV; V_{max} is the saturated response amplitude; R is the reciprocal of the intensity yielding a response of 50% V_{max}; n is a constant determining the slope of the function; see Lipetz 1971; Matic and Laughlin 1981) was fitted to the responses of a series of monochromatic or white light stimuli of various intensities by adjusting V_{max} and n through an interactive program on an Apple II computer. Spectral sensitivity was calculated by the computer from responses to the 19 spectral lights using the fitted V/log I curve.

Ramp method. A logarithmic neutral density wedge (Melles Griot) mounted on a fast stepping motor was used to produce a ramp of monochromatic light. When the light was switched on, the wedge started at its highest optical density and ran to a lower optical density with a speed of 3 log I units/200 ms. When the response of the cell reached a certain preselected DC potential, the light was switched off, the stepping motor stopped, and calculator (HP 25) read the number steps performed by the stepping motor. The spectral sensitivity was calculated by the HP 25 on-line with a set of hard-wired calibration factors which were read by the calculator for each of the wavelengths used. The $S(\lambda)$ function was displayed immediately after each spectral ramp flash on a storage oscilloscope (Menzel et al. 1978).

Spectral scanning method. Following Franceschini (1984), Smakman and Pijpker (1983) and Hardie and Kirschfeld (1983), we developed a fast spectral scanning method in which a photoreceptor is clamped to a certain preselected DC potential by adjusting the light flux to changes in spectral responsiveness. A grid monochromator (Schoeffel, GM 252/1) scans through the spectrum (300–700 nm) within 16 s. Any deviation of the receptor potential from a preselected value is monitored by a comparator and fed to the microprocessor which adjusts the circular neutral density wedge (Melles Griot 3 log units). The computer reads the new position of the stepping motor, which drives the neutral density wedge, and stores the value together with the wavelength. It takes 160 ms to run through this cycle, so a wavelength resolution of 4 nm is reached for a total scan time (400 nm) of 16 s. Faster or slower scans can be used, but all data reported here come from this setting. Spectral sensitivity is displayed a few seconds after the end of the scan. All cells were tested with spectral scans in both directions (300–700 nm, 700–300 nm), and any dependence on the directions of the scan was carefully inspected.

Calibration. The radiometer IL 700 with detector PM 270 D from International Light Corp. used for measuring light flux at the location of the eye was calibrated to a standard light source of known spectral emission at the Lichttechnisches Institut der Technischen Universität Berlin. The interference filters (Schott and Gen., Mainz) used in the flash and the ramp methods had half-band widths below 7 nm and side band transmission (within 300–700 nm) below 10^{-7} of peak transmission. Calibration for the scan method was performed by a special program of the microprocessor, which included the action spectrum of the photomultiplier tube. The radiometer signal was read during the spectral scan at various settings of the quartz neutral density wedge and stored as quantal flux in 4-nm intervals. The overall wavelength dependence of the neutral density wedge turned out to be considerable, but it did not have to be taken into account since combinations of wavelengths and wedge positions used in all experiments reduced the wavelength dependence to less than $\pm 5\%$ on a linear scale. Stray light from the monochromator was tested many times, both during the calibration and during cell recordings by inserting cut-off filters. Stray light is negligible above 315 nm, 5% at 310–315 nm and 10%–15% at 300–310 nm. We saw no significant effect of stray light during cell recordings.

Electrophysiology. Microelectrodes were pulled with a Kopf puller from Hilgenberg glass capillaries with filaments filled with 2.5 mol/l KCl (70–200 MΩ) or 5% Lucifer yellow ($\geq$ 150 MΩ). Receptor potentials were amplified with a P16 (Grass) or LH 1 (List, Darmstadt) amplifier and stored on FM tape or processed on-line. Criteria for the acceptance of intracellular recordings are discussed under Results but were, in

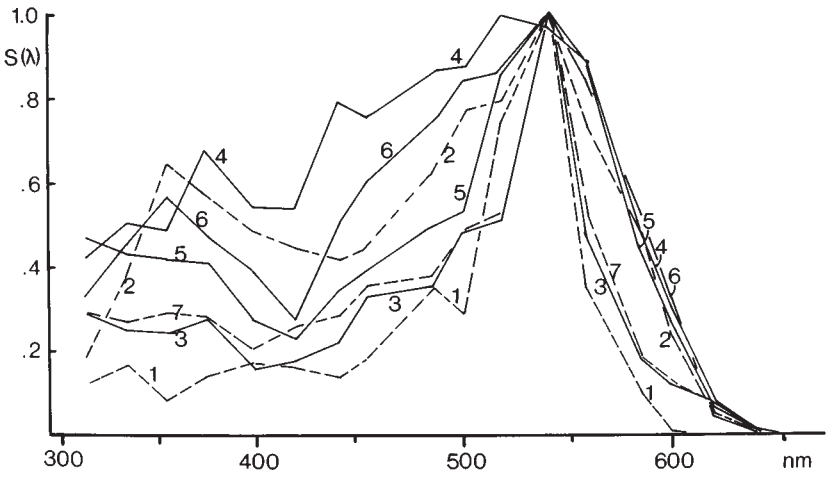

Fig. 1. Spectral sensitivity curves of 7 individual green receptors in the compound eye of the stingless worker bee, *Melipona quadrifasciata*, determined by the flash method. Note the large scatter of the sensitivity data

general, resting potential ≥ -40 mV, maximal light response ≥ 20 mV, resting potential stable within ± 5 mV longer than 30 min. The indifferent electrode was inserted either in the other eye or in the thorax (see Results for further details).

Results

Variability of $S(\lambda)$ measurements based on the flash method

Although the variability of $S(\lambda)$ measurements is well documented in the literature, we shall give a few more examples of recordings in the compound eye of the stingless bee *Melipona quadrifasciata*. Several individual $S(\lambda)$ functions of receptor cells with λ_{max} around 540 nm are given in Fig. 1. These seven examples were selected randomly from a total of 128 stable recordings from green receptors and do not represent the extremes of spectral sensitivities. It is obvious that selection of "acceptable" measurements and averaging selected $S(\lambda)$ functions strongly affect results. This is demonstrated in Fig. 2, which gives the average $S(\lambda)$ functions for nine different groups of green receptors together with the total average $S(\lambda)$ in Fig. 2e. The UV and blue receptors in the eye of *Melipona* have been analysed in the same way (Fig. 3).

