The Macular Pigment. I. Absorbance Spectra, Localization, and Discrimination from Other Yellow Pigments in Primate Retinas

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The nonbleaching yellow pigments of the primate fovea were studied by microspectrophotometry (MSP). Retinas fixed with glutaraldehyde/paraformaldehyde mixtures retained yellow pigments with absorbance spectra very similar to those recorded by MSP in fresh retinas. This allowed the authors to prepare retinal sections for localization of the pigments. The spectrum of the macular pigment in fixed tissue is shifted slightly (about 6 nm) toward longer wavelengths, with maximum absorbance at 460 nm. Two short-wavelength yellow pigments also have been identified, with absorbance maxima at 410 nm (P410) and 435 nm (P435), respectively. All three yellow pigments are present in the fovea. The short-wavelength pigments are detected more easily outside the central foveal region because the macular pigment does not obscure them there. They are especially apparent when the MSP beam is confined to the outer nuclear layer or the inner segment layer of retinal sections. The macular pigment is most dense in the fiber layers (receptor axon layer and inner plexiform layer); its density declines markedly with retinal eccentricity. The maximal absorbance of P410 and P435 is usually lower than that of the macular pigment in the central fovea, but their densities and relative proportions change more gradually with eccentricity. Consequently, their maximal absorbance is higher than that of the macular pigment outside the foveal center. The P410 and P435 pigments may be two different oxidation states of one or more respiratory hemoproteins. Commonly used procedures for estimating the absorbance spectrum of the macular pigment by comparing the foveal center with a parafoveal region may be influenced by the amounts and the oxidation states of the short-wavelength pigments in the living eye.


The central retina of diurnal primates has a yellow region known as the macula lutea that surrounds and includes the fovea. The color is due primarily to a carotenoid that can be extracted from the retina with lipid solvents. High concentrations of this carotenoid are present in the foveal region, and only 1/5 to 1/20 as much pigment per unit area is found in other parts of the retina.1 On the basis of its spectral properties, Wald1,2 identified the macular pigment as lutein, (3,3′ dihydroxy-α-carotene), a xanthophyll originating in the leaves of green plants.3

The absorbance spectrum of lutein has a main peak flanked by two side bands, all of which are dependent on the chemical environment and the isomeric configuration of the molecule. In organic solvents, the main peak of all-trans lutein can shift from 440 nm in hexane4 to around 455 nm in chloroform.5 The spectrum of the human macular pigment also has been measured in situ in a retinal flat-mount.6 A difference spectrum was derived, using a microspectrophotometer, by subtracting the absorbance of a peripheral retinal region from that of the fovea. Although the λ max of the macular pigment in situ is within the range exhibited by all-trans lutein in solution, the spectrum is smoother and the side bands are reduced to gradual shoulders or plateaus. However, pure lutein can be made to reproduce the shape of the macular pigment spectrum by heat-isomerizing the lutein and dissolving it in a mixture of carbon disulfide and chloroform (20:80).7 This suggests that the local chemical environment of lutein within the retinal tissue may influence its filtering properties.

As yet, little information has been published on the anatomic localization of the pigment that has been
identified spectroscopically. Summaries of foveal structure\textsuperscript{6,7} have described the most intense yellow coloration along the foveal slope, or clivus, with little color being evident in the central fovea, or foveola. This is a curious conclusion, because it is inconsistent with the functional advantages thought to be conferred by macular pigmentation.

The most widely accepted role of the macular pigment is that of an optical filter to counteract the chromatic aberration of the eye and scattering in the ocular media.\textsuperscript{8,9} Since the center of the fovea has the closest receptor spacing and highest visual acuity, one would expect its function to be the most vulnerable to image degradation of any retinal region. Thus, optical filtering by the macular pigment should be of greatest value in the foveal center.

It also has been suggested that the macular pigment has a metabolic role related to the reduced vascularity of the fovea and the presence of a central avascular zone.\textsuperscript{10} This suggestion was dismissed immediately\textsuperscript{11} because of the belief that macular pigment was absent from the foveola, where it would be needed to compensate for the lack of retinal vessels.

In contrast to the descriptive anatomy, but consonant with functional considerations, several lines of evidence from human psychophysics have converged to the conclusion that the foveola has the highest macular pigment density. Data from color-matching,\textsuperscript{12} two-color increment thresholds\textsuperscript{13} and flicker thresholds\textsuperscript{14} (and B. R. Wooten, personal communication), are all consistent with a pigment distribution that peaks in the central fovea and declines to a low, constant value within 5 deg eccentricity.

