Orientation of Intermediates in the Bleaching of Shear-Oriented Rhodopsin

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ABSTRACT Cattle rhodopsin can be highly oriented by shearing a wet paste of digitonin micelles of this visual pigment between two quartz slides. This orients the rhodopsin micelles so that their chromophores lie mainly parallel to the direction of shear. In such preparations the orientation of rhodopsin and intermediates of its bleaching by light have been measured with plane-polarized light from −195°C to room temperature. The chromophore maintains essentially the same orientation as in rhodopsin in all the intermediates of bleaching: bathorhodopsin (prelumirhodopsin), lumirhodopsin, and metarhodopsins I and II. When, however, the retinaldehyde chromophore is hydrolyzed from opsin in the presence of hydroxylamine, the retinaldehyde oxime that results rotates so as to lie mainly across the direction of shear. That is, the retinal oxime, though free, orients itself upon the oriented matrix of the opsin-digitonin micelles. These experiments show the rhodopsin-digitonin micelle to be markedly asymmetric, with the chromophore lying parallel to its long axis. The asymmetry could originate in the formation of the micelle, in rhodopsin itself, or by its linear polymerization under the conditions of the experiment. If rhodopsin itself is markedly asymmetric, for which there is some evidence, then, since in the rod outer segments its chromophores lie parallel to the disk membranes, the molecules themselves must lie with their long axes parallel to the membranes.

The function of rhodopsin in the retina appears to depend as much on its orientation as on its sensitivity to light. The outer segments of the retinal rods are made up of a great stack of double-membrane disks, essentially flattened sacs, enclosed within the plasma membrane that covers the entire rod. A single rod outer segment may contain about 1,000 disks, perhaps 2,000 membranes, all lying transversely to the anatomical and optical axis of the rod. The dry weight of the disk membranes is made up 40–50% of mixed phospholipids, and about 40% of protein. Of this protein, some 80–90% is rhodopsin. Rhodopsin itself is a conjugated protein, its protein component, opsin, bearing as chromophore the haplocarotenoid, 11-*cis* retinal.

Rhodopsin is highly oriented in the outer segment, so that its chromophores lie primarily in the planes of the disk membranes. In this position rhodopsin

absorbs light about 50% more effectively than if it were randomly oriented. In view of the somewhat bent and twisted shape of the chromophore, the orientation is nearly perfect. The ratio of orientation vectors, transverse:axial is about 9:1 (Wald et al., 1963). For some, perhaps all visual functions, this orientation is decisive; thus, for example, for the appearance of the early receptor potential (ERP) (Cone and Brown, 1967). Heating rat retinas to 58°C for 10 min completely abolishes the ERP. Under these circumstances the rhodopsin remains, but has been completely disoriented.

Highly oriented as the rhodopsin molecules are in the disk membranes, they are not fixed in position. Brown (1972) has shown that they are in constant Brownian rotation, a motion that is blocked by fixation of the retina with such a bifunctional reagent as glutaraldehyde. The relaxation time in the unfixed retina is about 20 μs, the rhodopsin molecules behaving as though they were floating in an oily medium about as viscous as olive oil (Cone, 1972; cf. also Blasie, 1972). The fatty acids of the disk membrane phospholipids are unusually unsaturated, among the most unsaturated found in any biological membranes, and are correspondingly fluid (Anderson and Sperling, 1971). Also the amino acids of opsin in the immediate neighborhood of the chromophore are highly lipophilic (Bownds, 1967). It seems probable that such a lipophilic area of rhodopsin in contact with or embedded in a phospholipid matrix keeps the rhodopsin oriented even while afloat, for throughout its rotations its chromophores remain in the planes of the disk membranes.[1]

We have recently described two methods for orienting rhodopsin outside the retina (Wright et al., 1972). Rhodopsin, as a typical membrane protein, is insoluble in water and all usual solvents. It is brought into aqueous solution by a variety of detergents, in our case, digitonin. In such digitonin solutions, it forms a large micelle of particle weight approximately 270,000 g/mol, each containing one rhodopsin molecule of molecular weight about 40,000 g/mol (Hubbard, 1953–54; Daemen et al., 1972).