The rationale behind such an analysis is manifold. First, we want to know whether the *Melipona* eye contains chromatically different types of photoreceptors. Three types can easily be demonstrated, even with the large scatter of data. Their λ_{max} are around 350, 440, and 540 nm, and the $S(\lambda)$ functions of individual cells with even extreme deviations from the average function of one group differ significantly from the other groups. Next we ask whether each group is coherent or is a collection of subgroups. The scatter bars (standard deviations) in Figs. 2 and 3 make it very unlikely that there are any statistically significant subgroups. Consider, for example, the green receptors for which most data were collected (Fig. 2a–e). All subgroups, except perhaps one, have highly overlapping scatter bars, although the individual $S(\lambda)$ functions were grouped such that they were closest to the average, particularly with respect to the bandwidth of the main sensitivity in green and to the sensitivity outside the main band (side or secondary sensitivity). The one exception may be the two cells in Fig. 2b (dotted line), with a λ_{max} at much longer wavelengths and very low UV sensitivity. When we recorded the first of the two cells, we were confident that *Melipona* had an additional long-wave receptor type, but we found only one other example in more than 200 recordings.

The next step in our analysis is to ask whether the large deviation is due to inaccuracy in our method. We have accepted only long-lasting stable recordings with low noise (see Methods). The recording noise was less than 0.2 mV. The reading error of the computer or of the human eye measuring the voltage of the light response is less than 0.5 mV. A maximal total error of ± 0.5 mV corresponds to a deviation of the sensitivity values of close to $\pm \log 0.07$ (the average slope of the $V/\log I$ function in *Melipona* is 0.7). The measuring error is, therefore, many times smaller than the observed within-group variability. What then are the sources of this variability?

Let us first consider factors under the control of the experimenter which may systematically affect $S(\lambda)$ of individual cells, e.g., state of dark adaptation, autonomous circadian change of sensitivity, effect of preceding light flashes, resting potential, maximal light response, sensitivity measured by the peak and the plateau potential, and the effect of the slope of the $V/\log I$ function. We take

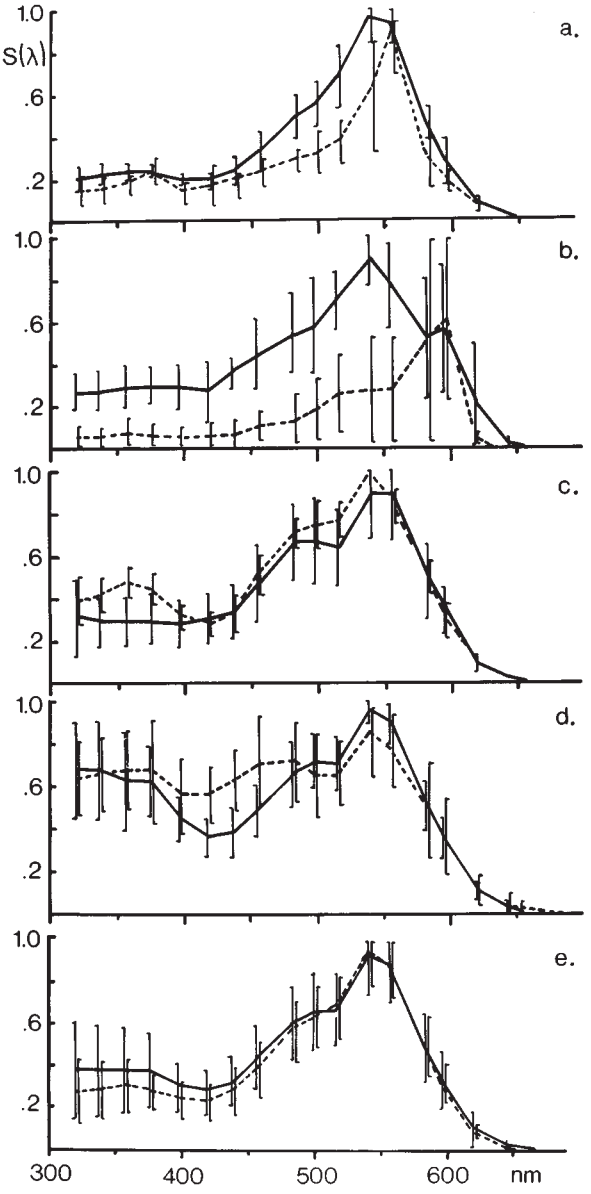

Fig. 2a–e. Average $S(\lambda)$ functions of different groups of green receptors of *Melipona quadrifasciata* determined by the flash method. Cells were grouped with respect to the spectral position of the main sensitivity peak, the width of the main peak and the height of the sensitivity in UV and in blue. Scatter bars give the standard deviation. **a** — 8 cells, -- 5 cells; **b** — 5 cells, -- 2 cells; **c** — 21 cells, -- 13 cells; **d** — 20 cells, -- 15 cells; **e** — total average 128 cells, -- average of 34 cells, whose individual $S(\lambda)$ were close to that of the total average

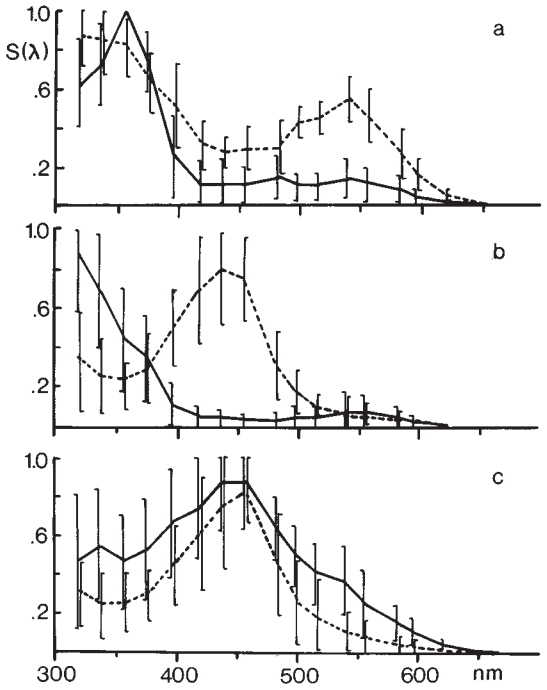

Fig. 3a–c. Average $S(\lambda)$ of UV and blue receptors of *Melipona quadrifasciata* determined by the flash method. **a** — 6 cells with λ_{max} at 360 nm, -- 9 cells with $\lambda_{max} \leq 350$ nm and high long wave sensitivity. **b** — 7 cells with $\lambda_{max} < 350$ nm and low long wave sensitivity, -- 9 cells with λ_{max} at 440 nm and narrow bandwidth of the main peak; **c** — 6 cells with λ_{max} at 440–455 nm and broad bandwidth of the main peak, -- 15 cells total average of all blue receptors

the large group of green receptors and ask whether any of these factors show a correlation between the width of the main peak and the side sensitivity in the UV.

