The Orientation of Rhodopsin and Other Pigments in Dry Films

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ABSTRACT When rhodopsin in a gelatin film is dried, the rhodopsin chromophores orient primarily in the plane of the film. When the film is wetted, the chromophores disorient. These changes are reversible. When rhodopsin in a wet film is bleached in the presence of hydroxylamine and redried, the retinal oxime which results is oriented more perpendicularly to the plane of the film. These orientations in dry gelatin films resemble those in the disc membranes of rod outer segments. A variety of other proteins are similarly oriented in dry gelatin films: methemoglobin, cytochrome c, phycocyanin. Films of methemoglobin and cytochrome c display prominently the high Soret band near 410 nm when measured with unpolarized light passing through the face of the film, but display no Soret band at all with light passing through the edge of the film. All of these orientations imply a large asymmetry of the protein micelles, perhaps conferred upon them by linear polymerization in the course of drying. Such asymmetry can be demonstrated directly with rhodopsin. A wet paste of rhodopsin-digitonin micelles, sheared between glass slides, becomes highly oriented, the rhodopsin chromophores lining up in the direction of shear, the retinal oxime produced by bleaching orienting more perpendicularly to the shear.

Rhodopsin, the pigment of rod vision, is composed of the 11-cis isomer of vitamin A aldehyde (11-cis retinal) linked to the protein, rod opsin. On exposure to light, the 11-cis chromophore is isomerized to the all-trans configuration. This initiates a succession of conformational changes in the opsin, in the course of which the rhodopsin bleaches, ending with the hydrolysis of the retinal from the opsin (see references 1–3).

Rhodopsin is the major protein of the outer segments of the rods, specifically of the disc membranes that principally compose the outer segment (4). It is a typical membrane protein, the first to be extensively studied. Like all such proteins it is insoluble and can be brought into aqueous solution only with the aid of a solubilizer.

Kühne was the first to extract rhodopsin from retinas, using bile salts as
solubilizer (5). He showed that in solution it bleaches much as in the retina. He found, however, that it did not bleach in dried retinas.

Wald et al. (6) studied the properties of rhodopsin in dried gelatin films, such as had been prepared earlier by Weigert and Nakashima (7). They observed that in dry films light converts rhodopsin over intermediate states to a stable, orange-red product with \( \lambda_{\text{max}} = 478 \) nm. The similarity of its color to that of rhodopsin explains why Kühne thought that in the light no change had occurred. Wald et al. called this product metarhodopsin; the yellow product to which it bleached upon being wetted in the dark they called retinal. The red-orange product of irradiation in solution (now metarhodopsin I) has since been found to be in tautomeric equilibrium with the very light yellow metarhodopsin II, with \( \lambda_{\text{max}} \) about 380 nm, close to that of free retinal, with which its spectrum is easily confused (9). We still have not identified finally the products of bleaching in dry films, but for the present we will refer to the stable orange-red product in the dry as metarhodopsin I, and the final product on wetting as retinal. When hydroxylamine is present, the final product of bleaching is unequivocally retinal oxime.

When rhodopsin (\( \lambda_{\text{max}} \) about 500 nm) is bleached in solution, as the absorbance at 500 nm falls that at 380 nm rises, owing to the formation of retinal. As has already been said, rhodopsin in gelatin films goes through a similar transition on irradiation and wetting. The absorption spectrum of the bleached solution or film, subtracted from that of the original rhodopsin, constitutes the difference spectrum.

We have recently noted that in earlier measurements of such difference spectra by Durell (8), the ratio of absorbances at 380 and 500 nm (K380/K500) was much smaller in films than in neutral solutions. The present experiments were undertaken to analyze the source of this discrepancy.

To prepare gelatin films of rhodopsin, cattle rod outer segments were isolated by a modification of the method of Matthews et al. (9), and the rhodopsin was extracted into 2% aqueous digitonin. To 4 ml of this solution, which had an absorbance at 500 nm (K500) of about 2, we added 1.5 ml of 10% calfskin gelatin (Eastman Organic Chemicals, Rochester, N. Y., lot 1066), warmed to 45°C. This mixture was poured into a rectangular mold (27 cm²) made by laying a paraffin wall on a cellulose acetate base. The film was dried for 3 days in a desiccator over CaSO₄ (Drierite, W. A. Hammond Drierite Co., Xenia, Ohio), and then peeled off the cellulose acetate backing. The dry film, which was about 0.1 mm thick, was cut into small squares (6 × 6 mm) and stored over Drierite. Spectra were recorded with a Cary Model 11 spectrophotometer, using gelatin films containing digitonin as blanks. All of these operations were performed in dim red light.