In our first method for orienting rhodopsin, a solution in digitonin was mixed with a gelatin solution, and the mixture poured onto acetate film or glass. When this film was dried, the rhodopsin molecules were found to be aligned with their chromophores in the plane of the gelatin film. When rhodopsin in a wet film was bleached in the presence of hydroxylamine and redried, the retinaldehyde oxime which resulted was oriented more perpen-

[1] Removing virtually all the phospholipids from rhodopsin preparations is a herculean task (Krinsky, 1958). This has now been done, however, in a number of laboratories (Zorn and Futterman, 1971; Borggreven et al., 1972; Hong and Hubbell, 1972). One particular incentive was to test the report of Poincelot et al. (1970) that in cattle rhodopsin the retinaldehyde chromophore is bound to phosphatidylethanolamine, rather than to an epsilon-amino group of lysine, as originally reported by Bownds (1967). In fact, rhodopsin can survive virtually complete delipidation; and the binding of retinal to lysine has lately been confirmed also by Prof. Abrahamson and co-workers (Fager et al., 1972).

dicularly to the plane of the film. These orientations resemble those in the disk membranes of rod outer segments.

The second method achieves a still higher degree of molecular orientation. When a wet paste of rhodopsin-digitonin micelles is sheared between glass or quartz slides, this simple mechanical procedure orients the rhodopsin chromophores parallel to the direction of shear. A high degree of orientation can be achieved in this way, the dichroic ratios (the ratios of absorbance of plane-polarized light at 500 nm, parallel versus perpendicular to the direction of shear) ranging up to 5. Clearly this behavior implies a large asymmetry of the rhodopsin-digitonin micelles under the conditions of preparation.

The only apparent action of light upon rhodopsin is to isomerize its chromophore from 11-*cis* to all-*trans*. The first product, bathorhodopsin (formerly prelumirhodopsin; cf. Yoshizawa, 1972), has λ_{max} about 543 nm, and is stable below −150°C (Yoshizawa and Wald, 1963; Busch et al., 1972). On warming in darkness, the bathorhodopsin bleaches over a sequence of intermediates, each of which represents a stage in the step-wise opening up of the tertiary structure of opsin: lumirhodopsin, stable between −140°C and −40°C; metarhodopsin I, stable from −35°C to −15°C; and metarhodopsin II, reasonably stable between −10° and 0°C (Matthews et al., 1963–64). Finally, on further warming, the all-*trans* retinal is hydrolyzed from opsin and in the presence of hydroxylamine is immediately converted to retinaldehyde oxime.

The present paper is concerned with the orientations of the chromophores in shear-oriented rhodopsin, the intermediates of its bleaching by light, and the free retinaldehyde oxime that results from bleaching wet rhodopsin in the presence of hydroxylamine.

METHODS

Preparation

Cattle rod outer segments were isolated using a modification of the method of Matthews et al. (1963–64), and the rhodopsin was extracted into 2% digitonin in 0.06 M phosphate buffer, pH 7.1. The extract was centrifuged for 16 h at about 105,000 *g* in a Spinco Model L Ultracentrifuge (Beckman Instruments Inc. Spinco Div., Palo Alto, Calif.). This treatment sediments the rhodopsin-digitonin micelles, which form a transparent gel at the bottom of the centrifuge tube. After the supernate was decanted, this gel was dried in a desiccator over calcium sulphate (Drierite) (W. A. Hammond Drierite Co., Xenia, Ohio) at room temperature. The dried gel was kept dark in a deep freeze. All operations were performed in dim red light.

Shear Orientation

For this, a few bits of dry rhodopsin-digitonin gel, about 0.5 mm in diameter, were moistened for ½–3 min with a drop of 1 M hydroxylamine (NH_2OH-HCl neutralized to pH 7 with concentrated NaOH). Excess hydroxylamine was drawn off with filter

paper, and the resulting rhodopsin-digitonin paste was covered with a few drops of glycerol for about 30 s. The glycerol keeps the paste from cracking and becoming turbid at liquid nitrogen temperature. Excess glycerol was drawn off with filter paper, and the paste was sandwiched between two quartz slides (2.5 × 1.0 × 0.1 cm).

