The visual pigments
Absorption spectra of isolated single frog retinal rods and cones

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The visual pigment complex within a single retinal rod and cone can be studied by microspectrophotometry in order to identify the pigments during the light $\leftrightarrow$ dark reactions. Specimen areas in the rods and cones of the order 2 $\mu^2$ were scanned at relatively low magnifications from 340 to 900 nm. Freshly isolated frog (Rana pipiens) retinal rods and cones were rapidly scanned with the microspectrophotometer at 22° C. The retinal rods showed a broad absorption peak near 500 nm, typical of extracted frog rhodopsin. However, additional peaks at 380, 440, 480, and 515 nm were also observed. Some of these absorption peaks may be products of bleaching. Upon light bleaching, the 380 nm peak substantially increases, whereas the other absorption peaks diminish. These spectral changes upon light bleaching are accompanied by structural changes in the retinal rod. The shifts in spectral peaks indicate a mixture of isomers which may be formed upon light bleaching. In addition, these studies show that rhodopsin is evenly distributed throughout the outer segment of the retinal rod.

From the absorption data and the volume of the retinal rod, the concentration of rhodopsin was calculated to be $3 \times 10^9$ molecules. The retinal cones show general absorption throughout the visible range, with major peaks near or at 420, 470, 540, 570, 610, 660, and 690 nm. These and other experimental data are presented and discussed.

In order to study the chemistry of single cells and their organelles, techniques in microspectrophotometry are being developed, and instruments have been designed which are being applied to the identification of pigments within photoreceptor structures. Previous studies on the retinal rods of the frog and the cones of the carp, and more recent reports on the frog retinal rods suggest that the absorption peaks of the visual pigment complex (e.g., the identification of the visual pigments and their isomers during the light $\leftrightarrow$ dark reactions) can be studied in single isolated retinal rods.

Rhodopsin, a photosensitive carotenoid-protein complex, is one of the principle components of the retinal rod, accounting for about 40 per cent of the dry weight of the outer segment of the frog rod, and 14 per cent of the dry weight in cattle rods. Spectrophotometric data obtained from aqueous digitonin (1.8 per cent) extracts of retinal rods and measurements of threshold retinal spectral sensitivity have led to the conclusion that rhodopsin is the light-absorbing pigment and is responsible for the "primary event" in visual excitation. Granit, however, has suggested that it is premature to assume from the facts emerg-
From one kind of technique, such as extraction by detergents, that no photochemically active substances are found in the retina other than the broad-band absorption curves (Fig. 4, b). Such studies should show whether there is fine structure in the spectra of the rods and cones. In addition, by comparing the absorption spectral data of freshly isolated retinal rods to the spectral bleaching characteristics of rhodopsin (Table I), information relative to the photosensitive visual pigment complex would be revealed.

The absorption spectra presented here for the retinal rods and cones of the frog were obtained with a microspectrophotometer developed in our laboratory. The design, electronics, and optical characteristics of this instrument have been described previously. The photoconductive cell, cadmium selenide (1/2 x 1 mm.), permits investigation of very small specimen areas, because of its size, at relatively low magnification. An optical magnification of x500 corresponds to a specimen area of < 2μ². The absorption spectrum of a frog retinal rod (approximately 6 μ wide and 50 μ long) can be easily obtained at an optical magnification of x175. At this setting, from the monochromator with a tungsten lamp, the instrument is usable over the wavelength range 340 to 900 mμ at an entering half-band width of 2.6 mμ.

In operation, the retinal rod or cone is focused on a ground glass screen, and the desired area is centered on two cross hairs set in the screen. By means of a sliding track, the photocell replaces the screen at the marked position. The monochromator is then set to the desired wavelengths, and readings of the photocell output (usually in the range from 0 to 100 mv.) are recorded. The reference area selected is either a clear area in the retinal cell, or in the suspending medium. At each wavelength the reading takes about 10 seconds. The per cent absorption for each wavelength is computed from 1 – I/I₀ x 100,

Table I. Products which may be formed on bleaching rhodopsin

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Products</th>
</tr>
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<tbody>
<tr>
<td>rhodopsin (500 mμ) + (350 mμ)</td>
<td>small band</td>
</tr>
<tr>
<td></td>
<td>lumirhodopsin (515 mμ)</td>
</tr>
<tr>
<td></td>
<td>trace amounts other isomers 9, 13, di-cis 13 cis, etc.</td>
</tr>
<tr>
<td></td>
<td>metarhodopsin (462 mμ)</td>
</tr>
<tr>
<td></td>
<td>isorhodopsin (490 ± 2 mμ)</td>
</tr>
<tr>
<td></td>
<td>acid indicator yellow (440 mμ)  pH</td>
</tr>
<tr>
<td></td>
<td>alkaline indicator yellow (355 mμ)</td>
</tr>
<tr>
<td></td>
<td>retinene (385 mμ) + protein (opsin)</td>
</tr>
<tr>
<td></td>
<td>Vitamin A (328 mμ) + protein (opsin)</td>
</tr>
<tr>
<td></td>
<td>in chloroform</td>
</tr>
</tbody>
</table>

*No attempt is made here to distinguish between photo, thermal, or enzymatic bleaching. The absorption peaks and products were taken in part from Dartnall. Wald recently reported a pre-lumirhodopsin at 540 mμ.
where $I$ is the sample (retinal rod or cone) and $I_0$ the reference.

