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Spectral Sensitivities of Seven Morphological Types of Photoreceptors in the Retina of the Turtle, *Geoclemys reevesii*

TERUYA OHTSUKA
Department of Information Physiology, National Institute for Physiological Sciences, Okazaki 444, Japan

ABSTRACT

Spectral sensitivities of photoreceptors in the turtle (*Geoclemys*) retina were studied by intracellular recording, and each cell was filled with Lucifer yellow (LY). Photoreceptors were classified into seven morphological types: rod, four types of single cones, and two members of a double cone. Single cones contained one of four different oil droplets: red, pale-green, orange, and clear. Double cones consisted of two apposed cones; principal members contained yellow oil droplets, while accessory members contained no oil droplet. Spectral sensitivities recorded from these seven types of photoreceptors were classified into one type of rod and three chromatic types of cones. Rods (n = 19) showed peak sensitivity at 520 nm. Single cones containing either a red (n = 51) or a pale-green (n = 9) oil droplet were red-sensitive (λmax at 620 nm). Single cones containing an orange oil droplet (n = 14) were green-sensitive (λmax at 540 nm). Single cones containing a clear oil droplet (n=3) were blue-sensitive (λmax at 460 nm). Both members of the double cone, principal (n = 22) and accessory (n = 15), were red-sensitive (λmax at 620 nm). No diffusion of LY was detected between the apposed members of double cones. Red-sensitive cones, therefore, consisted of four different morphological types of cones, and they occupy about 70% of the photoreceptor mosaic in the turtle retina.

Key words: turtle retina, oil droplet, rod, cone

In the retina of the red-eared turtle (*Pseudemys*), brillantly colored oil droplets found in the cone inner segments have been used to identify each chromatic type of cones in studies of the connections between photoreceptors and horizontal cells (Leeper, '78a,b) and in the electron microscopic analysis of photoreceptors (Kolb and Jones, '82a,b; Normann et al., '84). Assignment of each color of oil droplet to a specific chromatic type of cone in these anatomical studies was based on evidence obtained independently by microspectrophotometry (MSP) (Liebman and Granda, '71; Liebman, '72; Liebman and Granda, '75; Granda and Dvorak, '77) and by physiological experiments (Baylor and Hodgkin, '73; Richter and Simon, '74). In these experiments, red-sensitive cones were presumed to contain either a red or an orange oil droplet, green-sensitive cones a yellow oil droplet, and blue-sensitive cones a colorless oil droplet.

Recently, an MSP study (Lipetz and MacNichol, '82) reported that both members of double cones contained red-absorbing visual pigments, despite the widespread belief that double cones were composed of red- and green-sensitive cones (Richter and Simon, '74; Baylor and Fettiplace, '75). Moreover, some cones which would be predicted to be blue-sensitive by previously proposed criteria, i.e., on the basis of containing colorless oil droplets (Liebman, '72), have in fact been found to be red-sensitive (Ohtsuka, '84). Photoreceptors in the turtle retina, therefore, need to be re-evaluated with regard to correlations between spectral sensitivities and anatomical characteristics such as oil droplet colors.

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In the freshwater Reeves' turtle, *Geoclemys reevesii*, five different types of oil droplet have been characterized by MSP (Fujimoto et al., '57). Each of these can be unequivocally distinguished by visual inspection of isolated and flat-mounted retinas. In the present study, an intracellular electrode was used to determine both the peak spectral sensitivity of these cells, and, by dye injection, their morphological types of photoreceptors. The results presented are supplementary to those of previous morphological studies of horizontal cells in this species (Ohtsuka and Kouyama, '82; Ohtsuka, '83).

MATERIALS AND METHODS

Preparation

Eyes of Reeves' turtle, *Geoclemys reevesii*, were used. Adult specimens (carapace length = 20–23 cm) were kept either in darkness or under a room light (400 lux) for 2 hours prior to decapitation and enucleation as described elsewhere (Ohtsuka, '83).

Light and fluorescence microscopy of unfixed retina

Eyecup preparations, i.e., the posterior halves of freshly dissected eyeballs, were immersed in a turtle Ringer's solution, and the dorsomedial retina was dissected out and detached from the sclera. In each piece of retina placed photoreceptor-side upward on a glass slide, a mosaic of photoreceptors and oil droplets was observed under a light microscope (Biophoto, Nikon). Five kinds of oil droplets, i.e., red, orange, yellow, pale-green, and all-transmittable (Fujimoto et al., '57), were distinguishable under these conditions. Oil droplets were photographed, and their diameters measured in an image processor (IBAS II, Kontron, München). The "all-transmittable" oil droplets will be referred to in the present paper simply as "clear."

Pale-green oil droplets were similar to the clear type when viewed under light microscopy. However, these two types of oil droplets are dramatically different under fluorescence microscopy (Ohtsuka, '84), when near-UV excitation was provided with near-UV bandpass filter (330–380 nm), and autofluorescence was observed through a blocking filter (cutoff wavelength, 430 nm).

Intracellular recording

Details of eyecup preparation and photostimulation have been described elsewhere (Ohtsuka, '83). Glass microelectrodes were filled with 5% w/v aqueous solution of Lucifer yellow CH (Aldrich Chemical Co.) and electrode resistance was about 700 MΩ. In some experiments, a 5% w/v aqueous solution of Procion yellow MX4R (Ohtsuka, '78) was also used. Photoreceptors, located at the medial retina 2–3 mm dorsal from the visual streak, were identified by the following two criteria: (1) the recording depth (about 150 μm) from the inner limiting membrane, and (2) equal peak response amplitudes to two spot stimuli (white light, 0.56 and 10 mm in diameter). These, however, included some displaced bipolar cells whose somata were also located at the outer nuclear layer. Therefore, further morphological inspections (see below) were performed to discriminate these two different groups of retinal cells.

When a photoreceptor was impaled, whole-field illumination by white light at various light intensities was applied to obtain intensity-response amplitude relationships. Unattenuated white light measured 117 μW cm^{-2} at the level of the retina. Then, spectral responses to whole-field illumination of monochromatic stimuli provided by interference filters (420–700 nm, bandwidth about 10 nm) were recorded. The photon fluxes of the monochromatic test stimuli were equalized with neutral density filters to 1.4×10^{12} photons cm^{-2} second^{-1}. The photostimulator was connected to a microcomputer (M223 Mark III, SORD, Tokyo), and stimulating paradigms were controlled by interactive programs.

