J. Physiol. (1965), **178**, *pp.* 14–32
With 1 *plate and* 6 *text-figures*
Printed in Great Britain

VISUAL PIGMENTS OF SINGLE GOLDFISH CONES

BY W. B. MARKS

From the Department of Biophysics, Johns Hopkins University, Baltimore, Md. 21218, *U.S.A.*

(*Received* 30 *June* 1964)

Thomas Young (1802) suggested that colour vision depends on the presence in the retina of a limited number, perhaps three, of substances with differing preferences for catching lights of different wave-lengths, situated so that the nervous system can distinguish their relative excitation by light. These substances, the visual pigments, are now known to be in the outer segments of the rods and cones. The simplest form of Young's hypothesis is that each of these photoreceptors contains one of a number of different pigments. The first example of two visual pigments segregated among the receptors in one eye was seen by Boll (1877) and by Kühne (1878) as the pink and green rods in the frog's eye. Denton & Wyllie (1955) photographed the mosaic of pink and green frog rods, and measured the approximate absorption spectrum of rhodopsin in single rods, photographically.

Frog rods are comparatively large. For most receptors the greater sensitivity of a photomultiplier is required to measure the light absorbed. Hanaoka & Fujimoto (1957), with a microspectrophotometer, detected light absorption by visual pigments in frog rods, carp rods, and carp cones lying on their sides. Also in single frog rods, Liebman (1961) measured the absorption spectrum of metarhodopsin, and Brown (1961), Liebman (1962), and Wald, Brown & Gibbons (1963) measured the spectrum of rhodopsin. Wolken (1963) detected visual pigments in single frog rods and possibly also in frog cones. The present paper is taken from the dissertation of Marks (1963) preliminary reports of which have been published (Marks & MacNichol, 1962, 1963). Marks, Dobelle & MacNichol (1964) and Brown & Wald (1964) have since measured difference spectra of visual pigments of single primate cones.

The purpose of the experiments reported here was to measure the absorption spectra of single cones in the goldfish and the amount of pigment each contains. To find the absorption spectrum, lights of various wave-lengths must be passed through the pigment of the cone, and for each wave-length the number of photons transmitted must be measured.

The absorption spectrum measures the light absorbed, and since the pigment is photosensitive the absorbed light bleaches the pigment and makes it no longer available for further measurement. The investigation therefore presents this dilemma: if the measuring light is strong the pigment will have all been bleached away before more than a few spectral wave-lengths have been sampled, but if the light is weak so few photons will be used that their inevitable random fluctuation will limit the precision of measurements. A compromise was reached in which the light intensity used was sufficient to bleach about one third of the pigment in the course of a traverse through the spectrum. At each stage in the traverse the total pigment lost at that moment was estimated, and the *extinction* was taken as the change in transmissivity measured at this wave-length after bleaching, divided by the estimate of the fraction of the pigment bleached. The measured density of the pigments was always less than 0·03; thus self-screening was negligible. A few measurements were made with still dimmer light so that no correction for bleaching was required. These gave results that differed in no significant way from those reported in this paper except that they were noisier. Liebman (personal communication) has repeated these experiments using light weak enough to cause negligible bleaching of the pigment and has obtained very similar results.

For the examination of small receptors it is clearly important to minimize the quantum fluctuations. The fluctuations recorded were from 4 to 7 times the minimum theoretically possible. Almost all the excess over the theoretical minimum is accounted for by the inefficiency of the best available photomultiplier.

METHODS

Instrument. A complete description of the instrument will be published elsewhere (MacNichol & Marks, 1965). The microspectrophotometer is essentially a standard microscope having a photographic tube with a photomultiplier in place of the camera. Illumination is monochromatic, of constant quantum flux, and chopped into two alternating beams. The beams are demagnified to receptor dimensions by an inverted microscope objective in place of a condenser, and one of them passes through a receptor and the other through a clear region in the neighbourhood (see the two arrows in Pl. 1). The intensities that reach the photomultiplier are measured by a feed-back circuit, and at each wave-length the ratio of intensity of the two beams, equal to the fraction of light transmitted by the specimen, is plotted and punched on to paper tape.