Our recording experiments with *Melipona* were performed in the afternoon and early evening at São Paulo, Brazil. Sunset is at 18:30 hours. When the stingless bees, about to fly out on a foraging trip, were removed from the entrance of a colony they were adapted to the natural cycle of daylight. We may expect an increase in sensitivity after 18:30 hours, if the eye changes its sensitivity in darkness according to an internal circadian rhythm. Circadian sensitivity changes under constant darkness have been demonstrated in insects (see Fleissner 1982). In particular, the honeybee's eyes are 1.5–2 log units more sensitive at night as measured with the ERG (Milde 1982). There was no correlation between individual $S(\lambda)$ in *Melipona* and the time of day. In the honeybee, the same question was studied with ERG recordings and stimuli of different wavelengths (Menzel, unpubl.). Again no change in the relative sensitivity to various wavelengths was found. The absolute sensitivity of the cells may depend on the time of the day, but we were not able to determine this accurately enough in our intracellular recording experiments.

The eye was kept in the dark for an hour or longer before data were collected. Illumination of the part of the eye from which we later recorded

was avoided. We found no correlation between dark adaptation time and $S(\lambda)$. Particularly, even in cases of a short dark adaptation time (30 min) or a short illumination with the dissecting lamp during the dark adaptation time the $S(\lambda)$ functions were not significantly different from those cells which had been dark-adapted longer than 2 h.

The resting potential should be a reliable indicator of the quality of an intracellular recording. Unfortunately, it can rarely be measured in absolute terms because the electrode seems to jump from one intracellular space to another in the densely packed retina. We analysed, therefore, any dependence $S(\lambda)$ might have on the maximal light response of the cell. The maximal response was determined with white light in order to make sure that intensity was high enough. Again we found no correlation between side band sensitivity or half-bandwidth with the maximal light response of those cells, which we included in our analysis and which we regard as good recordings. (For further discussion see below).

The light response recorded intracellularly in insect photoreceptors is a phasic-tonic depolarization. $S(\lambda)$ was calculated for all receptors for the phasic (peak) potential and for the tonic (plateau) potential (see Methods), through the respective $V/\log I$ functions. In all cells, $S(\lambda)$ based on the plateau potential is much more variable than that based on the peak potential. In addition, calculation of $S(\lambda)$ by the plateau potential is complicated by its narrow intensity range. A close inspection of these sensitivity data, in which saturation of the plateau response is not a problem, reveals that a strong light flash (causing a response above half the maximal response) reduces the sensitivity to the next test flash noticeably, although the test flashes are separated by 10–15 s in the dark. Such an effect was not detected for the peak potential but may still be an important factor in variability (see below).

The spectral light flashes were presented in a sequence from short to long (up-run) or long to short (down-run) wavelengths. Such a sequence allows us quickly to identify to which type a receptor belongs, but it may have the disadvantage of biasing the sensitivity by cumulative adaptation. Again, sensitivity measured with the peak potential did not depend on the spectral sequence of the lights as long as the intensity of the test flashes was kept low, but the plateau potentials were more sensitive to the sequence and showed a somewhat higher sensitivity to short wavelengths in up-runs and higher sensitivity to longer wavelengths in down-runs. Notice that we are searching for subtle differences in sensitivity. Any obvious and stronger effects are eliminated by separating the short test flashes by a relatively long period in the dark (10–15 s) and by keeping the test intensity low.

We have to conclude that the very procedure of the flash method introduces unavoidable variability. (a) The interflash interval has to be kept short, otherwise the spectral run lasts too long and the cell may change its physiological status. (b) The test intensity should be low to avoid adaptation, but high enough to measure sensitivity sufficiently accurately. Since the spectral sensitivity of the cell is unknown, the experimenter has to adjust the quantal flux from flash to flash on the basis of his experience and his expectations as to what kind of cell he may be recording from. It is obvious that light flashes with stronger effects are unavoidable, and more or less effective light flashes may alternate in quasi-random fashion. (c) Fluctuations of the resting potential occur even in good recordings. We have rejected any cells which changed their resting potential by more than 5 mV, but even smaller fluctuations may affect sensitivity. This question was studied in a number of recordings from retinula cells of *Osmia rufa* (Fig. 4). All cells were green receptors. A spectral light of 500 nm was used to establish the $V/\log I$ function and to test sensitivity with a random succession of different intensities. The interflash interval was 15 s, and the test intensity was always less than that necessary to produce a half-maximal response. Notice that the sensitivity scale in Fig. 4 is logarithmic (± 0.1 log S) and differs in the three examples. We find that even small changes in the resting potential are accompanied by considerable changes in sensitivity. This is most obvious for relatively fast changes (notice time scale in Fig. 4). Depolarization by a few millivolts reduces sensitivity by more than 0.2 log units and partial (Fig. 4a) or full (Fig. 4c) repolarizations re-establish sensitivity. Even at relatively constant resting potential, sensitivity may fluctuate by 0.1 log units or more (compare, e.g., the first 10 min of trace a).