In cells whose peak response amplitudes to saturating white light intensity were more than 5 mV and the membrane potentials in darkness (−10 to −30 mV) were stable, spectral responses were recorded to monochromatic flashes (duration, 0.3 second) from 420 to 700 nm, in 40-nm steps, at various light intensities. To study rods, the flash duration was extended to 0.6 seconds and stimulus wavelengths were changed by 20-nm steps. Spectral sensitivity was measured as the inverse of the light intensity which produced a threshold response amplitude (2–5 mV at response peak) at each stimulus wavelength. Spectral sensitivity curves were compared to the absorption spectra of the retinene$_2$-based pigments calculated from the nomogram (Munz and Schwanzara, '67) in combination with transmission spectra of oil droplets (Fujimoto et al., '57). Since absorption spectra of visual pigments in the *Geoclemys* retina are not available, the wavelengths of maximum absorption by three visual pigments in the *Pseudemys* retina (460, 520, and 620 nm; Lipetz and MacNichol, '82) were used.

Most of the photoreceptor responses were small in amplitude (< 5 mV) to saturating light intensities, and the intracellular microelectrode was easily dislodged within a few minutes. Therefore, spectral sensitivities were determined by the following simplified paradigms described by Tomita et al. ('67). At first, photoreceptor responses to a series of monochromatic flashes were recorded at the light intensity which elicited response peak amplitudes less than half of the maximal amplitude. Then several responses to white light stimuli at various light intensities were recorded. All these responses were stored on an FM tape recorder (NFR-3000, Sony) and displayed later on a chart recorder. The light intensity which would elicit a given response amplitude for certain monochromatic stimuli was obtained by interpolation of the peak response amplitudes to white light illuminations. The inverse of this light intensity was considered as the sensitivity to the monochromatic stimulus.

Lucifer-yellow-filled photoreceptors

After intracellular recording, LY was injected by rectangular current pulses (-3 nA, 2 Hz) for 1–2 minutes. In some experiments, axon terminals of luminosity-type horizontal cells were injected with LY to test the diffusion of LY through gap junctions in the present experimental condition. Eyecup preparations containing these dye-filled cells were immersed in Ringer's solution containing 5% w/v collagenase (type I, Sigma Chemical Co.) for 30 minutes, which facilitated detachment of retina from pigment epithelium (Ohtsuka, '78). Then a piece of retina containing several LY-filled cells was dissected and fixed in 0.1 M phosphate buffer (pH 7.4) containing 8% formaldehyde and 10% ethanol for 2–6 hours at 4°C. Such a low concentration of ethanol only hardened the retinal tissue, but color and size of the oil droplets did not change. This procedure prevented loss of oil droplets during subsequent observations.

The retinal piece was mounted photoreceptor-side up on a glass slide. LY-filled photoreceptors were unequivocally identified by yellow fluorescence under violet exciting light

TABLE 1. Spectral Sensitivities of Seven Morphological Types of Photoreceptors in the *Geoclemys* Retina[1]

Morphological type	Oil droplet Color	λ½ (nm)	Diameter (μm)	Density (%)	Spectral type	λ max (nm)	Cell no.
Rod	None			10	Rod	520	19
Single cone	Red	572	8.5 ± 0.3	19	Red	620	51
	Pale-green	413	7.3 ± 0.4	10	Red	620	9
	Orange	530	7.4 ± 0.4	13	Green	540	14
	Clear	—	5.6 ± 0.4	6	Blue	460	3
Double cone							
Pr. memb.	Yellow	495	7.0 ± 0.2	21	Red	620	22
Acc. memb.	None			21	Red	620	15

[1]Half-transmission wavelength (λ½) of the oil droplets was from Fujimoto et al. ('57). Oil droplet diameters (mean ± S.D.) were measured in cones located at 2 mm dorsal from the visual streak at the medial retina. Values indicate mean ± S.D. of about 30 oil droplets of each type. Carapace length of this turtle was 19 cm. Number of photoreceptors within a unit area (0.047 mm^2) in the same retina was calculated into density per 1 mm^2. One percent corresponds to 110 photoreceptors/mm^2. Each member of the double cone was counted separately. The wavelength of peak spectral sensitivity (λmax) was measured in the number of photoreceptors shown in the rightmost column.

(400–420 nm) (Stewart, '78). LY-filled displaced bipolar cells gave diffuse fluorescence spanning a diameter of several photoreceptors and also along the Landolt club in the interreceptor space. Fluorescent micrographs of LY-filled photoreceptor cells were taken on color film (Fujichrome RHP, ASA 400), and immediately thereafter the oil droplet contained in each photoreceptor was photographed by changing the light source to standard visible illumination.

In some preparations, oil droplets inevitably detached during histological procedures before the light microscopic inspection. In such preparations, cones would be mistaken as rods. Therefore, LY-filled photoreceptors were also examined along their inner segments in the radial sections. The retinal piece was dehydrated and embedded in modified Spurr's medium (Quetol 653, Nisshin EM, Tokyo). Before baking, details of LY-filled photoreceptors in the cleared preparation, especially at apposed regions of the members of double cones, were again observed. Serial radial sections (10 μm thickness) were made perpendicular to this apposed region in the double cones by a microtome (JB-4, Sorvall).

A total of 347 cells located in the outer nuclear layer were studied by intracellular recording of spectral responses and filling with LY, in 52 eyecup preparations from 34 turtles. In about half of them (n = 168), definite identification was difficult because LY leaked into neighboring Müller cells or displaced bipolar cells, or because cells were filled incompletely. In the remaining 179 cells, LY filled only photoreceptors (n = 166) or displaced bipolar cells (n = 13). As 33 photoreceptors showed small responses (< 3 mV) to saturating light intensity, spectral responses were analyzed from 133 photoreceptors (Table 1).

RESULTS

Seven morphological types of photoreceptor

Photoreceptors in the turtle retina have been morphologically classified by the presence and color of oil droplet (Kolb and Jones, '82a; Ohtsuka, '84). Figure 1A shows a flat-mount of the dorsomedial retina of *Geoclemys*, where seven morphological types of photoreceptors were distinguishable. Pairs of photoreceptors apposed in a "theta"-like configuration were double cones; cones containing yellow oil droplets were principal members; while apposed cones which have no oil droplet were the accessory members. Of the single photoreceptors, rods lacked oil droplets, while single cones contained one of four different oil droplets—red, pale-green, orange, or clear (Fujimoto et al., '57). Since absorption spectra of oil droplets are species specific (Liebman and Granda, '71), some combinations of oil droplet and morphological types of photoreceptor are different in *Geoclemys* as compared to *Pseudemys* retina (Kolb and Jones, '82a).