In the present paper, we describe the nonbleaching yellow pigments in the primate fovea measured by microspectrophotometry (MSP). We find that the macular pigment can be identified spectroscopically in each layer of the retina, and it is most dense in the foveola, as the proposed functional roles require. In addition to lutein, we also have detected two other yellow pigments with absorbance maxima at shorter wavelengths. These measurements provide a schematic description of the screening pigments in the primate fovea. They will serve as the basis for a quantitative description of the macular pigment distribution by two-wavelength microdensitometry in the companion paper.\textsuperscript{15}

Materials and Methods

Tissue Preparation

In the initial exploratory experiments, we anesthetized the monkey with 35 mg/kg sodium pentobarbital, enucleated the eye, removed the vitreous, and dissected the retina free from the pigment epithelium in order to examine the foveal region for yellow pigmentation. We always observed swelling and distortion of the retina, often near the rim of the foveal depression where the ganglion cell layer is especially thick. This deterioration of the tissue made it difficult to localize the pigmentation to specific layers or regions. We, therefore, turned to fixing the retina with paraformaldehyde, glutaraldehyde, or mixtures of the two. As long as the solutions were freshly prepared so that no alcohols or acids were given time to form, the yellow pigmentation remained conspicuous after exposure to the fixatives.

Most of the microspectrophotometric measurements were done on retinas from two animals perfused with one-quarter strength Karnovsky’s fixative (1.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4). This was the best-preserved tissue in our series. Some measurements also were performed on one retina that was first dissected and then fixed in full-strength Karnovsky’s fixative. This retina was swollen on one side of the fovea, so only the undistorted side was studied.

In addition, we visually examined foveal sections from other eyes fixed in different ways: (1) by immersion in quarter strength Karnovsky’s fixative after enucleation or (2) by perfusion of the animal with glutaraldehyde or paraformaldehyde alone. Qualitatively similar patterns of pigmentation were visible in all cases, but the immersion-fixed tissue was mechanically distorted, and the tissue perfused with single fixatives tore or folded during sectioning. We concluded that perfusion of the animal was advantageous because it fixed the tissue before it could swell and become distorted. The glutaraldehyde/paraformaldehyde mixture was preferable to single fixatives because it imparted appropriate toughness for cutting tissue in the flexible aqueous gelatin used for embedding.

After fixation of the eye, either by perfusion or immersion, all adherent tissue was dissected free from the scleral surface until it was smooth. The eye was cut open around the equator and as much vitreous as possible was removed. Then a square 1 cm on a side, centered on the fovea, was cut out gently with small scissors. In order to indicate the orientation of the tissue, a wedge-shaped notch, oriented vertically upward, was cut about 2 mm from the center of the fovea. A disk of tissue 4 mm in diameter centered on the fovea was isolated by tapping a sharp metal punch through the tissue while it lay on a firm but resilient vinyl sheet. The sclera was peeled from the resulting disk, leaving only soft retinal and choroidal tissue for cutting.

Since standard embedding and sectioning techniques require the use of lipid solvents that would extract the
macular pigment, we could not use them. Instead, the tissue was embedded in gelatin and, in the early experiments, frozen-sectioned. Later, we found that section cutting with a Vibratome (Model G, Oxford Laboratories; Foster City, CA) was more reliable and the procedures finally adopted were as follows:

Purified calf skin gelatin (Polysciences) 25% w/v in distilled water was allowed to gel and form a flat surface. The disk of fixed retinal tissue was placed on this surface and an additional aliquot of the liquid gelatin was poured over the tissue to surround it with an aqueous supporting matrix. A small (6 mm) cubical block was cut from the gelatin so that the retinal tissue was parallel to one of the faces. The block was fixed at 4°C for 5–6 hr in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 to stiffen the gelatin. This caused some distortion of the block, so the sides were trimmed again to ensure that the vitreal surface of the retina was perpendicular to the sectioning plane.

The block was glued with cyanoacrylate to the bottom of a small plastic tray so that it could be kept immersed during cutting. The tray was filled with 1 M phosphate buffer, pH 7.4 and it was surrounded by ice to keep the tissue cold. Serial sections nominally 40–50 μm thick were cut through the foveal region. Because of the fragility of the specimen, best results were obtained with the blade angle set at 25 deg, and the blade vibration amplitude and cutting speed at their minimum values.

The sections were rinsed with distilled water and placed on microscope slides in Kaiser's glycerol jelly, an aqueous mounting medium of gelatin and glycerol, with benzalkonium chloride (Zephiran) as a preservative. Coverslips were placed over the sections and sealed with lacquer (clear fingernail polish). All slides were conducted by hemolyzing both human and macaque red blood cell preparations and measuring the spectrum of blood in the retinal vessels was measured by positioning an 8-μm beam on the vessel and subtracting a similar absorbance measurement immediately adjacent to the vessel. Additional control experiments on the effects of fixatives on hemoglobin were conducted by hemolyzing both human and macaque red blood cell preparations and measuring the spectra in solution with a DK 2 recording spectrophotometer (Beckman Instruments, Fullerton, CA).

