IN SITU MICROSPECTROPHOTOMETRIC STUDIES ON THE PIGMENTS OF SINGLE RETINAL RODS

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ABSTRACT Three spectral entities have been observed in single intact frog rod outer segments at 506 mμ, 480 mμ and 380 mμ. It is likely that the peak of 506 mμ was somewhat altered by bleaching reactions and originated at about 510 mμ. This is identified with the 502 mμ frog rhodopsin of digitonin extracts. Spectra in polarized light have the same maximum, identifying the dichroism of rods with rhodopsin. The dichroic ratio is around 6, giving the outer segment an axial density of 0.09/5μ or 0.9 OD total, with a pigment concentration of 2 to 3 mM. The dichroism data are used to compute the angle separating the rhodopsin molecular absorption vectors in rods from perfect restriction to a plane. This angle is 16° or 23° depending on which of two assumptions one chooses for the type of molecular ordering. The spectral peaks at 480 mμ and 380 mμ are thought to correspond respectively to metarhodopsin and retinene. Disappearance of the former is accompanied by accumulation of the latter. This reaction seems to occur more slowly in the intact outer segment than the corresponding reaction in solution. Spread of bleaching spectra from illuminated to dark areas of the same rod did not occur over distances of 2 μ or greater. Spectra were similar from rod to rod and from point to point on the same rod showing that frog rods are spectrally homogeneous both individually and collectively.

INTRODUCTION

The chemistry of the visual pigment rhodopsin and its intermediate and final products of bleaching has been much studied in solution (1, 2). The structure of the retinal rod which contains the pigment has likewise been examined by electron microscopy (3–5). The molecular events leading to the visual impulse have, however, remained obscure. The time seems ripe for more detailed study of the area common to both structure and chemistry. The importance of the relation of structure to chemistry for vision has been confirmed by the finding that the absorption spectrum of rhodopsin in rods may differ from that in solution (6–9). That this difference is associated with the state of aggregation of the rhodopsin molecules is supported by the finding of identical spectral shifts on sonic disintegration and

digitonin extraction of rods (8). The discovery that human rhodopsin absorbs maximally at a longer wavelength in rods (503 mμ) than in solution (496 mμ) clarified the long-puzzling discrepancy between the absorption spectrum of rhodopsin solutions and the luminosity function of the human eye (9). Moreover, such findings remind us that much of our chemical information from the extracted system may need modification before it can be successfully used to explain the phenomenon of visual excitation.

Methods that have been used to study the pigments of intact receptor cells or their outer segments are spectrophotometry of cell suspensions (6), photographic densitometry of whole retina or cells (7, 10), microphotometry (11), and reflection photometry on the living retina (12). These methods have enjoyed a certain amount of success in studies of large numbers of cells. The method of cell suspension spectrophotometry is limited by settling artifacts and high light-scattering which necessitate difference spectra and provide relatively low sensitivity. The presence of heterogeneous cell and pigment populations has frequently made results difficult to interpret and has necessitated selective wavelength bleaching and hydroxylamine difference spectra as auxiliaries. The only methods allowing one to study individual cells are photographic densitometry and microspectrophotometry. The former leaves much to be desired in flexibility, sensitivity, and ease of application (7). The latter has heretofore only been used to study small groups of retinal rods and to make semiquantitative measurements (11).

Recently, a scanning microspectrophotometer was constructed in this laboratory, having sufficient sensitivity and stability to make measurements on living cells of absorbancies in the 0.1 − 1 per cent range (13). This instrument has heretofore been used to study respiratory enzymes in single cells (14, 15). Its high sensitivity makes it suitable also for the study of visual pigments in individual retinal rods. Using such an instrument, a number of the properties of the intact pigment-structure relationship can be examined directly.[1] First, spectra of rhodopsin can be obtained *in situ*. Second, the spectra of products of bleaching can be obtained *in situ* and the stability of intermediates formed can be compared with that described for extracted systems. Third, the homogeneity of the pigment can be studied from point to point in a single rod and from rod to rod. Fourth, the possibility of energy migration from an illuminated part of the rod to a non-illuminated part can be examined experimentally. Fifth, one may measure directly the photodichroism of rhodopsin in the intact outer segment and from these data compute the probable extent of molecular orientation. Finally, the density of rhodopsin in rods can be determined directly.

METHOD

The instrument is a revised model (17) of a sensitive scanning microspectrophotometer

[1] Preliminary experiments were begun by N. Chalazonitis in this laboratory during the winter 1959–60 using an earlier form of the apparatus (16).

described previously (13). Light from a 6 volt, 8 amp General Electric ribbon filament is dispersed by a Bausch and Lomb 250 mm grating monochromator (1200 lines/mm) which illuminates a microscope by the Koehler method. Zeiss ultrafluar optics (320×) were used in a Leitz microscope stand. A microphotographic beam splitter was placed above the ocular. The specimen was viewed through one light path of the beam splitter and photometry performed through the other. In the photometry path, an image is produced in the plane of a pair of circular defining apertures (6.5 μ diameter in the object plane) which were opened alternately at 60 cycles per second by a single aperture vibrating to and fro. This latter chopping aperture is driven by a Brown converter as described for a sensitive microfluorometer (18). The pair of defining apertures projected back to the object plane correspond to a reference and an experimental cuvette of conventional spectrophotometers. A lens above the aperture plane insures that the image of each aperture falls at the same spot on the photocathode of an end-on photomultiplier. This optical and chopping arrangement differs from that used previously and was introduced to allow easier viewing of the specimen during a spectral recording and to reduce the considerable vibration caused by the chopper driving mechanism of the previous system. The position of the pair of defining apertures was identified on an ocular reticule by placing human red blood cells (6 to 8 μ) in the object plane and noting their coordinates when a maximum signal was obtained from the photometer. A specimen could then be identically placed in the microscope field and measurements made as with the sensitive microfluorometer (18).