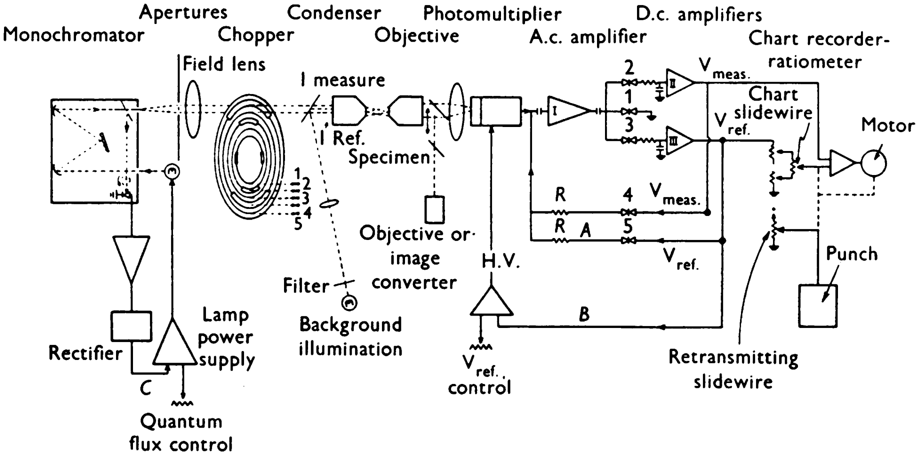
The flow of light and electric current is shown diagrammatically in Text-fig. 1. The filament of a tight-coiled projection lamp is imaged on the entrance slit of a Czerny–Turner type grating monochromator. The exit slit is a round aperture in a mirror that reflects some of the light through a chopper on to a germanium photodiode. The response of the photodiode (RCA 7467) is a fairly good measure of the quantum flux. By keeping this response constant by feed-back control of the lamp voltage by loop *C*, the quantum flux emerging from the exit slit is held constant within 20 % as the wave-length changes, and much more precisely as the lamp filament moves or the bulb blackens. This flux uniformly illuminates two approximately 0·1 mm apertures, forming two beams. These are selected alternately by a chopper disk rotating at 25 rev/sec. Each beam is on for $\frac{3}{8}$ of a cycle, separated by $\frac{1}{8}$ cycle of darkness. A field lens immediately in front of the chopper projects the two beams into the substage condenser of the microscope. The condenser, an inverted apochromatic objective, forms images of the two apertures in the specimen plane. As the wave-length varies between 450 and 650 mμ these images, the 'measuring' beam and the 'reference' beam, move vertically about 2 μ. The beams can be varied in shape to fit the specimen and can be made as small as 1 μ. The specimen, held between two cover-slips because of the lenses' short working distance, can be placed in either beam.

Auxiliary illumination from a tungsten lamp and filter for viewing the entire field of the microscope is reflected into the condenser by a thin glass plate between it and the chopper disk. An apochromatic objective collects the beams and images the specimen for viewing. A standard photomonocular tube (Leitz) with sliding prism permits the light passing through the specimen to fall on to the photomultiplier or to be deflected for viewing into an ocular or an infra-red image converter tube. For photometry the two beams pass through a single lens which images the objective lens stop on the photomultiplier surface. The photosurface of this photomultiplier, E.M.I. 9558A, is a tri-alkali, S-20 type, available since 1960 and of crucial importance because of its high quantum efficiency throughout the visible spectrum, e.g. 12 % at 500 mμ and 5 % at 600 mμ, and low dark current.

In order to provide drift-free amplification and to prevent saturation of the amplifiers, the feed-back circuit shown in Text-fig. 1 was used. Amplifier I produces at its output a voltage proportional to the difference between the photomultiplier anode current and a feed-back current from the output of the measuring circuit. This voltage is switched by photodiodes 1–5 (T.I. 1N2175), which effectively become short circuits when illuminated through slots in the chopper disk. Their actual position is illustrated next to the chopper where each is opposite a slot. Photodiode 1 acts as a 'dc-restorer', zeroing the output of amplifier I between light pulses by shorting the output plate of the coupling capacitor to ground. When the light pulses are on, the output voltages proportional to the error current are switched by photodiodes 2 and 3 to RC low pass filters at the inputs of amplifiers II and III, producing slowly varying voltages there equal to the average height of the error voltage pulses. These voltages, amplified by amplifiers II and III, are the output voltages V_{measure} and $V_{\text{reference}}$. When the measuring beam or reference beam turns on, photodiode 4 or 5 connects the corresponding output voltage to the photomultiplier through one of the resistors R. The differences between the currents that flow, $V_{\text{meas.}}/R$ or $V_{\text{ref.}}/R$, and the photomultiplier currents constitute the error signals. The negative feed-back adjusts the voltages so that these errors are very small, and consequently the output voltages $V_{\text{meas.}}$ and $V_{\text{ref.}}$ are almost exactly equal to R times the increase in photomultiplier currents caused by the light signals.