After we realized how sensitive $S(\lambda)$ measurements were to small changes in the resting potential, we re-analysed our data on *Melipona* and searched for a correlation between the shape of the $S(\lambda)$ function (e.g., the height of the secondary sensitivity) and the maximal fluctuations of the resting potential during the period of the $V/\log I$ and spectral measurements. An example is given in Fig. 5 for UV, blue, and green receptors in both *Melipona* and *Osmia*. Although there are recordings with low secondary sensitivity at relatively high DC fluctuations and high secondary sensitivi-

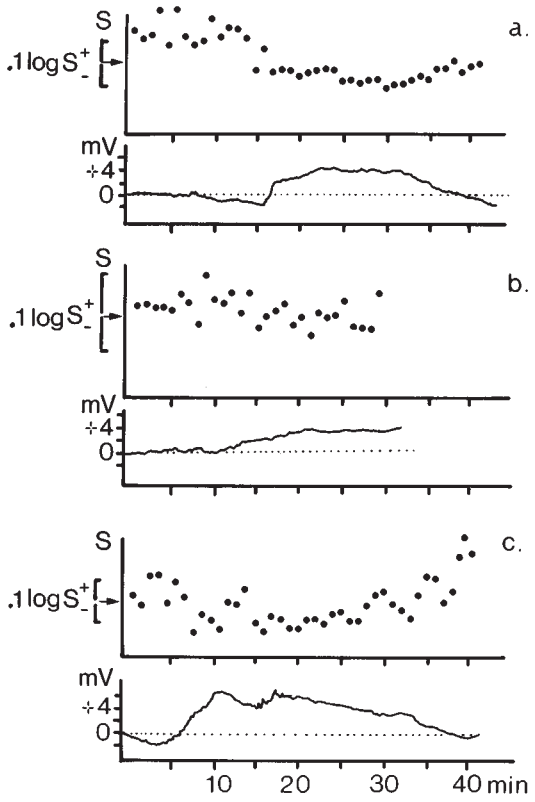

Fig. 4a–c. Effect of small changes of resting potential on the sensitivity of green receptors in the compound eye of *Osmia rufa*. Three examples are given (**a–c**). The dots in the upper part of each example gives the sensitivity on a log scale (see unit ± 1 log on the left side, note different scale in the 3 examples) determined every minute by a flash of 532 nm at various intensities. The continuous line below gives the resting potential with 0 mV set to the intracellular potential at the beginning of each experiment

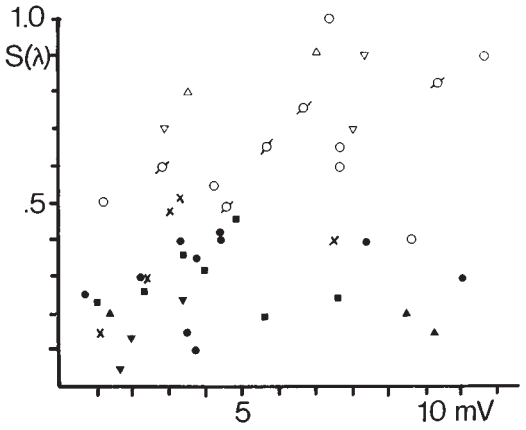

Fig. 5. Dependence of spectral sensitivity on the stability of the resting potential during recording from green, blue and UV receptors in *Melipona* and *Osmia*. Abscissa maximal DC fluctuation of the resting potential during the test period in which spectral measurements were taken with the flash method; ordinate plots $S(\lambda)$ of the secondary sensitivity relative to the main sensitivity ($S(\lambda)$ in the UV for green receptors, in the green for UV receptors, and in the UV for blue receptors). Symbols: *Melipona:* ● green receptors, × blue receptors, ▲ UV receptors, ○ UV-green, △ green-blue, ▽ blue-UV, □ broad band (see Figs. 2 and 3); *Osmia:* ■ green receptors, ◇ blue receptors, ▽ UV receptors

ty at low DC fluctuations, the main trend is a positive correlation between these two factors. This means that a high DC fluctuation indicating an imperfect intracellular recording results in a distortion of the $S(\lambda)$ function towards an increase in the sensitivity outside the main peak.

If the variability of the $S(\lambda)$ functions of individual receptors were an intrinsic property of the receptor, independent of the quality of the recording, one would not expect such a correlation. Since the correlation that is found is not very strong, there have to be other factors, artifactual and/or functional ones, which influence the shape of $S(\lambda)$. If DC fluctuations indicate mainly electrode damage to the membrane, one would expect variable, low-resistance pathways, not only to other spectral receptor types (causing an increase in sensitivity to wavelengths outside the main sensitivity), but also to receptors of the same spectral type, to the extracellular space, and to pigment cells. Artifactual coupling to the same spectral receptor type is very unlikely, because neighboring cells in a bee ommatidium are of different spectral type (apart from one common border between the two green receptors: Menzel and Blakers 1976; Wehner and Bernard 1980). The effect of coupling to a pigment cell is unknown, because there are no measurements of the electrical coupling between receptor cells and pigment cells, although glia cells (probably pigment cells) have been recorded by Coles and Tsacopoulos (1979) in the drone bee. Coupling to the extracellular space may sharpen the $S(\lambda)$ because of the effect of the hyperpolarizing field potential (ERG). If the field potential plays a major role, one would expect a different dependence on green and UV receptors because the ERG is dominated by the green receptors. This was not found. We think that artifactual couplings to the extracellular space also have little (if any) influence, because any hyperpolarizing response at light-off (particularly off axis) was interpreted as a sign of a bad recording, and data were not collected under such conditions. Hyperpolarizing off responses are well known to originate from the ERG (Burkhardt and Autrum 1960). Other aspects of field potentials are studied below.

It is obvious from our analysis that sensitivity measurements over several minutes are impaired by sudden or slow changes in the resting potential,