Absorbance spectra were recorded on the instrument previously described. The measuring beam in most cases was a spot of about 10 μm in diameter that could be restricted to a single layer of a retinal section. For one set of measurements, the beam was a slit 18 μm wide whose length was adjusted at each eccentricity to span the retina from the vitreal edge to the pigment epithelium, parallel to the axis of the receptors. A baseline was recorded outside the retina in the clear gelatin mounting medium for each beam geometry.

In some control experiments on both fresh and fixed tissue, absorbance spectra of retinal flat mounts were measured with the beam traversing the retina from the vitreal surface to the receptors (i.e., along the normal optical path). For these measurements, the retina was dissected free from the pigment epithelium and placed in a microcell consisting of a 0.05 × 1 cm acrylic ring mounted on a 22-mm diameter pyrex coverslip with high vacuum grease (Dow Corning; Midland, MI). This microcell was filled with mammalian Ringer's solution and sealed with another coverslip and more vacuum grease. Spectra were measured with a 10 μm diameter beam at different eccentricities from the center of the fovea.

The spectrum of blood in the retinal vessels was measured by positioning an 8-μm beam on the vessel and subtracting a similar absorbance measurement immediately adjacent to the vessel. Additional control experiments on the effects of fixatives on hemoglobin were conducted by hemolyzing both human and macaque red blood cell preparations and measuring the spectra in solution with a DK 2 recording spectrophotometer (Beckman Instruments, Fullerton, CA).

Computer Processing

Absorbance values at 5-nm intervals from 400 to 500 nm and at 10 nm intervals from 500 to 600 nm were read manually from the instrument chart records and typed into computer files. These numbers were appropriately subtracted and scaled to create the difference spectra and template spectra plotted in the figures. They also were used for curve-fitting procedures to specify the amount of each pigment needed in a mixture to match the measured absorbance spectra of retinal tissue. The curve fitting was done on a PDP 11/70 computer (Digital Equipment Corp; Maynard, MA) by a program that iteratively changes the values of each of the unknown parameters in a random se-
Localization of the Yellow Pigments of the Fovea

When sections of primate foveas are examined in white light, intense yellow bands can be seen in the receptor axon layer and often in the inner plexiform layer as well. This pattern has been observed in seven Old World macaque monkeys (four *Macaca fascicularis*, two *Macaca mulatta*, and one *Macaca nemestrina*) and two New World monkeys (one *Saimiri* [species unknown] and one *Cebus apella*). It therefore appears to be a general feature of primate foveas.

For illustration, the macular pigment is visualized most easily by illuminating the section with blue light, which is absorbed strongly by the pigment. This produces a pattern of density quite different from that seen in green light, which is weakly absorbed by the pigment. Such a comparison is shown in Figure 1 for two sections, each from a different *M. fascicularis* monkey. The top pair of photos are from an animal that had a very high density of pigmentation in the receptor axons but much less density in other layers of the retina. The bottom pair of photos are from another animal that had dense pigmentation in the receptor axons, as well as an additional dense band in the inner plexiform layer. These two sections represent the extreme cases of the retinas that we examined. (The variation in pigmentation from animal to animal is discussed more fully in the companion paper.15)

The density patterns of most retinas are intermediate between the two shown here, with dense pigmentation in the receptor axon layer and less dense, but distinct pigmentation in the inner plexiform layer. An example of the intermediate case is illustrated in Figure 2 of the companion paper,15 where quantitative estimates of intraretinal macular pigment density are reported.

Identification of the Yellow Pigments

*Measurement of spectra in single layers:* The fact that pigmentation often can be observed in more than one layer of the retina naturally raises the question whether there are more yellow pigments than the lutein that Wald1,2 extracted from human maculas. In order to address this question we measured the absorbance spectrum of single layers in several retinas from four macaque monkeys (two *M. fascicularis*, one *M. mulatta*, and one *M. nemestrina*). The most detailed measurements were from *M. fascicularis* and *M. mulatta* retinas fixed in glutaraldehyde/paraformaldehyde mixtures (see Materials and Methods). Results from control experiments on two unfixed retinas from *M. fascicularis* and some earlier unpublished data on unfixed retinas of *M. mulatta* (from PKB) are introduced later in the Results section to show that fixation causes little change in the photostable pigments of the retina.

Absorbance spectra from a retina with intense coloring in both the receptor axons and the inner plexiform layer are shown in Figure 2. Fixed tissue scatters more light than fresh tissue, so the molecular absorbance spectra are superimposed on an apparent increase in density as the measurements approach shorter wavelengths. This has been compensated for partially in the figure by adjusting the instrument baseline. Note that the spectrum of both fiber layers is practically identical, which suggests that the same pigments are present in both layers. However, the absorbance spectrum of the inner layer between the two fiber layers has a peak at much shorter wavelengths. We will present evidence that this apparent spectral shift is due to the presence of additional pigments that peak at shorter wavelengths.