$V_{\text{meas.}}$ is applied to the input of a potentiometric chart recorder, and a portion of $V_{\text{ref.}}$ is applied across its slide wire, so that the pen position is determined by the ratio $V_{\text{meas.}}/V_{\text{ref.}}$, in which a 10 % change causes a full-scale deflexion. Since the two beams are treated identically except that one goes through the receptor, this ratio is independent of photomultiplier gain, light intensity, etc., and is proportional only to the fraction of light transmitted by the receptor. To keep the sensitivity of the recorder constant and to eliminate transients, feed-back loop B continuously adjusts the high voltage supply to the photomultiplier to keep $V_{\text{ref.}}$ constant. The pen position is coded on to paper tape by a retransmitting slide wire, digitizer, and paper-tape punch permitting direct analysis by an LGP-30 digital computer.

Performance of the instrument. The transmissivity of the specimen is the ratio of the number of photons transmitted through it to the number incident upon it. The number of transmitted photons is measured, but the number incident cannot be since they must be allowed to strike the specimen. Instead, the incident flux is estimated by measuring the reference flux, and the transmissivity is set proportional to the ratio of these two measured fluxes. However, because the photons are emitted independently into the two beams, their numbers in the two beams vary independently so that the ratio between them fluctuates randomly. Greater numbers of photons in the two beams produce smaller fluctuations in the ratio; the fractional root-mean-square fluctuation for each beam can be no less than the reciprocal of the square root of the number of photons converted into electrical signals while recording one independent datum point. Hence, if the reference beam contains much more flux than the measuring beam the photon noise contributed by the reference beam is negligible. The noise factor for the instrument is the ratio of the actual r.m.s. fluctuations in the records to those caused by the photon fluctuations in the measuring beam.



Text-fig. 1. Schematic diagram of microspectrophotometer. Monochromatic light of constant quantum flux controlled by loop C illuminates two apertures. The beams so formed are alternately allowed by the chopper disk to strike the condenser, which forms demagnified images of the two apertures in the specimen plane. One beam penetrates the specimen. When the measuring beam is turned on, light through the small slots in the chopper disk causes photodiodes 2 and 4 to conduct, closing the upper branch of feed-back loop A, which adjusts the slowly changing voltage $V_{meas.}$ toward $R \times$ photomultiplier current. When the reference beam is on, $V_{ref.}$ is adjusted. Loop B controls the photomultiplier gain to keep $V_{ref.}$ constant. The transmissivity of the specimen, $V_{meas.}/V_{ref.}$, is recorded directly by the chart recorder and also punched as digital information on paper tape.

If the pigment under study is photolabile, a limited number of photons can be passed through it before its amount has been reduced by the factor e. This number is $A/\gamma\alpha$, the area of the beam divided by the photosensitivity of the pigment, here about 10^9 photons. The associated fluctuations limit the possible accuracy of measurement. The efficiency of the instrument in gathering and amplifying photons determines how closely this limit can be approached.

The instrument described above gathers into the photomultiplier 80 % of the photons that pass through the receptor, but at 600 mμ the photomultiplier converts only 5 % of these into photo-electrons. These losses cause a fivefold increase in the fluctuations ($[0{\cdot}8 \times 0{\cdot}05]^{-\frac{1}{2}} = 5$), and the noise generated in the amplifiers and recorder increases this noise factor to about 7. The corresponding noise factor for 500 mμ light is about 4, because of the greater quantum efficiency of the photomultiplier at this wave-length. (Because of the square root relation, the relatively low quantum efficiency of the IP 22 photomultiplier used by Hanaoka & Fujimoto (1957), about 0·0016 at 600 mμ, could still have given a noise factor of 25 ($= 0{\cdot}0016^{-\frac{1}{2}}$).)

