Human Rhodopsin

George Wald and Paul K. Brown

The first measurement of the absorption spectrum of rhodopsin involved a human preparation (*1*). Arthur König had asked his colleagues Köttgen and Abelsdorff to measure the absorption spectrum of frog rhodopsin, hoping that the experience gained with this material might eventually be turned to the measurement of human rhodopsin. Before any frog pigment was measured, however, König unexpectedly received the gift of a single human eye. This was extracted with bile salts, and it yielded enough solution for three fillings of a microabsorption vessel. With the first filling König and Köttgen measured the absorption spectrum of human rhodopsin; with the second, the spectrum of a partly bleached solution. The third portion had begun to rot and was discarded.

König and Köttgen measured what we should now call the difference spectrum of rhodopsin—the difference in absorption spectrum before and after bleaching—in the mistaken belief that it was the true absorption spectrum. They found this to be maximal near 500 millimicrons (mμ). Assuming that the rhodopsin had been evenly spread over the rod-containing area of the retina, König computed that its absorption coefficient *in situ* was about 0.038 at 500 mμ, corresponding to an extinction of 0.0165.

König proceeded to compare this difference spectrum with the spectral sensitivity of human rod vision, measured in a totally color-blind subject and in the normal eye. Both sensitivity functions were corrected for the energy distribution of the source and for losses of light in the eye caused by absorption by the yellow lens and the pigment of the *macula lutea*. In this way König derived spectral sensitivity functions for rod vision, as though measured at the receptor layer. These functions corresponded closely with the difference spectrum of human rhodopsin. In this experiment, therefore, König and Köttgen provided the first direct demonstration that rhodopsin is a *visual* pigment and set the main themes for this type of investigation.

Recently Crescitelli and Dartnall (*2*) measured a second preparation of human rhodopsin, again obtained from a single retina. The difference spectrum at *p*H 8.2 was found to be maximal at 497 ± 2 mμ. The extinction of this pigment in the retina (assuming it to be evenly distributed over the rod surface) was estimated to be about 0.016, almost exactly König's result. The absorption spectrum inferred tentatively on the basis of these measurements was found to agree reasonably well with the spectral sensitivity of human rod vision, calculated on a quantum basis and corrected for the transmission of the ocular tissues. In accordance with a system of nomenclature proposed earlier by Dartnall, it was suggested that human rhodopsin be called "visual pigment 497."

Preparation

We have recently obtained a number of human eyes, within several hours following enucleation (*3*). No special precautions were taken to adapt them to the dark. The retinas were removed under dim red light and stored in the dark at − 10°C until used. The retinal tissue was ground with a mortar and pestle in 40-percent sucrose solution, and the outer segments of the rods were separated from the remaining retinal tissues by differential centrifugation, as described earlier (*4*). The rods were washed free from sugar and buffer and were hardened by treatment with 4-percent alum solution for 15 minutes. They were washed once again with distilled water and neutral phosphate buffer, frozen-dried, and extracted with petroleum ether to remove fat-soluble material. The residue was then extracted with 2 percent digitonin in phosphate buffer at *p*H 6.5 to solubilize the rhodopsin and opsin.

Absorption Spectrum

The absorption spectrum of a solution of human rhodopsin is shown in Fig. 1. It displays the three usual absorption bands. The α-band, which dominates the visible spectrum, is maximal at 493 mμ. This is an extraordinarily short $\lambda_{max.}$, shorter than that of any vertebrate rhodopsin previously examined, with the exception of that of certain deep-sea fishes. Indeed, it corresponds in this regard to the rhodopsin of a marine fish living at a mean depth of about 60 fathoms (*5*). The spectrum continues with a minimum at about 390 mμ, a small β-band at about 350 mμ, and the tall γ or opsin band at 278 mμ. The ratio of extinctions at 390 and 493 mμ ($E_{min.}/E_{max.}$) is 0.24. We believe that this spectrum is virtually that of the pure pigment down to about 320 mμ. The γ-band probably involves, in part, other proteins. When this pigment is bleached in orange—nonisomerizing—light, the α- and β-bands are replaced by the absorption band of all-*trans* retinene, maximal at about 380 mμ.

