

Visual Pigments of Goldfish Cones

Spectral Properties and Dichroism

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ABSTRACT Freshly isolated retinal photoreceptors of goldfish were studied microspectrophotometrically. Absolute absorbance spectra obtained from dark-adapted cone outer segments reaffirm the existence of three spectrally distinct cone types with absorption maxima at 455 ± 3 , 530 ± 3 , and 625 ± 5 nm. These types were found often recognizable by gross cellular morphology. Side-illuminated cone outer segments were dichroic. The measured dichroic ratio for the main absorption band of each type was 2–3:1. Rapidly bleached cells revealed spectral and dichroic transitions in regions near 400–410, 435–455, and 350–360 nm. These photoproducts decay about fivefold as fast as the intermediates in frog rods. The spectral maxima of photoproducts, combined with other evidence, indicate that retinene₂ is the chromophore of all three cone pigments. The average specific optical density for goldfish cone outer segments was found to be $0.0124 \pm 0.0015/\mu\text{m}$. The spectra of the blue-, and green-absorbing cones appeared to match porphyropsin standards with half-band width $\Delta\nu = 4,832 \pm 100 \text{ cm}^{-1}$. The red-absorbing spectrum was found narrower, having $\Delta\nu = 3,625 \pm 100 \text{ cm}^{-1}$. The results are consistent with the notion that visual pigment concentration within the outer segments is about the same for frog rods and goldfish cones, but that the blue-, and green-absorbing pigments possess molar extinctions of 30,000 liter/mol cm. The red-absorbing pigment was found to have extinction of 40,000 liter/mol cm, assuming invariance of oscillator strength among the three cone spectra.

INTRODUCTION

Visual pigment extraction from retinas of cyprinids, such as the goldfish and the closely related carp, has been successfully accomplished by several investigators. For example, solutions of carp visual pigment were prepared by Köttgen and Abelsdorff (1896), Saito (1938), Wald (1939 *b*), Crescitelli and Dartnall (1954), and Bridges (1967); and of the goldfish pigment by Schwanzara (1967). The wavelength of peak absorption (λ_{max}) of these light-sensitive extracts had remarkably similar values and, according to the more recent determinations, it is near 522 nm. The substance responsible for the

absorption band with λ_{max} at 522 nm was called porphyropsin by Wald. He demonstrated that porphyropsin is also a chromoprotein, and it is, in fact, quite similar to rhodopsin. Another important contribution of Wald (1939 *a*) in this connection was the demonstration that, whereas the carp's liver contains a mixture of the A₁ and A₂ vitamins, its retina and other eye tissues contain only vitamin A₂.

The homogeneity tests of Crescitelli and Dartnall (1954), consisting of partial bleaches brought about by colored light exposures, were important in that they established, for the first time, that carp retinas extracted by conventional procedures contain single bleachable pigments. The question from which type of photoreceptor the extracted visual pigment originated could not be answered unequivocally at that time. The pigment (porphyropsin) was assigned to the rod cells, and it is usually assumed that the cone pigments either (*a*) are not extracted, or (*b*) are destroyed by the usual extraction methods, or (*c*) are present in such small quantities as to escape detection. The correctness of attributing porphyropsin to retinal rod cells (in goldfish and other vertebrates) was later confirmed by microspectrophotometric measurements (see review by Liebman, 1972), and the lack of cone pigment contamination in these extracts still awaits an adequate explanation.

The fact remains, however, that cone pigments cannot as yet be routinely extracted as numerous rhodopsins and porphyropsins can. Among the exceptions are the chicken cone pigment, iodopsin (Wald, 1937; Wald et al., 1955), a presumptive cone pigment from the pigeon (Bridges, 1962), and an iodopsin-like pigment extracted from the turkey retina (Crescitelli et al., 1964). In view of this failure of solution chemistry, microspectrophotometry seems at present to be the best and perhaps the only tool available to study the spectral and chemical properties of the visual pigments residing in cone cells.

The pioneering attempt of using microspectrophotometry for measuring vertebrate photoreceptors is due to Hanaoka and Fujimoto (1957). They constructed a microspectrophotometer and used it to detect bleachable pigments in frog and carp rods and cones. During the ensuing few years a number of similar instruments were made, and their users all contributed to establishing microspectrophotometry as a valid and valuable technique (see review by MacNichol et al., 1973).

Evidence for the existence of three spectrally distinct pigments occurring in different cones of goldfish (*Carassius auratus*) has been obtained by Marks (1963, 1965 *a*, 1965 *b*), Liebman and Entine (1964) and Svaetichin et al. (1965). The wavelengths of peak absorption determined by these investigators agreed quite well. Furthermore, confirmatory results have been obtained by electrophysiological methods. Thus, Tomita et al. (1967) by re-

cording from single carp (*Cyprinus carpio*) cones determined three classes of spectral response curves which showed reasonably good agreement with the microspectrophotometric results.

Despite the obvious successes of the microspectrophotometric investigations of goldfish cones, a number of issues remained unsettled. Marks (1965 *a*), for example, reported an apparent lack of photoproducts in cones, and could not rule out the possible admixture of a red-labile pigment in blue receptors. His final conclusion that "goldfish cone pigments resemble the frog rod pigment in spectral shape, photosensitivity, and concentration within the outer segment" had to be reexamined for two reasons. One of these was the problem raised by Bridges (1965): "the difference spectra (obtained by Marks) are not accurate enough for us to decide whether they are of the retinene₂ width or whether they correspond more closely to the retinene₁ pigment spectrum." The other reason is the problem which arises with the proposition (reinforced by Liebman, 1972) that the rhodopsin containing frog rods exhibit the same density per micrometer thickness (specific density) as goldfish cones whose pigments belong most likely to the porphyropsin series. If this were true, it would imply either that the two kinds of pigments have the same molar extinction coefficient and equal concentration, or that the dehydroretinal-based (porphyropsin-type) pigments are more concentrated than the retinal-based (rhodopsin-type) counterparts to make up for their roughly 30% lower molar extinction coefficient (Bridges, 1965, 1967). While the first alternative defies all the available information, the second is contrary to intuition.

Improved instrumentation make it possible to obtain absolute dichroic adsorption spectra from goldfish cones (and from frog rods) which are believed to be more accurate than earlier results. In the present communication new data are presented, and on their basis clarifications are sought for the issues referred to above.

METHODS

Experimental Material

Two variants of goldfish (*C. auratus*) were used: orange colored (comet) and black (black moor). These were purchased from nearby suppliers in Maryland, kept in an aerated aquarium, and dark adapted for a few hours prior to use. The overall fish size varied from 6 to 10 inches. Although the black moors made up the lower end of the range, their larger eyes compensated for their smaller body size. In addition to large eyes, the black moor has a much more fluid vitreous humor than the comet; this property renders the vitreous to be less bothersome and the removal of the retina easier and cleaner. Both kinds of fish yielded retinal preparations which we could not distinguish from one another either by visual inspection of the shapes of cells occurring in them or by spectroscopic measurements of their visual pigments.

Preparation

Dissection and other preparatory work were done at dim red light of a Kodak Safe-light Lamp (equipped with a 15 W bulb and No. 2 filter, Eastman Kodak Co., Rochester, N. Y.). The procedure for making preparations was similar to that of others (e.g., Marks, 1965 *a*). It consisted of enucleation, hemisection of the eye, and the transfer of pieces of retina into a small volume of the desired suspending solution (at room temperature). A small retinal fragment was then teased apart on a cover glass wetted with the same solution. After placing a second cover glass on top, the two were gently squeezed together, blotted along the edges, and finally sealed with a molten paraffin wax and Vaseline (Chesebrough-Ponds Inc., New York) mixture (cf. Dobelle et al., 1969). Efforts were made to mount the preparations in as nearly physiological solutions as were feasible and to measure the cells in as fresh states as possible. No gelatin was used in these experiments. The preparation consisting of a glass sandwich was firmly affixed to the gliding stage of the measuring microscope so that thermal equilibrium could establish between them. The temperature of the stationary part of the microscope stage was monitored with a Telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 43TD). Searching for cells (oriented sideways) and their alignment with the measuring beam was performed with the aid of dim red light derived from a microscope illuminator so filtered (peak transmission at 650 nm, 20-nm spectral bandwidth).