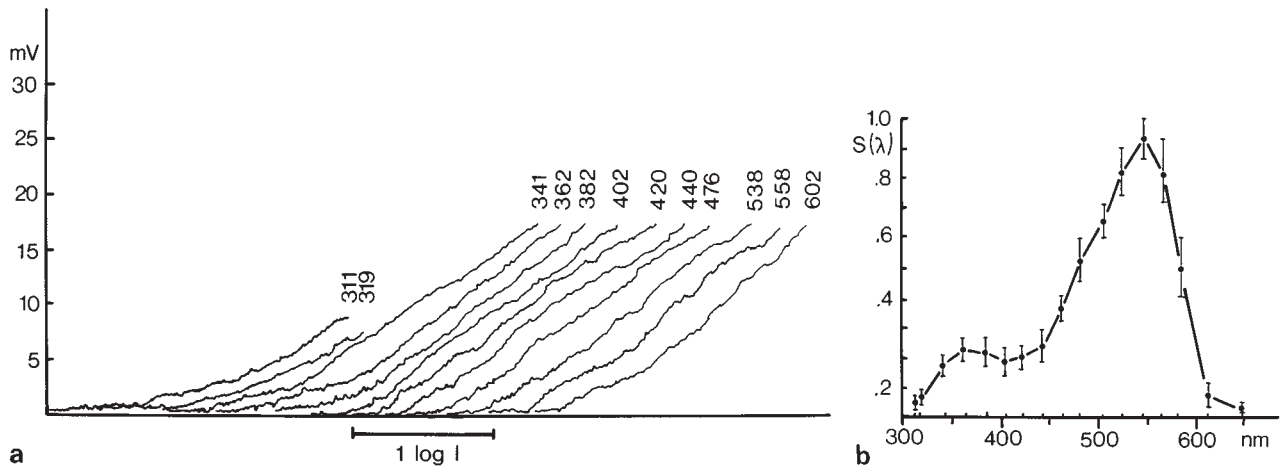

Fig. 6a, b. Ramp method. **a** Recordings of the receptor potential of a green cell (*Apis*) to a ramp stimulus at 12 wavelengths. Responses are shifted along log I axis for a better comparison. Comparator was set to 20 mV. When the response reached 20 mV the wedge stoped, the shutter closed and the computer read the position of the stepping motor. The highest intensity at 311 and 319 nm was too low to reach the criterion of 20 mV. **b** Average $S(\lambda)$ function of 16 runs in green receptors (*Apis*) determined with the ramp method. Scatter is given in $\pm$standard deviation

which have a strong impact on receptor sensitivity, and adaptive effects due to the test stimuli themselves. More reliable results might be obtained by using a constant, effective light, thus eliminating different amounts of long lasting sensitivity changes, and by decreasing measuring time, thus reducing the effect of resting potential fluctuations.

The ramp method

Constant-effective light flashes are produced with the ramp method (Fig. 6a, b). A light flash starts at a very low light intensity which causes no response in the cell, and light flux increases logarithmically at high speed (1 log unit increase per 65 ms). The light is switched off when a certain preselected DC value of the receptor potential is reached, and sensitivity is calculated on-line on the basis of the number of steps needed by the motor which drives the logarithmic neutral density wedge and a calibration factor assigned to the interference filter in use. We found that the spectral run could also be speeded up considerably without intensifying the adaptation effect, because the cell is exposed to much less effective light than in the flash method. The ramp speed was adjusted to requirements of the retinula cells empirically. A ramp speed of less than 100 ms/log I unit reduces the V/log I function, particularly at higher intensities due to the fast adaptation processes in the photoreceptors; a ramp speed above 50 ms/log I unit gives a less reliable sensitivity reading because of the lag between response and ramp position due to the latency of the receptor. A spectral sensitivity function as in Fig. 6b, based on 17 monochromatic ramp flashes, is established within 1 min. A comparison with the flash data shows that variability is very much reduced. Another advantage of the method is the on-line data evaluation. The spectral sensitivity function appears on the screen of a storage scope during the measurement process. Monitoring the voltage change of the cell during the ramp flashes indicates any wavelength dependence on the V/log I function. We found no such wavelength dependence in either UV or green receptors of the honeybee, and this confirms the result found by the flash method that the photoreceptor in the bee eye follows the univariance rule.

Disadvantages of the method are that considerable time is still needed to establish a $S(\lambda)$ function and that the spectral resolution is low (e.g., 17 filters).

The spectral scan method

The sensitivity of a photoreceptor is determined by the reciprocal of the number of quanta needed to elicit a constant response. Such a definition is necessary to account for the nonlinearities of the V/log I function and allows a designation of sensitivity without reference to the gain of the cell as long as the univariance principle holds. Univariance with respect to quantal flux has been established for several insect photoreceptors, including those of the honeybee (see above, and rev. Menzel 1979).

In essence, sensitivity measurements are homologous to voltage-clamp recordings if one uses the voltage deviation from any preselected value to ad-

just the quantum flux such that the membrane voltage does not change. Franceschini (1984) and Smakman and Pijpker (1983) were the first to realize this and to build a fast-acting control unit to 'light-clamp' the cell during a scan through the spectrum. Our system works with a grid monochromator and delivers sensitivity values every 4 nm in a continuous spectral scan (300–700 nm) within 16 s. Higher spectral resolution needs, accordingly, more time. The method combines short recording times, constant-effect illumination, and high spectral resolution. A few precautions have to be taken to collect reliable results. Adaptation effects are negligible for clamped light responses below 10 mV. The initial phasic response component at the start of the scan has to be separated from the spectral run. To avoid spectral adaptation effects, we start the spectral run at 500 nm, apply a half spectrum pre-run (upwards or downwards) which is not stored by the computer, and follow this with a whole spectral run when measurements are made. Fast, on-line computing displays the result as a $S(\lambda)$ function within seconds and allows one to detect any effects of the direction of a spectral run, or other parameters of interest.

The spectral scan method has been applied to several hymenopteran species. Figure 7 gives the average results for workers and drones of *Apis mellifera* and *Osmia rufa* females. The results on *Melipona marginata* and *Trigona spinipes* are reported by Hertel and Ventura (1985). Compared with the flash data, the variability is much smaller, the averaged $S(\lambda)$ functions are very close to rhodopsin absorption functions (except $S(\lambda)$ of the blue receptors in drones), and the side band sensitivity is much lower. The workers of the three species have very similar $S(\lambda)$ of UV, blue, and green receptors, and one may expect very similar color discriminations in these species.

The spectral scan method is an interactive on-line method. It immediately informs the experimenter during recording about the $S(\lambda)$, whereas the flash method leaves the experimenter unaware of the result. This may cause a bias towards 'clean' $S(\lambda)$ functions because of selective sampling of 'clean' recordings and thus may lead to exclusion of functionally significant results. Indeed, we have measured many more spectral scans than we have actually stored, processed, and included in Fig. 7. Those we excluded were always characterized by an unstable baseline, differences in $S(\lambda)$ for upruns and downruns, and/or ERG components in the flash response, particularly to off-axis light. We are confident that our criteria exclude only those recordings that have been affected by the intracellular recording technique.