The microscope condenser and objective were used at equal aperture (NA = 0.4) and the monochromator exit slit was opened to just fill the condenser back aperture. This required an exit slit of 1.8 mm corresponding to a spectral bandwidth of 5 mμ. Polaroids and neutral density attenuators were introduced at the condenser back aperture where they were out of focus for the specimen plane of the microscope.

A cooling device was designed and constructed consisting of two hollow nylon collars which fit snugly around objective and condenser. Through these collars and their nozzles which formed a concentric ring about the front lenses of the optics, cooled nitrogen gas could be forced over the specimen. A microthermocouple sealed between slide and coverslip monitored temperatures which were controlled from + 27°C. to − 5°C. This method was developed to allow all parts of the slide to be examined at low temperatures without the problem of condensation of water vapor from the air which would obscure the specimen.

The electric signal from the photomultiplier consists of a sequence of pulses which proceed: measure, dark, reference, dark, measure, dark, etc. and represent by their pulse height the magnitude of signal coming from the photomultiplier during these respective times in the chopping cycle. The amplified difference between the measure and reference pulses operates a Varian recorder whose chart drive is synchronized with the wavelength drive of the monochromator. This chart drive is advanced manually through a gear train allowing one to vary the wavelength-scanning rate at will during a recording. The reference pulse is also compared to a standard pulse which by means of a feedback mechanism controls multiplier dynode voltage to normalize continuously the gain of the system against variation of light intensity, multiplier characteristics, etc. A time constant of 2 or 5 seconds was used, depending on the requirements of the particular experiment. Spectra are recorded directly in per cent absorption. The approximation OD $\cong$ 0.43× per cent absorption is accurate to 10 per cent at an optical density of 0.1 and is more accurate for smaller optical densities. The spectra recorded in these experiments were all at extinctions less than 0.1 optical density.

MATERIALS

Winter frogs (*Rana pipiens*) which were kept in hibernation at 10°C. were placed in total darkness at room temperature for 1 to 2 hours before each experiment. A frog was pithed and decapitated and the removed ocular globe was hemisected at the ora terminalis. The retina was carefully lifted away from the pigment epithelium in a watchglass filled with frog-Ringer's solution, pH 6.8. The retina was then moved with forceps to a quartz slide and tapped against the slide several times. This procedure liberates the outer segments of the rods, leaving a small drop of outer segment suspension in Ringer's solution on the slide. The slide was immediately closed with a coverslip and the preparation blotted with a piece of filter paper. With experience, sufficient pressure can be applied to the coverslip in blotting to compress the preparation to a separation of slide and coverslip slightly less than the outer segment thickness while avoiding compression sufficient to crush the segments. This is done to impede motion under the microscope and to provide nearly flat surfaces for the light beam to pass through for the measurements. The coverslip edges were sealed to the slide with wax in order to prevent evaporation of the Ringer's. These preparations always contained a few frog red blood cells whose spectra could be measured to ascertain the oxygen tension (19). They showed the system to be anaerobic. All preparations were made in red light provided by a microscope lamp filtered by a Corning number 2030 filter, transmitting wavelengths greater than 630 mμ.

The width of the slightly compressed outer segments was 8 to 10 μ. This was adequate to allow a defining aperture to be used for photometry which measured 6.5 μ in the object plane. The recordings were made with this aperture centered between the two highly refractile long edges of the outer segment in order to avoid optical artifacts. The spectra represent the difference between rod (outer segment) and non-rod containing adjacent space ("absolute" spectra). Wavelength was scanned between 350 and 650 mμ from short to long wavelength at rates of around 50 mμ/minute.

RESULTS AND DISCUSSION

Control. Spectra were made of bleached, borohydride reduced outer segments in order to determine to what degree residual pigments or optical artifacts might distort the spectra of the photopigments and their products (20) (Fig. 1).

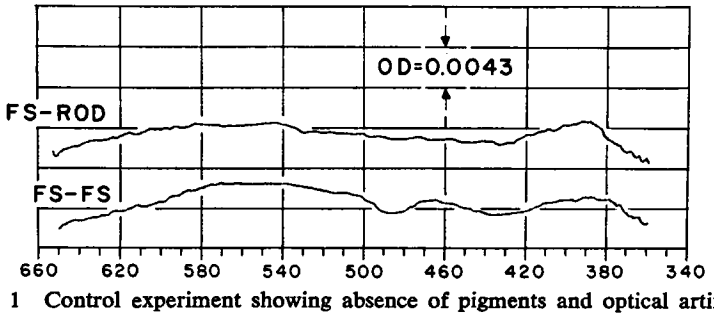

FIGURE 1 Control experiment showing absence of pigments and optical artifacts in bleached-reduced outer segments. FS-FS is a baseline and designates a recording made with both reference and measure beam passing part of the slide which is free of absorbing material (Free Space). FS-Rod is the spectrum of an outer segment which was exposed to intense light and reduced with borohydride before being detached from the retina.

Such outer segments were prepared by exposing a retina in Ringer's solution to bright light (focused B & L Nicolas illuminator) for 5 minutes and then to daylight for about an hour. Potassium borohydride was then added and slides prepared as described above. Spectra of such outer segments show the presence of no detectable pigments which absorb in the visible and near ultraviolet. Similar preparations were made without borohydride and were found to be identical. Spectra recorded at several different regions along the long axis of the rod showed that indeed the entire outer segment was empty of pigments to the limit of detectability of the instrument (ca. 0.1 per cent absorption).