Measurement

The instrument used for recording single-cell absorption spectra is of our design. Detailed descriptions are given elsewhere (Hárosi, 1971; Hárosi and MacNichol, in preparation). It is a rapid, wavelength-scanning and recording, single-beam microspectrophotometer which simultaneously resolves absorption into two mutually perpendicular polarized components. A salient feature of the instrument is that it performs rapid and repeated scanning of the spectrum with summation of photocurrents in corresponding wavelength regions of all scans. This allows the use of measuring light fluxes which bleach very little pigment during one scan and hence introduce negligible distortion into a recorded spectrum (because adjacent spectral regions are measured at nearly identical states of pigment bleaching). When the scans are taken in alternating directions and are summed, even the small spectral shifts of the individual scans average to zero. The final record yields the true wavelength of peak absorption, and an absorption spectrum which is very slightly broadened. Therefore, the need for the correction procedure of Marks (1965 *a*) is completely obviated even though a significant amount of pigment is bleached during the measurement. Another facet of the instrument is that the measuring light beam, by passing through a rotating polarizer prism, causes a modulation in the detected photocurrent which is proportional to the dichroic absorptance of the sample. Therefore, by appropriate signal processing and by the utilization of a small on-line digital computer, the instrument can calculate direct absorption spectra for polarized light. It resolves absorptance into two components, designated as A-perpendicular and A-parallel. The former means absorption when the light beam is polarized per-

pendicular to, and the latter, when the direction of polarization is parallel with the long dimension of the illuminated field.¹

The two curves it displays represent ratios of photocurrents (subtracted from unity) due to polarized light fluxes transmitted by the cell and by a cell-free area in the preparation (measured sequentially). Since the isolated photoreceptor is viewed transversely in the microscope, the measuring light traverses it at a right angle to the physiological direction of incidence. In this arrangement when the long dimension of the beam (with slit-shaped cross section) is made to coincide with the long axis of a cell, the recorded spectra become a measure of the cell's linear dichroism, which means preferential absorption of light that is linearly polarized in a particular direction. In order to characterize this dichroism quantitatively, it is customary to determine the dichroic ratio which, by definition, is the ratio of the (logarithmic) optical density values at a particular wavelength for two mutually orthogonal polarizations, one being parallel with and the other being perpendicular to the axis of maximal absorption. The dichroic ratio (or its approximation when "linear" absorbance ratios are used) in an absorption band furnishes information about the spatial arrangement (molecular order) of the chromophores.

Standard Curves

Standard extinction (= density) curves with which to compare the experimentally obtained spectra were derived from the standard spectrum for porphyropsins as compiled by Bridges (1967). The procedure for obtaining the standards is based on the principle underlying the nomogram constructed by Dartnall (1953), who found that the shape of the curve relating relative extinction to the reciprocal of wavelength is approximately invariant for visual pigments having the same chromophore regardless of the λ_{\max} (wavelength of maximum extinction). In case of the green-absorbing standard, with $\lambda_{\max} = 530$ nm, his extinction values (Bridges, 1967, Table 2, column A) were used directly when they were available at desired wavelengths, or else after linear interpolations to obtain intermediate points. The blue-, ($\lambda_{\max} = 455$ nm) and the red-absorbing ($\lambda_{\max} = 625$ nm) standard curves were derived by shifting his porphyropsin spectrum (Table 1 A, column A) in wave numbers to the desired peak, reconvertng to wavelengths, and interpolating the results for selected wavelengths. The extinction values thus determined were scaled appropriately, and placed in the computer's core memory when needed for simultaneous displays with experimental data. Errors resulting from the linear interpolation and from the failure of converting the measured absorbances to densities were considered insignificant as compared with the resolution of the display system (0.4% of its full scale range). The reasons for these simplifications were the following. First, the porphyropsin standard spectrum of Bridges (1967) is defined by closely spaced points such that the adjacent ones differ from one another by 2–3 nm in wavelength and 2–4% in relative extinction. Therefore, intermediate values of the curve within these narrow ranges would be expected to depart from those of the cord by no more than fractions of a percent. Second, it is well known that self-screening in

¹ The meaning of polarization throughout this paper is the plane of vibration of the electric vector.

any pigment is unimportant at low densities. Thus, when the peak absorbance is low (as in all the measurements of this work), say $A \leq 0.1$, the departure between it and the logarithmic density is less than 5% (e.g. Dartnall, 1957). In the course of normalization even this error dwindles and, although the relative absorbance spectrum is slightly broader than the relative extinction curve, the difference between the two would be imperceptible under the present conditions.

Standard extinction curves were also prepared from the nomograms of Dartnall (1953) and of Munz and Schwanzara (1967). These, however, were found of limited usefulness for reasons discussed in connection with the results.

RESULTS

The experiments were performed during the spring and fall of 1972. Absorption spectra were obtained from 55 isolated cone outer segments. Following spectral recordings (and bleaching) 15 of these cells were photographed through the measuring microscope.

In the decision whether to accept a record for further data processing or to reject it, the following selection rule was used: a spectral curve may be regarded free of distortions if it falls within about $\pm 1\%$ of the zero line, before as well as after bleaching, in spectral regions where no absorption is expected. (Red-absorbing cones could meet this requirement only for post-bleach recordings, for the red pigment absorbs considerably throughout the instrument's spectral range.) Although this selection rule may appear arbitrary, we formulated it in order to make the choosing of records more systematic. The rule actually is an empirical one which has grown out of the experience of others as well as our own. The individual spectra of the record collection of Marks (1965 *a*), for example, show considerable variability in the wavelength of peak absorption within each cone class. Liebman (1972), on the other hand, has stated that his measurements show no such variation. He attributes the variability in λ_{max} to mechanical motion of the receptors during the recording. We confirm this explanation, for motion of the receptor during measurement invariably distorts the recorded absorption spectrum, furthermore, there is usually an attendant failure of the base line to return to zero in spectral regions which normally reveal negligible absorption. The zero base line criterion also proved to be a sensitive measure of other conditions. Thus, whenever focusing was improper (partial) though stable, or when defocusing changes occurring while recording could directly be verified, or, when the measuring beam passed too close to the cell perimeters, the absorbance traces failed to meet the aforesaid requirement. The criterion set forth by the selection rule is, in fact, so demanding that no more than 20–30% of our records can comply with it.

The numerical distribution of types among the 55 cells, according to the peak of their absorption spectra, were: 8 blue (B), 27 green (G), and 20 red (R). Of the latter two types, nine were definitely identified as members of

twin cones. Since it was possible with experience to recognize the types upon visual inspection, cell selection was nonrandom and hence no statistical significance should be attached to these numbers.

Morphology

Under the light microscope, at a magnification of $\times 1,000$, goldfish cones appear in various forms. The shape of the inner segment may look spherical, slightly ellipsoidal, or extensively elongated so that the step in diameter at the junction of the inner and outer segments is obliterated. In addition to the shape of the inner segment, the relative diameter of the outer segment at its base and the tapering of the outer limb are features which may be related to pigment content. The observations were as follows: (a) Blue-absorbing cones have spherically shaped inner segments. No exception was ever found to this rule and, because of it, we probably found a larger number of type B cells than if selection had proceeded at random. (b) Red-absorbing cones appear to have elongated inner segments and broad-based and slowly tapered outer segments. A few examples also suggested that the type R cone outer limbs may also be longer than the B and G counterparts. The most ellipsoidal, the longest, and those cells which have the smallest step in diameter between the inner and outer segments turned out to be type R. (c) Green-absorbing cones were found in the range intermediate between B and R. Type G outer segments are similar to those of B: they taper more rapidly and show a significant discontinuity in diameter at the junction of the two limbs. The G cone inner segments are more spherical than those of R and thus they may invade the B range. On one occasion a cone thought to be type B turned out to have type G pigment. (d) Twin cones, by definition, are made up of two equal cones joined at the inner segment. Their two outer segments contain dissimilar pigments of R-G combination. Although the shape of the two inner segments is usually similar, the outer segments can often be distinguished on the same basis as the single R and G types. The outer limb of the G member of twins tends to taper more rapidly whereas the R member tapers more slowly and has a broader base. In a few instances the twins were truly identical. Thus we were unable to distinguish visually one from the other even though they contained dissimilar (R-G) visual pigments.