This sandwich was placed in the shearing apparatus shown in Fig. 1, which held the two slides tightly together while one could be pushed across the other. To perform the shearing, this apparatus was placed in the sample beam of a Cary 11 recording

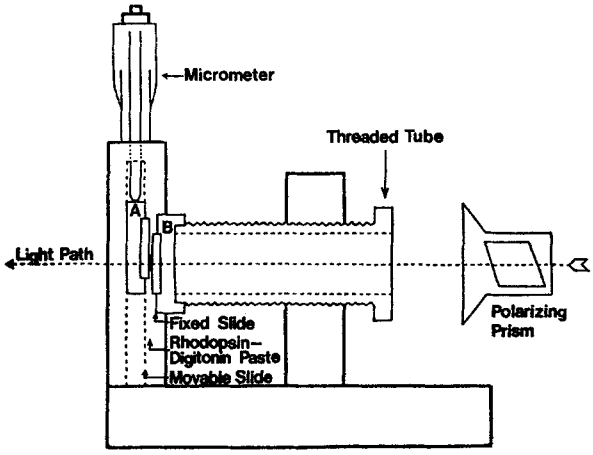

FIGURE 1

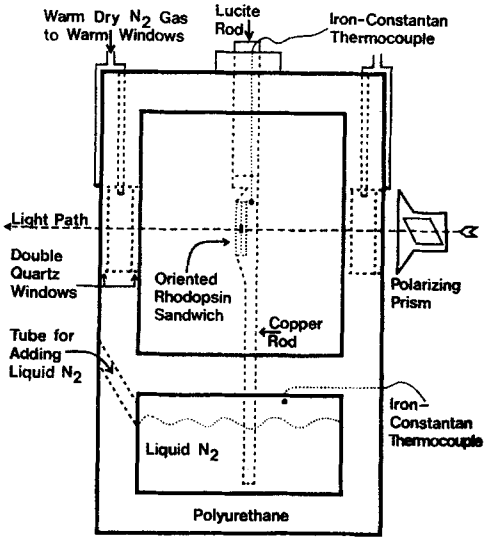

FIGURE 2

FIGURE 1. Rig for orienting rhodopsin. Two quartz slides enclosing a small amount of rhodopsin-digitonin paste are fitted into slots on the faces of plates A and B. Plate A is mounted on a track so that it can be pushed downward by turning the micrometer. Plate B is mounted with a Teflon bearing on the end of a threaded brass tube, on a track that keeps it from rotating when the tube is turned. Screwing in the tube presses plate B evenly against plate A. The shearing forces created when the micrometer screw is advanced, pushing plate A downward across plate B, orient the rhodopsin. Light for monitoring the degree of orientation passes through the polarizing prism, the hollow tube, holes cut in plates A and B, and the rhodopsin sandwich. For this the rig was mounted in the sample compartment of a Cary Model 11 Spectrophotometer, and the orientation of rhodopsin checked by measuring its absorbance at 500 nm in plane-polarized light parallel (0°) and perpendicular (90°) to the direction of shear.

FIGURE 2. Polyurethane cold unit for examining shear-oriented rhodopsin at −195°C and higher temperatures. The rhodopsin sandwich is taped to a copper rod that connects the upper sample compartment with the lower cold well. Liquid nitrogen is poured into the lower compartment until the iron-constantan thermocouple at the top of it registers near −195°C. Another iron-constantan thermocouple is taped to the side of the rhodopsin sandwich to register its temperature. After measurements have been completed at −195°C, the temperature of the unit can be raised by blowing dry room air into the opening for filling the lower compartment. Warmed dry nitrogen gas is blown continuously between the double quartz windows to retard frosting.

spectrophotometer (Cary Instruments, Monrovia, Calif.) so that light passed through the plane of the film. The absorption at 500 nm was monitored in plane-polarized light with its electric vector either parallel (0°) or perpendicular (90°) to the direction of shear. One slide was pushed across the other until the dichroic ratio, measured in these planes, had risen to 3–5. Then the slide sandwich was removed from the apparatus and the edges were sealed with Duco cement.

The length of time the dry rhodopsin gel was moistened with buffered hydroxylamine solution was critical for the degree of orientation obtained. Since the granules of dry gel varied widely in size and shape, this had to be estimated by trial and error. Also the distance one slide was pushed across the other to obtain the largest dichroic ratios varied widely. Ordinarily 1 mm of shear was needed to obtain a dichroic ratio of about 3; whereas 1 cm displacement was required to reach a dichroic ratio up to 4 or 5. As the slides were pushed across each other, the layer of rhodopsin between them, of course, grew thinner, and samples with the highest dichroic ratios often had absorbances too low to permit accurate measurement. Consequently several such sandwiches were sometimes taped together to increase the absorbance.