*Spectra of single layers at different eccentricities:* A complete set of absorbance spectra for single layers at the center of the fovea and at 1 mm eccentricity is shown in Figure 3. A great variety of shapes is evident. In the foveal center, the absorbance of the receptor axons looks very similar to the macular pigment curves derived by other authors (for review see reference 19). A prominent feature of the macular pigment spectrum easily identifiable in the other layers is the plateau between 480 and 490 nm. All of the layers in the foveal center exhibit this plateau and contain substantial amounts of macular pigment.

Contrast these curves with spectra measured in the same layers at 1 mm from the foveal center. Here there is much less macular pigment, but there are still definite peaks at the shorter wavelengths. The absorbance of the outer nuclear layer has a particularly sharp peak at 435 nm that is completely different from the macular pigment curve. Note, however, that a mixture of a pigment (P435, λ max = 435 nm) with this shape together with the macular pigment could produce the curve measured in the inner segment layer in the central fovea. This led us to the hypothesis that the absorbance spectra at the center of the retina might be the result of mixtures of the macular pigment with other yellow pigments.

Derivation of Template Spectra

In order to test the hypothesis that the retinal layers contain mixtures of pigments, we first had to derive a good estimate of the spectrum of each pigment individually. We call these the “template” spectra. The
FOVEAL CENTER

FOVEAL SLOPE
Fig. 1. Micrographs in monochromatic blue or green light of unstained horizontal sections through the foveas of two *M. fascicularis* monkeys. The optic disk is to the left. Macular pigment is indicated by dark regions in the blue-light photos where there are no corresponding dark regions in green light. Retinal layers are: PE, pigment epithelium; OS, outer segments; IS, inner segments; ON, outer nuclear layer; RA, receptor axons; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer. Nomenclature follows the convention established by Boycott and Dowling and adopted by Rodieck with the exception that their "receptor fiber layer" is referred to as receptor axons (RA).

In the foveal region, the bipolar and horizontal cell dendrites occupy such a small part of the outer fiber layer that we are not able to resolve them in our MSP measurements, and we have not labelled them separately. The optic nerve fiber layer is also too thin to be distinguished here. The top photo shows a 47-μm section through the center of the fovea of the left eye of an adult male taken in blue (460 nm) light. Second panel is a composite of two micrographs of the same section taken in green light (525 nm). The right half of the picture was taken with the substage condenser in its normal position, and it delineates well IN, IP, and GC. For the left half of the picture, the substage condenser was moved laterally to provide oblique illumination that increases the textural contrast of OS, IS, and ON. The apparent low density in IP on the left side is a result of the variation in illumination. The bottom pair of micrographs show a 56-μm section slightly eccentric from the foveal center of an adult female monkey taken with standard illumination. The section intersects the beginning of the foveal slope, where the inner nuclear layer is once again continuous across the fovea. The cell nuclei can be seen in blue light as a single row of light disks across the dark floor of the foveal depression. Since this eye was fixed by immersion, a row of erythrocytes also can be seen in a capillary crossing IP on the left at a very gradual angle.

Template spectra were computed by taking the difference in absorbance between two nearby locations in a retinal section according to the following criteria:

1. The two locations should have about the same density outside the absorption bands of the pigments. This minimizes differences in nonspecific absorption and scattering at the two locations. To meet this criterion, we chose locations that had about the same absorbance at 600 nm.
2. The section should be relatively thin to minimize possible overlap between the retinal layers and scattering of the measuring beam between layers. All sections used for deriving template spectra were less than 50 μm thick.
3. The two locations chosen should have a large difference in pigment density to minimize measurement error.

The spectra shown in Figure 3, along with single-layer spectra from the other eye of the same animal and from one eye of a rhesus monkey were used to derive the template spectra. The templates are plotted in the top panel of Figure 4. All of the template curves.

Fig. 2. Tracings from MSP records of absorbance spectra of single retinal layers. Abbreviations same as Figure 1. All curves set to zero at 600 nm but otherwise unaltered. The measuring beam was 7.5 μm in diameter and was confined to one retinal layer at a time, about 140 μm from the foveal center. These data are from a 65-μm thick section through the fovea of an adult female *M. fascicularis* fixed with Karnovsky's fixative. The baseline was recorded with the MSP beam passing through a nearby area of the clear gelatin medium in which the section was mounted.
Fig. 3. Absorbance difference spectra of single retinal layers in the center of the fovea (top panel) and 1 mm from the foveal center (bottom two panels). These spectra are from the 47-μm section illustrated in the top two panels of Figure 1. The measuring beam was 10 μm in diameter. The baseline in clear gelatin already has been subtracted. The spectrum in the outer segment layer at 1 mm eccentricity was not measured because the layer is too thin there.

have been set to zero at 600 nm, but none of the curves in the top panel have been scaled; these are the actual density differences measured in the tissue.