Figure 2 shows the absorption spectrum of another such preparation of human rhodopsin, measured in the presence of 0.1*M* hydroxylamine (*6*). When this solution is bleached with orange light, the retinene reacts rapidly with hydroxylamine to form all-*trans* retinene oxime ($\lambda_{max.}$ 367 mμ). This possesses a molar extinction of 51,600 in digitonin solution. In several such experiments we have found the $E_{max.}$ of human rhodopsin to be 0.76 to 0.79 times as high as the $E_{max.}$ of the retinene oxime formed by its bleaching. Therefore, its molar extinction $\varepsilon_{max.}$ must be 0.76 to $0.79 \times 51{,}600$, or $40{,}000 \pm 800$—close to that of cattle rhodopsin (*6*).

The difference spectrum of human rhodopsin, bleached in the presence of hydroxylamine, is also shown in Fig. 2. Since the absorption of light by retinene oxime is negligible at wavelengths longer than 450 mμ, the difference spectrum is identical with the true absorption spectrum of rhodopsin above this wavelength. The hydroxylamine difference spectrum represents an absolute measurement, independent of the presence of colored impurities and invariant with *p*H. It offers the opportunity to obtain the absorption spectrum of pure rhodopsin above 450 mμ from preparations however impure. It shares an inter-

The authors are members of the staff of the Biological Laboratories, Harvard University, Cambridge, Mass.

esting empirical characteristic with cattle rhodopsin: the change in extinction at the maximum almost exactly equals the change in extinction at the minimum ($\Delta E_{max.} = \Delta E_{min.}$).

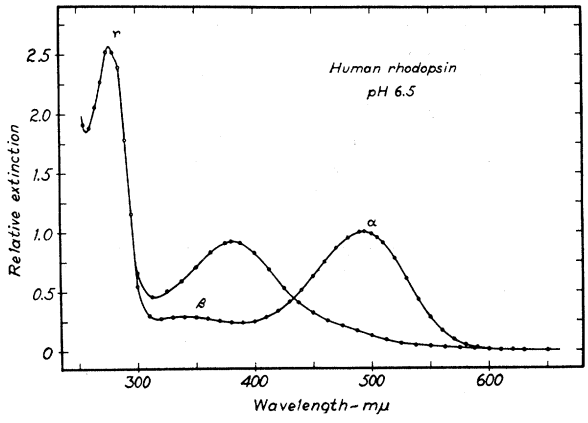

Fig. 1. Absorption spectra of human rhodopsin and the product of its bleaching in 2 percent aqueous digitonin solution. The α-band of rhodopsin has been set arbitrarily at $E_{max.} = 1$. On bleaching in orange (nonisomerizing) light, the α- and β-bands of rhodopsin are replaced by the absorption band of all-*trans* retinene ($\lambda_{max.}$ 380 mμ). The γ- or opsin band at 278 mμ remains unchanged.

Homogeneity

We have found that a useful characterization of both the shape and position of the rhodopsin spectrum is provided by the ratio of the extinction at 540 mμ to that at the maximum ($E_{540}/E_{max.}$) (*5*). For human rhodopsin this ratio is 0.436. (For a rhodopsin with $\lambda_{max.}$ 497 it would have been 0.55.) To test the homogeneity of the preparation from which Fig. 2 was derived, it was partly bleached, successively, with near-ultraviolet light containing wavelengths shorter than 400 mμ (Wratten 18A filter) and with orange light containing wavelengths longer than 550 mμ (Jena OG2 filter), all in the presence of hydroxylamine. The $\lambda_{max.}$ of the difference spectra for both types of bleach is about 493 mμ. The $E_{540}/E_{max.}$ ratios were essentially identical: 0.443 for bleaching in blue light and 0.436 for bleaching in orange light.

A further evidence of homogeneity is found in the spectra shown in Fig. 3. In this case a rhodopsin preparation was bleached in the deep orange of a Jena OG3 filter (wavelengths greater than 565 mμ). The sharp isosbestic point at about 432 mμ shows that a single photosensitive pigment bleached to a single light-absorbing product (all-*trans* retinene). The kinetics of bleaching follows the course of a first-order reaction, proportional in rate to the intensity of the light.