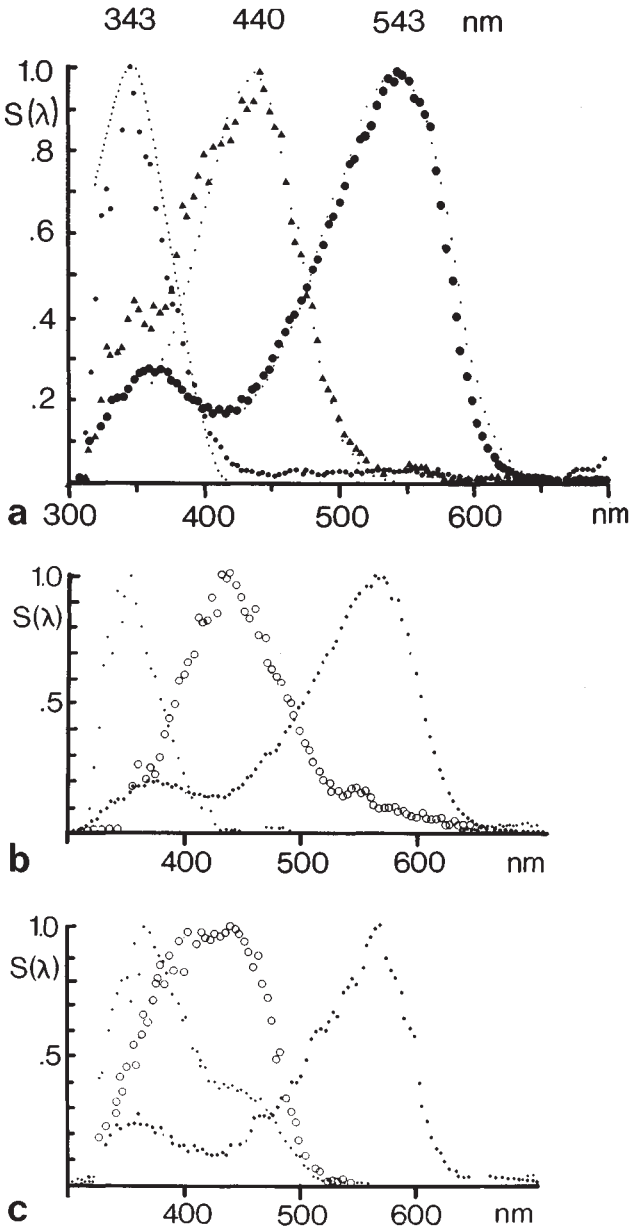

Fig. 7a–c. Average $S(\lambda)$ determined with spectral scan method. **a** *Apis mellifera* worker bee: · UV receptor (10 cells), ▲ blue receptor (5 cells), ● green receptor (39 cells). *Dotted lines:* absorbance spectrum of rhodopsin pigments with corresponding λ_{max} (blue and green receptor), and that of the UV pigment in the *Ascalaphus* eye (Hamdorf et al. 1973) for the UV receptor. **b** *Osmia rufa:* · UV receptor (3 cells), ○ blue receptor (3 cells), ● green receptor (26 cells). **c** *Apis mellifera* drone bee: · UV receptor (5 cells), ○ blue receptor (4 cells), ● green receptor (2 cells)

Field potentials and $S(\lambda)$

Shaw (1975, 1981) proposed a model of current flow in the fly retina which would affect the $S(\lambda)$ function in photoreceptors of hymenopteran insects if it also applied to them. Shaw's central argument is that a high resistance surrounding the reti-

na blocks current flow back into the retina along an extracellular pathway, and thus forces current through the axon terminals of less activated cells and along their axons and somata back into the extracellular space of the retina above the resistance barrier. The high resistance barrier uncouples the retina and each lamina cartridge from the extracellular space of the animal's body, and thus a reference electrode somewhere in the main extracellular space prevents the actual potential at the photoreceptor membrane from being measured. Other authors argue that $S(\lambda)$ in insect photoreceptors may indeed be sharpened by the current flow, because it originates from other spectral types (Horridge et al. 1983, 1984). Since we were the first to demonstrate a chromatically antagonistic voltage response in the bee retina, which could be interpreted as the result of such a recurrent flow (Menzel and Blakers 1976, Fig. 12), we were particularly interested in finding out whether (a) chromatic antagonism is a mechanism of color coding in the bee retina, (b) Shaw's proposal explains this effect, and (c) spectral responses without an antagonism of de- and hyperpolarizations are affected by current pathways as proposed by Shaw. It should be realized that experimental proof of Shaw's model is extremely difficult to obtain, particularly the separation of extracellular current flow from a synaptic pathway between photoreceptors. We report here cumulative evidence which supports the notion that field potentials and recurrent current flow have no significant effect in bees.

Although we have recorded from many hundreds of hymenopteran retinula cells over the last 10 years, we have seen just five cells which responded with de- and hyperpolarization to different spectral lights. In all cases, these cells were UV receptors (depolarization in the UV) with hyperpolarizing responses to wavelengths above 420 nm. Because of the rarity of such recordings, we could not analyse the source of the hyperpolarizing potential. Prominent features of all these recordings were the stable resting potential, the higher noise component which is typical for UV receptors, and a fast and noisy hyperpolarizing response to low intensity flashes of long wavelength light. In our opinion, the latter finding makes it unlikely that potentials caused by green light come from a current which moves along two axons to reach the recording site, because the long pathway should attenuate the high frequency components of the bumpy, low-light responses.

In bees, the basement membrane and the glia sheath around the cartridges are highly permeable to Lucifer yellow and thus may also be permeable to ion flow. This is obviously different from the fly (Shaw 1977). The retina and the lamina of the bee are intensely and evenly stained with Lucifer yellow less than 10 min after an injection of a small volume (1 µl) of 2% Lucifer yellow into the abdomen. The staining is not facilitated if the basement membrane is punctured with sharpened steel needles. The same applies if the Lucifer yellow is injected into the retina. Recording of the intracellular responses to spectral light flashes in an eye with a punctuated basement membrane gave results not different from those found in the untouched basement membrane. The $S(\lambda)$ of green receptors have also been determined by recordings of the axon in the lamina (Fig. 8). There is no indication of different $S(\lambda)$ in green receptors in the various recording conditions. Other receptor types have not yet been successfully recorded in the axon region. All these observations make it unlikely that field potentials due to a high resistance barrier significantly influence the spectral response of photoreceptors in bees.