Dark-Adapted Rods. Spectrophotometry of photosensitive pigments in low concentration is difficult because one faces the mutually contradictory necessities of maintaining the light intensity sufficiently high to obtain adequate signal-to-noise ratios for accurate recordings, and yet maintaining it sufficiently low to avoid bleaching the photosensitive pigment. In spectrophotometry of extracted rhodopsin, the optical density is usually high enough to obtain adequate signal-to-noise ratios and accurate spectra at low light levels with insignificant bleaching. At the approximately 0.05 OD across a single outer segment, it is necessary to use greater light intensity to obtain adequate signal-to-noise ratios for accurate spectra. Since initial experiments showed that spectral changes in dark-adapted rods were rapid at the unattenuated intensity of the tungsten source which had been used to measure respiratory enzymes with this apparatus (1 lambert), it was necessary to limit recording intensity to about 10 millilamberts at 500 mμ. This was achieved by the use of neutral density filters (21). Such an intensity, when used with a time constant of 5 seconds, was adequate to obtain spectra having large enough signal-to-noise ratios to make them readable to about 1 mμ in the region of 500 mμ. Under these conditions, the second and third repetitions of spectra from most rods showed little change from the first recording. There was, however, some variability in spectral stability from rod to rod.

The wavelength of maximum absorption for the majority of rods was 506 mμ with variations from 500 mμ to 511 mμ in the minority. This result is similar to the findings of Sidman (8) for smears and suspensions of frog rod outer segments where, in the apparent absence of pigment heterogeneity, an absorption peak was found at 508 mμ compared to 502 mμ for the extracted or sonicated rods. Arden (6) previously found that frog rod suspensions had their maximum absorption at 510 mμ but this result was difficult to interpret because of pigment heterogeneity. The variability in wavelength of peak absorption found from rod to rod in the present measurements may have been due to variation in the amount of bleaching-measuring light striking the photopigment.

In Fig. 2 is seen a series of recordings from an outer segment in which the photopigment was not so stable. Here the initial peak was at 508 mμ and spectral changes are initiated by the measuring light only. Judging from the magnitude of the shift

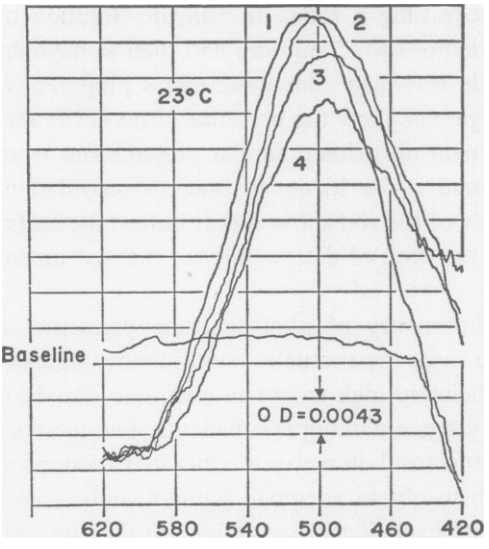

FIGURE 2 Spectra of rhodopsin in a single outer segment showing rapidity with which spectral changes may occur even at minimal light intensity. Initial peak at 508 mμ (Curve 1). Time between curves is about 2 minutes. The absorption peak shift from curve to curve suggests the admixture of a lower wavelength, photoproduct. The peak for rhodopsin is probably lowered from 510 mμ in Curve 1.

of the spectral peak on successive scans, the initial peak at 508 mμ may already have been shifted 2 or 3 mμ by exposure to wavelengths from 420 mμ to 508 mμ in the first half of the recording. As no absorption peaks were observed at longer than 511 mμ, it is likely that the true *in situ* absorption maximum of frog rhodopsin is 510 to 511 mμ.

Spectral Intermediate. The shifting spectral peak seen in Fig. 2 belies the presence of a product spectrum in the visible which mixes with the spectrum of the remaining rhodopsin. Fig. 3 shows a series of spectra which were recorded to shorter wavelengths in order to evaluate the formation of such products and done at low temperatures to retard thermal reactions that might lead to their disappearance. The first two curves whose peaks are marked "0 time" were recorded at the same

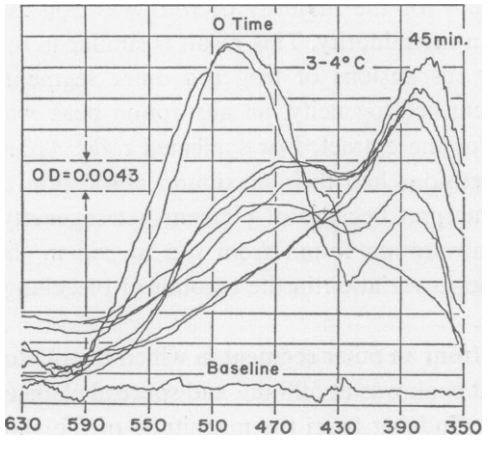

FIGURE 3 Spectral transitions in a single outer segment suggesting formation of an intermediate at 460 to 490 mμ. The product at 380 mμ required 45 minutes to form to completion at 3°C. Minimal bleaching light was insured by inserting a neutral density filter when the scan reached 410 mμ for the first two peaks at "0 time" only. Some of the curves do not come to the same zero because the cell moved in the course of the lengthy series of recordings.

reduced light level as in Fig. 2. The light attenuator was then removed and the reaction followed in succeeding spectra over 45 minutes. An intermediate peak is seen at about 460 mμ which gradually disappears as the stable product is formed at 380 mμ. Experiments at higher light intensity (to initially photolyse all the rhodopsin) were required to determine whether this 460 mμ peak was due to a chemical intermediate or was just some mixture of the 506 mμ reactant peak with the 380 mμ product peak. Fig. 4 shows a series of spectra done on a previously dark outer segment with