Photoproducts

Prebleach and postbleach spectra of a red-absorbing cone are depicted in Fig. 1. As seen in part *a*, the main band of the visible spectral region is dichroic: the cell exhibits preferential absorption for transversely polarized light. Here, as well as in our other records, the extinction ratio at 625 nm is between 2:1 and 3:1. The remarkable features of the shortwave end of the spectrum ($\lambda < 500$ nm) are relatively high absorption and reversed dichroism. Al-

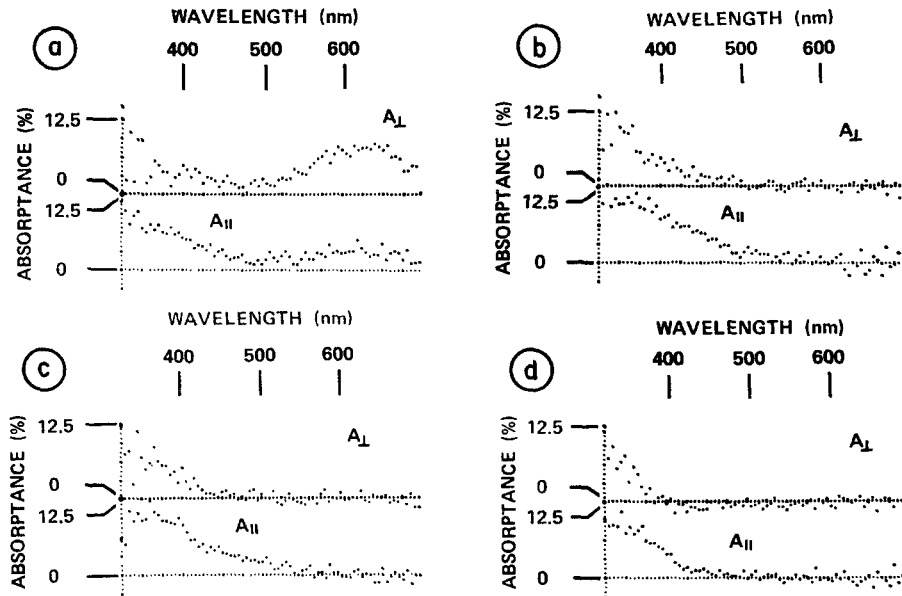


FIGURE 1. Prebleach and postbleach absorption spectra for polarized light of a red-absorbing goldfish cone outer segment (with visually estimated dimensions of $10\ \mu\text{m}$ length and $4\ \mu\text{m}$ average diameter). The suspending medium contained 110 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , and 10 mM HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer at pH 7.4. The temperature of the microscope stage was 22.0°C . The measuring light beam was delimited with an adjustable rectangular diaphragm; its image extended about $2 \times 8\ \mu\text{m}$ in the specimen's plane. The flux density of the beam was indirectly determined and found to vary between approximately 2×10^{18} – 7×10^{18} quanta/ cm^2 s in the wavelength range of 400–700 nm. Each record depicts average spectra for 32 scans (performed in alternating directions between 325 and 695 nm) which were obtained at a scanning rate of 500 nm/s. The time of completion of such a recording is about 40 s. The spectra for transversely polarized light (A_{\perp}) are displaced along the ordinates by +12.5% so that the individual traces be readily distinguishable. (a) Slightly light-adapted spectra as recorded 57 min following the removal of the eye (some inadvertent bleaching took place during dissection, searching and alignment, and measurement). (b) Postbleach spectra as recorded following a white flash (derived from a heat-filtered, type M3, clear photographic flashbulb). The first scan was initiated 30 ms after end of flash. The duration of bleaching exposure was set to 150 ms and its peak flux density was estimated to reach about 1×10^{18} "average visible quanta"/ cm^2 s. (c) Postbleach spectra for a recording initiated 2 min after the bleach. (d) Postbleach recording obtained 11–12 min after the flash-bleach. Spectra, in this case, are averages of 64 bidirectional scans.

though most of the absorption in this spectral range is probably due to photo-products, elevated β -band extinction may well be a contributing factor. Parts *b*, *c*, and *d* show a sequence of postbleach recordings. The rapid increase in axial absorption at shorter wavelengths is to be noted. These spectra suggest the existence of three regions of transition: 400–410, 435–455,

and 350–360 nm. The records reveal that within 12 min the chromophores are transformed into the last absorbing molecular species which exhibit predominantly axial orientation. This bleaching process appears to be analogous to that observable in the rhodopsin-containing (“red”) frog rods. The spectral locations of the photoproduct peaks in such frog rods are of course different (near 380, 480, and 330 nm), but there are two additional differences: (a) it takes about 60 min in a moderately fresh frog red rod to convert all the bleached intermediates to the final photoproduct and (b) the orange intermediate (480-nm peak) remains transversely dichroic throughout its lifetime (Hárosi, 1971). Thus it appears that when tested under similar external conditions the interconversion of photoproducts as measured by spectral and dichroic changes proceeds about five times faster in goldfish cones than in frog rods.

Spectral Shapes and Dichroism

Average absorption spectra are depicted for three blue-absorbing cones in Fig. 2. Note in part *a* the single and transversely dichroic peak in the visible spectrum. In part *b* the average transverse absorption spectrum (from part *a*) and ± 1 SD are shown. Since the standard deviation was computed between “unnormalized” spectra, the scatter of points is due not only to noise but also to variability in cell size, differences in state of bleaching, and perhaps to slight, undetected, mechanical motions. Part *c* shows the scatter of the axial component.

Fig. 3 depicts a comparison between the normalized average absorption spectrum (the transverse component from Fig. 2 *a*) and our porphyropsin standard curve of 455-nm peak. The agreement between the two is rather good. When the same experimental spectrum was compared with a curve derived from Dartnall’s nomogram (based upon rhodopsin instead of porphyropsin), the match was noticeably poorer (not illustrated). We found the rhodopsin standard to be too narrow for the blue cone spectrum. The Munz-Schwanzara nomogram, on the other hand, yielded a standard which closely followed the experimental spectrum at wavelengths longer than 410 nm (not illustrated). Since this nomogram is based upon a porphyropsin difference spectrum, the obtained partial match was to be expected. (See Table I subsequently for quantitative comparison of these spectral curves in terms of their half-band widths.)

Average absorption spectra for three green-absorbing cones are shown in Fig. 4. It may be observed in part *a* that the main absorption band is transversely dichroic. Part *b* again illustrates the scatter between the transverse spectral components. The two regions of increased scatter of points are located near 405 and 355 nm. Part *c* depicts the scatter between the axial components.

