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Filtering Effect of Cone Oil Droplets Detected in the P-III Response Spectra of Japanese Quail

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While absorption spectra of bird cone visual pigments have been well studied, physiological study of bird cone cells has been less advanced owing to their small sizes. We measured the P-III components of electroretinograms (ERG) from isolated retinas of Japanese quail. We recorded responses to monochromatic flashes of equal photon numbers, and found that the shape of the response spectrum is dependent on the incident direction of the flashes. The spectrum obtained with the flashes from the cornea side had a steeper peak around 500 nm than that with flashes from the receptor side. This is clear electrophysiological evidence of the filtering effect of the oil droplets in the cone cells, which has long been suspected. We analyzed these spectra with respect to the absorption spectra of cone pigments and transmittance spectra of oil droplets. © 1997 Elsevier Science Ltd

Bird retina Cone pigment Oil droplet Spectral response

INTRODUCTION

It is generally believed that a combination of several kinds of cone photoreceptors of different absorption spectra is responsible for the color sensation in vertebrates. However, the study of cones is less advanced than that of rods owing to their smaller sizes and populations.

It was relatively recently that highly improved purifying techniques allowed the recording of precise absorption spectra of the four different chicken cone pigments (for a review, see Okano *et al.*, 1995). On the other hand, bird cone cells contain respective oil droplets which have been presumed to work as cut-off filters. Their transmittance spectra were reported for chickens (Bowmaker & Knowles, 1977), pigeons (Bowmaker, 1977), Japanese quails (Konishi, 1965; Partridge, 1989) and some wild birds (Partridge, 1989; Gondo & Ando, 1995). Pairing of oil droplets and visual pigments was known from microphotometric study in turtle retinas (Granda & Dovorak, 1977) and demonstrated by histochemistry in bird retinas (Oishi *et al.*, 1990). The cut-off filter effect of oil droplets has been proposed by behavioral study on turtles (Neumeyer & Jager, 1985), by ERG action spectra of a light-adapted pigeon (Wortel & Nuboer, 1986), and by the action spectra of individual turtle cone photoreceptors (Ohtsuka, 1985a, b). However, those reports only examined the natural action spectra as compared with the expected absorption spectra of cone pigments. To make the filtering effect clearer, it is important to compare the natural responses to the responses without the effect of oil droplets. Therefore, we compared the spectral responses of isolated retina between the incident directions of test flashes, namely from the cornea side and the receptor side. We recorded the P-III responses from isolated retinas of Japanese quails (*Coturnix coturnix japonica*). Then we tried to analyze these spectra with regard to the absorption spectra of the pigments and the transmittance spectra of the oil droplets.

METHODS AND MATERIALS

Japanese quails were selected as convenient experimental animals, and purchased from a local farm (Tokai Yuki, Toyohashi). After dark adaptation in a dark box for at least 1 hr, birds were anesthetized by a few drops of chloroform in a glass jar, and were quickly decapitated. A dissected eye ball was then cut in half by small scissors. The obtained eye cup was further cut into a few pieces in the saline (in mM: $NaHCO_3$, 10; glucose, 11; sodium aspartate, 25; NaCl, 96.4; KCl, 5.6; $CaCl_2$, 2.2; $BaCl_2$, 0.01). The retina was detached from the pigment epithelium on an inside wall of the eye cup with forceps under the deep red light and set to cover a small hole (2 mm diameter) on a partition wall of the double chambers so that each side of the retina was soaked in the respective solution in the chamber. The gap in the

