Visual Pigments in Single Rods and Cones of the Human Retina

Direct measurements reveal mechanisms of human night and color vision.

Paul K. Brown and George Wald

Abstract. *Difference spectra of the visual pigments have been measured in single rods and cones of a parafoveal region of the human retina. Rods display an absorption maximum (λ_{max}) at about 505 mμ, associated with rhodopsin. Three kinds of cones were measured: a blue-sensitive cone with λ_{max} about 450 mμ; two green-sensitive cones with λ_{max} about 525 mμ; and a red-sensitive cone with λ_{max} about 555 mμ. These are presumably samples of the three types of cone responsible for human color vision.*

In this report we present measurements of the difference spectra of visual pigments in single rods and cones of the human retina (*1*). Such measurements are still in progress in our laboratory, and we expect eventually to extend and improve them considerably. This work develops slowly, however, largely for lack of suitable material. The measurements were made with the recording microspectrophotometer described earlier (*2*), which was used for measuring the difference spectra of rhodopsin in single rods of the frog retina (*2, 3*), and of the red- and green-sensitive pigments in the cones of human and monkey foveas (*4*).

Procedures

In the present arrangement of this instrument, a Zeiss W opton microscope with a 100× apochromatic objective and a photographic tube with a 20× eyepiece is fitted in the sample beam of a Cary model 14 recording spectrophotometer. The reference beam passes through a compensatory array of lenses and stops, so as to bring both beams into approximate balance. Final baseline adjustments are made with the "multipot" of the spectrophotometer. For these experiments the standard light source of the spectrophotometer was replaced by a 600-watt quartz iodine lamp (DWL), and the phototube (R136) was set at its highest voltage, both arrangements for keeping the slit-widths minimal during the recording of spectra. All recordings were run at 25 Å per second, the standard 0 to 1 absorbance scale and slide-wire of the spectrophotometer being used.

After much preliminary work with human and monkey retinas, the measurements presented here were made with a single retina from a 63-year-old man. This was removed from the eye, which had been kept dark and refrigerated in dim red light for about 10 hours after death. A piece of the retina including the fovea was mounted in a microcell consisting of two quartz coverslips held apart by a narrow ring of lucite and filled with a solution of 55-percent glycerol in 0.9-percent sodium chloride.

The retina was mounted on the stage of the microscope with the visual cells pointing upward, and the measurements were made with the light passing through them axially, the normal direction of incidence in the living eye. The image of a portion of the retina was projected in dim red light (680 to 690 mμ) at a magnification of 2000× at the level of the entrance to the phototube housing of the instrument. The projected image of the retina was placed in position relative to a small circular diaphragm at this level, so that the light entering the phototube passed only through a single rod or cone. The retina in place on the microscope stage could be observed visually through a viewing ocular, at a magnification of 1600×. A cross-hair in this ocular was aligned beforehand so as to center on the exit diaphragm at the phototube housing. To get the retina in position for a measurement, this cross-hair had only to be centered on a rod or cone, a relatively rapid operation.

The absorption spectrum was recorded in an otherwise dark room, first from 650 to 380 mμ, then immediately again in the reverse direction. This was done because the visual pigments are bleached to some degree during the recording of the spectrum, so distorting its shape somewhat. By recording the spectrum from both directions one obtains curves distorted in opposite ways, which can be averaged to yield a compensated spectrum. These are the "dark" spectra.

Then the rod or cone, after being checked for alignment, was bleached immediately by one or two flash exposures (General Electric M3 flash bulbs), transmitted through a Jena KG1 heat filter and a yellow Corning 3385 filter that transmitted all wavelengths above about 465 mμ, and the spectrum was again immediately recorded. The difference spectra were obtained by subtracting such "bleached" spectra from the "dark" spectra.