Composition and Regeneration

The bleaching of human rhodopsin in nonisomerizing light results in an inactive mixture of all-*trans* retinene and opsin. If neo-*b* (11-*cis*) retinene is incubated with this mixture in the dark, rhodopsin is regenerated (*7*). Human opsin has also been prepared from bleached retinas; it forms rhodopsin when it is incubated in the dark with neo-*b* retinene. All such preparations synthesized or regenerated *in vitro* have $\lambda_{max.}$ about 493 mμ and are indistinguishable from the rhodopsin extracted from human retinas (Fig. 4, right). It is clear that the exceptionally low $\lambda_{max.}$ of human rhodopsin derives from its peculiar opsin and not from an unusual prosthetic group.

The kinetics of regeneration of human rhodopsin in solution is shown in Fig. 4. The reaction mixture, containing 3.90 μmoles of opsin and 25.6 μmoles of neo-*b* retinene per liter, was incubated in the dark at *p*H 6.5 and 29.5°C. The synthesis of rhodopsin was complete in about 30 minutes and half-complete in about 2.5 minutes. As is usual, the regeneration follows the course of a second-order reaction The rate constant *k* equals 0.0084 liter per μmole minute, or 39 liters per mole second.

The regeneration of rhodopsin has recently been measured in the living human eye by Campbell and Rushton (*8*). It pursues much the same course as human rod dark adaptation, when the latter is plotted as change of log sensitivity with time. Both processes are nearly complete in 30 to 45 minutes and half complete in 4 to 8 minutes. The synthesis of rhodopsin shown in Fig. 4 is a much more rapid reaction; at human body temperature it would have been half completed in about 1 minute.

The synthesis of rhodopsin *in vivo* involves a chain of reactions in which the combination of neo-*b* retinene with opsin is only the last step.Earlier steps include the hydrolysis of vitamin A esters, the isomerization of all-*trans* vitamin A or retinene to the neo-*b* configuration, and the diffusion of vitamin A from the pigment epithelium into the rods; the subsequent oxidation of vitamin A to reti-

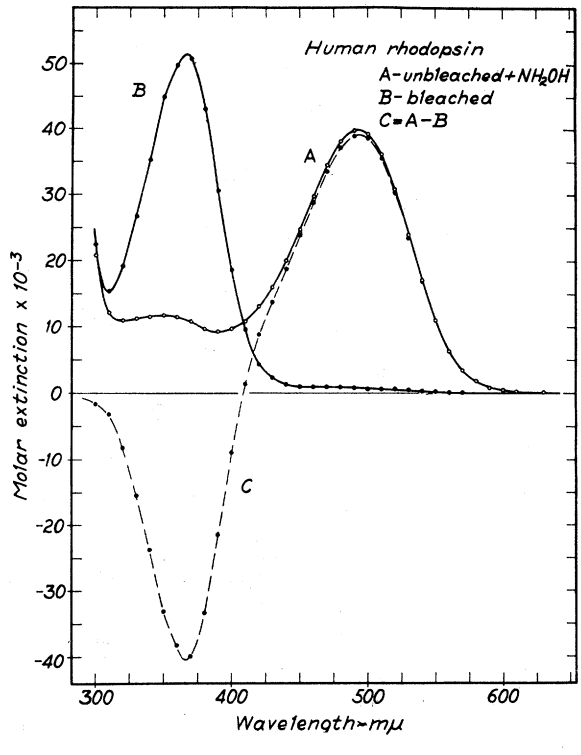

Fig. 2. Absorption spectra of human rhodopsin (curve *A*) and the product of its bleaching (curve *B*) in the presence of 0.1*M* hydroxylamine. Under these circumstances the product of bleaching is all-*trans* retinene oxime ($\lambda_{max.}$ 367 mμ). This compound does not absorb appreciably above 450 mμ; the small absorption of curve *B* above this wavelength is caused by a residue of unbleached rhodopsin. For this reason, also, the difference spectrum (curve *C*) is not quite identical with curve *A* above 450 mμ.

nene is coupled directly with the combination of retinene and opsin and must go at the same rate. The rate of synthesis of rhodopsin *in vivo* may be limited in part by the initial reactions. It should not be forgotten, also, that the synthesis *in vivo* occurs, not in free solution, but in the virtually solid-state structure of the rods, in which opsin is oriented and fixed in position and in which even vitamin A and retinene must move in restricted ways. This condition introduces steric and diffusion factors which may profoundly influence the kinetics of the reaction.