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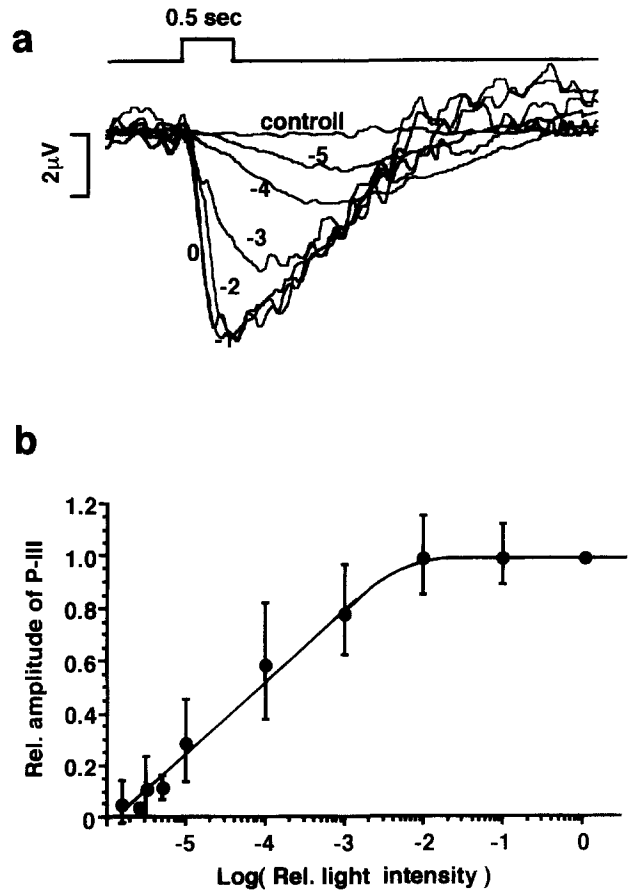

FIGURE 1. P-III responses from isolated retinas of Japanese quail. (a) Typical records of P-III generated by various intensities of stimuli from an isolated retina are superimposed. Relative intensities of the flashes are shown in log unit near the traces. Timings of the flashes are shown at the top. (b) The relationship between the light intensity and the amplitude of P-III.

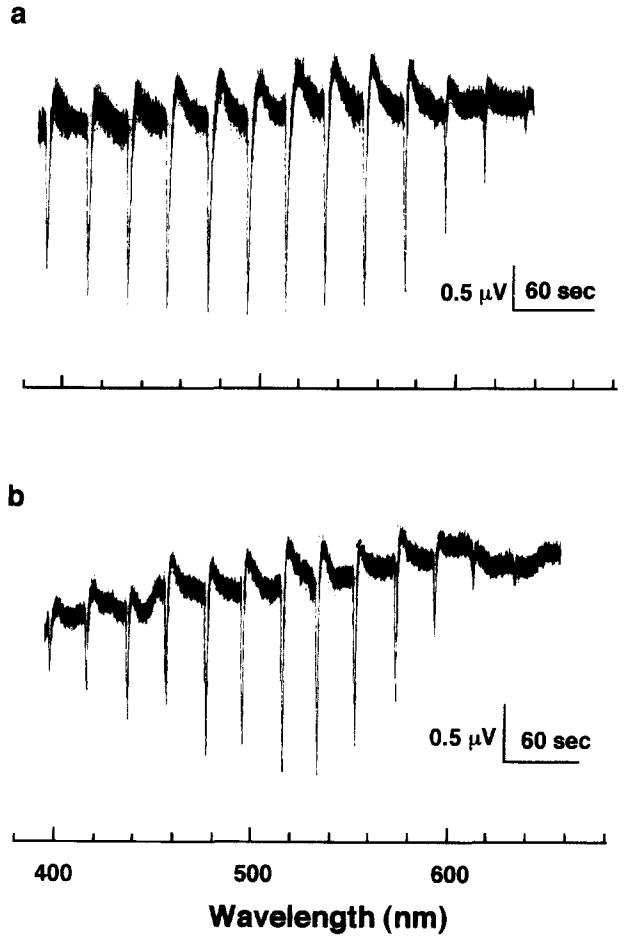

FIGURE 2. Direct response spectra of P-III responses. Series of P-III were generated by the monochromatic light flashes of equal photon numbers shifting their wavelengths at 20 nm interval. The records were displayed consecutively in a reduced time scale, which serve as the response spectra. (a) Thirteen records with flashes from the receptor side. (b) Fourteen records with flashes from the cornea side. Undetectable responses at 670–710 nm (a) or 690–710 nm (b) are not shown in these traces.