For measurement, one edge of a piece of dry film was cemented so as to lie flat on a brass plate, 1 × 1 cm, with a 3 mm circular hole at its center. This
was placed between two glass slides separated by a rubber washer. The space between the slides was filled with 0.1 M neutral hydroxylamine solution (NH₂OH-HCl, neutralized to pH 7 with concentrated NaOH).

In solution or in wet films, the spectrum of the final product of bleaching, and hence the difference spectrum, varies greatly with pH. For this reason in the present experiments we bleached in the presence of hydroxylamine, which reacts rapidly with retinal to form retinal oxime (λ_max = 367 nm), a stable product invariant with pH. Under such conditions the difference spectrum also is invariant; in solution the ratio K₃₆₇/K₅₀₀ is 1.0.

Fig. 1 shows a typical experiment with a gelatin film of cattle rhodopsin. The absorption spectrum of the wet film was recorded against a dry blank. Then the rhodopsin film was exposed for 15 min to the orange light of a 160 w tungsten filament lamp passing through 2 inches of water to absorb heat and through a Jena OG 2 filter, transmitting wavelengths longer than 550 nm (i.e., light which would not isomerize retinal). The spectrum of the bleached film was recorded, and subtracted from that of the unbleached film. The resulting difference spectrum is shown as curve 1 in Fig. 1.

Another piece of the same rhodopsin film was similarly mounted and its spectrum was recorded dry against a dry blank. The film was then wetted

![Figure 1](image_url)

**Figure 1.** Difference spectra (unbleached minus bleached) of rhodopsin bleached in the presence of 0.1 M hydroxylamine, in which the product of bleaching is retinal oxime (λ_max = 367 nm). 1, wet gelatin film; 2, dry gelatin film, wetted for bleaching and redried; 3, solution in 2% aqueous digitonin. The difference spectrum of rhodopsin is much the same in a wet gelatin film as in solution, with the ratio of absorbances at 367 and 500 nm (K₃₆₇/K₅₀₀) equal to about 1.0. In the dry film, however, this ratio is only 0.35–0.4, owing to the orientation of the rhodopsin chromophores in the plane of the film, and of the retinal oxime more perpendicularly to this plane.
with hydroxylamine solution, bleached 15 min with orange light, washed with distilled water, and redried over Drierite. The spectrum of the dry, bleached film, subtracted from the dry unbleached film, yielded difference spectrum curve 2 in Fig. 1.

The K367/K500 ratio for the wet film was 0.9–1.1, virtually the same as in rhodopsin solutions (curve 3). The ratio for the dry film, however, was only 0.35–0.45.

We have encountered such low K367/K500 ratios before, when measuring rhodopsin in retinas, in which the visual pigment is highly oriented (10). In the outer segments of the rods, the rhodopsin chromophores lie almost wholly in the planes of the transverse disc membranes. The conjugated system of the chromophore is highly asymmetric, and absorbs light most strongly when its long axis lies parallel to the electric vector of the incident polarized light (see Fig. 2). Its orientation in the transverse plane in the outer segments spreads

![Figure 2](image)

**Figure 2.** Relative orientations of a chromophore for maximal and minimal absorptions of incident light, unpolarized and polarized. The plane of polarization is that of the electric vector.

the chromophores in the position most advantageous for absorbing light—polarized or not—passing down the axis of the outer segments as it does ordinarily in the eye. Rhodopsin, if oriented perfectly in this way, might absorb unpolarized light $\frac{3}{4}$ as strongly as when randomly oriented, its condition in solution. (This ratio can be thought of most simply as the result of compressing the three planes of random orientation into two.) Hence, if the product of bleaching, in this case retinal oxime, were completely disoriented, the K367/K500 ratio in the difference spectrum, instead of being 1.0 as in solution, might be $\frac{3}{4}$ or 0.67. Actually, in freshly excised retinas we have found this ratio to be about 0.47 (Wald et al. [10]), implying that the retinal oxime, rather
than being completely disoriented, tends to lie more perpendicularly to the planes of the transverse rod membranes, and so absorbs light passing down the axes of the rods less efficiently than if randomly oriented (Fig. 2).

This is just the situation we have encountered in dry rhodopsin films. As Fig. 1 shows, the K367/K500 ratio of about 0.4 in the difference spectrum implies not only that the rhodopsin chromophores are oriented in the plane of the film, but that the retinal oxime that results from bleaching is preferentially oriented more perpendicularly to this plane, in both cases much as in the retina. On the other hand, in wet gelatin films both of these molecules seem to be randomly oriented, as in solution.

If this is a correct description, one should be able to take a wet rhodopsin film, dry it, and watch the absorption at 500 nm rise as the rhodopsin orients. The spectra of Fig. 3 demonstrate this effect.