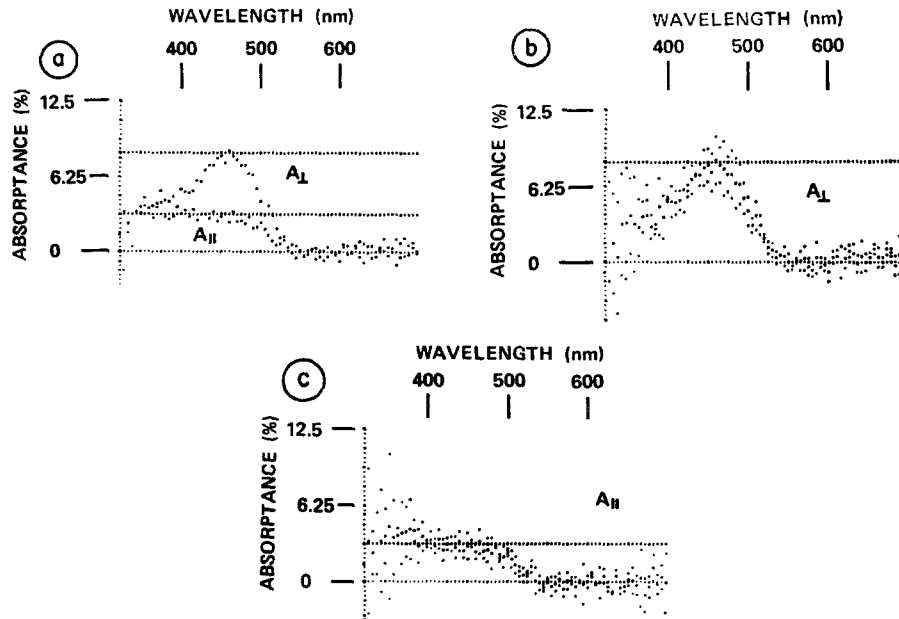


FIGURE 2. Average absorption spectra for three dark-adapted blue-absorbing goldfish cone outer segments. The spectra were obtained by averaging the first 32 bidirectional scan averages from each cell's recording. Postmortem ages of the cells were 60, 72, and 130 min. The experimental conditions were as for Fig. 1, with the exception that the preparation temperatures for the three experiments were 20°, 23°, and 21°C. (a) Absolute absorption spectra for polarized light. Auxiliary scales are set to the peaks of the main band. (b) Average absorption spectrum for transversely polarized light and the same ± 1 SD (three traces). The standard deviation has the usual meaning; it was calculated by the computer in the course of the point-by-point averaging of the appropriate spectra. (c) Same as b, except for axially polarized light.

Fig. 5 illustrates a comparison between the normalized average absorption spectrum (the transverse component from Fig. 4 a) and the porphyropsin standard curve of 530-nm peak. The correspondence between the two curves is excellent. When we tried the rhodopsin standard of the same peak wavelength, it proved to be too narrow for the green cone spectrum (not illustrated).

Average absorption spectra for three red-absorbing cones are depicted in Fig. 6. Part a illustrates the transverse dichroism of the main band. The scatter of points in part b (and part c) again suggests two minor peaks at about the same spectral locations as those observed in blue and green cones. Although we cannot deny the possibility that the postbleach absorptions we detect are of nonchromophore origin, we think that unlikely. First, visual cells devoid of chromophores, when measured with our microspectrophotometer, show negligible absorption throughout the scanned portion of

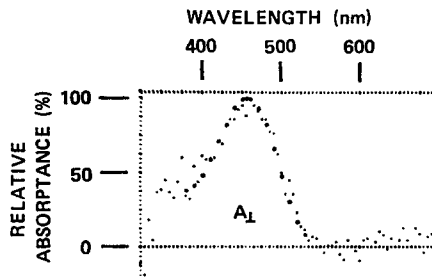


FIGURE 3. Normalized absorption spectrum of blue-absorbing goldfish cones for transversely polarized light (small filled circles) and a porphyropsin standard curve of 455-nm peak (large filled circles). Data were taken from the record depicted in Fig. 2 *a*. The standard curve was obtained as described in the text.

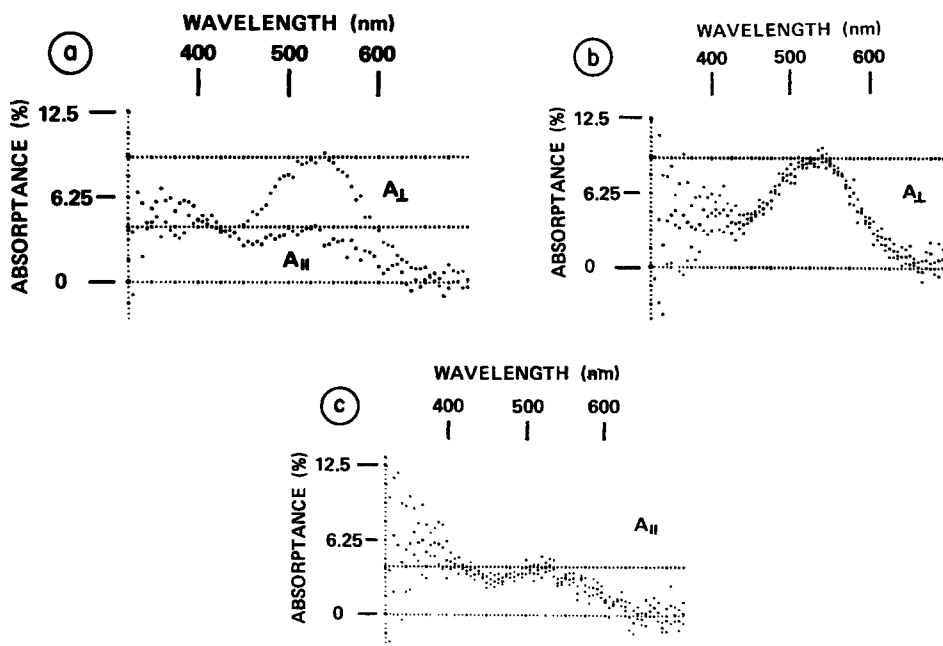


FIGURE 4. Average absorption spectra for three dark-adapted green-absorbing goldfish cone outer segments. The spectra were obtained by averaging the first 32 bidirectional scan averages from each cell's recording. Postmortem ages of the cells were 35, 30, and 53 min. The experimental conditions were as for Fig. 1, except that the preparation temperatures for the three experiments were 23°, 22°, and 22°C, and that the suspending medium contained 110 mM NaCl, 2 mM KCl, and 1 mM EGTA at pH 7.4 for one of the experiments. (*a*) Absolute absorption spectra for polarized light. Auxiliary scales are set to the peaks of the main band. (*b*) Average absorption spectrum for transversely polarized light and the same ± 1 SD (three traces). (*c*) Same as *d*, except for axially polarized light.

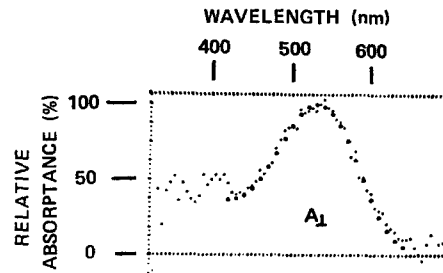


FIGURE 5. Normalized absorption spectrum of green-absorbing goldfish cones for transversely polarized light (small filled circles) and a porphyropsin standard curve of 530-nm peak (large filled circles). Data were taken from the record depicted in Fig. 4 *a*. The standard curve was obtained as described in the text.

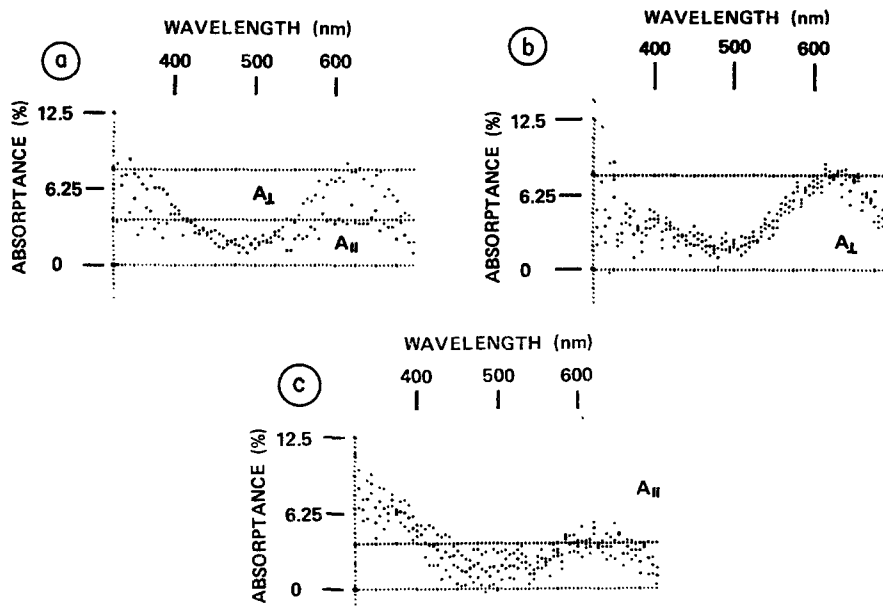


FIGURE 6. Average absorption spectra for three dark-adapted red-absorbing goldfish cone outer segments. The spectra were obtained by averaging the first 32 bidirectional scan averages from each cell's recording. Postmortem ages of the cells were 59, 20, and 57 min. The experimental conditions were as for Fig. 1, with the exception that the preparation temperatures for the three experiments were 22°, 23°, and 22°C. (*a*) Absolute absorption spectra for polarized light. Auxiliary scales are set to the peaks of the main band. (*b*) Average absorption spectrum for transversely polarized light and the same ± 1 SD (three traces). (*c*) Same as *b*, except for axially polarized light.