Low Temperature Experiments

Fig. 2 shows the arrangement for measuring chromophore orientations at liquid nitrogen temperature (−195°C, 78°K). Two compartments were hollowed out of a polyurethane block: a sample chamber and a liquid nitrogen well. A copper rod holding the sample ran through the partition so as to dip into the liquid nitrogen. Double quartz windows were fitted into the sample chamber, and warmed, dry nitrogen gas was blown over them throughout the experiment to retard frosting. Iron-constantan thermocouples were used to monitor the temperatures of both the sample and the liquid nitrogen well. The entire unit was wrapped with black masking tape to keep polyurethane dust out of the spectrophotometer. At −195°C, changes in absorption owing to frosting of the windows were small and about the same from one experiment to another. The film of rhodopsin-digitonin gel remained optically clear at this temperature.

For experiments between room temperature and −75°C, a copper, low temperature cell holder was used (Matthews et al., 1963–64), the temperature of which could be set by adjusting the flow from two constant temperature baths. One bath (Neslab Instruments, Inc., Portsmouth, N. H. 03801) containing methyl Cellosolve (Union Carbide, Corp., New York) (2-methoxyethanol) could be held at −20°C to ambient temperatures; the second bath contained methyl Cellosolve and dry ice (—80°C). By switching between both baths, the sample could be held at any desired intermediate temperature. Temperatures were monitored with a "Thermistemp" telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio) in the cell holder.

Spectrophotometry

All spectra were recorded with a Cary 14 Recording Spectrophotometer fitted with Bausch and Lomb microscope polarizing prisms (Glan-Thompson) (Bausch & Lomb Incorporated, Rochester, N. Y.) in both the sample and reference beams. The polarization of the reference beam, in terms of its electric vector, was always set parallel to

that of the sample beam. This was done because the optical system in a Cary 14 Spectrophotometer involves small wavelength-dependent polarizations that distort the base line. Such distortions are minimized by inserting polarizers in both beams.

OBSERVATIONS

Rhodopsin and Bathorhodopsin; Retinal Oxime

An experiment performed at −195°C is shown in Fig. 3. A shear-oriented rhodopsin film, mounted in the cold unit shown in Fig. 2, was brought to this temperature with liquid nitrogen.[2] Absorption spectra of the unbleached rhodopsin were recorded with the polarizing prisms set parallel to the direction of shear (0°), then perpendicular to it (90°) (Fig. 3, curves 1). The rhodopsin was then irradiated at 465 nm with the unpolarized light of the Cary sample beam with fully open (3 mm) slits for 15 min. This converted the rhodopsin to a steady-state mixture of about 70% rhodopsin and 30% bathorhodopsin (cf. Yoshizawa and Wald, 1963). The spectra were again recorded at 0° and 90° settings (curves 2).

The sample was then allowed to warm to room temperature during about 45 min in darkness in which dry room air was blown through the liquid nitrogen well of the cold unit. As the sample temperature rose, the bathorhodopsin went through the sequence of intermediate stages of bleaching, ending with the hydrolysis of retinal from opsin, and its reaction with hydroxylamine to form retinal oxime (λ_{max} 367 nm). The sample was then recooled to −195°C, and spectra again recorded (curves 3). 15 min later, curves 4 were recorded. Curves 3 and 4 show the absorption of rhodopsin left from the steady-state mixture (curves 2) after the bathorhodopsin had bleached to retinal oxime, and of the retinal oxime, which is responsible for the rise of absorbance in the neighborhood of 370 nm.

Frosting of the windows of the absorption chamber caused some distortion of spectra in the course of these measurements, owing to a general Rayleigh scattering that rises as $1/\lambda^4$, and hence is most serious at short wavelengths. This accounts for most of the rise of absorption at wavelengths shorter than 400 nm between curves 1 and 2 and for all the increase between curves 3 and 4. 15 min having elapsed between the recording of curve 1 and curve 2, we allowed just the same length of time between curves 3 and 4. In the latter instance no actual change of spectrum occurred, so that the changes recorded were caused entirely by window frosting. Consequently, they could be used

[2] On cooling rhodopsin to liquid nitrogen temperature in a 1:1 glycerol-water mixture, Yoshizawa and Wald (1963) found its absorbance at 505 nm to rise about 40%. Later, Yoshizawa (1972) found that when microcrystallization (cf. Yoshizawa and Wald, 1966) and crazing of the vitrified solvent are avoided, the rise in absorbance on cooling is only 10–15%. Our measurements on the present shear-oriented films involve rises of absorbance at 505 nm on cooling of only 8–13%.