The retinas were removed from the eyes of *Rana pipiens* that had been dark-adapted for longer than 4 hours. The dissected retinas were agitated to isolate the rods free of pigment epithelium. The free rods were then placed in a drop of frog Ringer's solution on a microscope slide, with a cover glass that was sealed with petrolatum to prevent evaporation (Figs. 1 and 2). A slight pressure was applied to prevent movement of the rods. The cones were isolated microscopically from a mixture of rods and aligned by proper focus (Fig. 2, a). All retinal measurements recorded here were made at 22° C, although data were also taken at 0° C. All operations, including the preparation, focus, and location of the rod or cone, were carried out within 10 minutes after dissection in dim red light.

The retina of the frog contains about 60 per cent rods ("red") of which about 8 per cent are "green" rods. Fig. 2, b shows a freshly isolated unbleached retinal rod outer segment taken in red light; Fig. 2, c shows the retinal rod bleached by white light and rephotographed. An average spectrum for the "red" rods from 360 to 590 mμ is illustrated in Fig. 3, which shows peaks at 380, 490, 500, and 515 mμ. Some spectrums showed a peak at 440 mμ, which is reduced since the curve is smoothed in the process of averaging the results. Upon bleaching with strong white light until the rod showed signs of transverse striations (Fig. 2, c), the absorption peaks at 480, 500, and 515 mμ are diminished, and the 380 mμ peak is substantially increased. Since the rods are extremely photosensitive, exposure of the rods during the process of taking the absorption spectrum results in some bleaching as Brown has shown. As a result, readings were taken at only one wavelength for each rod, which was exposed for less than 10 seconds, even at the most sensitive wavelengths in the region near 500 mμ. Measurement of more than 100 individuals rods was necessary to construct the composite spectrum (Fig. 4, a) of the "red" frog rod. Its similarity to the absorption curve of rhodopsin (Fig. 4, b) is unmistakable. The large average deviation in the region of 380 mμ indicates that a variation in the amount of vitamin A and/or retinene (vitamin A aldehyde) is present in the in situ rod. The series of curves in Fig. 5, a, b, c, show additional details. Bleaching, in the absence of hydroxylamine (NH₂OH) results in a reduction of absorption at 500 mμ, indicating the disappearance of rhodopsin. The increase at 380 mμ and at about 480 mμ indicates an increase in retinene and possible isorhodopsin (λ max. at about 490 ± 2 mμ). What might be called background absorption runs high, as is indicated by the spectrum (Fig. 5, c), of a rod bleached in the presence of NH₂OH. Subtracting the spectrum of Fig. 5, b from the composite Fig. 4, a, gives a difference spectrum nearly
Fig. 2. a, Frog retinal cone, photographed in green light. (×1900.) b, Frog retinal rod, photographed in red light. (×1600.) c, Frog retinal rod, bleaching with white light; note the rapid swelling and the destruction of the rod. (×1700.) d, Sweep of rod 2b along length and width at 500 m.µ.
identical to those recorded for rhodopsin (Fig. 4, b). \(^1\)

The results of a sweep at 500 m\(\mu\) along the length and width of the rod at intervals of 0.5 m\(\mu\) (Fig. 2, d) indicate that the pigment (rhodopsin) is evenly distributed throughout the whole outer segment of the rod as we have visualized in our retinal rod model. \(^18\) Knowing the volume of the retinal rod, its absorption maximum at 500 m\(\mu\), and the molar extinction coefficient, an estimate of the number of rhodopsin molecules can be calculated. From our data, this turns out to be \(3 \times 10^9\) molecules per retinal rod, which is in agreement with previous calculations for frog rhodopsin based on the optical density of the extracted rhodopsin and the number of retinal rods extracted.

The "green" frog rods, which are more difficult to identify, \(^10\) show spectra with peaks at 460 to 470 m\(\mu\), 530 to 540 m\(\mu\), and a maximum at 610 m\(\mu\), which is in agreement with the data of Granit\(^4\) and Arden. \(^12\)

The frog cones are more difficult to study. They are fewer in number, smaller in size, and much more photosensitive than the retinal rods. However, they can be isolated microscopically in suspensions with the rods, and their absorption spectra obtained in a very similar manner to that of the retinal rods (compare Fig. 2, a with 2, b).

An average absorption curve for the frog retinal cone is illustrated in Fig. 6. It will be immediately noted that there is general
absorption throughout the whole of the visible spectrum. The average curve does show absorption peaks near or at 420, 470, 540, 570, 610, 680, and 690 μm, with a considerable scattering of points showing the extremes in the data. Some of these peaks may be products of bleaching since the cones appear to disintegrate more rapidly than the rods when hit with light. It is of interest to compare this curve with that obtained for the carp cone by Hanaoka and Fujimoto who found peaks in the regions of 420 to 430, 490 to 500, 520 to 540, 560 to 580, 620 to 640, and 670 to 680 μm. Similarities to the photopic spectral sensitivity curves are also to be noted.

It is difficult at present to speculate on the meaning of these absorption curves. However, the spectral data indicating the pigment complexes within a single rod or cone can be compared to the extracted visual complexes in solution and, in particular, rhodopsin. As our instrumentation becomes more refined, we shall be able to learn more of the biochemistry of retinal cells.

The gold fish cone peaks were recently reported to be at 460 ± 15, 535 ± 10, and 610 ± 5 μm by Marks and MacNichol in Abstracts of the Sixth Biophysical Society, TE-2, Washington, D. C., 1962.

REFERENCES