As has been shown by MSP (Fujimoto et al., '57), pale-green oil droplets were distinguishable from clear ones by their tint under light microscopy. In addition, the diameters of pale-green oil droplets were significantly larger than those of nearby clear ones at any given retinal area, al-

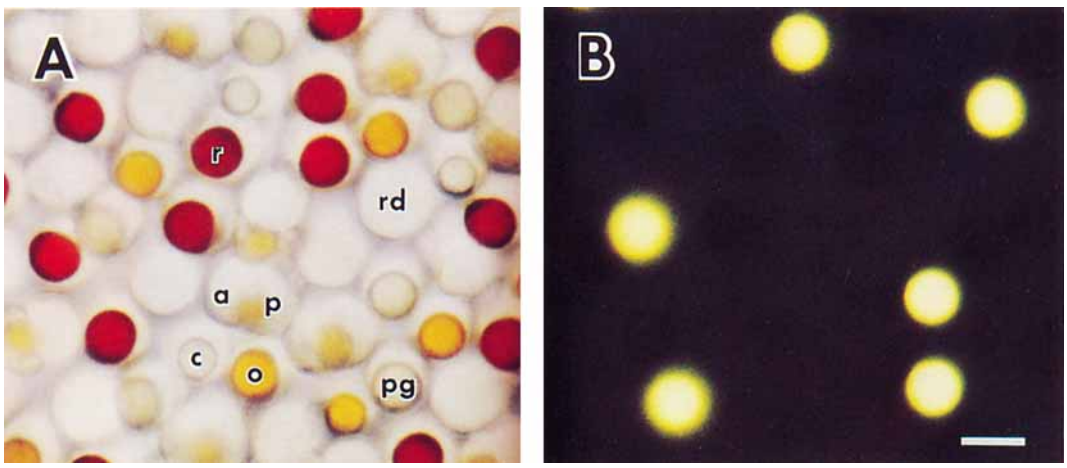

Fig. 1. Photomicrographs showing flat-mount of isolated unfixed retina of the Reeves' turtle. A. Seven morphological types of photoreceptors were distinguishing by the presence and color of their oil droplets. Rods (rd) have no oil droplet. Double cones show a figure "theta." Principal member (p) contained yellow oil droplets (out of focus in this picture), which were located about 5 μm sclerad to other oil droplets; accessory members (a) have no oil droplets. Four different oil droplets were found in the single cones: red (r), orange (o), pale-green (pg), and clear (c) ones. B. Identical preparation under a fluorescence microscope revealed that only cones with pale-green oil droplets fluoresced upon near-UV irradiation (380–400 nm). Fluorescence photomicrograph was taken through a blocking filter (cutoff wavelength, 460 nm). Scale bar in B = 10 μm.