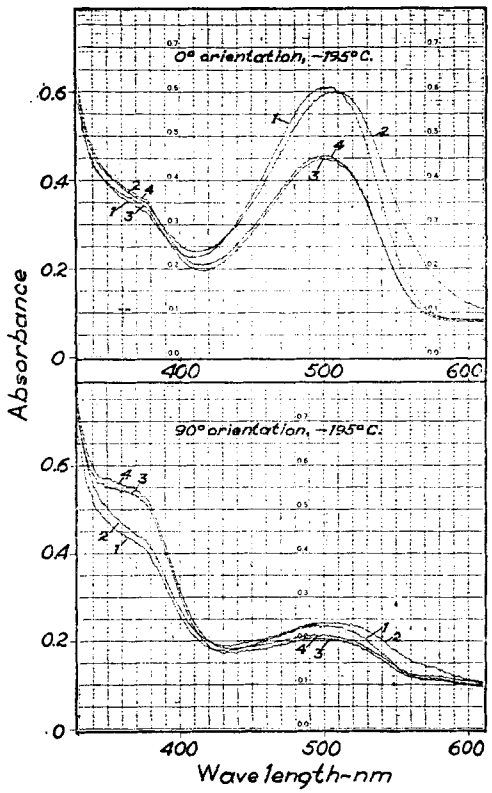

FIGURE 3. Absorption spectra in plane-polarized light of shear-oriented rhodopsin and products of its irradiation and bleaching. Curves 1 show spectra at −195°C of the shear-oriented rhodopsin in light polarized parallel (0°) and perpendicular (90°) to the direction of shear. The sample was irradiated with unpolarized light at 465 nm for 15 min, and curves 2 recorded in the same way. The irradiation had converted the rhodopsin to a steady-state mixture of about 75% rhodopsin and 25% bathorhodopsin (formerly prelumirhodopsin). The sample was then warmed to room temperature in the dark to allow the bathorhodopsin in this mixture to bleach completely to opsin and retinal oxime. Then the sample was recooled to −195°C and curves 3 recorded. 15 min later, curves 4 were recorded. The changes recorded between curves 3 and 4 are due wholly to frosting of the windows of the cold unit, and are used to correct for the frosting that occurred in the 15 min between recording curves 1 and 2.

to correct for the comparable changes that developed between recording curves 1 and 2.

Fig. 4 shows difference spectra computed from the curves of Fig. 3. By subtracting curves 3 from 1 in Fig. 3, we obtained the difference spectra of rhodopsin at the two polarizer settings (curves *a* and *c*). Similarly, subtracting curves 4 from 2 in Fig. 3 yielded the difference spectra of bathorhodopsin in both planes of polarized light (curves *b* and *d*). The fact that the difference

spectra of rhodopsin and bathorhodopsin in Fig. 4 coincide below 400 nm shows that this procedure compensated adequately for the light scattering which developed in the course of the measurements. Experiments that did not meet this criterion were discarded.