Obviously, it is of great advantage to restrict the bleaching light to a limited area of retina in the immediate neighborhood of the receptor being measured, so that one can go on making measurements on other receptors in the same specimen. We do this by taking advantage of the fact that any optical system is reversible—that is, the rays of light describe the same path in either direction; and so we introduce the flashes through the field-limiting diaphragm at the level of the phototube entrance. The bleaching light therefore traverses the system in the reverse direction to the measuring light. Owing to the fact that we have not yet arranged the bleaching radiation to enter the system in just the way the measuring radiation emerges, it spreads at the level of the specimen beyond the cell being measured, so as to expose the entire field seen at 1000× magnification in the

The authors are affiliated with the Biological Laboratories of Harvard University. Dr. Wald is professor of biology at Harvard and is currently a Guggenheim Fellow in the department of zoology, Cambridge University. Paul Brown is his assistant. This report was originally scheduled to be published with that of W. B. Marks, W. H. Dobelle, and E. F. MacNichol, Jr. (13 Mar. 1964, p. 1181), but was inadvertently delayed until this issue.

microscope. Most of the retina, however, is left in darkness, so that by moving from one field to another one can obtain a number of recordings from the same specimen.

Observations

The measurements reported here were obtained from a parafoveal region of the retina, 1 to 2 mm from the fovea (that is, about 3.5° to 7° from the fixation point). This lay outside the area of the yellow macular pigmentation. In this region cones are completely surrounded by rods, with, on the average, two rods between adjacent cones. Since the cones are considerably thicker than the rods, both types of receptor are easily distinguished and isolated for measurement. The cone inner segments are about 5 μ thick, and the bases of the outer segments 2 to 2.5 μ thick; the rods are on the average about 1.5 μ thick (*5*).

Figure 1 shows measurements made as described on a single rod, recording the spectrum first from the red to the ultraviolet, then from the ultraviolet back to the red. About 25 percent bleaching occurred during the recording of both "dark" spectra. This caused only small distortions in the curves and

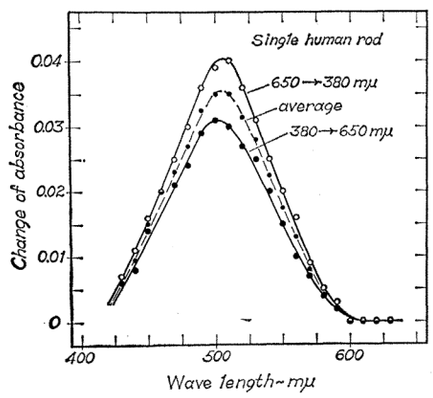

Fig. 1. Difference spectra of the visual pigment in a single rod in the parafoveal region of the human retina. The difference spectrum was measured, first from 650 to 380 mμ, then in the reverse direction. These two recordings involved about 25 percent bleaching. The averaged curve has λ_{max} 505 mμ, and resembles closely the difference spectrum of human rhodopsin measured in relatively large areas of retina (*5*) and in rod suspensions (*7*), where no bleaching occurred in the course of the measurements; though the present λ_{max} lies about 5 mμ further toward the red. The pigment in this rod is primarily or wholly rhodopsin.

wavelength maxima (λ_{max}) of the spectra; the run from 380 to 650 mμ is displaced slightly toward the red, and the run from 380 to 650 mμ slightly toward the blue. The average of the two traverses, whether they are averaged directly as in Fig. 1, or are brought to the same height and then averaged, agrees well with difference spectra of human rhodopsin measured earlier in suspensions of rod outer segments (*6*) and in relatively large areas of retina (*4*), where no appreciable bleaching occurred during the measurements. Figure 1, therefore, yields some idea of errors associated with the procedure, and offers some assurance that these do not greatly affect the first absorbances measured, and still less the shapes of the difference spectra.

Figure 2 shows the averaged difference spectrum obtained similarly from a second rod. Both these spectra have λ_{max} at about 505 mμ, displaced about 5 mμ toward the red as compared with our previous measurements on human rhodopsin in rod suspensions (*6*) and in the retina (*4*). In part, this discrepancy may be associated with incomplete compensation of the effects of bleaching during measurement; more important is the fact that the earlier measurements were made in the presence of hydroxylamine, and so yielded the colorless retinene oxime as a product of bleaching; whereas the yellow retinene formed by bleaching in the present experiments, with its absorption rising into the blue and violet, causes some displacement of the difference spectrum toward the red.