Measurements with plane-polarized light passing through the edge of a dry rhodopsin film provide direct evidence that the chromophores lie in the plane of the film. Since the dry films are only 0.1 mm thick, the edge-on measurements were made with the microspectrophotometer developed by Brown (11). For this purpose, dry pieces of rhodopsin film were suspended in water vapor at room temperature for 1 hr. This softens the film without disorienting the rhodopsin. Thin strips, 0.1 × 0.05–0.2 × 6 mm, were cut with a razor blade and redried over Drierite. A microcell was made by lightly coating the edge

![Figure 3. Reversibility of orientation of rhodopsin in gelatin films: 1, film wetted 1 min in distilled water; 2, the same film, dried for 3/2 hr in a vacuum desiccator; 3, same film, rewetted 10 min in distilled water; 4, same film, redried 3/2 hr in the vacuum desiccator. Wetting lowers the absorbance by disorienting the rhodopsin chromophores: drying raises the absorbance by reorienting them in the plane of the film. All measurements were performed in unpolarized light.](image-url)
of a Lucite ring (i.d. 10 mm, 1 mm thick) (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.) with Vaseline (Chesebrough-Ponds Inc., New York) and sandwiching it between two quartz cover slips. The strip of rhodopsin film was anchored in the microcell either face on or edge on with a small amount of Vaseline, and the microcell was filled with immersion oil to cut down refraction by the strip (refractive indices: gelatin, 1.52–1.53; immersion oil, 1.515). Under these conditions the strip, both edge on and face on, appeared smooth and transparent. Spectra of rhodopsin strips were recorded face on and edge on in plane-polarized light against a clear area of microcell as blank.

Fig. 4 shows a typical result. Measured edge on, the absorbance of dry rhodopsin films at 500 nm is about twice as great for light polarized parallel to the plane of the film as for light polarized perpendicular to this plane. This is direct evidence that in dry gelatin films the chromophores lie primarily in the plane of the film. The light paths both edge on and face on were about 0.1 mm, but were not accurately measured.

Throughout this paper the plane of polarization is taken to be that of the electric vector. This is now common practice (see R. W. Wood. 1934. Physical Optics. Macmillan and Co., New York. 329, and W. A. Shurcliff. 1962. Polarized Light. Harvard University Press, Cambridge, Mass. 3–4.).
plane of the film. On the other hand, for light passing face on through the film, the absorbance is the same in all planes of polarization. This indicates that within the plane of the film the rhodopsin chromophores are randomly oriented, as they are in the disc membranes of the rod outer segments.

To determine whether digitonin plays a specific role in the orientation of rhodopsin in dry films, these experiments were repeated with two other solubilizers, cationic cetyltrimethylammonium bromide (12) (CTAB, Eastman Organic Chemicals) and nonionic alkoxy(polyethylene) oxyethanol (13) (Emulphogene BC-720, General Aniline Film Corp., New York). In both cases the rhodopsin chromophores proved again to be oriented in the plane of the film.

Also, since the cellulose acetate used mainly as a base for the films itself possesses some degree of orientation which it might transmit to the films, a few trial rhodopsin films were cast on glass, which is wholly amorphous. These still displayed the characteristic orientation in the dry state.

To what degree is this orientation specific for rhodopsin? To look into this question, gelatin films containing methemoglobin (Nutritional Biochemical Corp., Cleveland, Ohio), cytochrome c (Nutritional Biochemical Corp.), or phycocyanin (kindly supplied by Allen Bennett) were prepared in the same manner as rhodopsin films, but without digitonin. A film made with β-carotene in 2% digitonin was also examined. The absorption spectra of strips of such films were measured face on and edge on in polarized and unpolarized light. The edge-on spectra were recorded in polarized light after determining the polarizer positions for maximum and minimum absorption for a specific absorption peak. All these molecules were found to be oriented in the dry film to various degrees.

The spectra of methemoglobin and cytochrome c in unpolarized light are particularly interesting in that the Soret bands at about 410 nm appear only in the spectra recorded face on, and are absent in the edge-on spectra (Fig. 5). In 1934 Adams et al. (14) observed that the Soret band was lacking in suspensions of red blood corpuscles, but appeared upon hemolysis. Adams (15) and Keilin and Hartree (16) did what they could to analyze this effect. The latter authors concluded that some “purely optical phenomenon” was responsible, “brought into play by the properties of surfaces separating haemoglobin from the surrounding medium.” Our observations in films suggest that the orientation of hemoglobin in the erythrocytes, aided perhaps by some orientation of the disc-shaped corpuscles, may be responsible for this effect.

Because of their intrinsic interest, we have prepared gelatin films of methemoglobin and cytochrome c repeatedly, with and without digitonin, and made similar observations, always with the same results. In every case the Soret band was prominent face on and absent edge on. The almost total disappearance of the band edge on is hard to explain as due only to molecular orienta-
tion, since the circular porphyrin chromophores should absorb light to some degree in all orientations. Perhaps molecular interactions also play a role, with consequent smearing of the spectrum.