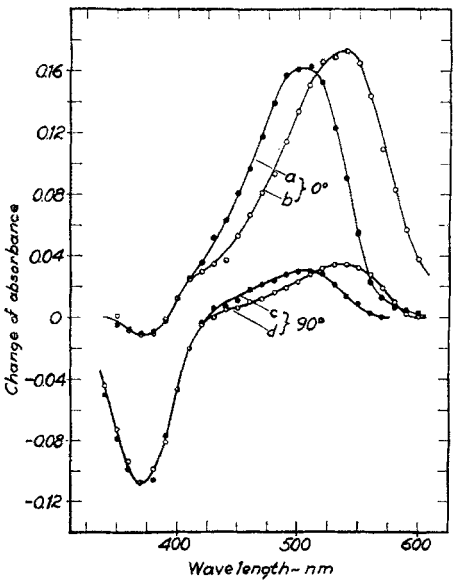

FIGURE 4

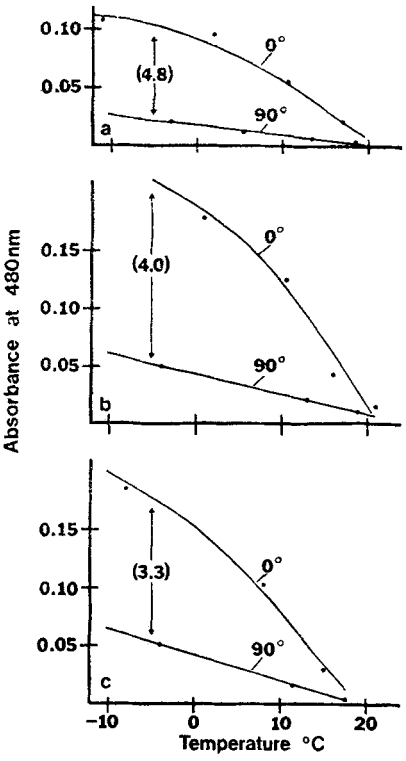

FIGURE 5

FIGURE 4. Difference spectra of shear-oriented rhodopsin (λ_{max} 505 nm) and products of its irradiation and bleaching, bathorhodopsin (λ_{max} 543 nm) and retinal oxime (λ_{min} 370 nm), measured in plane-polarized light at −195°C., computed from the data of Fig. 3. The measuring light was polarized either parallel (0°) or perpendicular (90°) to the direction of shear. The chromophores of rhodopsin and bathorhodopsin are highly oriented parallel to the direction of shear; their dichroic ratios (K_{max} 0°/K_{max} 90°) are, respectively, 5.4 and 4.9. Retinal oxime exhibits the reverse orientation. After correction for the residual absorption of rhodopsin at 370 nm, its dichroic ratio (K_{min} 0°/K_{min} 90°) is 0.47.

FIGURE 5. Decay of metarhodopsin I, derived from shear-oriented rhodopsin, and measured in plane-polarized light at temperatures above −10°C. The metarhodopsin I is going to metarhodopsin II and retinal oxime, neither of which absorbs appreciably at 480 nm. The measuring light was polarized either parallel (0°) or perpendicular (90°) to the direction of shear. Clearly the chromophore of metarhodopsin I maintains a high degree of orientation parallel to the direction of shear throughout its lifetime. The numbers in parentheses indicate the dichroic ratios measured at −6°C in these three preparations.

In these difference spectra the minima at about 370 nm represent the inverted and slightly shifted spectra of retinal oxime. Since retinal oxime does not absorb light significantly above 450 nm, at all longer wavelengths the difference spectra of rhodopsin and bathorhodopsin are equivalent to their true absorption spectra.

Fig. 4, typical of all such experiments that we have performed, shows that rhodopsin is oriented by mechanical shear so that its chromophores lie predominantly parallel to the direction of shear. The dichroic ratio in this instance (ratio of peak absorbances, curves *a/c*) is 5.4. Fig. 4 shows also that the bathorhodopsin obtained by irradiating rhodopsin maintains the same chromophore orientation. In this instance the dichroic ratio (peak absorbances, curves *b/d*) is 4.9. Retinal oxime, however, takes up a contrary orientation mainly perpendicular to the direction of shear. In Fig. 4 its dichroic ratio (ratio of absorbances, corrected for the absorbances of rhodopsin, at 370 nm, curves *a*, *b/c*, *d*) is about 0.47.

Lumirhodopsin and Metarhodopsins I and II

In the experiments begun at liquid nitrogen temperature, absorption spectra were recorded during warming, thus yielding data on lumirhodopsin and the metarhodopsins. In the range −100°C to −40°C, however, microcrystals tended to form (Yoshizawa and Wald, 1966), so distorting some of the spectra as to make them unusable. Reliable data on lumirhodopsin and metarhodopsin I were obtained in only a few such experiments.

For this reason we undertook a separate series of experiments using the copper cooling unit, regulated with two constant temperature baths, described above. In this procedure, shear-oriented rhodopsin sandwiches were cemented onto the copper block of the cell holder and brought to −75°C. Microcrystals do not form on cooling to this temperature. Spectra were recorded at 0° and 90° settings of the polarizer. Then the oriented rhodopsin was irradiated with the unpolarized light of the Cary 14 sample beam with fully open slits at 550 nm for 15 min, and the spectra recorded again in both orientations of polarized light. These are spectra of lumirhodopsin, the stable intermediate of bleaching at −75°C.