For the macular pigment template (MP) we calculated the difference between two locations at different eccentricities in the layer of receptor axons. Difference spectra for three pairs of locations 323–1000 μm apart in the section shown in the top panels of Figure 1 were averaged. This average spectrum from one eye was averaged again with difference spectra from two pairs of locations 700–1000 μm apart in the receptor axon layer of the other eye of the same animal. The resulting curve is our template for the macular pigment shown in Figure 4 (squares). The greatest dispersions of absorbance values occurred at 410 and at 520 nm, where the standard deviations were 0.035 and 0.031, respectively.

The second template, P435, was derived by taking the difference between the absorbance spectrum of the outer nuclear layer and the receptor axon layer at 1 mm from the foveal center in the section judged most suitable by the above criteria. The layer spectra are shown in the second panel of Figure 3 and the difference spectrum is plotted in Figure 4 (third panel).

Comparison of the shapes of the absorbance curves measured in the inner segment layer and the outer nuclear layer at 1 mm eccentricity suggests that still another pigment must be present. We will argue later that this may be an oxidized state of the molecule responsible for the P435 peak. We derived the template spectrum for this third pigment by subtracting the spectrum in the outer nuclear layer from that in the inner segments at 1 mm eccentricity. The difference spectrum obtained from the two curves shown in Figure 3 was averaged with a similar difference spectrum obtained from a section through the fovea of a rhesus macaque monkey.

Fig. 4. Template spectra for the blue-absorbing screening pigments in fixed primate retinas. Top panel: macular pigment, MP, and two other pigments, P410 and P435. Data points are tabulated in Table 1. Other panels compare the template spectra with those of known retinal pigments. In the lower three panels, the comparison curves are set to zero at 600 nm and scaled to the same maximum absorbance as the templates. Second panel: superposition of the MP template curve (squares) on an estimate of the macular pigment spectrum (triangles) derived from unfixed tissue (P. K. Brown, unpublished data). Both curves have been scaled to 0.5 maximal absorbance. Third panel: comparison of P435 with reduced hemoglobin (triangles) measured in a capillary of an unfixed retina of a macaque monkey. Fourth panel (bottom): comparison of P410 (open circles) with fixed, oxygenated macaque hemoglobin in a retinal capillary (X's) and reduced cytochrome C (diamonds) as measured by Keilin and Slater.
monkey (*M. mulatta*) to arrive at the P410 template plotted in Figure 4 (bottom panel). Numerical values of all the template spectra are presented in Table 1.

**Identification of the Different Pigments**

It was clear from the beginning that the major yellow pigment in the fiber layers of the fovea must be the macular pigment. The second panel of Figure 4 plots the macular pigment spectrum derived from fixed tissue against a measurement in unfixed tissue (P. K. Brown, unpublished data). The measurements from unfixed tissue were made on an excised retinal flat-mount of an adult female rhesus monkey. The difference in absorbance between the central fovea and a parafoveal region set to zero at 600 nm and scaled to 0.5 peak absorbance is shown here (data from March 31, 1965). Note that the MP template curve for fixed tissue is shifted slightly toward longer wavelengths and the plateau at 480–490 nm is slightly more prominent. Otherwise the fixation of the tissue appears to have had little effect on the pigment.

Identifying the pigments corresponding to the other template curves is more difficult. The absorption maxima are in the range that one might expect for the γ or Soret band of hemoproteins, such as hemoglobin or the cytochromes. We did a series of control experiments to examine the effects of our fixative on the hemoglobin γ band. When the fixative was made from fresh stock chemicals, it did not shift the hemoglobin spectrum. In addition, the spectral differences that are associated with different oxidation states of the molecule were preserved, ie, oxidized hemoglobin peaked at a shorter wavelength (415 nm) than reduced hemoglobin (431 nm). One batch of fixative made from older stock chemicals, however, converted reduced hemoglobin to the oxidized form (ie, shifted the peak to shorter wavelengths). In no case was the 431 peak shifted to longer wavelengths. We conclude that P435 is similar but not identical to the γ band of reduced hemoglobin. This comparison is plotted in the third panel of Figure 4. P435 may be the same pigment found by Bowmaker et al in the unidentified “photosensitive, blue-absorbing structures” of the rhesus retina.

In the case of P410, there are two hemoproteins with absorption peaks in the appropriate spectral location. They are oxidized hemoglobin and reduced cytochrome C (bottom panel, Fig. 4). Of course, we can not rule out the possibility of mixtures of several pigments. Mollon and Bowmaker have reported a pigment in the inner segments of macaque rods with a peak absorbance of 420 nm. As yet, we are unable to identify this pigment in our records.

The results of measurements on unfixed tissue indicate that the short-wavelength peaks shift in situ with exposure to oxygen (see below, control experiments with fresh tissue). This suggests that these pigments are probably hemoproteins related to tissue respiration. For a better understanding of their function, they will have to be characterized biochemically.