partition wall between the two chambers was sealed with silicone grease. The outer wall of the double chambers had two windows of cover glasses (0.17 mm thickness) through which stimulus test flashes illuminated the retina. The change of voltage across the retina was detected by a couple of salt bridges connected to Ag/AgCl electrodes in each chamber, amplified by an AC-coupled amplifier (AVB-8, Nihon Kohden, Tokyo, band pass: 0.1–16 Hz) and recorded with an FM tape recorder (FRC 1402N, SONY, Tokyo). Saline solution aerated with a mixture of 98% O_2 and 2% CO_2 (pH was 7.2– 7.5) was drawn to the double chamber by gravity. A flat light beam was obtained through a light diffuser, a mirror and lenses from a 100 V/150 W halogen lamp (Iwasaki, Tokyo). Monochromatic light was obtained by passing the beam through one of 16 interference filters (MIF-WL series, Koshin Kogyo, Tokyo) held on a motorized rotating disk. Half-second light pulses were obtained by an electromagnetic shutter. Light intensity was measured by a filter-coupled photodiode (UDT-PIN10DF, United Detector Technology, USA) calibrated by Hamamatsu Photonics (Hamamatsu), and adjusted by neutral density filters in the light path. Analysis of the P-III response spectra into the absorption spectra of pigments and oil droplets was carried out with an aid of a program written by Turbo Pascal (version 6, Borland, USA).

RESULTS

We recorded the P-III responses from isolated quail retinas which had been dark adapted for more than 30 min in saline with 25 mM aspartate following the method for amphibian P-III. At room temperature (20°C), we could record them repeatedly for up to 1 hr. The P-III amplitudes varied from preparation to preparation. The retina was easier to damage compared with the amphibian's.

Figure 1(a) shows the superimposed P-III waveforms induced by white test flashes of various intensities. Their time courses were rather slow, especially at low stimuli probably because rod components dominated in those responses since the retina was dark adapted. The low temperature for the warm-blooded animal might also explain the slow time courses.

The relationship between the light intensity and

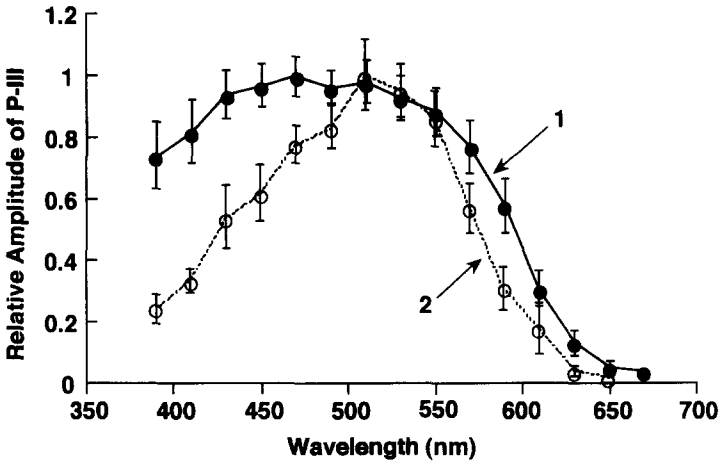

FIGURE 3. Average response spectra of P-III responses. The amplitude ratio of each record to the sum of a series of 16 records was first calculated. Then the average ratio at each wavelength was calculated from many series of recordings, and was further expressed relative to the maximal value. The P-IIIs were recorded with flashes from the receptor side of the retina (closed circles, $n = 31$) or from the cornea side of the retina (open circles, $n = 11$).

amplitude of P-III is shown in Fig. 1(b). The amplitude of the P-III was proportional to the flash intensity over 4 log unit. The intensity of the strongest flash (0 log unit) corresponds to 9.9×10^{16} photons/mm^2 of 500 nm light. Then we set 16 monochromatic test flashes to an identical number of photons (0.5 sec pulse of 3.5×10^{11} photons/mm^2/sec), which was sufficiently high to induce the distinguishable responses but low enough to permit the repetitive recordings. Monochromatic flashes at intervals of 20 nm were displayed to the retina every 30 sec. A typical series of P-III traces are displayed on a reduced time scale in Fig. 2.