By switching between the baths, the temperature of the sample was raised in the dark to −15°C, converting the lumirhodopsin to metarhodopsin I, then recooled to −75°C, and a third pair of spectra recorded in both orientations of polarized light. Finally the sample was warmed in the dark to room temperature, yielding retinal oxime, then recooled to −75°C and a fourth pair of spectra recorded. Since no significant frosting occurred during any of these coolings, the final polarized light spectra were used as base lines for determining the amount of rhodopsin bleached, the lumirhodopsin formed at −75°C, and the metarhodopsin I formed at −15°C.

In several of the experiments begun at liquid nitrogen temperature, we followed the dichroic ratios of metarhodopsin I as it was converted at temperatures above −10°C to metarhodopsin II and retinal oxime. These measurements were made at 480 mm, where neither of the latter products absorbs appreciably.

Such measurements are shown in Fig. 5. Each point involves the difference in absorbance at 480 nm measured as the sample warmed, and the final measurement made at room temperature. It is apparent that metarhodopsin I retains its orientation parallel to the direction of shear throughout its existence. Actually the dichroic ratios calculated from Fig. 5 tended to decline somewhat at the higher temperatures, possibly because some pararhodopsin

TABLE I

SUMMARY OF MEASUREMENTS ON THE ORIENTATION OF THE CHROMOPHORES OF SHEAR-ORIENTED CATTLE RHODOPSIN AND INTERMEDIATES OF ITS BLEACHING BY LIGHT

The dichroic ratios are ratios of peak absorbance in plane-polarized light in the direction of shear as compared with perpendicular to that direction.

	Dichroic ratio				Ratio as fraction of rhodopsin ratio			
Experiment	Rhodopsin	Batho-	Lumi-	Meta I	Batho-	Lumi-	Meta I	Cold unit
1	4.4	4.1	—	—	0.93	—	—	Polyurethane
2	5.4	4.9	—	—	0.92	—	—	"
3	4.5	3.9	—	4.8	0.89	—	1.07	"
4	3.5	3.3	—	4.0	0.94	—	1.14	"
5	3.3	2.9	3.5	3.3	0.88	1.06	1.00	"
6	3.8	—	3.8	4.4	—	1.00	1.16	Copper
7	5.2	—	5.3	5.5	—	1.02	1.08	"
Average	—	—	—	—	0.91	1.03	1.09	
Standard error	—	—	—	—	0.03	0.03	0.06	

(λ_{max} 465 nm) may have formed as a side product at the higher temperatures. −6°C was found to be the highest temperature at which the dichroic ratios in these three experiments could be reliably ascribed to metarhodopsin I.

Table I summarizes the results of all these experiments. It shows that the chromophores of bathorhodopsin, lumirhodopsin, and metarhodopsin I retain essentially the same orientation as rhodopsin, parallel to the direction of shear. The dichroic ratios, expressed as fractions of those found in shear-oriented rhodopsin, remain close to 1. In view of the standard errors which are associated with these experiments, as shown in Table I, we doubt that any of these differences imply significant changes in the orientation of the chromophores. From this point of view it should be remembered that between rhodopsin and bathorhodopsin there is a large change in the shape of the chromophore as it is photoisomerized from 11-*cis* to all-*trans*. The 9% average de-

crease in dichroic ratio as compared with rhodopsin (Table I) may reflect this change owing to isomerization rather than disorientation. Also the changes in opsin configuration among the subsequent intermediates may have small effects on the orientation of the chromophore, but clearly no large changes in orientation occur. Later experiments convinced us that metarhodopsin II also exhibits substantially the same orientation, parallel to the direction of shear.

DISCUSSION

The shear-oriented preparations represent the highest degree of orientation of rhodopsin yet observed. In the rod disk membranes the rhodopsin chromophores, though they lie in the plane of the membranes, are randomly disposed in that plane. The same is true of rhodopsin in dry gelatin films (Wright et al., 1972). These are therefore states of one-dimensional orientation. In the shear-oriented preparations the rhodopsin chromophores lie not only in the plane of the film, but parallel to one another in the direction of shear, hence in two-dimensional orientation.

The present experiments show that as long as the chromophores remain bound to opsin, they maintain this orientation. Whatever changes in configuration of the chromophore or opsin characterize the intermediates of bleaching do not greatly alter this relationship.