The fact that such diverse molecules as we have examined in dry gelatin films all display some degree of orientation suggests a physical rather than a chemical basis for this phenomenon. It seems possible that the orientations may be caused by stretching and shearing forces in the gelatin during the drying process. As a solution of gelatin cools and then dries, it first forms a soft gel, which rigidifies to a hard structure upon complete drying. In our films the wet gel is about 1 mm thick and the dry film is about 0.1 mm thick. As the film dries, the upper surface of the gel moves downward at the rate of approximately 1 mm per day, while the lower surface on the cellulose acetate backing remains stationary. This creates a "velocity of contraction" gradient, which could exert shearing forces on any asymmetric structure within the gelatin matrix. Shearing forces are directly proportional to the velocity gradient and to the viscosity of the medium. Although the velocity of contraction gra-

![Figure 5](image-url)

**Figure 5.** Measurements with unpolarized light of the spectra of cytochrome c and methemoglobin in dry gelatin films. In each case a narrow strip of film was cut, and the spectrum was measured either face on or edge on. Light paths were not measured, so these spectra are comparable only in relative shape, not in height. For light normal to the plane of the films (face on) the Soret band at about 410 nm is the most prominent feature of the spectrum, but in the edge-on measurements the Soret band does not appear at all.
dient in this case is very small, the viscosity becomes very great as the gelatin solidifies. The shearing forces might therefore become quite large. If an asymmetric molecule lies at an angle in the gelatin, its upper end would move downward at a faster rate than its lower end, and it would thus tend to become oriented in the plane of the film. When a dry rhodopsin film is wetted, it swells (thus reversing the velocity of contraction gradient) and the rhodopsin becomes disoriented. It is reoriented when it is redried (see Fig. 3).

In order to show that shearing forces are capable of orienting rhodopsin in the absence of gelatin, the following experiment was performed. A solution of rhodopsin in aqueous 2% digitonin, dried in a desiccator, forms a clear, transparent "glass." A few small pieces of such a preparation were moistened with distilled water, and the excess water was removed after 1 min. The rhodopsin was now a very stiff paste. Shearing forces were applied by sandwiching this rhodopsin paste between two microscope slides which could be slid over each other. This sandwich was placed in the spectrophotometer and spectra were recorded face on in plane-polarized light as one slide was
pushed across the other. The distance between the two slides was approximately 0.05 mm. As is shown in Fig. 6, light polarized parallel to the direction of shear was absorbed five times as strongly as light polarized at right angles to the shear. Wet rhodopsin micelles can therefore be oriented by purely mechanical means. On drying such a sandwich in the desiccator over Drierite, the orientation is maintained. One can then separate the slides and remove leaflets of dry, oriented rhodopsin.

Shear-oriented rhodopsin was prepared as described above, but with the rhodopsin "glass" wetted with 1 M hydroxylamine instead of water. Face-on spectra were recorded in plane-polarized light before and after bleaching (Fig. 6).

Fig. 7 shows the difference spectra from this experiment. Whereas the rhodopsin chromophores as before were oriented in the direction of shear, the retinal oxime formed by bleaching was oriented perpendicular to this direction. The rhodopsin chromophore must take on an orientation imposed on it by the opsin; oriented opsin may also provide a matrix that orients the free retinal oxime differently from the chromophore. On the other hand, retinal oxime may be independently oriented by shear. We are pursuing such experiments further.
It is not yet clear what forces determine the "spontaneous" orientations in dry films. Clearly, in rhodopsin they imply a large degree of asymmetry, and one that finds the long axis of the chromophore parallel with the long axis of the protein micelle. The mechanical shear experiments provide the plainest evidence for this. It must be the oriented protein micelle that orients the chromophore, and in the gelatin films this probably also determines the quite different orientation of free retinal oxime after bleaching.

Since there is no other evidence of great asymmetry in rhodopsin micelles in solution (see reference 17) or in cytochrome c, methemoglobin, and phyco- cyanin, it seems possible that in all these cases, perhaps associated with the drying of the films, monomeric units in solution may have polymerized linearly to long fibrils. Numbers of proteins polymerize in this way under a variety of simple treatments, so many as to suggest that all proteins may have this potentiality (18–23). Insulin, for example, a much smaller molecule than rhodopsin, can polymerize to fibrils so long as to be retained by filter paper (Waugh [23]; personal communication). Obviously such fibril formation would go a long way toward explaining our observations. Yet preliminary examination of our dry rhodopsin films in the electron microscope has not yet revealed fibrils.

For the rest, the drying of the films exerts stretching forces that would tend to orient highly asymmetric particles in the plane of the films. Surface and boundary forces may also play a role. The phenomena described here seem to be sufficiently general to call for the operation of fairly general mechanisms, but it will take much more searching analysis to determine what these are.

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