the spectrum. Second, we are unaware of the presence in rod and cone outer segments of any other substance in sufficient concentration which could account for our measured values (cf. Denton, 1959). Third, it is well known that visual pigments in solution following photolysis have products with

various blue-shifted spectra. Thus, the similarities we observe among the postbleach spectra of the three cone types may be ascribed to the similarities of photoproducts. Consequently, we are inclined to believe that the chromophores present in all three types of goldfish cones are either identical or at least very similar molecules.

Fig. 7 depicts a comparison between the normalized average absorption spectrum (the transverse component from Fig. 6 *a*) and our porphyropsin standard curve of 625-nm peak. As is plainly visible, the match between the two is poor. When compared with a rhodopsin standard curve of the same peak wavelength, the red cone pigment spectrum proved to be still too nar-

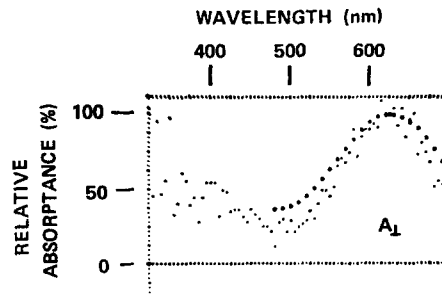


FIGURE 7. Normalized absorption spectrum of red-absorbing goldfish cones for transversely polarized light (small filled circles) and a porphyropsin standard curve of 625-nm peak (large filled circles). Data were taken from the record depicted in Fig. 6 *a*. The standard curve was obtained as described in the text.

row (not illustrated). This result agrees with that of Marks (1965 *a*). Interestingly enough, as was pointed out by Bridges (1967), the cyanopsin spectrum of Wald et al. (1953) is also narrower than the appropriate porphyropsin standard. Since cyanopsin was synthesized with the chromophore of porphyropsin and because we have reasons to believe that the same chromophore is present in the goldfish cone pigments (discussed below), these independent observations suggested to us the possibility that the dehydroretinal-based red-absorbing pigments may have exceptional spectral properties.

Specific Density

The specific optical densities of cone outer segments were determined on the basis of Figs. 2 *a*, 4 *a*, and 6 *a*. First the peak transverse absorbances were read from the average absolute spectra of the three types of cones (7.2% at 455 nm, 9.2% at 530 nm, 9.5% at 625 nm). These absorbances were then converted to optical densities by the relation $D = \log_{10}(1/1 - A)$, and corrected for bleaching caused by the measurement by adding 6.7% of the peak density to the measured peak density figures in all three types. The correction factor was derived from sequential recordings of a blue-absorbing

cone (not illustrated), which indicated that the average transverse density loss at λ_{\max} for a 32-scan measurement was 6.7% of the initial value.² The absolute density figures thus obtained were 0.0346, 0.0447, and 0.0463 for the B, G, and R types, respectively. The photographically measured dimensions of 10 typical cones permitted the determination of cylindrical equivalents to the conical outer segments. The averages of these equivalent diameters for the three classes yielded values of 3.62, 3.24, and 3.62 μm for the same sequence. By dividing the corresponding average diameters into the average peak transverse densities, the three specific densities obtain for the B, G, and R classes as 0.0106/, 0.0138/, and 0.0128/ μm . The overall average of these specific densities was calculated to be 0.0124/ μm , with an estimated error margin of $\pm 0.0015/\mu\text{m}$. In the judgment of accuracy we considered the possible absolute value errors allowed by the selection rule and the uncertainty associated with cellular dimensions due to blurred contours in the photographic images. We did not attempt to account for the inadequacy of bleaching corrections, the possible differences in pigment extinction between the three cone types, or the possible biases introduced by the fact that the optical density values and the cellular dimensions were obtained from different cells.

DISCUSSION

Morphology

We confirm the early observation of Marks (1965 *b*) in goldfish that "blue receptors seem to have more rounded ellipsoids." Svaetichin et al. (1965), in their studies of nine different species of fish, also agreed with Marks' description and stated that receptors with shortwave absorption belong to the type called "additional short single cone." Since we did not study retinal patterns of receptors, we cannot comment on Svaetichin's other observation concerning the type called "central long single cone" (referring to the center of a mosaic unit), which was found to be green-absorbing. However, their other observation on the pigments of the two members of the "unequal" goldfish double cones being red-green pairs agrees with ours on twin cones. Marks (1965 *a*) came essentially to the same conclusion, though he reported the finding of one blue-green pair out of the 30 pairs of twins he examined.

² The corrections for bleaching by this method are highly approximate and should be regarded as conservative estimates of the losses. The amount of pigment photolyzed in each cell during a recording is proportional to the convolution of the energy spectrum of the measuring light and the absorption spectrum of the pigment. Since the flux density of the microbeam increases toward longer wavelengths (see caption for Fig. 1), the blue-absorbing pigment should suffer the least exposure while the green-, and red-absorbing pigments would be more affected (during scans of constant rate and equal duration). We adopted the present method due to lack of pertinent measurements or adequate information for more satisfactory computations.

The recent report of Scholes and Morris (1973) concerning the cones of rudd, a common European cyprinid, as far as it is possible to judge from a brief description, appears to be in agreement with our morphological description of goldfish cones. A more complete discussion will be possible after their full report is published. We also intend to give a fuller account of our results elsewhere (Stell and Hárosi, in preparation).

Dichroism

Visual cell dichroism (for transverse illumination) was first demonstrated in frog rod outer segments by Schmidt (1938) and in other vertebrate rods by Denton (1959). Dichroism of goldfish cones was demonstrated by Marks (1963, 1965 *a*), Liebman and Entine (1964), and Svaetichin et al. (1965). Although these workers did not determine dichroic ratios, they generally agreed that transversely polarized light is absorbed more strongly, which implies a lamellar arrangement of visual pigment chromophores in vertebrate cones as well as in rods. Liebman and Entine (1964) went so far as to claim that "the parallel component has no detectible absorption. These cells therefore have a very high dichroic ratio." Our measurements do not support that contention. The published spectral absorption recordings from isolated fish receptors of Svaetichin et al. (1965), on the other hand, indicate dichroic ratios of 2–3:1, in good agreement with our own results.

It is difficult to evaluate the measured dichroic ratios obtained for the various photoreceptors because of the uncertainties associated with the microspectrophotometric technique. The instrumental limitations and the interpretation of results we have discussed elsewhere in more detail (Hárosi, 1971; MacNichol et al., 1973). The various aspects of the numerical apertures of condenser and objective, for instance, were considered from the viewpoint of diffraction, scattering, and the conical shape of the wave-front impinging on the specimen. We concluded that, the last phenomenon in particular, would yield underestimation of the intrinsic dichroic ratios. Scattering at optical interfaces would cause the same. Furthermore, the various aberrations of the optical parts and the possible nonperfect alignment of the cell with respect to the reference direction in the measuring beam would introduce limitations on both absolute values and dichroic ratios. A case in point is the fact that we measure dichroic ratios with the same instrument for cells of diminishing diameter, such as the frog red rod (4–5:1 at 502 nm; $d \simeq 6 \mu\text{m}$), the frog green rod (3–4:1 at 435 nm; $d \simeq 4 \mu\text{m}$), and the goldfish rod (1–2:1 at 525–530 nm; $d \simeq 2 \mu\text{m}$), which reveal an approximately direct relationship between apparent dichroic ratio and cell diameter. Although these results could be interpreted as the manifestation of reduced molecular order in smaller receptors, it is more likely that they are due to loss of light from the measuring beam by polarization-dependent refraction and light

scattering within the cell. Thinner cells probably have higher absolute refractiveness. Furthermore, the relative error due to even 1% refraction loss becomes significant when the uncollected fraction of light (which is the sum of the absorbed, scattered, and refracted fractions) is only 2–3%. We would think this the reason for the diminution of the measured dichroic ratios in thinner cells. Until we find a method, however, whereby the instrument can be tested with known dichroic samples mimicking photoreceptors, a satisfactory interpretation of these results seems unattainable.