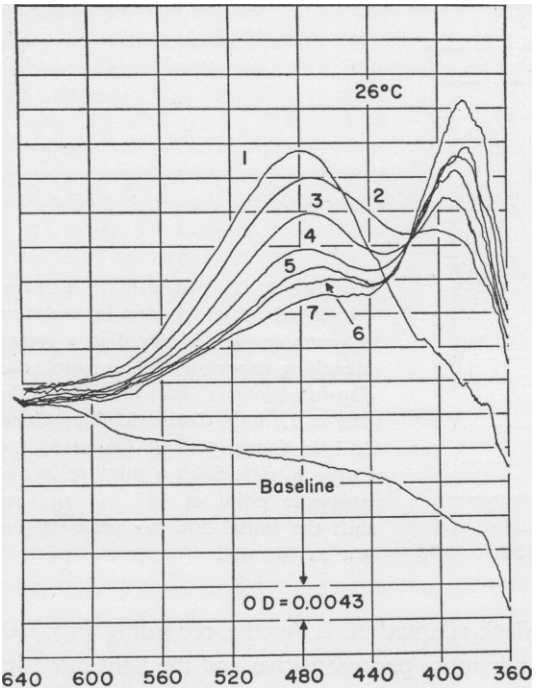

FIGURE 4 Spectral transition when recording was done with bright light at room temperature. The initial reactant seems to be at 480 mμ and an isosbestic point occurs at about 420 mμ. The reason Curve 1 does not pass through the isosbestic point is discussed in the text.

recording light 17 × the intensity used in Fig. 2. Here the initial spectrum shows a lone peak at 480 mμ. The disappearance of this peak is associated with the formation of the stable product at 380 mμ. One sees what appears to be an isosbestic point at 415 to 420 mμ.

An isosbestic point can exist if there are only two overlapping spectral species present, one giving rise to the other. Since the first curve (Fig. 4) did not pass through the isosbestic point and an isosbestic point for such a series of spectra had not been described for extracted pigments, the nature of the longer wavelength peak at first remained uncertain. In order to clarify this point, Fig. 5 was constructed. Curve 1 and curve 6 are, respectively, actual initial dark-adapted and final product curves obtained in an experiment bleaching dark-adapted rods. The intermediate curves were obtained by graphically combining various portions of curves 1 and 6 to simulate spectra expected for the simple reaction 506 mμ → 380 mμ. The isos-

bestic point for this hypothetical series of spectra is 447 mμ (compared with 415 to 420 mμ for the rod) and the most extreme peak shift from 506 mμ is to about 490 mμ (compared to about 460 mμ for the rod). It therefore seems the initial reactant in the series recorded in Fig. 4 must have a peak some 30 mμ shorter than rhodopsin, and this set of curves cannot represent simply a mixture of a 506 mμ reactant with a 380 mμ product. Curve 1 of Fig. 4 no doubt fails to pass through the isosbestic point because the reactant that belongs to the isosbestic point had not yet formed when this curve was begun; *i.e.*, the part of curve 1 from 360 mμ to about

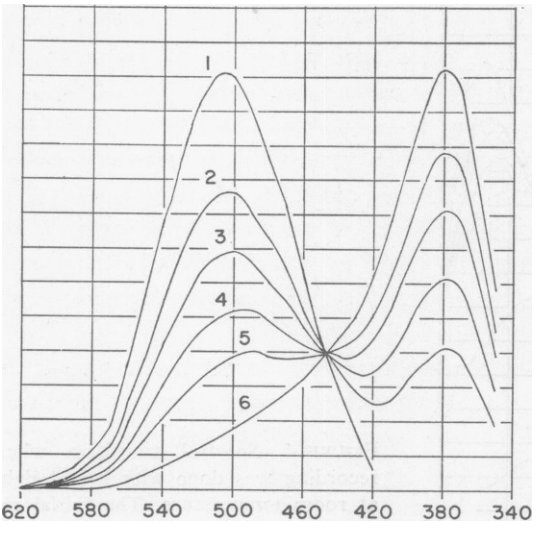

FIGURE 5 Hypothetical mixture curves of initial rhodopsin and final retinene spectra observed in a typical bleaching experiment. The ratio (rhodopsin/retinene) for the curves is: Curve 1, 1/0; Curve 2, 2/1; Curve 3, 1/1; Curve 4, 1/2; Curve 5, 1/4; Curve 6, 0/1. Such a mixture has an isosbestic point at 447 mμ and can shift the initial 506 mμ peak only as low as 490 mμ.

460 mμ is from rhodopsin in the dark-adapted rod. As the recording proceeds through wavelengths where rhodopsin is more photosensitive and the light more intense, the photopigment is photolyzed. Because of the intensity of the recording light, the photolysis has occurred during the first half of a single scan (about 1 minute exposure) and nearly to completion. For the above reasons it is concluded that a chemical intermediate has been demonstrated in intact outer segments that is similar to or identical with metarhodopsin in low temperature solutions and in gels (22). The wavelength 480 mμ is not to be regarded, however, as an accurate peak for metarhodopsin *in situ* because of the rapidity with which the spectra were changing.

It is of interest to compare Fig. 4 with an experiment done by Wald and Hubbard (23) on rhodopsin in solution under similar conditions. An initial spectrum was recorded at 26°C by these workers 30 seconds after one-half minute of bleaching light which was adequate to complete the light reaction. The shape and wavelength maxima of the curves in each set of data go through a similar sequence, but the sequence takes about 5 times as long in the intact outer segment. This might be due to the relative unavailability of water for addition across the chromophore Schiff

base linkage to the protein. This should be the case if chromophore contact with the aqueous phase is hindered by a lipid layer.