Procedure. Goldfish from a variety of sources were used. All seemed to possess the same visual pigments. Large (2 lb (0·9 kg)) wild goldfish were usually healthiest and had the largest cone outer segments, as large as 7 μ long and 5 μ wide at the base. Retinas were removed and dissected under deep red (greater than 600 mμ) or deep blue (less than 450 mμ) light. The colour of the light used for dissection had little effect on the results provided the light was dim. Pieces of the retina about $\frac{1}{2}$ mm^2 were teased apart on a cover-slip and stirred in physiological saline (NaCl, 110 mM; KCl, 2 mM; $CaCl_2$, 0·5 mM) containing 5 % gelatin, which melts at about 30° and gels at about 25° C. The pH of the gelatin solution was about 5·5. (On those occasions when they did not drift, receptors suspended in their own vitreous fluid at pH 7·0 without gelatin gave seemingly identical results.) A second cover-slip was squeezed down on top to mash the retina, and the edges were sealed with paraffin. This preparation was placed in the microscope and the whole field was illuminated with blue (2 % of maximum flux at 450 mμ), red (2 % of the maximum at 600 mμ) or infra-red (greater than 700 mμ) background light. The infra-red light was visualized with an RCA 6914-A image converter tube. When a detached cone was found in a clear spot, the beams were focused, polarized perpendicularly to the receptor axis, and the reference beam set in a clear spot. Transversely polarized light gave a higher signal-to-noise ratio than unpolarized light, probably because the cone pigment molecules, like those in rods (Schmidt, 1938; Denton, 1954*a*, *b*, 1955*a*, *b*), are oriented transversely to the receptor axis.

Before the measuring beam was adjusted to pass through a receptor, its colour was set to that of the background light (blue, red, or infra-red) since pigments sensitive to that colour would already be bleached away. When both lights were infra-red, no pigment loss was detected. The measuring beam was adjusted to fill as much as possible of the outer segment, usually one third to two thirds of it (Pl. 1), and the background light turned off. The spectrum was scanned in either direction at 2·3 mμ/sec, which was slow enough to avoid distortion of the spectra by the RC smoothing circuit (τ = 2·2 sec). The absorption spectra were recorded and punched on to paper tape. Each scan took about 150 sec, during which time data points were punched on to tape every 3·0 mμ.

Often 'odd' spectra occurred with fresh slides. These were caused by the motion of the receptors. When motion ceased (shown by perfect coincidence of successive absorption spectra through bleached outer segments or cone ellipsoids) outer segments gave records like those of Text-fig. 2, or did not bleach.

In the total absorption spectrum of a receptor, refraction, diffraction, and scattering effects add directly to the absorption by the pigments. These effects were reduced by taking the difference spectrum: the decrease in absorption at each wave-length after bleaching with light.

The difference spectra were not measured in the usual manner using beam intensities that cause negligible bleaching of the pigment. Instead spectra were recorded with a brighter measuring beam that bleached as it measured, and afterwards the difference spectra were corrected for bleaching. The brighter beams gave lower photon noise, and the distortion in the spectra due to bleaching that remained after correction was small (see Discussion). The correction for bleaching was based on an estimate of the fraction of pigment bleached during each scan through the spectrum. This estimate of the rate of bleaching was obtained from repeated scans with the measuring beam without any auxiliary bleaching light until the pigment was bleached almost completely away. The correction was applied to the difference spectrum: last scan minus first scan.

If the intensity of the measuring beam was too great, the gain through photon noise reduction was offset by the early loss of pigment. An intensity that caused about half the pigment to bleach away during each scan gave the greatest ratio of change in absorption to photon noise. However, then the effects of photoproducts and variations in the rate of bleaching became important, so in practice an intensity was used that bleached about one third of the pigment per scan. For the usual scan speed of 2·3 mμ/sec this intensity was approximately 10^7 photons per μ^2. sec.

Correction for distortion caused by bleaching. The following symbols are used:

M number of molecules of visual pigment present at any time;
M_0 number of molecules of visual pigment present initially;
T transmissivity, i.e. the fraction of incident photons transmitted;
T_λ transmissivity at wave-length λ before bleaching;
$T_{B\lambda}$ transmissivity after bleaching;
dN_λ number of incident photons of wave-length λ;
ϵ_λ extinction of the pigment;
K effective quantum efficiency of bleaching;
λ_0, λ_f wave-lengths at the beginning and end of the scan through the spectrum.