Concentration in the Retina

We have made preliminary attempts to measure the concentration of rhodopsin in the outer segments of the rods. Outer segments were isolated from the other retinal tissues by differential centrifugation and packed by centrifuging into the bottom of a hematocrit tube, and their volume was measured. They were then extracted, as has already been described. The measurements are summarized in Table 1. In four out of five determinations, performed with rods from three different groups of retinas, the values clustered about a mean extinction per rod of 0.015; in the fifth experiment the value obtained was 0.042. These values were computed on the assumption that the average length of the outer segment of a human rod in the parafoveal region is about 25 microns (*9*). No allowance was made for the orientation of rhodopsin molecules in the rod structure, which, if complete, might raise the extinction for light passing down the rod axis as much as 1.5 times (*10*). Since our retinas were not dark-adapted, these values should be taken as minimal. For this reason also only the highest value, 0.042, may be important.

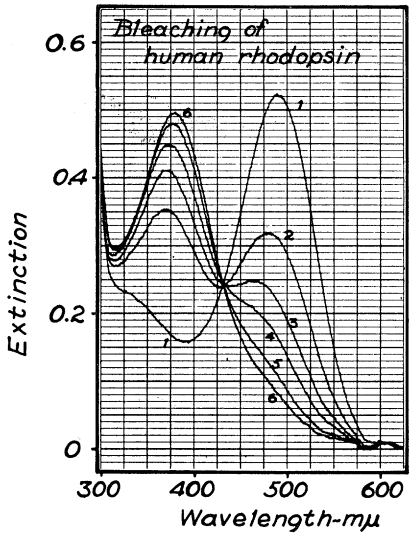

Fig. 3. The bleaching of human rhodopsin in aqueous digitonin solution at *p*H 6.45. The rhodopsin solution (curve 1) was exposed to the concentrated light of a 160-watt tungsten lamp passing through an orange filter (Jena OG3; $\lambda > 565$ mμ), and the spectrum was recorded after total irradiations of 5 minutes (curve 2), 10 minutes (curve 3), 15 minutes (curve 4), 25 minutes (curve 5), and 35 minutes (curve 6). The curves shown were drawn with a recording photoelectric spectrophotometer. The fairly sharp isosbestic point at about 432 mμ shows that, to a first approximation, only the single photopigment rhodopsin bleaches to the single light-absorbing product, all-*trans* retinene. Bleaching follows the kinetics of a first-order reaction.

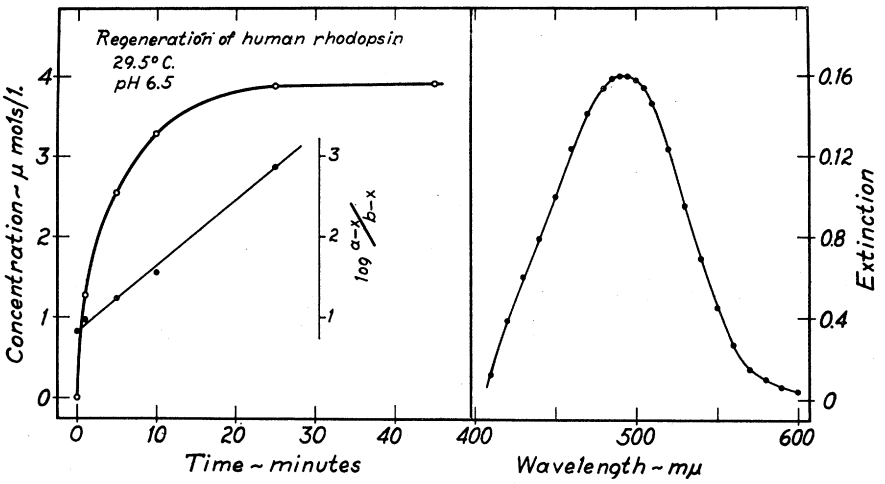

Fig. 4. Regeneration of human rhodopsin in aqueous solution. A preparation of rhodopsin was bleached in nonisomerizing light to an inactive mixture of all-*trans* retinene and opsin. This was incubated with neo-*b* retinene in the dark. The regeneration of rhodopsin followed the kinetics of a second-order reaction, as is shown by the linearity of the plot of log $[(a-x)/(b-x)]$ against time in the dark (a = initial concentration of neo-*b* retinene = 25.6 μmoles per liter; b = initial concentration of opsin = 3.90 μmoles per liter; x = concentration of rhodopsin at time t). At the right is shown the difference spectrum of the regenerated rhodopsin, measured in the presence of hydroxylamine. Its $\lambda_{max.}$ of 493 mμ is identical with that of the original pigment.