The intermediate lumirhodopsin (515 mμ) (24) was probably too unstable, if it exists *in situ* to be seen over the time course of these spectra. The significance of indicator yellow has been clarified *in vitro* (25, 26) but whether it exists and has some function *in situ* has not been demonstrated. Neither acid (440 mμ) nor alkaline (365 mμ) indicator yellow (27) was identified in the present experiments. The acid form occurs below pH 4 and is stable for hours *in vitro* (28). Alkaline indicator yellow at near neutral pH or in alkali is less stable (28) and in aqueous solution hydrolyzes to form retinene or some steady-state mixture of retinene and alkaline indicator yellow (2). If the *in vitro* information applies, one would not expect to see acid indicator yellow formed at the pH of these experiments. If the chemical mechanism of rhodopsin's thermal reactions involving hydrolysis of a Schiff base holds, alkaline indicator yellow must necessarily be formed before retinene is liberated (2). However, only the stable peak at 380 mμ indicative of retinene was seen. If the peak of alkaline indicator yellow is at 365 mμ in the intact outer segment, its cleavage after hydration may be faster than could be detected by kinetic spectra separated by about one minute, or its steady state concentration may have been too low to detect using this technique.

Homogeneity. Except for the variability in the initial spectral peak of rhodopsin discussed above, results obtained from different outer segments and from various points along the long axis of a single segment did not differ significantly. Frog rod outer segments therefore seem to be spectrally homogeneous individually and collectively, corroborating selective bleaching experiments (8) done on suspensions of frog rod outer segments.

No spectra of the "green rods" as seen on Denton and Wyllie's photographs (10) were recorded in more than 100 segments examined.[2] This may have been just a question of probability or it may be that "green" outer segments are more tightly attached to their inner segments and were not liberated by the preparation procedure as were the "pink" ones.

Microbeam Illumination. The ability of three or fewer photons to produce visual excitation in the human eye suggests that at least one in every three rhodopsin molecules couples to a nerve impulse. The presence of a nerve impulse implies change at the cell membrane. The photon-absorbing rhodopsin molecule which triggers this impulse may, however, be situated one or more micra away from the cell surface (4). There must, therefore, be a mechanism of transferring the excitation from the rhodopsin molecule to the cell membrane over considerable distances compared to molecular dimensions.

Electron micrographs show the rod outer segment consists of stacks of discs (3, 4), the lamellae, which are of macromolecular thickness and probably con-

[2] Several have been seen by Chalazonitis (16).

tain the rhodopsin. The concentration of rhodopsin in rods is great enough to suggest a crystalline structure. These data led Hagins and Jennings to investigate radiationless migration of electronic excitation (29) as a mechanism of energy transfer from the photon recipient rhodopsin molecule to the cell membrane. They approached this problem indirectly by looking for spread of fluorescence excitation in bleached rods and by a theoretical analysis of rod photodichroism. The results lead them to conclude that radiationless migration of electronic excitation probably does not occur in rods. No one, however, has been able to examine this possibility directly by looking for spread of bleaching changes from an illuminated to an unilluminated part of the same outer segment. This experiment was therefore done.

Parts of outer segments were strongly stimulated by illumination with a 3 μ diameter microbeam while spectra with weak light were followed in other parts of the same rod and compared with spectra obtained from the stimulated area following stimulation. The full intensity of the source was used at 500 mμ (1 lambert, 5 mμ bandwidth) for periods of 15 to 120 seconds for microbeam stimulation. Some rods were examined for spread of bleaching spectra in the direction of the rod long axis (Fig. 7), *i.e.* between the lamellae. Others were examined for the more likely possibility of spread of bleaching spectra perpendicular to the rod long axis, *i.e.* within the same group of stimulated lamellae. For this later measurement it was necessary to compress the rods to a width of about 12 μ to accommodate the adjacent microbeam and measuring beam.

No spread of bleaching spectra was observed either along or perpendicular to the outer segment long axis at distances greater than 2 μ from the stimulated area. Inaccuracies in aperture positioning and light-scattering within the outer segment made it impossible to obtain reliable results over shorter distances. Failure to observe spread perpendicular to the long axis could be inconclusive because compression of the outer segments might alter the contiguity necessary for energy migration.

Dichroism of Rhodopsin In Situ. Dichroism in retinal rod outer segments was described by Schmidt (30) in 1929. This phenomenon was again described by Denton (31) and recently associated more elegantly by him with the visual pigment rhodopsin (11). The observation of dichroism makes it clear that the absorption of light along the rod long axis by rhodopsin *in situ* is increased by some factor over that which the identical pathlength and concentration of pigment would absorb if randomly oriented as in solution. The value of this factor has been estimated, for instance, by Hagins (32) for rabbit rhodopsin in rods compared to solution and was found to be 1.6. Since this experimental estimate exceeds the theoretical maximum value of 1.5 for perfect orientation in two dimensions (33), it is desirable to determine the orientation by a more direct method not involving possible losses of pigment in an extraction procedure.