If the pigment is bleached by the beam that measures it, its spectrum will be distorted by continual loss of visual pigment and by the continual production of photoproducts. It was possible to correct the spectra for the loss of primary pigment but not for distortion by photoproducts, since the properties of the photoproducts were unknown. Over most of the visible spectral range the absorptive loss of the visual pigment, however, considerably exceeds the production of photoproducts. Distortions that remain after the correction for loss of visual pigment probably dislocate the spectral maximum no more than about 5 mμ (see Discussion).

Three assumptions were made in correcting the difference spectra for loss of visual pigment during the scans.

Assumption 1. The number of visual pigment molecules dM bleached by dN photons is proportional to the number of these absorbed by bleachable substance:

$$-dM_\lambda = K(T_{B\lambda}-T_\lambda)\,dN_\lambda,$$

since $T_{B\lambda}-T_\lambda$, the difference between the fraction of photons transmitted before and after bleaching, the difference spectrum, is the fraction of incident photons absorbed by bleachable substance. The proportionality constant does not equal the quantum efficiency of bleaching because photoproducts and original pigment regenerated from photoproducts absorb additional photons.

Assumption 2. At the end of the first scan the fraction of pigment remaining unbleached is the ratio of the 'areas' under the first two scans (e.g. areas under curves 1 and 2, Text-fig. 2*c*).

$$S = \int_{\lambda_0}^{\lambda_f}(T_{B\lambda}-T_{2\lambda})\,dN_\lambda \Big/ \int_{\lambda_0}^{\lambda_f}(T_{B\lambda}-T_{1\lambda})\,dN_\lambda.$$

The value for the fraction of the original pigment remaining at each wave-length that satisfies these two assumptions is:

$$M_\lambda/M_0 = 1-(1-S)x_\lambda, \tag{1}$$

where

$$x_\lambda = \frac{\int_{\lambda_0}^{\lambda}(T_{B\lambda}-T_\lambda)\,dN_\lambda}{\int_{\lambda_0}^{\lambda_f}(T_{B\lambda}-T_\lambda)\,dN_\lambda}. \tag{2}$$

This is clearly the correct function, for, when λ increases a small amount, x_λ (and therefore also M_λ according to (2)) changes by an amount proportional to $(T_{B\lambda}-T_\lambda)\,dN_\lambda$. Since x_λ is normalized so that it runs from 0 when $\lambda = \lambda_0$ at the beginning of a scan to 1 when $\lambda = \lambda_f$ at the end of a scan, the fraction of pigment remaining moves from 1 to S during a scan, in agreement with assumption 2.

Assumption 3. The extinction ϵ_λ of the pigment is proportional to the ratio of the amount of light absorbed by pigment to the amount of pigment present:

$$\epsilon_\lambda \propto \frac{T_{B\lambda}-T_\lambda}{1-(1-S)x_\lambda}. \tag{3}$$

In the actual calculation S^{x_λ} was used in the denominator of (3) instead of $1-(1-S)x_\lambda$. Since x runs from 0 to 1 during a scan, S^{x_λ} like $1-(1-S)x_\lambda$, also runs from 1 to S. However, instead of moving in a straight line when plotted against x, S^{x_λ} is concave downwards, so that for $S = \frac{1}{2}$ it is about 10 % too low in the middle of the spectrum. This causes about a 5 % narrowing of the spectra when half the pigment is bleached away on each scan and mathematically restored, and is not a serious error. More serious is the difficulty in estimating S when S is less than about $\frac{3}{4}$. Then the ratio of areas under successive scans is seriously affected by the accumulation of photoproducts or the quicker depletion of those molecules in the brighter part of the beam, or perhaps by quicker depletion of those molecules oriented more nearly transversely to the beam. When many scans are made, as in these experiments, the ratio of areas between successive scans should also equal S, but because of these effects the ratios varied, and S was taken as a weighted average of them. The exact procedure followed was to compute a list of the ratios of areas between successive scans and to average these ratios with weights proportional to the second area in each pair, to obtain S. Then x_λ was computed as in (2) as the fraction of the total area between the first and last scans covered when the scan has reached the wave-length λ. Then, $M_\lambda/M_0 = S^{x_\lambda}$ was computed, and the difference spectrum between the first and last scans at each wave-length λ was divided by M_λ/M_0.