The maximum absorbances of rhodopsin in these two rods are in the range 0.04 to 0.05. These are not improbable values for single rods, since the largest rhodopsin absorbance we have measured in large fields of the human retina was about 0.15 (*4*). Yet it should be noted also that this was not a wholly dark-adapted retina, and looked rather pale in dim white light, indicating that some of its rhodopsin may have been bleached to begin with.

Figure 3 shows difference spectra of the photosensitive pigments in four cones. From comparisons with earlier measurements of different types, these seem clearly to represent one blue-receptor, two green-receptors and one red-receptor. The λ_{max} in these spectra fall at about 450, 525, and 555 mμ.

The difference spectrum of the blue-receptor, together with the similar measurements on one human and one monkey cone (*1*), represent the first

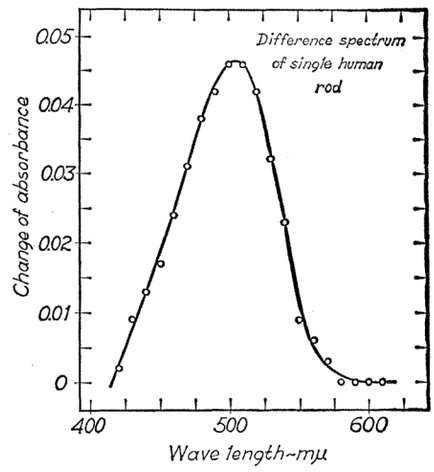

Fig. 2. Difference spectrum of visual pigment in a single rod of the human parafoveal retina. The spectrum was recorded first in the dark, then again after bleaching with a flash of yellow light. The difference between these spectra is shown. It has λ_{max} about 505 mμ, and closely resembles the difference spectrum of human rhodopsin measured earlier in suspensions of rod outer segments (*7*) and in relatively large areas of retina (*5*), though displaced about 5 mμ toward the red.

such measurements on this type of unit in the primate retina. The agreement is reasonably close, our λ_{max} at 450 mμ falling between that for the monkey cone (about 442 mμ) and that for the human cone (about 457 mμ) measured by Marks *et al.* (*1*). The λ_{max} of our green- and red-receptors lie at somewhat shorter wavelengths than the difference spectra of the green- and red-sensitive pigments measured earlier in relatively large patches of the human fovea (535 mμ for the green- and 565 to 570 mμ for the red-sensitive pigment) (*4*). A factor contributing to these displacements is that though the spectra of Fig. 3 were recorded as usual by traversing the spectrum in both directions, only the first recordings, from the red to the violet, proved useable. Whatever bleaching occurred in the course of these measurements would have caused small displacements of spectrum toward the red, increasing slightly, therefore, the disparity with our earlier measurements on the green- and red-sensitive pigments.

The absorbances of individual cones come out in about the same range as our earlier measurements (*4*). In the latter we found the red-sensitive pigment to have a maximum absorbance of about 0.03, the green-sensitive pigment about 0.015. In the measurements re-

ported here these proportions are reversed, the two green-sensitive cones having almost exactly equal absorbances of about 0.025, the blue-receptor nearly as high, and the red-receptor 0.013. Such measurements in individual cones may depart considerably from the averages of large numbers involved in our earlier work. It should be remembered also that these are parafoveal cones, which differ anatomically and perhaps in other ways from the foveal elements measured previously. As already said, this retina was not wholly dark adapted, its rhodopsin appearing to have been partly bleached. The same may have been true of the cone pigments, yet not necessarily, since their rapid regeneration relative to rhodopsin gives them first access to whatever 11-*cis* vitamin A is available.

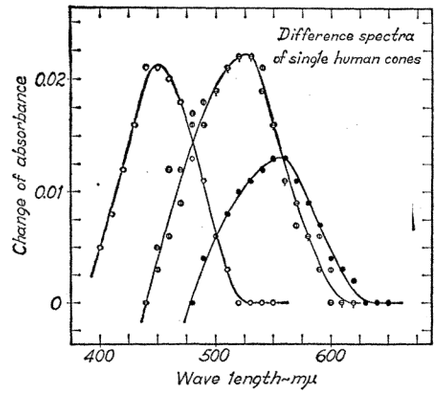

Fig. 3. Difference spectra of visual pigments in single cones of the parafoveal region of the human retina. In each case the absorption spectrum was recorded in the dark from 650 to 380 mμ, then again after bleaching with a flash of yellow light. The differences between these spectra are shown. One of these cones, apparently a blue receptor, has λ_{max} about 450 mμ; two cones, apparently green-receptors, have λ_{max} about 525 mμ; and one, apparently a red-receptor, has λ_{max} about 555 mμ. In making these measurements light passed through the cones axially, in the direction of incidence normal in the living eye.