The preceding data in this paper were obtained without regard for the relation between the rod long axis and the major axis of the partially polarized light pro-

duced by the Bausch and Lomb grating monochromator. The maximum absorptions measured, therefore, vary with the angle between these axes. In addition, no single measurement of maximum absorption thus recorded could give sufficient information to compute the concentration of pigment in the rod as can be done with measurements of extinction in solution from the Beer-Lambert law. One is obliged to make two measurements at each position to determine the rhodopsin concentration in the dichroic rod. In the following experiments, these two measurements are made in linearly polarized light, the electric vector of which is oriented first parallel ($\|$) and then perpendicular ($\perp$) to the long axis of the outer segment (Fig. 6). As

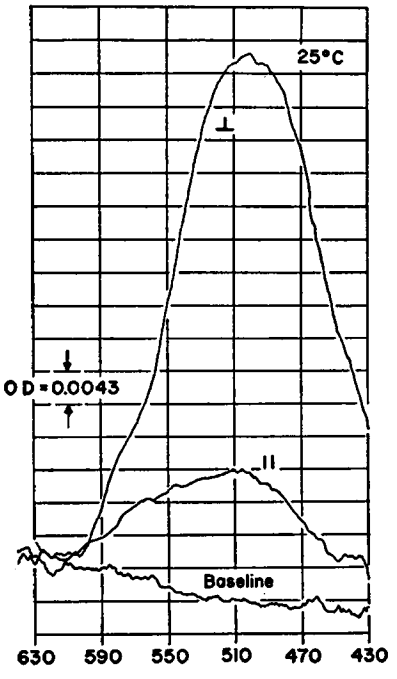

FIGURE 6 Spectra recorded in polarized light. || denotes recording made with light electric vector parallel to the rod long axis. ⊥ was recorded after || with light electric vector perpendicular to the rod long axis. The ratio $\epsilon_\perp/\epsilon_\parallel$ is 5.3.

in the previous experiments, it is desired to obtain spectra which are as little altered as possible by the measuring light. To fulfill that need for this experiment is one step more difficult than in the previous experiments, since one needs to record two nearly unaltered photopigment spectra at the same point on an outer segment. Since these spectra are recorded one after the other, the possibility exists that some of the light from the first recording will photolyse some of the pigment to be measured on the second recording, especially if the rod axis and light electric vector are not perfectly aligned. The dichroic ratio $\epsilon_\perp/\epsilon_\parallel$ measured in Fig. 6 therefore represents a lower limit for this quantity since $\epsilon_\parallel$ was measured first, giving $\epsilon_\perp$ the possibility of losing relatively more of its extinction than $\epsilon_\parallel$. The dichroic ratio from Fig. 6 is 5.3. This corresponds to an increase in absorption by a factor of 1.4 due to the

orientation in the rod as compared to 1.5 for perfect orientation in two dimensions. The wavelength of maximum absorption of these spectra tend to be nearer 500 mμ than those recorded with unpolarized light. This is probably due to slight bleaching as discussed under *Dark-Adapted Rods.* In other recordings in polarized light, dichroic ratios varied from about 3 to 6. The lower ratios were invariably associated with lower values for both extinctions, indicating those ratios were artifactually lowered by too great compression of the rods. These spectra in polarized light identify the dichroism of rods with rhodopsin as shown by Denton (11).

Molecular Orientation and Rhodopsin Concentration. It is of interest to know both the concentration of rhodopsin and the orientation of rhodopsin molecules in the intact rod. Since the computation of these quantities involves one as a function of the other, we cannot avoid knowing something about the molecular orientation if we try to compute the pigment concentration. It is for this reason, in fact, that two measurements ($\epsilon_\perp$ and $\epsilon_\parallel$) are needed for each determination. The derivation of the simultaneous equations relating the desired concentration and orientation to the measured extinctions, $\epsilon_\perp$ and $\epsilon_\parallel$, is given in the Appendix. One must assume some characteristic about the distribution of the orientation of the individual molecular absorptions to make the computation. The outer segment is considered to be a cylinder (Fig. 7*a*) composed of a stack of flat circular discs (the lamellae). The protein component of the lamellae is supposed to contain the rhodopsin molecules in close packed or nearly close packed array. The membranes of which the rhodopsin is presumably a part are only about 40A. thick (3, 4), nearly the dimensions of a spherical molecule the molecular weight of rhodopsin. One may consider each of these membranes as a circular cylinder or disc and draw a picture of how the dichroic molecules might be expected to lie in one such disc (Fig. 7*b*). To abstract somewhat, we are considering a field of vectors (molecular absorption vectors) of restricted orientation freedom. Since these are vectors, they are not altered by arranging them so as to have all their tails meet as in Fig. 7*c*. Looking down the long axis of the outer segment perpendicular to the discs, no preferred direction of orientation is seen; that is, the number of vectors in each arbitrarily small plane angle is the same. This effect could conceivably be produced by superimposing many individual discs whose molecules do have a preferred orientation when one looks along the axis of symmetry (as in Fig. 7*d*). If these discs have their preferred orientation axes crossed with respect to each other, an appearance of randomness could result in the stack which is the outer segment. The calculation to follow does not distinguish this possibility from that illustrated in Fig. 7*c*, upon which the calculation is based.

If one looks at a section through the edge of a disc (Fig. 7*e*), it is seen that the experimentally measured dichroism could be produced if the vectors either lay all at the same angle θ_1, with respect to the plane of the disc (Model 1) or if the vectors were distributed in some way over an angle θ_1 as in Model 2. If in the rod the rhodopsin molecules are contained between two other structures which limit their

Brownian rotation (Fig. 7*f*) so that the molecules behave like sticks in a box that is too low to allow them to stand upright, one might expect the situation depicted in Model 2 to obtain. If the rhodopsin molecules are bound together in a rigid structure (Fig. 7*g*) which does not allow Brownian rotation, the situation depicted in Model 1 may be more accurate. The angle θ_1 to which the molecules would be restricted is computed to be 16° for Model 1 and 23° for Model 2, assuming a dichroic ratio of 6 (Table 1). The rhodopsin concentration is computed assuming the molar