For two experiments in which the scans were in opposite directions for which $S = 0{\cdot}6$ this method reduced a 30 mμ disparity in the absorption maxima to 5 mμ, by moving a peak at 610 to 630 mμ, and a peak at 640 to 625 mμ. Similarly, the peak in the difference spectrum of a frog rod was moved on correction from 510 to 504 mμ. 504 mμ was found to be the maximum of the difference spectrum of single frog rods when bleaching was negligible, in agreement with Wald *et al.* (1963), and with Dartnall (1961).

MacNichol & Marks (1965) derive a relation between the signal-to-noise ratio and the amount of pigment bleached by the measuring scans. There the maximum possible signal-to-noise ratio is assigned to the weighted average of many undistorted scans made in dim light. It is then shown that the method employed here can achieve nearly this maximum signal-to-noise ratio, and that a single scan which bleaches a negligible amount (2–5 %) of the pigment can achieve about one third the maximum. Hence, methods which bleach an appreciable amount of pigment are mainly applicable in those situations where it is important to achieve a factor of 2 to 3 in signal-to-noise ratio, such as in the study of very small receptors. The method described here has, in fact, been applied to fragments of grey squirrel receptors only 2 μ long and 1 μ in diameter, to give an absorption maximum of 502 ± 4 mμ, the same as that of a pigment extracted from the grey squirrel retina by Dartnall (1960).

Simple estimate of displacement of spectral maximum caused by bleaching. S, the attenuation in the amount of visual pigment caused by one scan, can be estimated by eye as the ratio of the areas between successive scans, since the area between the scans is approximately proportional to the amount of pigment present to be bleached. This estimate can be used to estimate the displacement of the spectral maximum due to bleaching, without attending to the actual shape of the recorded spectrum, by assuming that the correct shape when plotted against wave number is that of rhodopsin (Dartnall, 1953). This shape can be approximated as a Gaussian function near the spectral maximum. The Gaussian approximation is multiplied by the rate of loss of pigment derived above, to obtain an approximation to a spectrum distorted by bleaching

$$\frac{\exp\{-[(f-f_0)/W]^2\}}{\sqrt{\pi}\,W}[1-(1-S)x_f],$$

where f stands for wave number, x_f is (from 3) the integral of the (normalized) Gaussian function up to the wave number f, and W is the width that fits the rhodopsin spectrum. By differentiating this function with respect to f and equating the result to 0 the spectral maximum is found to be displaced by

$$\Delta f = \frac{W(1-S)}{\sqrt{\pi}(1+S)}.$$

Now $\Delta f = \Delta\lambda/\lambda^2$, and from the Dartnall (1953) nomogram, $W = 0{\cdot}24u^{-1}$, so that finally