The amplitude measured from the baseline to the peak of each response was dependent of wavelength, and its maximum was located around 500 nm. We tested the effect of the oil droplets on such P-III spectra by comparing the shapes of the spectra obtained by the test flashes from the receptor side [Fig. 2(a)] and cornea side [Fig. 2(b)] of the retina. Apparently, the peak around 500 nm of the spectrum in Fig. 2(a) is not as steep as that in Fig. 2(b), but rather forms a pseudo-plateau. This difference between the two spectra appeared to be due to the cutting-off effect of oil droplets in the cones.

We repeated the same measurement on many retinas to obtain averaged spectra in order to confirm this observation. Following a previous report (Nakamura *et al.*, 1978), we calculated ratios of amplitudes at each wavelength to the sum of those of all records in one series of experiments. Next, the ratios at each wavelength were averaged from different series of experiments. Figure 3 shows such averaged spectra.

In Fig. 3, open circles show the averaged responses to test flashes from the cornea side ($n = 11$), and filled circles show those to flashes from the receptor side ($n = 31$). The shape of the spectrum is clearly dependent of the incident direction of the test flashes, confirming the direct P-III spectra in Fig. 2. Figure 3 carries the characteristics observed in Fig. 2: (1) The response maximum is around 500 nm; (2) Its peak is sharp when test flashes are from the cornea side, but rather rounded for a wide wavelength region when test flashes are from the receptor side. The difference between the two spectra is readily attributed to the filter effect of oil droplets in the inner segments of the photoreceptors. If it is so, the response spectra with receptor-side flashes can be expressed as the sum of the original absorption spectra of all the visual pigments with their proper population ratio in the retina, and the response spectra with cornea-side flashes can be expressed as the sum of the absorption spectra corrected by respective oil droplets. The responses presented here were all obtained within the range in which amplitudes were linear to the flash intensities [see Fig. 1(b)]. Therefore, we compared the normalized response spectra directly with the absorption spectra of pigments, because the absolute spectral sensitivity of single receptors calculated from similar measurements were also reported not to coincide perfectly with the absorption spectra (Tomita *et al.*, 1967; Ohtsuka, 1985b). Unfortunately, so far absorption spectra of visual pigments of Japanese quails have not been reported, and those of oil droplets were hav only partly been reported (Konishi, 1965; Partridge, 1989). However, since most birds are likely to have similar sets of pigments and oil droplets (see Partridge, 1989), it is worth trying to reconstitute those spectra from the available data to see the merit of our method. For this purpose, we tested published data of cone pigments of chicken (Okano *et al.*, 1989) and of oil droplets of chicken (Bowmaker & Knowles, 1977) and Japanese quail (Konishi, 1965).

First, we tried to reconstitute the response spectrum measured by receptor-side flashes [Fig. 4(a), curve 1] as the sum of five absorption spectra of visual pigments in the retina. The closest spectrum to the response spectrum [curve 1 in Fig. 4(a)] was obtained as curve 2 in Fig. 4(a), when their population ratio was (rod + green cone): red cone:blue cone:violet cone = 31:29:19:21.