Photoproducts

The existence of photoproducts in cone cells, although suggested in a record published by Wald and Brown (1965), have not been clearly demonstrated by previous investigators. Liebman (1972), for example, stated that he was unable to observe any visible-absorbing photoproduct in goldfish cones even when the entire spectrum was scanned in as little as 15 s. He therefore concluded that any such product formed cannot persist for this long. In our experience postbleach intermediates of goldfish visual pigments can be detected if both the bleaching and the recording are performed rapidly. Although the visible-absorbing photoproducts indeed disappear faster from goldfish cones than from frog rods, their decay proceeds slowly enough to allow our instrument to record some of them. As the spectra of Fig. 1 illustrate, elevated absorption in the visible region can be detected (at 22°C) not only during the first 40 s after a bleaching flash, but for several minutes thereafter.

Tentative identification of photoproducts appears possible on the basis of locating their maximal absorption and comparing them to that of known substances. According to Wald (1939 *a*) the absorption maxima of vitamin A₂ and retinene₂ in chloroform are at 355 and 405 nm, respectively. Bridges (1967), when identifying photoproducts in digitonin-solubilized porphyropsins, identified a λ_{\max} of about 360 nm as due to 3-dehydroretinol, and of about 408 nm as 3-dehydroretinaldehyde. It thus appears that two of the subsidiary peaks detected in goldfish cone spectra correspond to these substances (cf. Fig. 5). The suggestion of a third peak at about 435–455 nm (Fig. 1 *b*) we can identify less convincingly. By strict analogy with part of rhodopsin's bleaching sequence (\rightarrow Meta II \rightarrow Meta III or pararhodopsin \rightarrow vitamin A₁), this peak may correspond to the second of a homologous sequence (\rightarrow Meta II \rightarrow Meta III \rightarrow vitamin A₂).

Effect of Lack of Calcium

Since the important work of Hubbard and Kropf (1958) it has become known that the only direct effect of light on the visual pigments is to isomerize their chromophores from the *11-cis* to the *all-trans* configuration. All the subsequent steps of conversion appear to be due to thermal reactions which proceed

equally well in light or darkness. Though these principles were established by studies of rhodopsin extracts at low temperatures, it has been widely assumed that bleaching occurs no differently in the retina and at physiological temperatures (e.g. Hubbard et al., 1965). Some caution is advisable, however, so that the parallelism is not carried too far. It can be readily demonstrated, for example, that in freshly isolated frog rod outer segments the final photoproduct absorbs mostly at about 330 nm (and is presumably vitamin A₁), and there is no spectroscopic sign of evidence for the accumulation of "all-trans retinal + opsin," whereas this substance appears to be the final product in aged cells (Hárosi, 1971). Accordingly, therefore, the physiological process of visual pigment bleaching may be expected to differ in some details from the scheme established by solution chemistry. It seems relevant in this connection to note a phenomenon which partially manifests itself in the records of Figs. 4 and 5. It was observed (Hárosi, unpublished) that frog rods as well as goldfish cones when suspended in a salt solution containing no divalent cations (Ca²⁺ or Mg²⁺) and 1 mM EGTA exhibit bleaching properties which are different from those observable in the presence of calcium ions (when tested at the same room temperature and pH). The absence of calcium (assuming that 1 mM EGTA has no effect other than chelating most of the free Ca²⁺ originating from the receptors) seems to have two effects: (a) to prolong the decay of photoproducts and, (b) to protect the final chromophoric species (presumably the vitamins A₁ and A₂) from disappearance from the outer segments. The practical consequence of these effects is that the photoproducts remain more conspicuous in the cell's post-bleach absorption spectra under this condition than without EGTA (e.g. Fig. 5). Although much more experimentation will be necessary than what we have done to date before it can be interpreted, the observation raises the possibility that bleaching of vertebrate visual pigments in situ may be governed by facilitated (enzymatic) rather than purely thermal processes.

Specific Density

Our value of $0.0124 \pm 0.0015/\mu\text{m}$ for the specific optical density (transverse component) of goldfish cone outer segments is higher than that determined by Marks (1965 *a*), $0.008/\mu\text{m}$, and it is very close to the $0.013 \pm 0.002/\mu\text{m}$ figure of Liebman (1972). The more serious discrepancy between our determinations and those of Marks and Liebman arises regarding the specific density of the rhodopsin-containing frog rod outer segments. Whereas they both stated that goldfish cones and frog rods have about the same specific density, we find a significant difference between them. When corrected for bleaching due to the measurement, we obtain $0.0182 \pm 0.002/\mu\text{m}$ for the specific optical density (transverse component) of frog red rods (Hárosi and MacNichol, in preparation).

The disparity between our goldfish and frog results cannot be ascribed, in our opinion, to measurement errors. Although goldfish cone outer segments are somewhat smaller and are conically shaped structures which may introduce a slightly higher refraction error than the larger cylindrical frog rods, we would not expect the difference in measured specific absorptances to be as great as found. However, if the goldfish cone pigments were to have a lesser extinction due to the retinene₂ chromophore as compared with rhodopsin, a self-consistent explanation would obtain as follows. Brown et al. (1963) reported first that the molar extinction coefficient of a porphyropsin (unspecified type) is about 30,000 liter/mol cm, whereas the same for rhodopsin (cattle) is 40,600 liter/mol cm (Wald and Brown, 1953). The ratio of these two coefficients is 0.7389. If using the more recent value of 42,000 liter/mol cm (Matthews et al., 1963; Shichi et al., 1969) for rhodopsin, its ratio with 30,000 liter/mol cm yields 0.7143. Now the ratio of our measured specific densities for goldfish cones and frog rods gives 0.6813, which is within 5–8% of the above extinction ratios.³ The closeness of the agreement is probably fortuitous in view of the uncertainties associated with (a) the extinction coefficients of rhodopsin and porphyropsin, (b) the cellular diameters, (c) the conservative correction for bleaching for all cones based on measurements of a single blue-absorbing cell, (d) the inclusion of the deviant red-absorbing cones in the specific density average. Nevertheless, it appears justified to suggest that the

³ The procedure in taking the ratio of specific densities obtained with transversely polarized light from goldfish cone and frog rod outer segments involves two essential assumptions. These are: (a) the measured transverse specific optical densities of the cells in question are proportional to the molar extinction coefficient of the pigments they contain; (b) the visual pigment concentrations are the same in both cell types. Since the determination of a molar extinction coefficient (ϵ_{\max}) consists of measuring the optical density peak (D_{\max}) of a solution of known concentration (c) and path length (l) according to the relation ($D_{\max} = \epsilon_{\max} cl$), it is by necessity valid for a random collection of molecules. The specific density (S) of such a sample is related to the molecular extinction (M) as $S = D/l = \epsilon c = nM/3$, where (n) is the total number of molecules per unit volume. When the specific densities are determined at equal concentration for two pigments, they are directly proportional to the molecular extinctions as $S'/S'' = M'/M''$. When photoreceptors are measured in situ, due to their structural order and because of the use of polarized light, the simple relationship is modified by (k), a "geometrical factor," as $S_{\perp} = D_{\perp}/l = k\epsilon c$. Although the values of (k) are unknown, the information concerning their size must be reflected in the measured dichroic ratios. It seems intuitively obvious, for instance, that two cells with equal pigment concentration within and equal measured dichroic ratios would still allow the use of the above procedure as $S'_{\perp}/S''_{\perp} = k'\epsilon'/k''\epsilon'' = M'/M''$, for $k'/k'' = 1$. Although, for cells with unequal dichroic ratios the final relationship does not exactly hold, it provides with satisfactory accuracy for most of the dichroic ratios we attain. For example, it can be analytically shown by several rather simple ways that $k'/k'' > 0.94$ (instead of being 1), when S'_{\perp} is the transverse specific density of goldfish cones with measured dichroic ratio of 3 and S''_{\perp} is the transverse specific density of frog rods with measured dichroic ratio of 5, assuming that the conicity of the measuring beam is negligible. The smaller is the condenser's numerical aperture and the larger are the measured dichroic ratios, the closer is k'/k'' to one. As for the assumption of equal visual pigment concentration to exist in different vertebrate photoreceptors, we have no independent evidence. The remarkably close agreement in the specific densities of pigments measured in a number of different receptor types in different species, obtained in several laboratories, makes this appear to be a reasonable assumption.