Discussion

We have shown earlier that human rhodopsin (*6*) and the green- and red-sensitive pigments of human cones (*4*) are composed of the same chromophore, 11-*cis* retinene, bound to a variety of opsins. This has not yet been demonstrated for the blue-sensitive pigment, yet is in all likelihood the case. Retinene readily combines with opsin and other proteins to form light-sensitive artifacts with λ_{max} near 450 mμ (see *7*); 440 mμ is in fact the λ_{max} ordinarily associated with protonated Schiff bases of retinene (*8*). Furthermore, Goldsmith, in our laboratory, extracted from the eye of the honeybee a visual pigment with λ_{max} 440 mμ derived from retinene (*9*). If the bleaching of the blue-sensitive pigment yields retinene as product, this should considerably distort its difference spectrum, narrowing it and displacing it toward the red. We can expect, therefore, that the true absorption spectrum of the blue-sensitive pigment is broader and lies at shorter wavelengths than the difference spectrum shown in Fig. 3. Such effects are much smaller in the green- and red-sensitive pigments.

We have already mentioned that the λ_{max} of the green- and red-sensitive cones shown in Fig. 3 lie 10 to 15 mμ toward the blue from the difference spectra of the green- and red-sensitive pigments measured by differential bleaching in large portions of the human fovea (*4*). This difference may in part represent sampling errors; the cones of each type may differ enough in absorption spectrum so that no one cone adequately represents the class. It may be more to the point that the method of differential bleaching tends to single out the individual pigments, whereas the present measurements involve all the light-sensitive pigments in each cone. It is possible that the displacements of spectrum as between these two situations imply that though each green- or red-sensitive cone possesses primarily its appropriate visual pigment, this is mixed to a degree with others. Further experiments should clarify this issue.

Our measurements should be compared with Rushton's measurements of the difference spectra of the green- and red-sensitive pigments, obtained by reflection spectrophotometry in the living human eye. It is hard to know what to say about the latter, however, since Rushton has recently repudiated all such measurements that he had published prior to 1963 (*10*). He writes: "Attention has been drawn by Ripps and Weale [*11*] to the fact that during the past 6 years I have spoken at conferences and published short notes which show several inconsistencies. . . . At the beginning of the three papers that have just appeared [*12–14*] I expressly disclaim those preliminary suggestions and give no reference to them" (*10*). At present we are left with Rushton's new measurements on the green-sensitive pigment, which rise to a maximum absorbance near 555 mμ, but then do not descend significantly at shorter wavelengths—that is, do not display a peak (*12*). There is also a calculation "correcting" the absorbance of this pigment from a measured value of 0.07 to 0.35 (*13*), which judging from our experience may be 10 times too high.

Using a comparable procedure, Weale (*15*) has measured difference spectra of the green- and red-sensitive pigments in the living human eye, which he states to have λ_{max} near 540 and 600 mμ, though his data for the latter pigment seem to us to lie closer to 570 mμ. This method in the hands of Rushton and Weale has yielded valuable information on the rise and fall of visual pigment concentrations in the living eye, but does not seem capable of measuring reliably the spectra of the cone pigments.