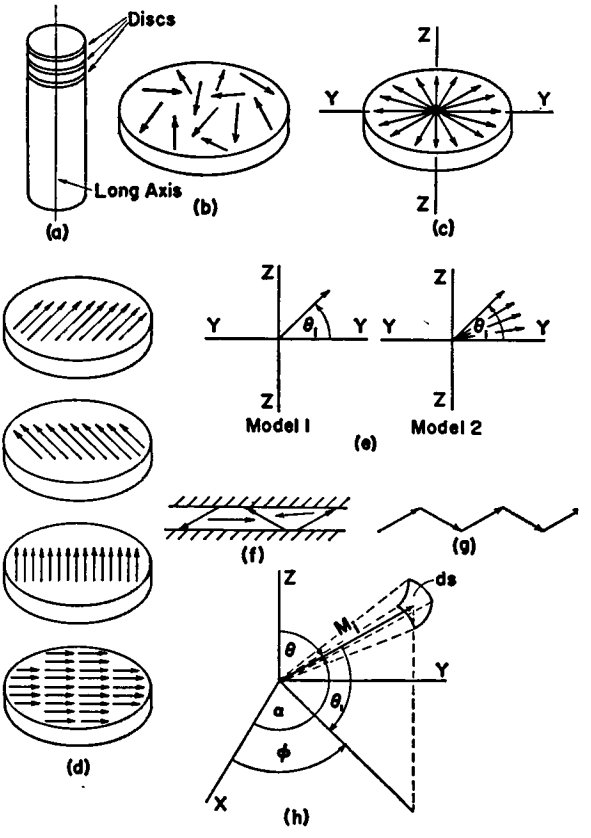

FIGURE 7 See text *Molecular Orientation and Rhodopsin Concentration* for description.

extinction for frog rhodopsin in rods is 46,000 as in solution and the path length through the rod is 5 μ. The pigment concentration given may be regarded accurate to about ± 20 per cent of the value 2.5 mM. This is due to the uncertainty in estimating the thickness (pathlength) of the outer segment wihout using interference microscopy. This concentration is slightly higher than values obtained by the extraction–cell count method (34). At this concentration and orientation of rhodopsin,

outer segments have an axial density of 0.09/5 μ or 0.9/50 μ showing that frog rods absorb nearly all of the light striking them.

A possible objection to the above computations is that the molecular extinction of rhodopsin may be a tensor quantity instead of a vector. This would not be surprising if the conjugation path of the rhodopsin absorption is as twisted as that of 11-cis retinene, its chromophore. If such were the case, the measured absorption of light polarized parallel to the rod long axis might be due to a tensor property of the

TABLE I

	$\epsilon_\perp/\epsilon_\parallel$	θ_1	Rhodopsin concentration	Per cent of maximum extinction for $\theta_1 = 0$
Model 1	6.0	16°	2.5mm	92
Model 2	6.0	23°	2.5mm	92

rhodopsin extinction and not to a deviation of vector absorptions from a plane. The degree of orientation computed may therefore be only a lower limit. It is difficult to evaluate this mater, however, since we know so little even about the electronic mechanism responsible for the 130 mμ shift from retinene's 380 mμ absorption maximum to the 510 mμ peak or rhodopsin.

CONCLUSION

The above studies demonstrate that in several ways the *in situ* behavior of the pigments of the frog photoreceptor differs from that in solution. The absorption maximum of rhodopsin is shifted about 8 mμ toward the red. The persistence of the intermediate, metarhodopsin, is increased by a factor of 5 over its lifetime in solution on bleaching rhodopsin. No other intermediate such as indicator yellow is seen in the conversion of metarhodopsin to retinene. Attempts made to measure energy migration in the form of spread of bleaching spectra from an illuminated to a dark part of a single photoreceptor showed that this does not occur over distances of 2μ or greater.

Spectra recorded with linearly polarized light show that nearly all of the rhodopsin absorption *in situ* can be attributed to interaction of the chromophore with the electric vector which vibrates in a plane perpendicular to the long axis of the photoreceptor cell. The experimentally determined dichroic ratio is $\epsilon_\perp/\epsilon_\parallel = 6$. If the absorption property of rhodopsin is treated mathematically as a vector, it can be concluded that the rotational freedom of the rhodopsin molecules in the intact retinal rod is severely restricted and that their absorption vectors must lie almost perfectly perpendicular to the long axis of the rod, *i.e.* in the planes of the lamellae seen by electron microscopy (4) and inferred from birefringence studies (30). The *in situ* concentration of rhodopsin for the rods measured is 2 to 3mM. The effect of

this concentration on the absorption of light would however be increased by a factor of 1.4 for light travelling axially down the rod due to the above measured orientation. It is alternatively suggested that the dichroic ratio measured could be caused by a tensor absorption by identically oriented chromophores instead of vector absorption by not quite identically oriented chromophores. Thus native rhodopsin may be crystalline.

Finally, several words of reservation should be observed regarding physiological usefulness of these data. It has been pointed out that light traveling axially down the external segment of rods or cones encounters steps of refractive index due to the alternation of aqueous and lipid layers (35). The present measurements would not encounter these steps since the direction of propagation was normal to the long axis. Theories concerning the mechanism of color vision have frequently included participation of a passively absorbing dye (*e.g.* indicator yellow, 440 mμ) which might alter the wavelength of maximum sensitivity of an "active" photopigment like rhodopsin or iodopsin in rods and cones. While the data of these experiments do not support such participation of indicator yellow at pH 6.8 in rods, they in no way rule it out, particularly since the stability and color of indicator yellow is pH-dependent. The *in vivo* environment of the pigment may be significantly more acid and could conceivably favor accumulation of this substance.