There is, however, a large change in orientation associated with the hydrolysis of retinal from opsin and its conversion to retinal oxime. As already noted, and shown in Fig. 4, the dichroic ratio of retinal oxime is reversed as compared with rhodopsin and the intermediates of bleaching, the retinal oxime having swung around so as to lie primarily *across the direction of shear.* Its dichroic ratio in Fig. 4 is 0.47; in a similar experiment reported earlier it was 0.43 (Wright et al., 1972, Fig. 7). Our earlier experiments with rhodopsin in dry gelatin films showed that retinal oxime rotates so as to rise somewhat out of the plane of the film, its net orientation vector lying at an angle to that plane. That is also its behavior in the rod disk membranes (Wald, et al., 1963). What we observe in the present experiments represents another type of motion, a rotation parallel to the plane of the shear-oriented preparation.

It is remarkable that retinal oxime, though free in the sense that it is not covalently bound, nevertheless finds a new orientation within the oriented matrix of the opsin-digitonin micelles.

How does shear orient the rhodopsin-digitonin micelles? As already said, this effect implies a high degree of asymmetry in the orienting particles. In default of precise information on the shape of cattle rhodopsin, it is generally assumed to be roughly spherical. With a molecular weight of about 40,000 g/mol, and an assumed density, usual for proteins, of about 1.2, rhodopsin should possess a molecular volume of about 55.3 nm^3, and if spherical its diameter should be about 4.72 nm.

One possible source of asymmetry, as we suggested earlier, is that rhodopsin

undergoes linear polymerization under the conditions of these experiments. For this we have as yet no evidence.

Recently Wu and Stryer (1972), having labeled three points in cattle rhodopsin with fluorescent probes, estimated, on the basis of the facility of energy transfer from them to the chromophore, that one of them lies about 7.5 nm from the chromophore. In that case the rhodopsin molecule must be at least that long. If we assume that rhodopsin has roughly the shape of an oblate ellipsoid, (i.e., spindle-shaped) retaining its volume of 55.3 nm^3, then with a long dimension of 8 nm, the ratio of its long-to-short axes should be 4.85:1. If its long dimension were 10 nm, which would accommodate Wu and Stryer's measurement more adequately, the ratio of long-to-short axes would be 9.45:1. Also,—still to accommodate Wu and Stryer's measurement—if the molecule were no longer than this, the chromophore would have to lie at one end of it.

The shape of rhodopsin itself however may have little relevance to the shape of the rhodopsin-digitonin micelle. Hubbard (1953–54) assigned to this a particle weight of 260,000–290,000, each micelle containing one rhodopsin molecule associated with 180–200 molecules of digitonin. Hubbard found the frictional ratio of the micelle to be about 1.2–1.4, indicating "a somewhat ellipsoidal shape." In fact if the micelle takes the form of an elongated ellipsoid, this range of frictional ratios implies ratios of major to minor axes of 4.2:1–7.4:1 (cf. Svedberg and Pedersen, 1940, p. 41). This is about as in rhodopsin itself, as though the coating with digitonin followed the contours of the rhodopsin molecule.

None of this information is as firm as one would like to have it. The argument of Wu and Stryer, interesting as it is, is not compelling. Nor does Hubbard attach great weight to her estimate of the frictional ratio of the micelle (personal communication). Yet if the molecule and micelle have anything like the shapes that these measurements imply, and if one assumes further that the chromophore lies approximately parallel to the long axis of the molecule or micelle, these degrees of asymmetry could account for the shear orientation observed.

This model however involves a feature that needs special emphasis. For the rhodopsin chromophores to behave as we have observed, it is essential that they lie *parallel to the long* axes of the molecules or micelles. Since, however, the chromophores lie parallel with the planes of the rod disk membranes, that means that if the rhodopsin itself is asymmetric, its long axis *must lie parallel with the disk membranes.*

Other evidence has shown that in the disk membranes rhodopsin is free to undergo both Brownian rotation (Brown, 1972; Cone, 1972) and translation (Poo and Cone, 1973). It appears to float on a lipid phase of about the viscosity of olive oil, presumably composed of the hydrocarbon chains of phos-

pholipid bilayers. If rhodopsin itself is markedly asymmetric, rather than penetrating the disk membranes as has been suggested (Wu and Stryer, 1972), the rhodopsin molecules float upon them like logs on a pond.

This investigation was supported in part with funds from the National Science Foundation and the National Institutes of Health.

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Received for publication 26 April 1973.

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