Then we corrected each absorption spectrum with the

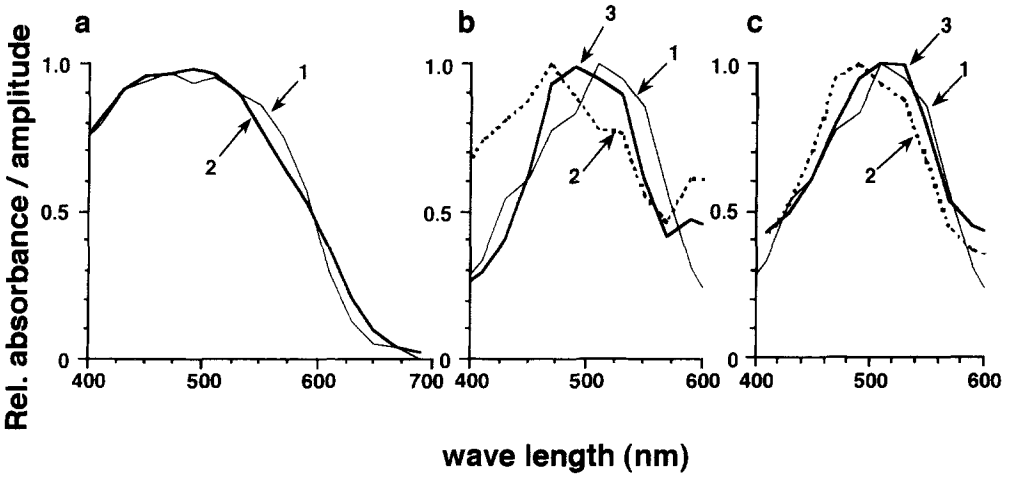

FIGURE 4. Trial to reconstitute the P-III response spectra by the sum of absorption spectra of visual pigments for the test flashes from receptor side (a) and cornea side (b, c). a-1: P-III response spectrum with the receptor-side flashes (same as Fig. 3-1). a-2: An absorption spectrum of (16% rod + 15% green cone + 29% red cone+19% blue cone + 21% violet cone) without the "oil droplet filters". b-1: P-III response spectrum with the cornea-side flashes (same as Fig. 3-2). b-2: An absorption spectrum of the same composition of pigments as a-2 under the filtering effect of chicken oil droplets (after Bowmaker & Knowles, 1977). b-3: b-2 curve was further modified by the attenuation of cone outputs (attenuation factors: 0.50, 0.58, 0.50 and 0.16 for red cones, green cones, blue cones and violet cones, respectively). c-1: Same as b-1. c-2: An absorption spectrum of the same composition of pigments as a-2 except violet pigments under the filtering effect of Japanese quail's oil droplets (after Konishi, 1965). Data of violet pigments were omitted. (See text.) c-3: c-2 was further modified: Receptor outputs were attenuated by factors of 1.0, 0.80, 0.20 for red, green, and blue cone, respectively. Then 15% of violet cones included in a-2 was further added.

transmittance of the respective oil droplet. The correction was done for the wavelength range 400–600 nm, as some data for oil droplets were available only for this range. We tested the transmittance data of chicken oil droplets by Bowmaker & Knowles (1977) at first. Curve 2 in Fig. 4(b) is the direct sum of the corrected absorption spectra of all the visual pigments when the population ratio of rod:green cone is 16:15, in addition to the ratio used for curve 2 in Fig. 4(a). This curve was very different from the response spectrum obtained with cornea-side flashes [curve 1 in Figs 4(b) 4(c)]. Note that absolute amplitudes around 600 nm were the same as that of curve 2 in Fig. 4(a) because we set 100% transmittance of the red oil droplets at those wavelengths. The observed attenuation of the P-III amplitudes around 600 nm (see Figs 2 and 3) suggests that practically the maximum transmittance of each oil droplet was not 100% but around 50%. It may not be too speculative to suppose that the maximum light intensities are attenuated respectively by the oil droplets and other structures in each type of cone. Then we tried to adjust the contribution ratio between each type of photoreceptor to bring the summed spectrum close to the observed response spectrum. The best summed spectrum from these corrected spectra was obtained when the attenuation factor was 0.50, 0.58, 0.50, and 0.16 for red cone, green cone, blue cone and violet cone, respectively. However, this spectrum [curve 2 in Fig. 4(b)] was still not close enough to the response spectrum measured with the cornea-side flashes [curve 1 in Fig. 4(b)].

Then, we tested the transmittance data of oil droplets of Japanese quails (Konishi, 1965) in this calculation. Because the data of clear oil droplets was not shown in the report, we calculated the direct sum of corrected absorption spectra [curve 2, Fig. 4(c)] without the violet cones at first. Note that this curve was already relatively close to the response spectrum with the cornea-side flashes. Then we adjusted the contribution ratio of each type of photoreceptors, as was done for Fig. 4(b). When the attenuation factors were 1.0, 0.80, 0.20 and 0.15 for red cone, green cone, blue cone and violet cone, respectively, the best curve [curve 3, Fig. 4(c)] was obtained. Curve 3 is closer in Fig. 4(c) than in Fig. 4(b) to the response spectrum [Figs 4(b), 4(c), curve 1]. Thus, certainly a better result was obtained with the oil droplet data of Japanese quail.