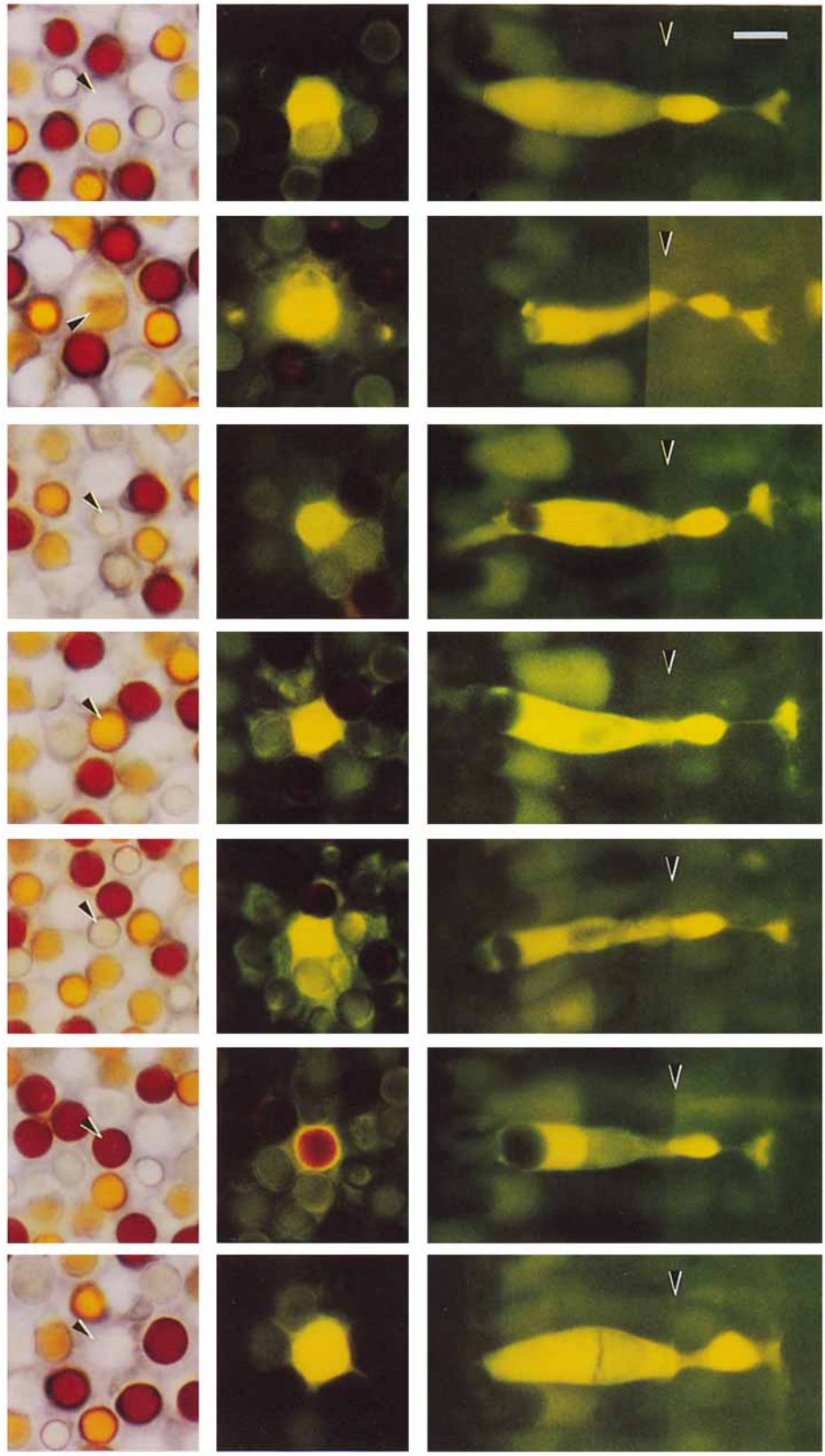

Fig. 2. Light and fluorescence photomicrographs showing seven morphological types of photoreceptors, which were intracellulary recorded and injected with LY. Each column indicates one of the seven morphological types of photoreceptors. Rod (A), single cones containing, respectively, a red (B), pale-green (C), orange (D), and clear (E) oil droplets, and principal (F) or accessory (G) members of double cones. Flat-mount view of each of these is shown at the arrowhead in the upper row of light micrographs. Middle row of photomicrographs shows the same LY-filled photoreceptors as in the upper row under fluorescence microscopy. Radial views of each type of photoreceptor are shown in the lower row. Arrowheads indicate the level of the outer limiting membrane. Magnification of all photographs was the same; scale bar shown in the lower photomicrograph of column (G) = 10 μm.

though the size of oil droplets increased with distance from the visual streak. A recent study (Ohtsuka, '84) has shown that only pale-green oil droplets autofluorescenced under near-UV irradiation (see also Fig. 1B).

The color and size of the five different oil droplets were unchanged by immersion of the retinal preparations in the formaldehyde-ethanol solution used in the present study (see Fig. 2, upper row). Therefore, seven morphological types of photoreceptors could be identified by the presence, color, and size of oil droplets in flat-mounted preparations (Table 1).

Spectral sensitivities of photoreceptors

Rods. Nineteen rods, characterized by the slow time course of response to dim stimuli (Baylor and Hodgkin, '73), were recorded in the dark-adapted retina. LY-filled rods in the flat-mounted retina showed no oil droplets, large-diameter (> 12 μm) inner segments, and large, thick outer segments (Fig. 2A). Radial sections illustrated that the thick inner segment narrowed at the outer limiting membrane and that a thick process (> 3 μm in diameter) led to the cell body located at the proximal portion of the outer nuclear layer. A short axon more than 4 μm in diameter was connected with the pediclelike terminal ending, where fine telodendria extended laterally up to 50 μm in the outer plexiform layer.

The peak sensitivity of 19 rods was at 520 nm, and spectral sensitivity curves (Fig. 3) fitted the absorption spectrum of the P520 pigment calculated from the nomogram (Munz and Schwanzara, '67).

Single cones. Spectral sensitivities were obtained from 77 LY-filled single cones of light-adapted retinas. Four different colors of oil droplets were identified in these LY-filled cones (Fig. 2B–E).

Cones containing red oil droplet. Red oil droplets were the most readily distinguishable among the five different oil droplets in *Geoclemys* retina. Under fluorescence microscopy, 51 cones contained a red oil droplet. Yellow fluorescent rings around each dense red carotinoid were observed as a corona (Fig. 2B). Since the cutoff wavelength, i.e., 5% of peak transmission, of red oil droplet was at 552 nm, most of the yellow fluorescence of LY was absorbed in whole-mount views by the interposing oil droplet. In the radial section, a large vacuole which had been filled with a red oil droplet before dehydration was found at the outermost inner segment. The cell body was located at the distal portion of the outer nuclear layer and sent a thin axon (< 1 μm) to the terminal ending, where telodendria extended laterally up to 15 μm in the outer plexiform layer.

The spectral sensitivity obtained from these 51 cones was maximum at 620 nm. Unlike the rods, cones containing densely colored oil droplets showed sharply tuned spectral sensitivities because shorter-wavelength incident light was absorbed by the interposing oil droplet. Half-power bandwidth (commonly called bandwidth), i.e., the breadth of wavelengths enclosed at half peak absorption, was 180 nm for the absorption spectrum of the P620 pigment (Munz and Schwanzara, '67), while that of the P620 pigment in combination with red oil droplet (Fujimoto et al., '57) was 114 nm. Spectral sensitivity curves obtained from nine cones (Fig. 4) fitted the latter absorption spectrum between 580 and 700 nm, and their bandwidth was about 85 nm. In spite of the dense red oil dropletes in LY-filled cones, the sensitivity to shorter wavelengths varied from cone to cone by more than 1 log unit. This large deviation of the sensitivity curve was not seen in the recordings from rods.

Cones containing pale-green oil droplet. Nine cones containing a pale-green oil droplet were filled with LY. Since pale-green oil droplets absorbed only violet light (cutoff wavelength, 404 nm), most of the yellow fluorescence of LY passed through this oil droplet and thus the entire profile of the inner segment was seen in the fluorescence micrograph (Fig. 2C) of the whole-mount. As the autofluorescence of the pale-green oil droplets was masked by the intense fluorescence of LY, this type of oil droplet was more

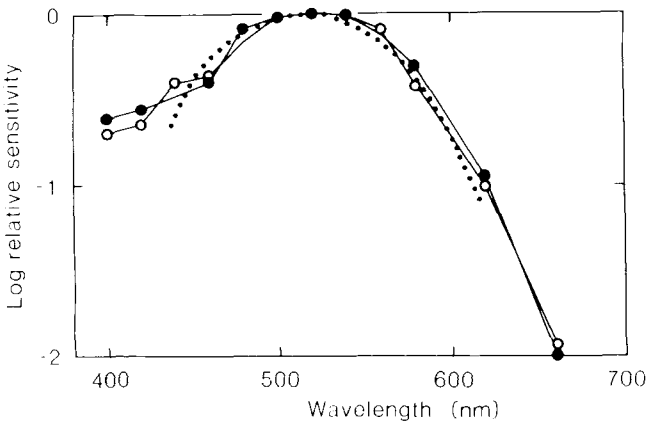

Fig. 3. Typipcal spectral sensitivity of two rods. Peak sensitivity is at 520 nm; the absorption spectrum of the P520 pigment calculated from the nomogram (see text) is indicated by the dotted line.