APPENDIX

The problem may be stated as follows: suppose the extinction of a solution of pigment at a given concentration is ϵ; the same in polarized and unpolarized light. If it were possible to align all of the pigment absorption axes in the same direction and measure again their extinction with linear polarized light whose electric vector is parallel to the absorption axes, we would find an extinction of $M = 3\epsilon$ for the same concentration. This result is intuitive but will be proved below as a special case in the more general formulation. If the electric light vector is perpendicular to such completely aligned absorption vectors, the extinction will, of course, be zero. There is, therefore, a range of extinctions from zero to three times the classic Beer's law extinction that might be obtained for the same concentration of pigment depending on how the molecular absorption axes are distributed in the structure being studied. One cannot, of course, expect to determine the concentration of a pigment by a simple Beer's law computation if it is not randomly distributed as in solution. What then is the relation between the concentration of the non-randomly oriented molecules and something we can measure? It will be shown that M can be derived from measurements if enough is known about the *degree* of molecular orientation. Since $M = 3\epsilon$, ϵ can be determined and hence the concentration also will be obtained.

Consider a field of vectors, $\overline{M}_i$, all of the same magnitude in the Cartesian coordinate system (x, y, z). Each vector and the element of solid angle, ds, which contains it has the direction (θ, α) or in spherical coordinates (θ, φ) (Fig. 7*f*). $\overline{M}_i$ represents the extinction of a molecule to linear polarized light whose electric vector points in the same direction as $\overline{M}_i$. If the extinction of this same molecule is measured in light polarized along the x or z axis, the extinction will be, respectively, $\epsilon i_x = |\overline{M}_i| \cos^2 \alpha$ or $\epsilon i_z = |\overline{M}_i| \cos^2 \theta$. The summed extinction due to all vectors in the space (x, y, z) will be

$$\epsilon_x = \frac{M \iint g(\theta, \phi) \cos^2 \alpha \, ds}{\iint ds}; \qquad \epsilon_z = \frac{M \iint g(\theta, \phi) \cos^2 \theta \, ds}{\iint ds} \tag{1}$$

where $M = n M_i$ and n is the number of molecules present. $g(\theta, \varphi)$ is a distribution function which tells whether some solid angles have more vectors in them than others. Since, for the computation to follow, the molecules are assumed distributed uniformly over the solid angle where they are defined, the number of vectors per solid angle is equal for all solid angles and $g(\theta, \varphi) = 1$.

Working in spherical coordinates, $\cos^2\alpha = \sin^2\theta \cos^2\varphi$ and $ds = \sin\theta d\theta d\varphi$. Therefore:

$$\epsilon_x = \frac{M \iint \cos^2 \alpha \, ds}{\iint ds} = \frac{M \iint \cos^2 \phi \sin^3 \theta \, d\theta \, d\phi}{\iint \sin \theta \, d\theta \, d\phi} \tag{2}$$

$$\epsilon_z = \frac{M \iint \cos^2 \theta \, ds}{\iint ds} = \frac{M \iint \cos^2 \theta \sin \theta \, d\theta \, d\phi}{\iint \sin \theta \, d\theta \, d\phi} \tag{3}$$

A third expression can be written for ϵ_y but since the rods have cylindrical symmetry, $\epsilon_x = \epsilon_y$. As will be seen, the angle ϕ drops out of the integration and equations (2) and (3) become a set of two simultaneous equations in the two unknowns M and θ. ϵ_x and ϵ_z are obtained by measurement. If M is obtained, ϵ, the Beer's law extinction can be determined from the conventional Beer-Lambert equation $\epsilon = \epsilon_0 cl$ where ϵ_0 is the molar extinction, c, the molar concentration, l, the pathlength, and ϵ, the measured extinction.

First, it may be demonstrated that these equations give the correct result $\epsilon = 1/3\ M$ for solutions of pigment (uniform 3-dimensional distribution). The limits for the integration should cover $0 \leq \theta \leq \pi$, $0 \leq \phi < 2\pi$. Because of spherical symmetry, $\epsilon_x = \epsilon_y = \epsilon_z$.

$$\epsilon_z = \frac{M \int_0^{2\pi} \int_0^{\pi} \cos^2 \theta \sin \theta \, d\theta \, d\phi}{\int_0^{2\pi} \int_0^{\pi} \sin \theta \, d\theta \, d\phi} = \frac{M}{3} \tag{4}$$

Now consider Model 1, Fig. 7*e*. The angle θ is a constant, θ_1, and is therefore not to be integrated over. From equation (2):

$$\epsilon_x = \epsilon_y = \frac{\sin^3 \theta M \int_0^{2\pi} \cos^2 \phi \, d\phi}{\sin \theta \int_0^{2\pi} d\phi} = \frac{M}{2} \cos^2 \theta_1 \tag{5}$$

From equation (3):

$$\epsilon_z = \frac{M \iint \cos^2 \theta \sin \theta \, d\theta \, d\phi}{\iint \sin \theta \, d\theta \, d\phi} = M \sin^2 \theta_1 \tag{6}$$

Finally, consider Model 2, Fig. 7*e*. The molecular vectors are random over some angle θ_1.

$$\epsilon_x = \epsilon_y = \frac{2M \int_0^{2\pi} \int_{\pi/2-\theta_1}^{\pi/2} \cos^2 \phi \sin^3 \theta \, d\theta \, d\phi}{2 \int_0^{2\pi} \int_{\pi/2-\theta_1}^{\pi/2} \sin \theta \, d\theta \, d\phi} = \frac{M}{2}\left(1 - \frac{\sin^2 \theta_1}{3}\right) \tag{7}$$

$$\epsilon_z = \frac{M \int_{\pi/2-\theta_1}^{\pi/2} \cos^2 \theta \sin \theta \, d\theta}{\int_{\pi/2-\theta_1}^{\pi/2} \sin \theta \, d\theta} = \frac{M}{3} \sin^2 \theta_1 \tag{8}$$

In the experiments described, ϵ_y and ϵ_z are measured. M and θ_1 are then determined by solving the set of simultaneous equations 5, 6 or 7, 8, respectively for Model 1 or Model 2.

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