goldfish cone pigments (at least the types B and G) have similar molar extinction coefficients to the porphyropsins and not to the rhodopsins, and this alone is sufficient to account for our observed difference in specific density of frog rod and goldfish cone outer segments.

Spectroscopic Properties

As discussed in detail by Bridges (1965, 1967), the replacement of the retinal chromophore of a rhodopsin with the dehydroretinal chromophore in order to make a porphyropsin has a threefold effect: (a) λ_{\max} moves to longer wavelengths (bathochromic effect); (b) the molar extinction coefficient is reduced (hypochromic effect); (c) the main band broadens and on its short-wave side the extinction increases (short-wave effect).

Although unequivocal demonstration of these effects can only be done by actually replacing the chromophores of one kind with the other in bleaching and regeneration studies, they can, nonetheless, be recognized individually. Whereas the second and third effects should readily be measurable, the first one is problematic due to the multiplicity of visual pigments and the spectral overlap of the retinene₁ and retinene₂ types. There appears to be no general rule whereby to recognize the type of pigment from its λ_{\max} . And yet, we suggest to do just that regarding those special retinas in which the 620–625-nm red pigment is present. It has occurred to us in the course of studying the available lists of vertebrate visual pigments (e.g. Lythgoe, 1972; Liebman, 1972) that the highest λ_{\max} , when the retinene₁ chromophore is present, is 560–570 nm. On the other hand, in the retinas of goldfish, turtle (*Pseudemys scripta*) and tadpole (*Rana pipiens*), which were shown to have receptors with long-wave pigments ($\lambda_{\max} = 620 - 625$ nm), the retinene₂ chromophore is implicated. If we now add to these the artificial pigment, cyanopsin, which can only be synthesized with 11-cis dehydroretinal and has a λ_{\max} of 620 nm, the following statement seems justified: The red-absorbing visual pigment with $\lambda_{\max} = 620-625$ nm must have dehydroretinal (retinene₂) as chromophore. Since no exception is known to this "empirical rule," we suggest its application as a test criterion in establishing the presence of retinene₂ based on the presence of a "bathochromic red pigment" with λ_{\max} of 620–625 nm. We make use of this criterion in the following summary.

Indications for the Presence of Dehydroretinal (Retinene₂) Chromophores in Goldfish Cone Pigments

(a) The presence of vitamin A₂ (and the lack of vitamin A₁) in eye tissues (Wald, 1939 a). (b) The presence of photoproducts in cone outer segments with absorption maxima near 355 and 405 nm. (c) The existence of a red pigment with $\lambda_{\max} = 625$ nm (bathochromic effect). (d) The diminution of specific optical density of outer segments, implying the presence of a visual

pigment with reduced molar extinction coefficient (hypochromic effect). (e) The breadth of the blue and green spectra (on a reciprocal wavelength scale) being equal to that of porphyropsin and the presence of elevated short-wave absorptions (short-wave effect). With the exception of the narrowness of the red pigment spectrum, therefore, all the experimental evidence fits into a logically complete picture in which dehydroretinal is implicated as the chromophore.

Spectral Width and Molar Extinction

In order to investigate the broadness of visual pigment spectra in the α -band, experimental data were tabulated for comparison (Table I). It is to be noted that spectral widths of visual pigments with various λ_{\max} can be meaningfully compared only when the *relative extinction* spectra are plotted on a *reciprocal*

TABLE I
EXPERIMENTAL DATA CONCERNING THE BROADNESS OF VISUAL PIGMENT SPECTRA

Widths are computed in wave numbers (cm^{-1}) at 50% of α -band peak extinction by the equation: $\Delta\nu = |10^7/\lambda_1 - 10^7/\lambda_2|$, where λ_1 and λ_2 are measured in nanometers at half of peak extinction. $\Delta\nu$ is called half-band width (e.g. Jaffé and Orchin, 1962).

Visual pigment	Wavelength of peak extinction λ_{\max}	Half-band width $\Delta\nu$	Estimated error for width determination	Source of information
	(nm)	(cm^{-1})	(cm^{-1})	
Frog rhodopsin	502	4,258	± 50	Fig. 1 (Nomogram) of Dartnall (1953)
Cattle rhodopsin	498	4,106	± 50	Fig. 3 of Shichi et al. (1969)*
Fish (seven species) porphyropsin	521.2-543.6	4,832	± 50	Table 1 A of Bridges (1967)
Salmon porphyropsin	527 \pm 1	4,533	± 50	Fig. 1 (Nomogram) of Munz and Schwanzara (1967)†
Cyanopsin (chicken cone opsin + 11-cis dehydroretinal)	620	3,544	± 100	Fig. 2 of Wald et al. (1953)
Goldfish red cone pigment in situ	625 \pm 5	3,818	± 200	Text-Fig. 5 of Marks (1965 a)
Goldfish red cone pigment in situ	625 \pm 5	3,625	± 100	Fig. 7 of this paper‡

* Dr. H. Shichi kindly provided us with one of his original records which allowed a more accurate determination than what would be possible to obtain from the printed spectrum.

† Since this nomogram yields bleaching difference spectra, which are narrower than absorption spectra on their short-wavelength side, the determined half-band width, $\Delta\nu$, is probably in considerable error.

‡ A photographically enlarged version of Fig. 7 was used first to construct a smooth curve. This was drawn by free-hand and by visually interpolating the experimental points. The resulting spectrum was then used for determining $\Delta\nu$.

wavelength (or wave number) scale. Although it would seem necessary, for that reason, to transform all the wavelength spectra to wave number spectra, it is sufficient for the present purpose to carry out the conversion at two selected wavelengths. The two wavelengths are customarily chosen to correspond to 50% of the peak extinction on the two slopes of an absorption band. The absolute difference of the reciprocal values of these two wavelengths, called half-band width ($\Delta\nu$), may then be used to characterize the broadness of the band. The ultimate use of $\Delta\nu$ obtains when estimating the intensity of an absorption band by approximating it with an isosceles triangle. In such case the area under the curve is equal to the product of peak extinction and half-band width.

The compiled data in Table I reveal two interesting features of the goldfish red-sensitive cone pigment spectrum: first, that it is remarkably similar to that of cyanopsin not only in λ_{max} but also in $\Delta\nu$; and second, that it is considerably narrower than that of rhodopsin, which in turn is also narrower than that of porphyropsin. Two possible explanations have already been advanced to account for the narrowness of the cyanopsin spectrum (Bridges, 1967): (a) the breakdown of Dartnall's principle, which states that the shapes of visual pigment spectra plotted on a reciprocal wavelength scale are invariant with respect to λ_{max} (Dartnall, 1953); and (b) error in the cyanopsin spectrum itself. In view of the experimental evidence obtained to date (Table I), we are inclined to conclude that although a single spectral template appears to suffice for describing all the rhodopsins and another template for describing all the porphyropsins, including the B and G type cone pigments of goldfish, it does not hold for the dehydroretinal-based red-absorbing pigments.