DISCUSSION

Previously reported spectral responses of birds were recorded from eye balls or living animals (Armington & Thiede, 1956; Armington & Crampton, 1958; Wortel & Nuboer, 1986; Wortel *et al.*, 1987). Spectral responses of individual photoreceptors were reported in amphibians (Perry & McNaughton, 1991), turtles (Baylor & Hodgkin, 1973; Ohtsuka, 1985a,b), monkeys (Baylor *et al.*, 1987) or humans (Schnapf *et al.*, 1987), but not in birds. Although bird retinas have been good resources for the biochemical or molecular biological study of cone pigments, they were difficult materials for electrophysiology.

In this report, by the use of isolated retinas, we obtained two different response spectra according to the

incident direction of the test flashes. Data from one retina (Fig. 2) and from numbers of measurements (Fig. 3) showed an obvious difference between them. The spectrum with the cornea-side flashes had a steeper peak around 500 nm than that with the receptor-side flashes. It is easy to suppose that the light at near ultraviolet region is cut off by oil droplets before reaching the outer segment of photoreceptors when it comes from the cornea side.

We observed that the responses to the longer wavelength light were also suppressed when flashes were given from the cornea side (Figs 2 and 3). One of the explanations for this is that responses of red cones whose absorption maximums are near infrared regions were strongly suppressed by the red oil droplets: suppression at shorter wavelengths than the cut-off wavelength of the oil droplets would lower the right wing of the spectrum of the whole retina. However, the response was still largely suppressed at the longer wavelengths than 600 nm (Figs 2 and 3) where suppression was not expected. This might be due to the light scattering by oil droplets and membrane structures in the retina. Our calculation done for Fig. 4 suggests that the attenuation due to this effect makes a particular contribution to shape the action spectrum of the whole retina. Similar deflections to sharpen the action spectra from the expected absorption spectra of the visual pigments were previously reported on the intracellular recordings from individual receptors, where the light path in the retina was suggested as the source of the deflection (Tomita *et al.*, 1967; Ohtsuka, 1985b).

On the other hand, recent data of the absorption spectra of the oil droplets measured up to 800 nm in some wild birds (Gondo & Ando, 1995) showed second peaks of absorption around 700 nm. Therefore, those droplets may work as band pass filters rather than simple cut-off filters. Unfortunately, available absorption spectra of oil droplets of Japanese quails are not complete: Konishi (1965) did not show the spectrum of clear droplets and Partridge (1989) did not show the spectrum of yellow (or orange) droplets. However, Konishi provided the relative intensities of absorbances of three types of oil droplets in the retina, which was directly reflected in the calculated "effective" spectral response [Fig. 4(c), curve 3]. Thus, for the present method, such data showing relative intensities between oil droplets are important.

Our calculation suggested that the ratio of receptor outputs included in the P-III responses is rod:red cone:green cone:blue cone:violet cone = 16:29:15:19:21. It is largely different from the ratio of pigment concentrations in the extracts of chicken visual pigments (rhodopsin:red pigment:green pigment:blue pigment: violet pigment = 49:40:5:5:1) reported by Okano *et al.* (1989). The reason for this difference could be (1) a difference between the output amplitudes of each receptor cells and the pigment content in those cells; (2) the different denaturations of cone pigments during the purification; or (3) the difference between animal species. It is very important to obtain the precise absorption spectra of all the visual pigments and oil droplets of Japanese quails for the current issue. The electrophysiology of individual photoreceptors may be most necessary to improve the analysis.

We also tried the partial bleach or specific adaptation with colored background light to isolate the cone responses, as Wortel & Nuboer (1986) have shown in isolating the ERG-component of blue sensitive cones. However, probably owing to the absence of the pigment epithelium, such color adaptation has been, so far, very difficult to control: we usually lost all the responsiveness when the weak adaptation light was presented. This direction of research, however, may be still worth attempting.

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