### Table 1. Relative absorbance of pigments in sections of fixed retinas

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<th>Wavelength (nm)</th>
<th>MP*</th>
<th>P435</th>
<th>P410</th>
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<td>400</td>
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*The macular pigment (MP) has been arbitrarily set to a maximum absorbance of 0.5. The maximum absorbance of the other two pigments has been set arbitrarily to 0.2. The negative values are due to insignificant differences in absorbance between the two locations chosen to derive the difference spectra.

### Reconstruction of the Absorbance Spectra

We now are in a position to test the hypothesis that the absorbance of retinal tissue at any location can be accounted for by a mixture of the pigments for which we have derived the template curves. The total absorbance of the tissue $A(\lambda)$ can be expressed as the sum of the absorbance of each pigment separately plus a non specific term $S(\lambda)$ according to the equation:

$$A(\lambda) = aMP(\lambda) + bP435(\lambda) + cP410(\lambda) + S(\lambda)$$

where MP(\lambda), P435(\lambda) and P410(\lambda) are the template spectra, \( \lambda \) the wavelength, and a, b, and c are constants of proportionality that depend upon the density of each pigment.

The non specific term $S(\lambda)$ is given by:

$$S(\lambda) = d + g\lambda^{-k}.$$
The constant \( d \) represents the "remaining" absorbance of the tissue and \( g\lambda^{-k} \) estimates the effect of scattering of light in the tissue. Since we required that \( MP(\lambda), P435(\lambda) \) and \( P410(\lambda) \) be zero at 600 nm, the constant \( d \) can be eliminated by \( d = A(600) - g600^{-k} \).

The constants \( a, b, c, g, \) and \( k \) were calculated using the computer program described in Materials and Methods. The relative amount of each pigment is expressed as \( aMP(460), bP435(435) \) and \( cP410(410) \), ie, the maximum absorbance of each pigment. The scattering term \( S(\lambda) \) was usually small and unimportant to the interpretation of the results.

As a demonstration of the adequacy of representing tissue spectra by a sum of components, we show results in Figure 5 for spectra of two retinal layers of the other eye of the animal from which the data of Figure 3 were taken. The spectra in Figure 5 were measured at 400 \( \mu \)m from the center of the fovea, where significant amounts of all three pigments are found. They are from layers that were not used to derive the template spectra.

The maximum absorbances of the individual components estimated from fitting a sum of the template spectra to the curves of Figure 5 are given in Table 2. There is less macular pigment and \( P410 \) but more \( P435 \) in the inner nuclear layer than in the inner plexiform layer at this eccentricity. Since we know that the amount of macular pigment declines with eccentricity, the relative amounts of the different pigments will be different at other locations.

### Changes in Pigment Mixture with Eccentricity

The effect of eccentricity on the relative amounts of each pigment was studied in two ways. In one set of measurements, the absorbance spectrum of the whole retina was measured at closely spaced eccentricities with a narrow slit that extended from the vitreal edge to the pigment epithelium. This is the only practical way to measure changes in pigmentation with eccentricity with high spatial resolution. It was done only on the section shown in the top panels of Figure 1. The second approach was to measure absorbance spectra of single layers with a small spot at several widely spaced eccentricities. This same section and

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**Table 2. Maximum absorbance of each component of the pigment mixture fitted to the absorbance spectra of the inner nuclear layer and the inner plexiform layer of a macaque retina about 400 \( \mu \)m from the foveal center**

<table>
<thead>
<tr>
<th>Layer</th>
<th>( MP^* )</th>
<th>( P435 )</th>
<th>( P410 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN( ^* )</td>
<td>0.095</td>
<td>0.068</td>
<td>0.038</td>
</tr>
<tr>
<td>IP( ^\dagger )</td>
<td>0.138</td>
<td>0.051</td>
<td>0.060</td>
</tr>
</tbody>
</table>

* MP = macular pigment.

† IN = inner nuclear layer.

\( \dagger \) IP = inner plexiform layer.
Fig. 6. Variation with retinal eccentricity of the maximum absorbance (density at λ max) of each of the yellow pigments. Absorbance spectra were measured at closely spaced eccentricities with a slit 18 μm wide oriented parallel to the axes of the receptor inner segments and extending from the vitreal edge of the retina to the pigment epithelium. The estimated absorbance of each pigment was calculated from the best fitting sum of components. Bottom panel shows the calculated light scattering contribution at two wavelengths, 410 and 460 nm. Symbols have been omitted from these curves for clarity.

sections from three other retinas were studied in this manner.

In each case, the absorbance spectra were fitted with equation 1 as described above, and the relative amount of each pigment was calculated. The results for the axial slit measurements are plotted in Figure 6. They show that there are no abrupt or nonmonotonic changes in the density of any pigment with eccentricity. In the center of the fovea, the macular pigment has a higher density at all wavelengths than the short-wavelength pigments, but its density declines steadily with eccentricity. At 1 mm from the center of the fovea, the short-wavelength pigments are the most prominent contributors to the spectrum (Fig. 3, middle panel). The calculated light scattering makes only a small contribution to the tissue absorbance and it changes little with eccentricity, so it does not affect the overall picture very much (Fig. 6, lower panel).