In an attempt to elucidate this exceptional phenomenon, we have performed a few computations commonly used in spectroscopy (e.g., Jaffé and Orchin, 1962; Sandorfy, 1964). The results are summarized in Table II. A single hypothesis forms the basis of these calculations which may be stated as follows: *The oscillator strength⁴ of the main absorption bands of the three goldfish cone pigments are equal to that of porphyropsin.*⁵

⁴ Oscillator strength is a measure of the intensity of an absorption band which in turn is a manifestation of the strength of a molecular electronic transition. It is proportional to the area underlying the extinction curve when molar extinction is plotted as a function of wave number.

⁵ The assumption implied in the hypothesis is that the main band's oscillator strength is conserved upon going from one molecule to another within the same family of visual pigments. This is not what one should expect, perhaps, but rather that the sum of the strengths of all absorptions be maintained constant, and upon perturbation of the system the strength of one band would be redistributed throughout the remaining absorption bands. Solvent and temperature effects upon dyes tend to follow this rule. However, visual pigments have been proven to be unusual dyes. For example, they show no solvent effects and are insensitive to temperature and pH variations over wide limits. The Dartnall principle itself expresses an exceptional behavior which is yet unexplained. Although, based on theoretical considerations, it would seem unlikely that either Dartnall's principle or our hypothesis be true in the strictest sense, they both may well be phenomenologically

TABLE II
SPECTRAL PROPERTIES OF SOME VISUAL PIGMENTS IN THE MAIN ABSORPTION
(α) BAND

	Previously determined molar extinction coefficient ϵ_{\max}	Previously determined oscillator strength $f = 4.32 \times$ $10^{-9} \int \epsilon d\nu$	Measured half-band width (Table I) $\Delta\nu$	Computed oscillator strength (approx.) $f = 4.32 \times$ $10^{-9} \epsilon_{\max} \Delta\nu$	Assumed molar extinction coefficient ϵ_{\max}
	(liter/mol cm)		(cm^{-1})		(liter/mol cm)
Cattle rhodopsin	42,000*	0.75‡	4,106	0.745	42,000
Fish porphyropsin	30,000§	—	4,832	0.626	30,000
Goldfish cone pigments:					
Blue-sensitive ($\lambda_{\max} =$ 455 \pm 3nm)	—	—	4,832	0.626	30,000
Green-sensitive ($\lambda_{\max} = 530 \pm 3\text{nm}$)	—	—	4,832	0.626	30,000
Red-sensitive ($\lambda_{\max} =$ 625 \pm 5nm)	—	—	3,625	0.626¶¶	40,000**

* Matthews et al. (1963); Shichi et al. (1969).

‡ Shichi et al. (1969).

§ Brown et al. (1963), confirmed by Dartnall. See, Bridges (1967).

|| Assumed on the basis of good spectral match with the corresponding porphyropsin standard.

¶¶ Assumed on the basis of presumed molecular similarities.

** Computed by the approximate relationship: $\epsilon_{\max} = f/4.32 \times 10^{-9} \Delta\nu$.

As the major consequence of the hypothesis, by linking the goldfish cone pigments to porphyropsin and because of the existing relation between oscillator strength (f), half-band width ($\Delta\nu$), and peak molar extinction (ϵ_{\max}), one can calculate unknown extinction coefficients on the basis of measured half-band widths. According to these calculations (Table II), one would predict that, if the molar extinction of porphyropsin is 30,000 liter/mol cm, the B and G type pigments, since they both have the same half-band width, would be expected to possess the same peak extinction. On the other hand, the red-absorbing pigment, due to its narrower half-band width, should have a molar extinction of 40,000 liter/mol cm.

Because of this specific prediction, the hypothesis can be tested, at least in principle, by simply comparing experimentally determined extinction coefficients with the predicted ones. There appear to be two approaches for this test: (a) by measuring the specific optical density of red-absorbing goldfish cone outer segments and, (b) by determining the maximal molar extinction of cyanopsin in solution. The outcome of these (or similar) experiments will provide the necessary information, and on their basis it will be possible to

useful and even correct to a first approximation in most cases. We feel that their application can be justified on the basis of their predictive values and that they should be judged according to the validity of their predictions.

decide whether to accept, to modify, or to reject altogether our hypothesis. Meanwhile, we suggest its use insofar as its predictions explain other phenomena in a consistent manner. There are two examples discussed below indicating the possible usefulness of the hypothesis.

In the course of this work we found that the red-absorbing cone outer segments show consistently high UV absorbance when first measured (see Figs. 1 *a* and 6 *a, b, c*). The obvious explanation for this probably lies in the fact that red light was used for the preparatory work which must have bleached a substantial portion of the pigment originally present. Yet, when the specific densities were calculated for the main absorption bands of the various cells, the values were found essentially the same for all three cone types. Therefore, in spite of their partially bleached conditions, the red-absorbing cones remained optically dense, which result may readily be explained if this pigment possesses a higher molar extinction coefficient.⁶

The second example concerns electrophysiological results obtained from the turtle retina. The relevance of goldfish visual pigments to that of the swamp turtle (*P. scripta*) stems from the findings of Wald et al. (1953) and of Liebman and Granda (1971). The former investigators established that the retina of this species contains considerable amounts of vitamin A₂, and the latter work indicated that its visual pigments might be identical with, or at least spectrally very similar to, the goldfish visual pigments. This similarity bears upon a recent observation of Simon (1973). He found in the turtle retina that the luminosity-type (*L*-type) horizontal cells are maximally sensitive to red light, their hyperpolarizing response being maximal at about 600 nm. The explanation of this phenomenon is by no means an easy task for several factors may be involved. It is possible, for instance, that (*a*) the *L*-type horizontal cells are preferentially innervated by red-absorbing cones; (*b*) the type R cones are more abundant; (*c*) the type R cones collect more light than the B and G types by virtue of their larger ellipsoid and oil droplet diameters; (*d*) the type R cones may have longer outer segments and hence possess a thicker layer of pigment to catch light; (*e*) the type R pigment itself may be a more efficient absorber as a result of having a relatively high molar extinction. We cannot at present choose between these alternatives or to designate the extent to which each of these factors contribute to the response. However, this electrophysiological result is consistent with the proposition that the dehydroretinal-based red-sensitive visual pigments may have a higher molar extinction than the same type of blue-, and green-sensitive cone pigments.

⁶ The correctness of this explanation could be tested by the use of infrared illumination and image converter in all the stages of the preliminary experimental work. Due to technical difficulties, however, this has not been satisfactorily accomplished.

CONCLUSIONS

(a) We confirm the finding of three types of cones in the goldfish retina, each containing a spectrally pure visual pigment. Their absorption maxima and estimated error margins are: 455 ± 3 , 530 ± 3 , and 625 ± 5 nm. (b) Side-illuminated cone outer segments are dichroic. The apparent preference for absorbing transversely polarized light (measured dichroic ratio) is 2–3:1. (c) We confirm and extend earlier observations concerning correlations to exist between visual pigment content and gross cone morphology. (d) The spectral maxima of the detected photoproducts, taken together with other evidence, indicate that the aldehyde of vitamin A₂ is the chromophore of the cone pigments in the goldfish. (e) The rate of decay of photoproducts in goldfish cones is about five times as rapid as that in frog rods when measured under similar external conditions. (f) The average specific optical density for cone outer segments (in side view and for transversely polarized light) is found to be $0.0124 \pm 0.0015/\mu\text{m}$. (g) The half-band widths of cone pigment spectra are such that the types B and G match well the appropriate porphyropsin standards, having $\Delta\nu = 4,832 \pm 100 \text{ cm}^{-1}$. (h) The type R spectrum is significantly narrower than the standard and has $\Delta\nu = 3,625 \pm 100 \text{ cm}^{-1}$. (i) The specific density and spectral width determinations are consistent with the notion that the visual pigment packing (concentration) is about the same in frog rod and goldfish cone outer segments, but that the types B and G pigments have a molar extinction of only about 30,000 liter/mol cm. (j) Based on the hypothesis that the oscillator strength of the main band is invariant for the three types of cone spectra, the molar extinction for the type R pigment is calculated to be about 40,000 liter/mol cm.

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