The closest indirect approach to the absorption properties of the color vision mechanisms of the human eye is in the measurements of Stiles, who uses a two-color threshold method. Stiles's "mechanisms" are not necessarily individual cones; still less need they involve individual pigments. Nevertheless, three primary mechanisms emerge, with λ_{max} at about 440, 540, and 575 mμ. Correction for the transmission of light by the ocular media and the macular pigmentation changes the shapes of the curves, but does not greatly alter their λ_{max} (*16*). Using a method of differential adaptation, Auerbach and Wald (*17*) found the spectral sensitivity of the human blue-receptor to have λ_{max} about 440 mμ (uncorrected). Recently, one of us (G.W.) has attempted to measure the spectral sensitivities of the color vision pigments in the human fovea; after correction for ocular transmission they come out with λ_{max} about 430, 540, and 575 mμ (*18*). Altogether, we now seem close to having reliable information on the properties of these pigments.

References and Notes

1. W. B. Marks, W. H. Dobelle, E. F. MacNichol, *Science* **143**, 1181 (1964).
2. P. K. Brown, *J. Opt. Soc. Am.* **51**, 1000 (1961).
3. G. Wald, P. K. Brown, I. R. Gibbons, *ibid.* **53**, 20 (1963); also in "Biological receptor mechanisms," J. W. L. Beament, Ed., *Symp. Soc. Exptl. Biol.* **16**, 32 (1962).
4. P. K. Brown and G. Wald, *Nature* **200**, 37 (1963).
5. S. L. Polyak, *The Retina* (Univ. of Chicago Press, Chicago, 1941), pp. 213–214.
6. G. Wald and P. K. Brown, *Science* **127**, 222 (1958).

7. ———, *Nature* **177**, 174 (1956).
8. S. Ball, F. D. Collins, P. D. Dalvi, R. A. Morton, *Biochem. J.* **45**, 304 (1949).
9. T. H. Goldsmith, *Proc. Natl. Acad. Sci. U.S.* **44**, 123 (1958).
10. W. A. H. Rushton, *J. Opt. Soc. Am.* **54**, 273 (1964).
11. H. Ripps and R. A. Weale, *ibid.*, p. 272.
12. W. A. H. Rushton, *J. Physiol.* **168**, 345 (1963).
13. ———, *ibid.*, p. 360.
14. ———, *ibid.*, p. 374.
15. R. A. Weale, *Opt. Acta* **6**, 158 (1959).
16. W. S. Stiles, *Rev. Opt.* **28**, 215 (1949); *Proc. Natl. Acad. Sci. U.S.* **45**, 100 (1959).
17. E. Auerbach and G. Wald, *Science* **120**, 401 (1954); *Am. J. Ophthalmol.* **39**, 24 (1955).
18. G. Wald, in preparation.
19. This investigation was supported in part by funds from the National Science Foundation and the Office of Naval Research. We are greatly indebted to the Boston Eye Bank for donations of eyes.

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Urea: Apparent Carrier-Mediated Transport by Facilitated Diffusion in Dogfish Erythrocytes

Abstract. *The exposure of erythrocytes from the elasmobranch,* Squalus acanthias, *to solutions isosmotic with plasma (1M) but containing urea or hydroxyurea as the sole solute does not produce hemolysis. Exposure of these cells to 1M methylurea, thiourea and acetamide does produce hemolysis. Low concentrations of urea, which are associated with hemolysis, protect dogfish red cells against hemolysis by methylurea and thiourea. Dogfish red cells exposed to mediums containing high concentrations of urea, or no urea, reach 95 percent of their equilibrium concentration in less than 5 minutes.*

The presence of high concentrations of urea in the plasma (and presumably the tissues) of elasmobranchs is well known (*1*). This compound accounts for approximately one third of the total tonicity of plasma. Previous work has shown that the elasmobranch red cell is permeable to urea (*2*). By implication, it has been assumed that penetration occurs by means of simple passive diffusion. If this were true, then these erythrocytes, like mammalian erythrocytes, should be rapidly hemolyzed when exposed to solutions isosmotic with dogfish plasma, but containing urea as the sole solute. The experiments reported here demonstrate that there is no hemolysis of dogfish erythrocytes in isosmotic salt-free solutions of urea or hydroxyurea. Further, the behavior of dogfish erythrocytes when exposed to compounds chemically similar to urea suggests a carrier-mediated transport system for urea, similar to that described by a number of workers including LeFevre (*3*) and Wilbrandt (*4*) for glucose in primate erythrocytes.