In contrast to the macular pigment, the contributions of the other pigments to the spectrum change less with eccentricity. Nevertheless, there is a systematic trend. The amount of P435 decreases with eccentricity, while the amount of P410 increases. This is the behavior that would be expected if there were relatively constant amounts of tissue respiratory proteins that changed oxidation state as a function of eccentricity.

Since each of the pigments varies monotonically with eccentricity, we can attain a schematic picture of the overall pattern by examining the pigment mixtures in individual layers at a few selected eccentricities. Figure 7 is derived from measurements made on the section used for Figure 6 combined with data from the other eye of the same animal. We felt justified in pooling the data because the maximum absorbance of the macular pigment in the receptor axons of the central fovea was within 4% of the same value in the two eyes. The major patterns have been confirmed in measurements of single-layer spectra from another M. fascicularis and one M. mulatta, bearing in mind that foveal size and the lateral distribution of the pigments varies from animal to animal.15

The distribution of the macular pigment is illustrated in the top panel of Figure 7. It is most dense in the receptor axons in the center of the fovea and declines markedly with eccentricity. By the time we reach 400 μm from the foveal center, there is about as much macular pigment in most of the other layers of the retina as there is in the receptor axons. The outer nuclear layer has a comparatively low amount of macular pigment. A measurable, though small, amount of macular pigment can still be detected even at 1 mm from the center, especially in the inner plexiform layer.

The distribution pattern of P435 in the receptor layers (second panel, left column) is very different from that of the macular pigment, since it increases gradually with eccentricity. The inner layers (right column) never have larger amounts than the receptor layers. Since the inner layers increase in volume with eccentricity, this leads to the gradual net decline of P435 density with eccentricity that we observed in the slit measurement.

For P410, there is little or no pigment in the outer nuclear layer and decreasing amounts in the receptor axons with increasing eccentricity. The inner layers of the retina have more P410 than two of the receptor layers at eccentric locations. This is why there is a
Fig. 7. Maximum absorbance (density at $\lambda_{\text{max}}$) of each of the three yellow pigments in each layer of the retina at several eccentricities. Left column presents data from the receptor layers; right column shows data from the inner layers, which are not present in the foveola. Bottom panel is the calculated light scattering contribution at 435 nm. In cases where identifying symbols are omitted, hue type can be used to identify the retinal layer. These values are combined measurements from the eye used for Figures 3 and 6 and from the other eye of the same animal. The values at 0 $\mu$m (central fovea) are the averages of both eyes. Points at 161 and 1000 $\mu$m are data from the left eye only, and points at 400 $\mu$m are data from the right eye only.

Gradual net increase of P410 with increasing eccentricity in the axial slit measurement, which includes all of the layers.

The calculated contribution of light scattering to tissue absorbance at 435 nm is included in the bottom panel to show that it is small and has no obvious pattern.

Control Experiments with Fresh Tissue

When absorbance spectra are measured in flatmounts of primate retinas, the measuring beam passes through all the retinal layers. If the retina has a normal amount of macular pigment, it often obscures the contribution of the short-wavelength pigments to the total absorbance. In cases where identifying symbols are omitted, hue type can be used to identify the retinal layer.
absorbance of the tissue. This might lead one to question whether P435 and P410 exist in fresh tissue; they could be artifacts of fixation.

We know fixation is not necessary to observe short-wavelength pigments, because a peak at about 415 nm is readily seen in the parafovea of fresh, unfixed macaque retinas when flat-mounts are measured by MSP. We have also seen short-wavelength peaks in foveal spectra from the unfixed retinas of a female monkey (*M. fascicularis*) that had very little macular pigment. In those retinas, a small peak at about 430 nm was present in the center of the fovea, and it shifted to shorter wavelengths as the measuring beam was moved to more eccentric locations. This is consistent with the axial-slit and single-layer measurements from fixed retinas.

We suspected that these short-wavelength peaks might be due to respiratory hemoproteins. In that case, the unfixed tissue in the enclosed microcell might maintain the pigments in a partially reduced state. We, therefore, exposed the tissue to air in order to oxidize the pigment, and remeasured the spectrum at several retinal eccentricities. At 500 µm from the foveal center the "reduced" peak was at 423 nm. After exposure of the tissue to air, the λ max shifted to 412 nm.

In the intact, living eye the oxidation states of the respiratory pigments will presumably be determined by the proximity of blood vessels and the metabolic demands of the retinal tissue. The oxidation states and, thus, the retinal metabolism also may be modulated by incident light. Consequently, the description we have given here may need substantial revision when appropriate measurements under *in vivo* conditions are possible.