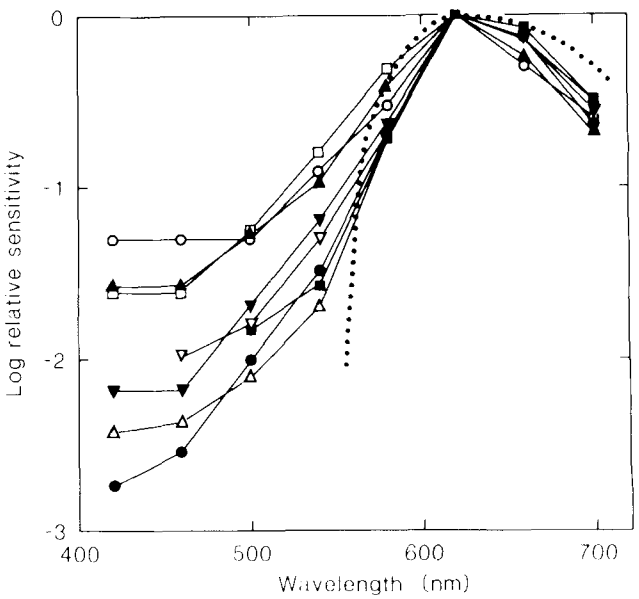

Fig. 4. Spectral sensitivity of nine single cones each of which contained a red oil droplet. Although their peak sensitivity was at 620 nm, a large variety of sensitivities to the shorter wavelengths was seen. Dotted line indicates the absorption spectrum of the P620 pigment calculated from the nomogram in combination with the red oil droplet. Bandwidths (see text) of this absorption spectrum and measured spectral sensitivity were 114 nm and about 85 nm.

easily identified by the tint under visible light, and could be confirmed because its size was larger than that of the clear oil droplet (Table 1). The radial profiles of these cones were similar to those of cones which contained red oil droplets, particularly concerning the vacuole of oil droplet, the cell body located at the distal portion of the outer nuclear layer, and the thin axon connecting with terminal ending.

The peak sensitivity of this group of cones was at 620 nm (Fig. 5), which was the same as the cones with red oil droplet. However, the decrease in sensitivity over the shorter-wavelength side of the spectrum was less than 1 log unit from the peak. Thus, the shorter cutoff wavelength (404 nm) of the pale-green oil droplets seemed to be less effective as a color filter over the red-absorbing visual pigment. The bandwidth (about 110 nm) of the spectral sensitivity curve was narrower than that (180 nm) of the absorption spectrum of the P620 pigment. Sensitivity lower than that predicted by the absorption spectrum might have resulted from self-screening by the retinal tissue.

Cones containing orange oil droplet. Fourteen cones contained orange oil droplets (Fig. 2D). As the orange oil droplet absorbed most of the yellow fluorescence of LY, fluorescent rings were also seen in axial views of this type of cone. Radial sections revealed that the cell body, thin axon, and terminal ending were similar to those of the other single cones.

The spectal sensitivity of these cones was maximal at either 540 nm (n = 11) or 580 nm (n = 3). As the cutoff wavelength (506 nm) of orange oil droplet absorption overlapped the peak absorption of the P520 pigment, the peak of the calculated absorption spectrum of the P520 pigment-oil droplet combination shifted to the longer wavelength (540 nm). Spectral sensitivities fitted this calculated absorption spectrum in the wavelengths longer than 540 nm, but large differences in sensitivity were seen in the shorter wavelength region (Fig. 6). The bandwidth of these spectral sensitivities was about 60 nm, which was roughly the same as that of the calculated absorption spectrum (80 nm) of the P520 pigment-oil droplet combination, and about half of that (116 nm) of the P520 pigment. Thus, because of the interposing orange oil droplet, the spectral sensitivity of green-sensitive cones in the *Geoclemys* retina was narrowly tuned to about half of the absorption spectrum of green-absorbing visual pigment.

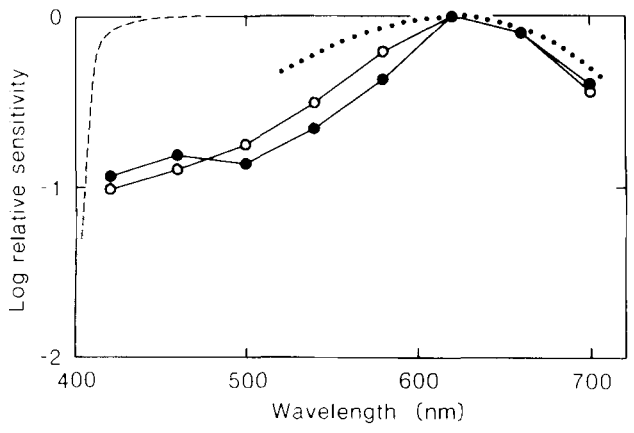

Fig. 5. Spectral sensitivity of two single cones each containing a pale-green oil droplet. Dotted line indicates absorption spectrum of the P620 pigment. Broken line is the transmission spectrum of pale-green oil droplets, whose cutoff wavelengths was 404 nm. Bandwidth of spectral sensitivity curve was about 110 nm.

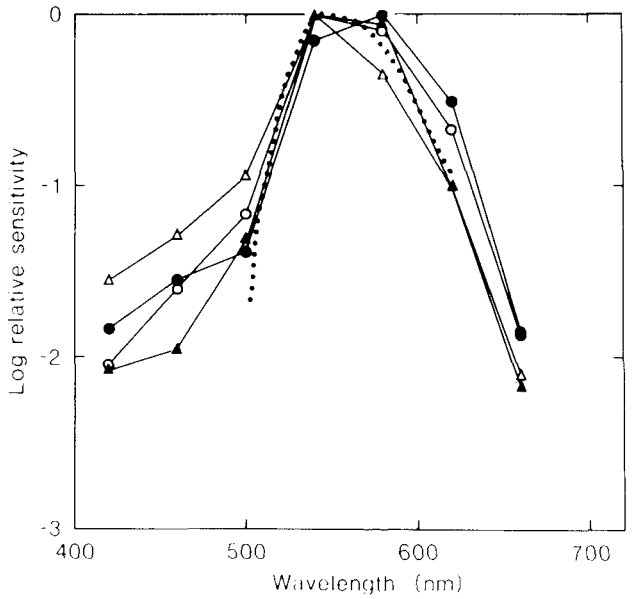

Fig. 6. Spectral sensitivity of four single cones each containing an orange oil droplet. Dotted line is the calculated absorption spectrum of the P520 pigment in combination with the orange oil droplet. The peak of this absorption spectrum appeared to be shifted 20 nm toward longer wavelengths. Bandwidth of the absorption spectrum (dotted line) and measured spectral sensitivity was 80 nm and about 60 nm.