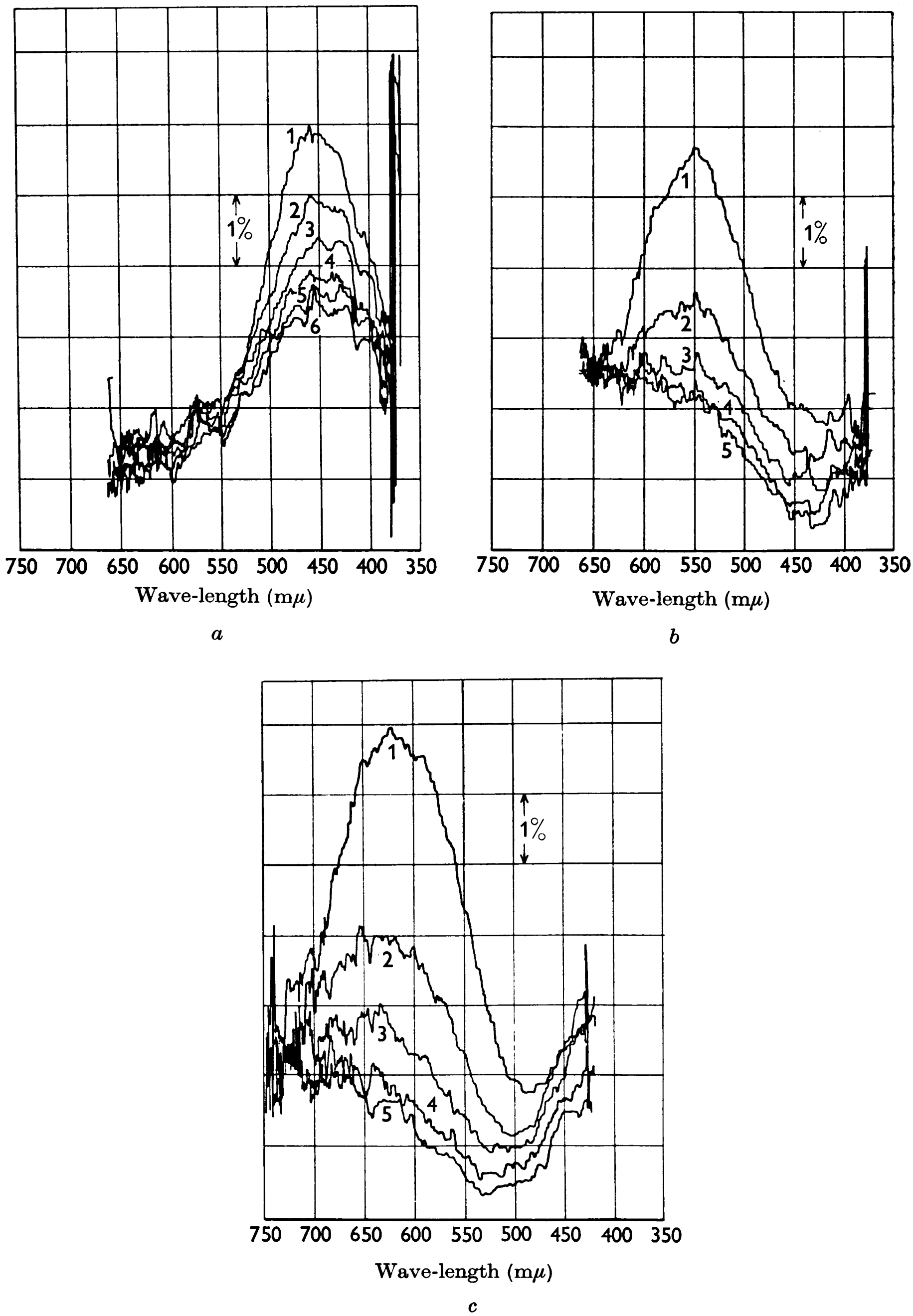
$$\Delta\lambda = 0{\cdot}14\lambda^2 \frac{(1-S)}{(1+S)} \cong 0{\cdot}07\lambda^2 \ln(1/S),$$

where λ and $\Delta\lambda$ are measured in microns.

RESULTS

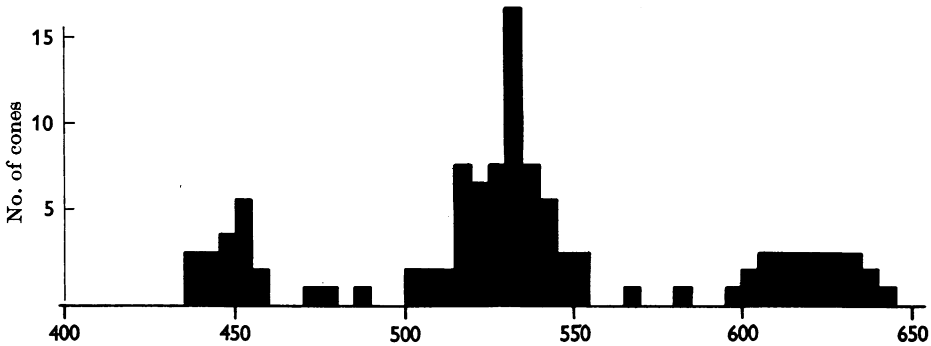
Text-figure 2 shows absorption spectra from three goldfish cone outer segments measured transversely as shown on the plate. Increasing absorption is plotted upward, 1 % per division. Each series was recorded successively without intervening bleaching. Text-figures 2*a*, *b* were scanned from long wave-lengths to short, and *c* from short wave-lengths to long. The successive decreases in absorption show the presence of pigments being bleached away by the measuring beam, which initially absorbed as much as 2·5, 3·5, or 5 % of the incident light. Some absorption, 2–6 %, remained after all the pigment was bleached away. Similar experiments done on other structures such as cone ellipsoids showed no such change in absorption. The wave-lengths of greatest absorption change differ somewhat from the absorption maxima of the pigments because the amount of pigment was decreasing continuously during the scans. In the absence of photoproducts and changes in pigment distribution each scan should attenuate the amount of pigment by a constant fraction equal to the ratio S of areas between successive scans. S is 0·8 in Text-fig. 2*a* and 0·5 in *b* and *c*. As derived in Methods, attenuating the amount of pigment by S on each scan would displace the absorption maximum of rhodopsin-like pigments roughly $0{\cdot}07\lambda^2 \log_e(1/S)\ \mu$ toward wave-lengths measured earlier, where λ is the wave-length maximum in μ. Applying this correction shifts the wave-length maxima of the experiments shown in Text-fig. 2 from 455 to 452 mμ, from 545 to 531 mμ, and from 610 to 630 mμ.