Earlier Comparisons with Spectral Sensitivity

The comparison between the absorption spectrum of rhodopsin and the spectral sensitivity of human rod vision, introduced by König, has had a peculiar history. Since the time of König it has been recognized that these two functions resemble each other closely; yet there has always been some fault in the data used to compare them. König's own comparison was vitiated in part by the use of the difference spectrum instead of the absorption spectrum of rhodopsin. Hecht and Williams restated this comparison on the basis of their new measurements of the human scotopic sensitivity, which they had found to be maximal at about 511 mμ. Again they employed the difference spectrum of rhodopsin [monkey and rabbit (*11*)], believing it to represent the absorption spectrum. They also omitted König's corrections for ocular transmission. The difference spectrum was maximal at about 503 mμ—that is, it was about 8 mμ shorter in wavelength than the visual sensitivity. Hecht and Williams suggested that this shift is caused by the fact that since the outer segments of the rods have a higher refractive index than aqueous solutions, rhodopsin in the former medium exhibits a shift of absorption toward the red in accordance with Kundt's rule (*12*).

The later development of this comparison succeeded in disposing of this difference in position between the rhodopsin absorption spectrum and spectral sensitivity, and hence of the necessity to invoke Kundt's rule. The data on spectral sensitivity were quantized (*13*) and were recorrected for ocular transmission (*14*), both operations shifting the curve toward shorter wavelengths. In place of the difference spectrum of rhodopsin employed earlier, a genuine absorption spectrum was substituted, and this was recomputed to correspond with estimates of the actual absorption of rhodopsin in the retina (*15*). When all these adjustments had been made, the spectral sensitivity computed as though at

the inner retinal surface agreed very well with the absorption spectrum of rhodopsin (*15*).

Such comparisons, however, involved until lately the rhodopsins of animals other than man: frog rhodopsin, with $\lambda_{max.}$ 502 mμ, or cattle rhodopsin, with $\lambda_{max.}$ 498 mμ. Crescitelli and Dartnall reintroduced an estimated spectrum of human rhodopsin, with $\lambda_{max.}$ 497 mμ; this agreed reasonably well with Crawford's (*18*) recent measurements of the scotopic sensitivity function.

The present measurements show, however, that the $\lambda_{max.}$ of human rhodopsin is at 493 mμ and that the entire absorption spectrum lies at distinctly shorter wavelengths than does the human scotopic sensitivity, however corrected. We are forced, therefore, to consider again Hecht's suggestion that, in the outer segments of the rods, the absorption spectrum of rhodopsin is shifted toward the red from the absorption spectrum in aqueous solution.

Absorption of Rhodopsin in Rods

To examine this possibility, we have measured the absorption spectrum of rhodopsin in suspensions of human rod particles. The particles from a fresh retina were separated from the remaining retinal tissues by differential centrifugation in 40 percent sucrose, as already described, and were examined without further treatment. The difference spectrum of such a suspension, measured in the presence of hydroxylamine, is compared in Fig. 5 with the difference spectrum of human rhodopsin in aqueous solution. Such data obtained with rod particles satisfy all the criteria of good difference spectra: for example, $\Delta E_{max.}$ almost exactly equals $\Delta E_{min.}$ However, $\lambda_{max.}$ lies at 500 mμ, shifted 7 mμ toward the red as compared with $\lambda_{max.}$ of the solution. The α-band of rhodopsin in the rod particles is also slightly *narrower* than it is in solution. Indeed, the spectrum in the rod particles exhibits much the same changes as are displayed by rhodopsin when it is measured at low temperatures in a rigid medium (*17*).

We have also recently measured directly the difference spectrum of the human retina. A portion of human retina, laid flat upon a microscope slide and backed with finely ground Vycor glass to diffuse the light, was measured against an identical microscope slide and ground glass as blank. Then the retina was bleached in position, and its

Table 1. Concentration and extinction of rhodopsin in outer segments of human rods. The average length of the outer segment of the rod in the parafoveal region is taken as 25 microns. No allowance is made for orientation of rhodopsin molecules in the rods, which may raise the extinction *in situ* as much as 1.5 times, or for other aspects of the solid state structure of the rods, which may raise the extinction still further.