Cones containing clear oil droplet. Because of the low density and small size of this group of cone (Table 1), only three were successfully recorded and filled with LY (Fig. 2E). Flat-mounted preparations showed transparent oil droplets. The radially viewed aspects of these cones were similar to the other type of single cones. Some axons of cones containing clear oil droplets projected laterally about 5 μm (corresponded to the width of one pedicle) in the outer nuclear layer, *en route* to connecting with the terminal ending. However, such short lateral displacement of the axon terminal was also seen in cones with the other types of oil droplets. Long oblique axons, which run laterally more than 10 μm (Leeper, '78b), were not observed in the present study. Such axons are found in less than 1% of all photoreceptors in the dorsomedial retina of *Geoclemys* (N. Kouyama and T. Ohtsuka, unpublished observation). The much higher occurrence of cones containing clear oil droplets (see Table 1) suggests that not all blue-sensitive cones have long oblique axons.

All three cones showed peak spectral sensitivity at 460 nm (Fig. 7). As clear oil droplets pass most of the visible light, the spectral sensitivity fitted the absorption spectrum of the P460 pigment (Munz and Schwanzara, '67) around the peak. Although the sensitivity curve in the longer wavelengths was always broader than the absorption spectrum, the bandwidth of spectral sensitivity curves (about 75 nm) was almost the same as the absorption spectrum of the P460 pigment.

Double cones. Since LY was only detected in one member of 37 LY-filled double cones, the site of intracellular recording was unequivocally identified (Fig. 2F,G).

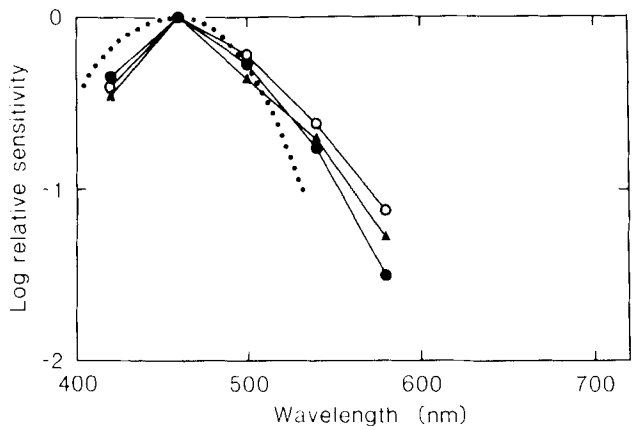

Fig. 7. Spectral sensitivity of three single cones each containing a clear oil droplet. Their peak sensitivity was at 460 nm. Dotted line is the absorption spectrum of the P460 pigment, where the absorption of the clear oil droplet was not considered. Bandwidths of the absorption spectrum of the P460 pigment and measured spectral sensitivity were 90 nm and about 75 nm.

Principal members. LY-filled principal members of double cones contained yellow oil droplets which were located about 5 μm sclerad to the other type of oil droplets (Fig. 2F). Under fluorescence microscopy, the profile of the LY-filled member was semicircular. The apposed region of two members were seen in the retina cleared by Spurr's medium. The radial section, cut perpendicularly to this apposed region, illustrated a skewed slender inner segment of LY-filled principal member adhered to the fat round inner segment of the accessory member. Only this group of cones showed the soma at the middle portion of the outer nuclear layer. The inner segment and the soma of the principal cone were connected by a thin process running along the soma of the apposed accessory member.

The spectral sensitivity of all studied principal members (n = 22) was maximal at 620 nm (Fig. 8). As the cutoff wavelength of yellow oil droplets was 474 nm, the visual pigment of this cone type seemed to absorb most of the incident light. Lower sensitivity to wavelengths less than 500 nm seemed to result from filtering by the yellow oil droplet.Thus, it could be concluded that the principal member contained red-absorbing pigment. As observed in the cones containing pale-green oil droplets, the bandwidth of the spectral sensitivity was 115 nm, i.e., narrower than that (180 nm) of the absorption spectrum of the P620 pigment.

Accessory members. Although both rod and accessory member have no oil droplets, accessory cones were obviously identified by their apposition with principal members in the flat-mounted preparations (Fig. 2G). Fifteen accessory members were filled with LY, and no diffusion into the apposed principal members was detected. This was also confirmed in the radial section cut perpendicular to the apposed region. The fat round inner segment of accessory members was similar to that of rods; it would be easy to mistake rods for the isolated accessory members of double cones (Liebman and Granda, '71). However, the somata of accessory members were located at the middle portion of the outer nuclear layer, and a long thin axon connected with the terminal ending. This radial appearance in the outer nuclear layer was easy to differentiate from that of rods (Fig. 2A).

All these accessory members showed peak sensitivity at 620 nm. As there were no oil droplets in this type of cones, the spectral sensitivity seemed to be similar to the absorption spectrum of endogeneous visual pigment. The absorption spectrum of the P620 pigment (Munz and Schwanzara, '67) fitted the spectral sensitivity obtained from the accessory member (Fig. 8). Sensitivity to the shorter wavelength was significantly lower than the absorption spectrum of the P620 pigment. As the optical properties and possible self-screening of this type of cone are not known, no explanation is available for deviation between absorption and spectral sensitivity. Nonetheless, it could be concluded that accessory members of double cones in the *Geoclemys* retina were red-sensitive.

Although both members of three double cones were filled with dye, they were not analyzed in the present study because these cells also leaked LY out to the neighboring photoreceptors and Müller cells. The possibility that dye diffusion through gap junction between two apposed members (Richter and Simon, '74; Kolb and Jones, '82b) was blocked in the present experimental condition was checked by comparison with LY injections into axon terminals of luminosity-type horizontal cells. Meshwork structures of interwoven axon terminals (Stewart, '78; Piccolino et al., '82; Ohtsuka, '83) were readily observed, while diffusion of LY between apposed members of double cones could not be detected in the same eyecup preparation. Furthermore, Procion yellow, which had been used to show coupling between the two members of double cones in *Pseudemys* retina (Richter and Simon, '74), was also intracellularly injected into nine principal and three accessory members of double cones. As in the experiments involving LY injections both members of double cones were red-sensitive, and no dye diffusion into the apposed member was detected.