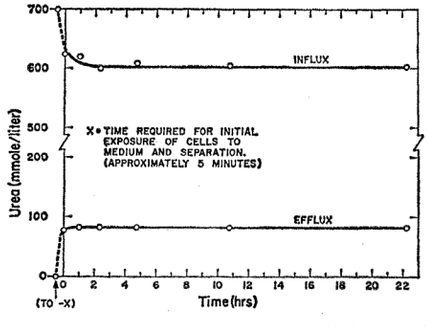

Fig. 1. Measurements of urea influx and efflux in dogfish red cells.

Erythrocytes from the spiny dogfish, *Squalus acanthias*, were used throughout this study. Blood was obtained from a caudal vessel in a syringe containing heparin. The red cells were separated from the plasma and white cells by means of centrifugation at 10°C in a refrigerated centrifuge. The erythrocytes were then washed once or twice and the red cells isolated by means of in elasmobranch Ringer solution (*5*) careful aspiration of the supernatant solution. Hemolysis was studied by adding approximately 0.1 ml of erythrocytes to 2 ml of the solution being tested. The red cell mixture was permitted to stand at room temperature for approximately 5 minutes; the mixture was then centrifuged for 2 minutes. The color of the supernatant was taken as an indicator of the degree of hemolysis (Table 1).

Solutions of the following substances (1.0 and 1.3*M*) were tested for their ability to produce hemolysis: urea (NH_2CONH_2), methylurea ($NH_2CONHCH_3$), thiourea (NH_2CSNH_2), acetamide (CH_3CONH_2), and hydroxyurea ($NH_2CONHOH$). A modified "urea osmotic fragility" test was performed by exposing dogfish erythrocytes to a graded series of urea concentrations varying from 0.1 to 1.0*M*.

Net urea influx was measured by suspending dogfish erythrocytes in a modified isosmotic dogfish Ringer solution containing twice the concentrations of urea normally found in dogfish erythrocytes (700 mmole/liter) (*5*) and determining the rate of decrease of urea concentration in the Ringer's solution. Net efflux of urea was measured by suspending dogfish erythrocytes in a modified isosmotic Ringer's solution containing no urea (*5*) and determining the rate of increase of urea concentration in the Ringer's solution. In these studies, the red cell hematocrit of the suspension was approximately 10 percent. Urea concentrations in the suspending media were determined in duplicate by the technique of Conway (*6*), an ammonia blank being used.

The results obtained, which were the same for three different animals, are shown in Table 1. The exposure of erythrocytes to 1*M* urea as the only solute produces no hemolysis. When the concentration of urea in the suspending media is reduced to 0.3*M* or less, hemolysis occurs in less than 5 minutes. There is slow hemolysis at intermediate urea concentrations. The behavior of hydroxyurea is similar to urea in that it produces no hemolysis at concentrations of 1.0*M* and above and causes rapid hemolysis at 0.3*M* concentrations or less. The three other urea-like compounds studied, when present as the sole solute in contradistinction to urea, produce rapid hemolysis although the degree of hemolysis produced by thiourea is somewhat less than that produced by methylurea and acetamide.

The addition of 0.3*M* urea to the suspending media protects the red cells

Table 1. Effect of urea and related compounds on the dogfish erythrocyte.

Solutes and concentrations used	Degree of hemolysis*
1.0 *M* urea	0
0.3 *M* urea	4
1.0 *M* hydroxyurea	0
0.3 *M* hydroxyurea	4
1.0 and 1.3 *M* methylurea	4
1.0 *M* methylurea + 0.3 *M* urea	4
1.0 and 1.3 *M* thiourea	3
1.0 *M* thiourea + 0.3 *M* urea	0
1.0 and 1.3 *M* acetamide	4
1.0 *M* acetamide + 0.3 *M* urea	4
1.0 *M* methylurea + 0.32 *M* hydroxyurea	0
1.0 *M* thiourea + 0.32 *M* hydroxyurea	0
1.0 *M* acetamide + 0.3 *M* hydroxyurea	4

* 0, No detectable pink color in the supernatant; 1, barely detectable pink color; 2, moderate pink to red color; 3, red color; 4, deep red color in the supernatant and no red cells in the sediment.