No. of retinas	Volume of outer segments of rods (mm^3)	Volume of extract (ml)	$E_{max.}$ per cm (solution)	$E_{max.}$ per cm (rods)	$E_{max.}$ per rod (25 μ)
			1 February 1957		
3	11	0.5	0.367	16.7	0.042
			24 July 1957		
2	75	0.5	0.875	5.83	0.015
1	47	0.5	0.319	3.39	0.0085
			15 August 1957		
2	23	0.5	0.257	5.59	0.014
2	20	0.5	0.312	7.80	0.020

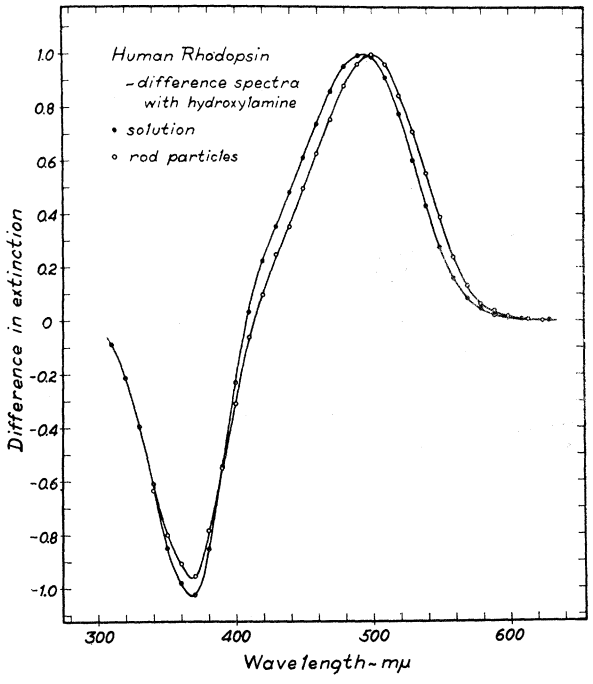

Fig. 5. Difference spectra of human rhodopsin in aqueous solution and in rod particles, measured in the presence of hydroxylamine. The $\lambda_{max.}$ in solution is 493 mμ; in a suspension of rod particles oriented at random it is 500 mμ. The spectrum in the particles is also narrower than it is in solution. At wavelengths longer than 450 mμ these difference spectra are identical with the absorption spectra.

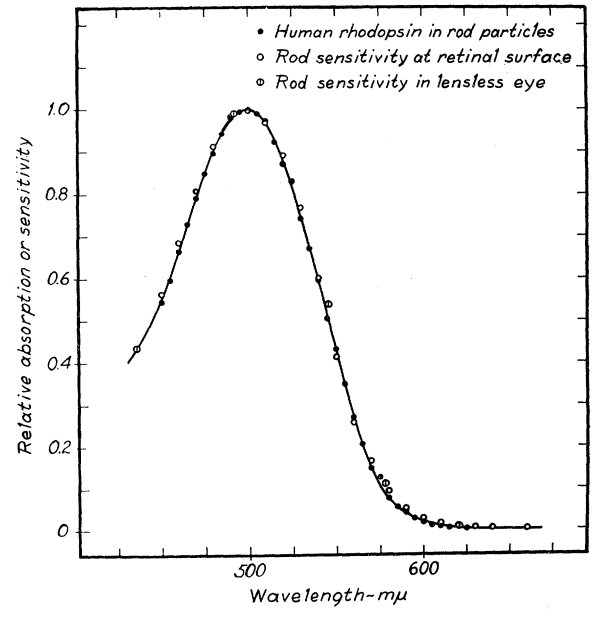

Fig. 6. Human rhodopsin and the spectral sensitivity of rod vision. The absorption spectrum of rhodopsin, measured in human rod particles, is compared with the scotopic (rod) luminosity function, quantized and corrected for ocular transmission so as to represent the sensitivity at the retinal surface, and with the scotopic luminosity (quantized) of the lensless human eye. In the latter, the principal colored structure of the eye, the yellow lens, having been removed in the operation for cataract, the luminosity function comes as close to the intrinsic sensitivity of the rods as one can approach *in vivo*.