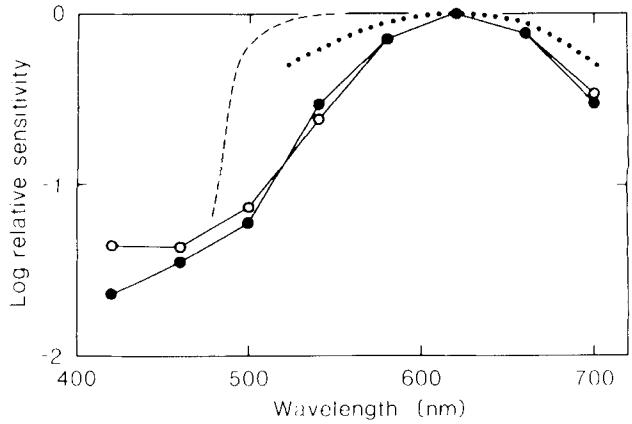

Fig. 8. Spectral sensitivities of a principal (filled circles) and an accessory member (open circles) of a double cone. Both members of double cones were maximally sensitive at 620 nm. The absorption spectrum of the P620 pigment is indicated by the dotted line between 520 and 700 nm. As the yellow oil droplet (broken line) contained in the principal member absorbed blue incident light, the sensitivity of the principal member was reduced in the shorter wavelengths less than 500 nm. Bandwidth of the absorption spectrum of the P620 pigment was 180 nm, while that of measured spectral sensitivities obtained from both members was about 115 nm.

Intensity-response curves

The different-colored oil droplets would *in situ* differentially attenuate a given stimulus. Responses of the various cones were therefore measured as a function of stimulus intensity, to determine if the filtering by oil droplets shaped the cone operating range. Peak response amplitude (V) was a function of applied white light intensity (I) described by the following equation modified from Naka and Rushton ('66):

$$V = Vmax \times I^n / (I^n + I\sigma^n)$$

where Vmax is the maximal peak amplitude to the saturating light intensity, and Iσ is the intensity which elicited half the maximal peak amplitude. Vmax was obtained directly from the experiment, and Iσ was graphically determined by interpolation of the data points. Exponent n, which indicates the steepness of the sigmodal curve of this equation, was determined by curve-fitting by eye. Figure 9 illustrates values of the exponent n of the seven types of photoreceptors. The mean of the exponents of all types of cones was 0.82, indicating that the operating range of the responses, i.e., 5–95% of Vmax, was roughly 3 log units. Furthermore, the intensity to elicit half-maximal response peak amplitudes was about 0.1 $\mu W/cm^2$ for all six different types of cones.

The variability in shorter-wavelength sensitivity of the cones containing red oil droplets (Fig. 4) was further studied. Figure 10 illustrates the difference in sensitivity at 500 and 620 nm, replotted against the half-saturating white light intensity of each cone. This plot showed that cones with higher sensitivity to green flashes needed more white light intensity to elicit half of the maximal peak amplitude. This suggested that the cones, illuminated by probably off-axis stimuli, presumably revealed higher sensitivity to green flashes because the incident light was less absorbed by the interposing oil droplet (Baylor and Hodgkin, '73). In response to white light stimulation, more light energy was required to produce the half-saturation.

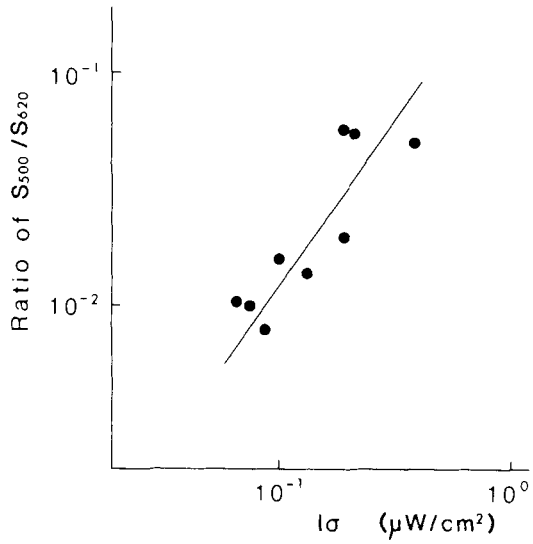

Fig. 10. Difference in sensitivity at 500 and 620 nm (S_{500}/S_{620}) shown in Figure 4, replotted against the half-saturating light intensity (Iσ) of each cone. Both axes are logarithmic scale. Cones showing higher sensitivity to 500 nm were less sensitive to white light. Straight line was drawn by eye.

DISCUSSION

Seven morphological types of photoreceptors in the *Geoclemys* retina have been identified in the present study and classified into one rod and three chromatic types of cones (Table 1). These results indicate that the number of blue-sensitive cones in the retina may have been overestimated in previous studies (Baylor and Fettiplace, '75; Granda and Dvorak, '77; Kolb and Jones, '82a; Leeper, '78b). The opposite is suggested regarding red-sensitive cones, since these constitute four different types of cones in *Geoclemys* and occupy more than 70% of the photoreceptor mosaic in the dorsal retina.

Oil droplet and spectral sensitivity

In spite of the different cutoff wavelengths of the oil droplets, the three chromatic types of cones responded to the same light intensity levels (Fig. 9B). Since no example of lower-sensitivity cones was found in the present experi-

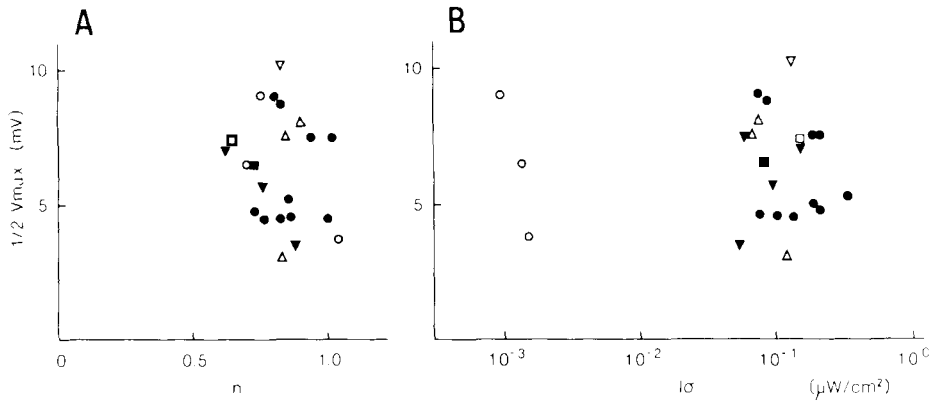

Fig. 9. A. Half-saturating peak amplitude (1/2 Vmax) and exponent (n) of the equation described in the text. Exponent n (mean ± S.D.) of cones was 0.82 ± 0.11 (n = 22); for comparison, n of rods was 0.83 ± 0.15 (n = 3). B. Light intensity (Iσ) which elicited half-saturating peak amplitude (1/2 Vmax). Mean and S.D. of half-saturating intensity (Iσ) of cones was $10^{-0.95 \pm 0.27}$ $\mu W/cm^2$ (n = 25); for comparison, that of rods was $10^{-2.90 \pm 0.09}$ $\mu W/cm^2$ (n = 3). Symbols represent seven morphological types of photoreceptors: single cone containing red (●), pale-green (▽), orange (▼), and clear (■) oil droplets; principal (□) and accessory (△) members of double cones; rod (○).