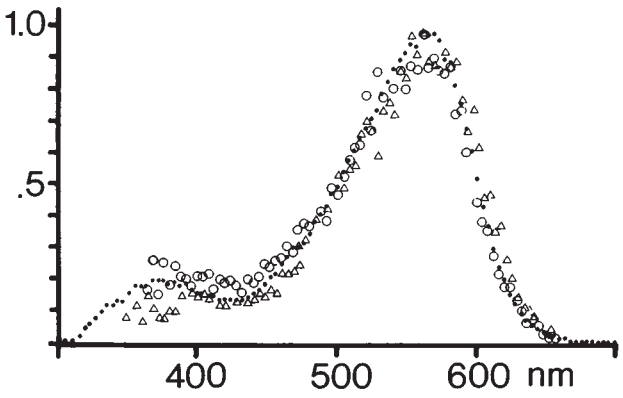

Fig. 8. $S(\lambda)$ of 2 green receptors (*Osmia rufa*) determined by the scan method based on intracellular recordings from the axon. Small dots: average $S(\lambda)$ of green receptors of recordings from the cell somata

To test the effect of field potentials in the retina more directly, we performed two other series of experiments. In the first, two microelectrodes were glued together tightly so that the tips were separated by about 20–30 µm. When the more advanced tip recorded a cell intracellularly, the point light source was centered to that cell and the response was monitored either relative to a far-removed ground electrode (a silver wire placed in the abdomen; Fig. 9a–d, recording A vs ground) or relative to the second microelectrode (Fig. 9a–d, recording A vs B). The examples given in Fig. 9a–d demonstrate that no difference is seen in $S(\lambda)$ with respect to the position of the reference electrode. This is true also for an extended light source which caused an ERG in the second electrode. Even in the few cases where both the A and B electrodes

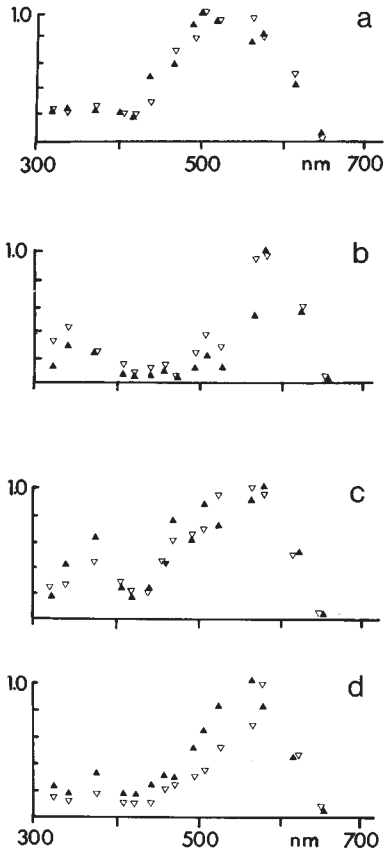

Fig. 9a–d. Four examples of double electrode experiments with green receptors in *Osmia rufa*. ▽ intracellular potential measured with respect to a ground electrode in the body (A vs ground), ▲ intracellular potential measured with respect to another microelectrode close to the recorded cell (A vs B)

were positioned intracellularly, the $S(\lambda)$ to a point light source was not changed in either A vs B, B vs A, or A or B vs ground. (An extended light source cancelled the response in the A vs B recording.)

In the second series of experiments, the effect of the size of the light stimulus was studied in an A vs ground recording situation. If the electrical field potential produced by the stimulated photoreceptors surrounding the recorded cell affects its $S(\lambda)$, one should find a change in $S(\lambda)$ when the field size and the wavelength of the surrounding field are altered. Since we did not see any change in $S(\lambda)$ when the size of the stimulating field was altered (0.8° to 30°), we then used two light sources, a parallel light beam (0.8° visual angle) which was well centered to the recording cell and a second light beam either as a point light source 2° off axis or as a ring illumination (outer diameter 5°, inner diameter 2°). Again we found no change in the $S(\lambda)$ within the accuracy of that found by using the ramp method. An example is given in

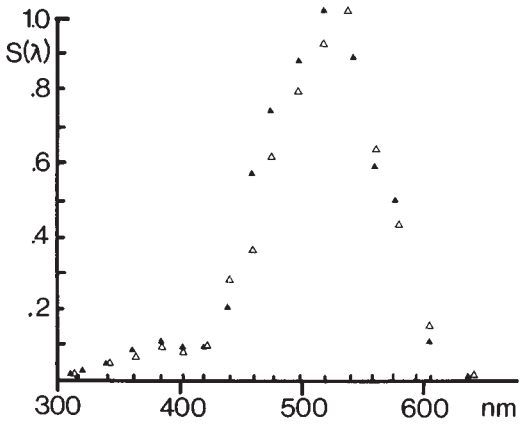

Fig. 10. Effect of UV light surrounding a green receptor (*Apis*). ▲ Test flashes from a point light source, △ same test flashes from the point light source delivered with ring flashes of UV light (ring 2°/5° visual angle centred around the point light source). There is no difference in $S(\lambda)$ between the two stimulations

Fig. 10, in which we used a ring of broad-band UV light surrounding the recorded cell. In this experiment the ring was flashed simultaneously with the test light in the center of the ring. The result was the same as when the ring was continuously switched on or off during the spectral run.

We conclude from these experiments that field potentials do not influence the spectral sensitivity of photoreceptors in the eyes of bees. The very rare chromatically antagonistic responses may not indicate an effect of retinal field potentials, but rather are probably due to a mechanism of antagonistic synaptic connections between UV and green receptors.

Discussion

Photoreceptor types with distinct different spectral sensitivities provide the nervous system with the differential information necessary to code chromatic contrasts of visual objects. Color vision depends, therefore, considerably on the spectral properties of these receptor types. Chromatic contrast, however, is only one of several parameters guiding visual orientation, and others such as intensity, polarization, spatial and temporal contrast are equally important. The primary response of photoreceptors compounds all these parameters in its sheer dependence on the number of quanta absorbed, and it is the task of the nervous system to extract information by the selective processing of parallel inputs from many receptors. This neural processing can be simplified by appropriate interactions at the level of the photoreceptors, e.g., color contrast could be enhanced by opponent pro-

cessing between receptors of different spectral type, but this would have the unavoidable consequence of the nervous system losing information on other parameters, e.g., intensity. The insect nervous system has to operate with a relatively small number of neurons, and these may process color contrast at a very peripheral level at the expense of other properties. It has been suspected that this may indeed be the case in the retina of butterflies (Horridge et al. 1983), although no evidence in favor of this interpretation exists on the level of visual interneurons and behavior (Horridge et al. 1984). In the honeybee, color vision (von Frisch 1914; Daumer 1956; von Helversen 1972; reviewed by Menzel 1979, 1985) and color coding (Kien and Menzel 1977a, b; Hertel 1980; Menzel 1985) are well studied, and thus the retinal contribution to the coding of chromatic contrast is of particular interest.