spectrum was remeasured. The difference spectrum ($\lambda_{max.}$ 502.5 mμ), as expected, was displaced slightly from the hydroxylamine difference spectrum, owing to the fact that, in the absence of hydroxylamine, retinene itself absorbs light in the region of the rhodopsin maximum. The change in extinction at 502.5 mμ was 0.067. This must be taken as a minimal value, since the retina was not dark-adapted and since no allowance was made for the passage of light through spaces between the rods. It corresponds almost exactly with the highest extinction we have measured in unoriented rod particles (0.042; Table 1) multiplied by the orientation factor 1.5 (see *10*).

As already explained, the difference spectrum measured in the presence of hydroxylamine is identical with the true absorption spectrum at wavelengths longer than 450 mμ. This portion of the difference spectrum for human rod particles is plotted in Fig. 6 in terms of percentage absorption; we assume a maximal extinction in the rods of 0.15, and hence a maximal absorption of 29 percent, as estimated by Rushton in the living human eye (*18*).

Comparison with Spectral Sensitivity

For comparison with these data we have plotted together measurements of scotopic sensitivity from five sources: Hecht and Williams (1922), Weaver (1937), Wald (1945), Flamant and Stiles (1948), and Crawford (1949) (*19*). Between 450 and 650 mμ, these measurements are in very good agreement, and a freehand curve was drawn through them. This curve was then quantized and corrected for ocular transmission with the data of Ludvigh and McCarthy (*14*). The resulting measurements, which represent the sensitivity of human rod vision as though measured at the inner retinal surface, are shown with open circles in Fig. 6. In this figure we have also plotted the direct measurements of human scotopic sensitivity in an extramacular area of the lensless ("aphakic") eye (*20*). Such measurements come as close as is possible to representing the intrinsic sensitivity of the human rods in the living eye. It is clear that all these data agree very well with one another. There is enough leeway in present measurements of the human scotopic sensitivity function, and in the correction for ocular transmission, to permit as much closer a fit to the absorption data as one takes the trouble to obtain; but this would be gilding the lily.

We can conclude that, when adequately measured, the absorption spectrum of human rhodopsin and the spectral sensitivity of human rod vision correspond exactly. It is now clear, however, that this correspondence hangs upon a peculiar phenomenon: the displacement of the absorption spectrum of rhodopsin toward the red in the outer segments of the rods, as compared with the spectrum in aqueous solution. In the highly organized microstructure of the outer segments, the molecules of rhodopsin are fixed in position and highly oriented. In this regard, rhodopsin is virtually in the solid state. In consequence of this condition, its absorption spectrum is displaced toward the red and is narrower than in solution. It also presents a higher extinction for light passing down the rod axis, the normal direction of incidence *in vivo*.

Summary

Human rhodopsin in aqueous solution has $\lambda_{max.}$ of 493 mμ and is lower in the spectrum than the rhodopsins of all other known vertebrates, with the exception of certain deep-sea fishes. Its molar extinction is $40{,}000 \pm 800$. Like other rhodopsins, it bleaches to a mixture of opsin and all-*trans* retinene and is resynthesized by incubating opsin with neo-*b* (11-*cis*) retinene. The regenerated rhodopsin has the same $\lambda_{max.}$ as the extracted pigment; this is due, therefore, not to an unusual retinene but to a characteristic human opsin. The regeneration in solution from opsin and neo-*b* retinene is a second-order reaction with a half-time, at 29.5°C, of about 2.5 minutes. This is much faster than the synthesis of rhodopsin in the living human eye, and faster than human rod dark-adaptation; the rate of both processes *in vivo* must be limited by reactions which precede the union of neo-*b* retinene with opsin, the final step in rhodopsin synthesis. In the rods, rhodopsin is virtually in the solid state—highly oriented in close relation with other highly oriented molecules. In this situation its spectrum is displaced toward the red ($\lambda_{max.}$ 500 mμ) and is narrower than in solution. For light entering the rods axially, rhodopsin has also a considerably increased extinction, some 1.5 times higher than when randomly oriented. The spectrum of rhodopsin in rods agrees well in form and position with the spectral sensitivity of human rod vision, measured at the retinal surface.

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