ment, or in previous studies (Balyor and Hodgkin, '73), it seems unlikely that cones containing green- or blue-absorbing visual pigment contained red oil droplet. Variance in the shorter wavelength sensitivity of red- and green-sensitive cones has been explained by off-axis illumination being less absorbed by the interposing oil droplet (Baylor and Hodgkin, '73; Baylor and Fettiplace, '75). Figures 4 and 6 of the present study show that cones containing the same kind of oil droplet could differ considerably in shorter-wavelength sensitivity. The angle between the stimulating light and impaled photoreceptors was not known in these experiments, but red-sensitive cones showing higher sensitivity to 500-nm flash needed a high light intensity to elicit half-saturating amplitude (Fig. 10). This relation could be explained by the directional sensitivity (Baylor and Fettiplace, '75) of cone photoreceptors. Similar results were also obtained in cones containing orange oil droplet (Fig. 6).

The present study suggests another factor underlying the variance in the shorter-wavelength sensitivity of the red-sensitive cones. In the *Geoclemys* retina, four different types of cones were considered of red-sensitive (Table 1). Differences in sensitivity to green flashes would be expected to result from the different absorption spectra of oil droplet contained in the various red sensitive cones (Figs. 4, 5, 8). The observed spectral sensitivities of red-sensitive cones in the *Geoclemys* retina appeared to fall into at least three groups: (1) broad bandwidth (cones with pale-green oil droplet), (2) medium, i.e., having a sharper cutoff than the broad bandwith in the shorter-wavelength region (principal and accessory members), and (3) narrowly tuned type (cones with red oil droplets).

Pale-green oil droplet

Fujimoto et al. ('57) reported two different transmission curves, i.e., pale-green and all-transmittable, of relatively colorless oil droplets. Since these two oil droplets were similar as compared to the other brillant-colored oil droplets, they have been grouped together under the name of "colorless" or "clear" oil droplet (Liebman, '72). In the light microscope, however, pale-green oil droplets can definitely be distinguished from clear ones in tint and size as shown in the present study.

Recently, these two different oil droplets have also been detected by MSP in the *Pseudemys* retina (Lipetz and MacNichol, '82). Pale-green oil droplets of the *Pseudemys* retina fluoresced upon near-UV irradiation, and cones with this type of oil droplet are red-sensitive (Ohtsuka, '84), as shown in the *Geoclemys* retina (Fig. 5). Cones containing pale-green and clear oil droplets in the turtle retina have been grouped together as "blue-sensitive cones" for the last decade (Liebman, '72; Baylor and Hodgkin,'73; Baylor and Fettiplace, '75; Granda and Dvorak, '77; Leeper, '78b; Ohtsuka, '78; Kolb and Jones, '82a). However, they now ought to be considered as cones which differ in chromatic sensitivity, and therefore the synaptic pathways involving blue-sensitive cones in the turtle retina remain to be re-examined.

After autofluorescent oil droplets were first reported in cones of the frog retina (Liebman and Leigh, '69), no similar investigation has been executed, so that little is known about the functional role of this organelle. Recently, UV absorption of this oil droplet was proposed to be a filter against near-UV light-induced photolysis of visual pigment (Kirschfeld, '82). Functional significance of autofluorescence emitted from the oil droplets found also in the avian retina (Ohtsuka, '84) is open for further study.

Two members of double cone

The present study also demonstrates that in double cones of the *Geoclemys* retina (1) no diffusion of either Lucifer yellow or Procion yellow was detected between two apposed members, (2) both members of double cone were red-sensitive, and (3) no signal characteristic of green-sensitive cone was recorded from either member. These results suggest that both members of double cones contained red-absorbing visual pigment and that they are electrically independent. A similar experiment performed in the retina of *Pseudemys* yielded the same results (T. Ohtsuka, in preparation).

Liebman and Granda ('71) first described the visual pigments of the *Pseudemys* retina. They reported that the accessory member contained green-absorbing visual pigment. However, their morphological identification of the accessory member was not clear. Recently, Lipetz and MacNichol ('82) measured the absorption of each outer segment of intact double cones of *Pseudemys*. These authors concluded that both members of double cones contained red-absorbing pigment, in full agreement with the results of the present experimental.

Two-peaked sensitivity curves were recorded in the present experiments, as had been reported previously (Richter and Simon, '74; Baylor and Fettiplace, '75; Normann et al., '84). Intracellular injection of LY, however, revealed that these responses were recorded only from displaced bipolar cells, whose somata were located at the outer nuclear layer. Thus, spectral sensitivity curves recorded from single photoreceptors of the turtle retina showed only single peaks.

More generally speaking, double cones in the vertebrate retina seem to be of two types. One, observed in some teleosts, is composed of one member containing green-sensitive visual pigment, and the other, red-sensitive visual pigment (Marc and Sperling, '76; Stell and Hárosi, '76; Loew and Lythgoe, '78). Elsewhere, e.g., in the avian retina, double cones are composed of principal and accessory members which contain the same type of visual pigment (Bowmaker, '77). Double cones of freshwater turtles appear to be of the latter sort. Recent electrophysiological studies reported that both members of apposed cones contained the same visual pigment in the twin cones of a fish (walleye) retina, and both members were electrically independent (Burkhardt et al., '80). Dissociated double cones of salamander retina also revealed no electrical connection between two apposed members (Attwell et al., '83).

Identification of cone chromatic subtypes provides a basis for morphological analysis to understand how neural pathways are organized in the vertebrate retina to convey color information between photoreceptors and second-order neurons such as horizontal and bipolar cells (Stell and Lightfoot, '75; Stell et al., '75; Scholes, '75; Stell and Hárosi, '76; Ishida et al., '80). The present study has classified the seven morphological types of photoreceptors in the *Geoclemys* retina into one rod and three chromatic types of cone. The single morphological characteristic which most decidedly identified these cones, the oil droplets in the cone inner segments, should provide a reliable landmark for further investigations of the neuronal network in this vertebrate retina.

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