**Discussion**

**Macular Pigment Localization**

Earlier authors have reported that the macular pigment is most dense in the receptor axons (also called the fibers of Henle, or called the outer plexiform layer). However, no one has described the pigmentation of the other layers, especially the high density of macular pigment in the inner plexiform layer. Near the edge of the foveal depression, the density of macular pigment can be even greater in the inner plexiform layer than in the receptor axon layer.

This distribution of macular pigment corresponds closely to the pattern of intraretinal light damage caused by photocoagulation of the macular region with blue (488 nm) argon laser light. Marshall and his associates reported that the greatest damage in the foveola was in the receptor axons (fibers of Henle). But at more eccentric locations, the severest damage occurred in the inner plexiform and inner nuclear layers. If the light damage is caused by absorption of the incident blue light by macular pigment, this is evidence that the multilayer pigment distribution we have described in excised retinas does exist in the living eye.

**Comparison of Macular Pigment with Oil Droplets**

In comparative treatments of ocular filters, the macular pigment is considered together with the oil droplets of nonprimate vertebrates as a prereceptoral filter. In two important respects, the macular pigment is similar to the oil droplet pigments: (1) both are lipid-soluble carotenoids present in high concentrations in the photoreceptor cells, and (2) both can be depleted by raising animals on diets lacking carotenoids. However, the topographic distribution of the macular pigment differs from that of the oil droplet pigments in several important ways. (1) The macular pigment is distributed over a large part of the photoreceptor cell instead of being sequestered in one organelle. As a result of the lateral course of the receptor axons in the fovea, each photoreceptor is screening other photoreceptors as well as itself. (2) Macular pigment can be present in high densities in retinal cells other than the photoreceptors. Macular pigment in an interneuron, therefore, could screen photoreceptors that have little or no macular pigment themselves. (3) High filtering densities of macular pigment are found only within and immediately surrounding the fovea, whereas filtering by oil droplets occurs in individual cones distributed over large peripheral fields of the retina.

**Short-Wavelength Pigments**

The two short-wavelength yellow pigments that we have described (P410 and P435) are most prominent in the inner segments and the outer nuclear layer. The amount of each pigment in each layer also varies with eccentricity. It is possible that these hemoprotein-like pigments are associated with some specific subcellular structures such as the mitochondria or the endoplasmic reticulum. For the human retina, Hogan and his associates have reported that mitochondria are less densely aggregated in the inner segments of the foveal cones than in the inner segments of more peripheral cones. This suggests that gradients in cellular structure and metabolism may occur along radii through the fovea that could provide a basis for the gradients in pigmentation that we find.

From a comparative point of view, it is interesting to note that a specialized organelle in the cone inner segments of certain fishes has a high density of a substance spectrscopically identical to reduced cytochrome C and, hence, very similar to P410. While no such discrete organelle has been identified in primates, they may have a special hemoprotein system more widely distributed within the retina.
Absorbance Spectrum of the Macular Pigment

The presence of additional yellow pigments in the retina requires reexamination of the process by which macular pigment spectra are derived. All current data are based on the assumption that only the macular pigment changes in density as one moves from the foveal center to the parafovea. This is true for both psychophysical,12,14 and spectrophotometric data.5 Although the changes with eccentricity in the density of the two short-wavelength pigments are gradual when compared with macular pigment, they are consistent, and they will contribute to absorbance differences between the fovea and parafovea. Fortunately, when the macular pigment density is high, the other pigments make only minor contributions to the short-wavelength limb of the absorbance spectrum. Under our conditions, their effect is small and the MP template curve is a good estimate of the macular pigment spectrum in fixed tissue.

For measurements in the living, intact eye, however, a word of caution seems in order. At short wavelengths (400–415 nm), the curves we derive by MSP from both fixed and fresh monkey tissue have higher values than the often cited average macular pigment curve of Wyszecki and Stiles19 for the human eye. This difference may be due to other pigments or optical filtering in the living eye that our procedure does not preserve. However, it also could be due to different oxidation states of the short-wavelength pigments, including steeper density gradients with retinal eccentricity. For example, if P410 were more dense in the parafovea of the living eye it would distort the macular pigment difference spectrum to produce the observed discrepancy.

From our results, we can suggest some approaches to optimize future measurements of the macular pigment spectrum, especially in the wavelength range 400–450 nm. Since the macular pigment declines markedly with eccentricity and the other pigments do not, errors introduced by the short-wavelength pigments would be minimized by deriving macular pigment difference spectra from retinal loci spaced close together. This is true whether the specific technique is spectrophotometry or psychophysics. There is no reason to use retinal microspectrophotometry for the fovea, primate retina, color vision

Key words: retinal pigments, microspectrophotometry, macular pigment, fovea, parafovea, color vision

Quantification of the Macular Pigment Distribution

For present purposes, our current estimate of the macular pigment spectrum in fixed tissue is adequate to calculate its density distribution within the retina. From our data, the errors introduced by the presence of the short-wavelength pigments also can be estimated. This is described in the next paper of this series.15

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