Although intracellular measurements of the spectral properties of the bee's photoreceptors are among the first successful attempts to study the retinal basis of color vision in the history of vision research (Autrum and von Zwehl 1964), the confusion of functionally significant spectral characteristics and the distortions introduced by the recording technique has not yet been resolved. In particular, it was unknown whether the high variability of $S(\lambda)$ measurements, the variable secondary spectral sensitivities, and the rarely recorded opponent responses to UV and green light are of functional significance. The experiments reported here are all in favor of Autrum and von Zwehl's (1964) suggestion that clean, rhodopsin-like spectral sensitivities characterize the three color receptor types in bees, and any deviations are recording artifacts. The honeybee is not different from other hymenopteran insects in this respect. We find similar results in several other species (*Melipona quadrifasciata, Osmia rufa* in this paper, and *Trigona spinipes* in Hertel and Ventura 1985; *Vespa vulgaris, Paravespula germanica, Bombus terrestris, B. hortorum,* Fietz and Menzel, unpubl.). Our results stress the importance of fast spectral recordings which avoid different states of sensitivity of the cell during the spectral run.

The picture emerging from our spectral measurements with minimum experimental distortion is as follows. (a) Each ommatidium, which contains all three color receptor types (Menzel and Blakers 1976), is optically and electrically isolated from neighboring ommatidia. Off-axis light has no influence on the $S(\lambda)$ of a cell. Bees differ in this respect from R7 and 8 in flies (Hardie et al. 1979), and from butterflies (Horridge et al. 1983). (b) The receptors within one ommatidium are functionally isolated from one another, at least from those of different spectral types. Some questions remain about the 1%–2% sensitivity of UV receptors at long wavelengths, because the UV photopigment in *Ascalaphus* (Hamdorf et al. 1973) absorbs less than 1%–2% above 450 nm. The variable sensitivity shoulder at 350–360 nm in the blue receptor might indicate some optical and/or electrical interaction within one ommatidium (see Fig. 7a, b, c) or an additional antennal pigment, but since blue receptors are rarely recorded in honeybees and are difficult to hold for longer than 10 min, we are unable to solve this question. In the drone bee, at least, the enhanced UV sensitivity of blue receptors is not artificial (see below). (c) Furthermore, $S(\lambda)$ does not change with light adaptation. This supports the interpretation of Menzel and Blakers (1976) that selective adaptation effects demonstrate electrical coupling. We now add: artifactual electrical coupling. Here again, the bee receptors behave differently from R1–6 in flies (Hardie 1979) that show a shift by 40–50 nm of their λ_{max} with light adaptation. The reasons for the difference between the bee and the fly are twofold: (1) The chromatic properties of screening pigment in bees are similar throughout the effective wavelength region (Langer and Schneider 1972); (2) in the fused rhabdom with three different spectral classes of receptors a median strong white or broad band chromatic adaptation does not significantly change the equilibrium between rhodopsin and metarhodopsin (Schlecht 1979). (d) Our spectral scan method makes it likely that there are no antennal pigments in the worker bees of the three hymenopteran species studied so far, at least those antennal pigments which because of their double bonds to the phenol ring exhibit fine structures in the UV. If the antennal pigment is free to rotate about one atomic bond, this fine structure may be lost (Vogt, pers. comm.). Our results support Vogt's (1984) finding that hymenopteran insects contain rhodopsin as photopigment. Male bees may differ in this respect because their blue receptors have a high UV sensitivity and their UV receptors have a high blue sensitivity. These $S(\lambda)$ functions are certainly not the result of artifactual recordings. Further experiments are needed to test whether drone bees contain a UV-absorbing antennal pigment in their blue receptors, or whether these two classes of receptors are strongly electrically coupled as suggested by Shaw (1969).

If a single electrode adequately records the receptor potentials relative to a distant reference electrode, why did five cells out of several hundred

respond with a depolarization to UV light and a hyperpolarization to wavelengths above 430 nm? We could not study this question in more detail because of the rarity of such recordings, but we want to direct attention to an important difference between such cells in the bee retina (see Fig. 12 in Menzel and Blakers 1976) and in the retina of the butterfly *Papilio* (see Fig. 12k in Horridge et al. 1983). The hyperpolarizing responses in *Papilio* are significantly slower and later than their depolarizing responses, whereas in the bee there is no such difference. A selective attenuation of the high frequency components in the DC potential caused by the return flow of the electric current would be expected since the current passes twice the length of the retinula cells and axons. In the absence of such an attenuation, it is unlikely that electric current has to flow via a long pathway. Chemical synapses have never been found in the bee retina, and electrical synapses which reverse the potential are unknown. It is, therefore, probable that the origin of the hyperpolarizing potential lies in the lamina. A chemical inhibitory synapse feeding back on the retinula axons seems most likely and would account for the fast response components of the hyperpolarizing potentials if it is accompanied by amplification. It is unclear, however, whether the rare cases of de- and hyperpolarizing UV receptors in the bee eye indicate a special mechanism of a particular class of UV receptors or a simple failure of the synaptic wiring in the lamina. The latter seems to be more likely, considering the frequency of about 1% of all receptors recorded.

We conclude that single-electrode recordings provide us with accurate information on the spectral sensitivity of individual receptors in the hymenopteran eye if the precautions described in this paper are observed. This conclusion is supported by interneuron recordings from various levels of visual integration in the optic ganglia of the bee (Kien and Menzel 1977a, b; Hertel 1980; Erber and Menzel 1977; Riehle 1981; Schäfer 1984; review Menzel, 1985). The simultaneous existence of broad band, narrow band, and chromatically antagonistic neurons with the spectral properties of just the three types of photoreceptors excludes the possibility of chromatic processing at the level of the retina and supports the interpretation that three separate wavelength channels with the spectral properties of their respective rhodopsin photopigments provide the visual ganglia with the information necessary for the neuronal mechanism of color coding and the enhancing of color contrast. Behavioral experiments on color vision of various hymenopteran species are in agreement with this conclusion, because they show that color discrimination can be predicted on the basis of the spectral sensitivity functions as measured by the spectral scan method (Menzel, Werner, and Backhaus, in prep.).

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