This correction, based on a visual estimate of S, the ratio of areas between successive scans, was applied to all the experiments done since the instrument was modified to irradiate the receptors with a constant quantum flux. The average magnitude of the correction was about 15 mμ. The result is shown in Text-fig. 3, where the wave-length of maximum absorption is plotted against the number of occurrences of each maximum. All experiments are included except those where no bleaching occurred or wherein the receptors moved, 113 experiments in all. Eighteen experiments fell in a group near 450 mμ, 66 near 530 mμ, 24 near 630 mμ, 2 near 570 mμ, and 3 near 480 mμ. The two entries near 570 mμ are not from spectra of single cones but are the maxima of composite spectra made on touching twin cones. The shape of the difference spectra having absorption maxima in the blue sometimes varies, possibly because of more prominent



Text-fig. 2. For legend see opposite page.

photoproducts or because misalignment causes more chromatic motion of the beam in the blue. This, rather than another cone type, probably caused the three entries near 480 mμ in Text-fig. 3. Most of the scatter of the three main groupings was caused by the difficulty of estimating by eye the peak wave-length and rate of bleaching of noisy spectra. The groupings in Text-fig. 3 suggest that there are just three kinds of cones in the goldfish retina.



Text-fig. 3. Histogram from 113 experiments on single cones such as those in Text-fig. 2 showing number of occurrences of difference spectra having each peak wave-length. Peak wave-lengths were shifted, by a simple estimate described in methods, by an average of 15 mμ to allow for distortion due to bleaching. The two entries at 565 mμ and 580 mμ are the peak wave-lengths of composite spectra of touching twin cones. The groupings imply there are three types of cone in the goldfish retina.

A plot of frequency of occurrence of various absorption maxima similar to Text-fig. 3 that included the experiments done before the quantum flux in the measuring beam was controlled contained about six peaks. These were absorption maxima scattered by distortion due to variable rates of bleaching.

Populations

Some 'bleached' cones are always observed when a visible background light is used to search for cones. Such experiments cannot exclude the possibility that in intact eyes there is a fourth class of cones that are not

---

Legend to Text-fig. 2.

Text-fig. 2. Series of absorption spectra recorded from three cone outer segments, made without auxiliary bleaching. The successive decreases in absorption show bleaching by the measuring beam in three different spectral regions. The photon noise fluctuations are evident. Each vertical division represents an absorption change of 1 %. Absorption or scattering of 2–6 % remained after bleaching. The absorption differences between the first and last scans are maximum at 455, 545, and 610 mμ. Correction for progressive bleaching moved the maxima to 452, 531 and 630 mμ. Records *a* (blue) and *b* (green) were scanned from long to short wave-lengths, and record *c* (red) from short to long.

bleached by the energies and wave-lengths of light in the measuring beam. However, of seventeen single cones (see below) in one fish found using an infra-red background light, eleven were green receptors, four were red receptors, two were blue receptors and none were unbleachable. Thus, all three cone types occur in one eye, and probably all the cones in the eye are one or another of the three types.

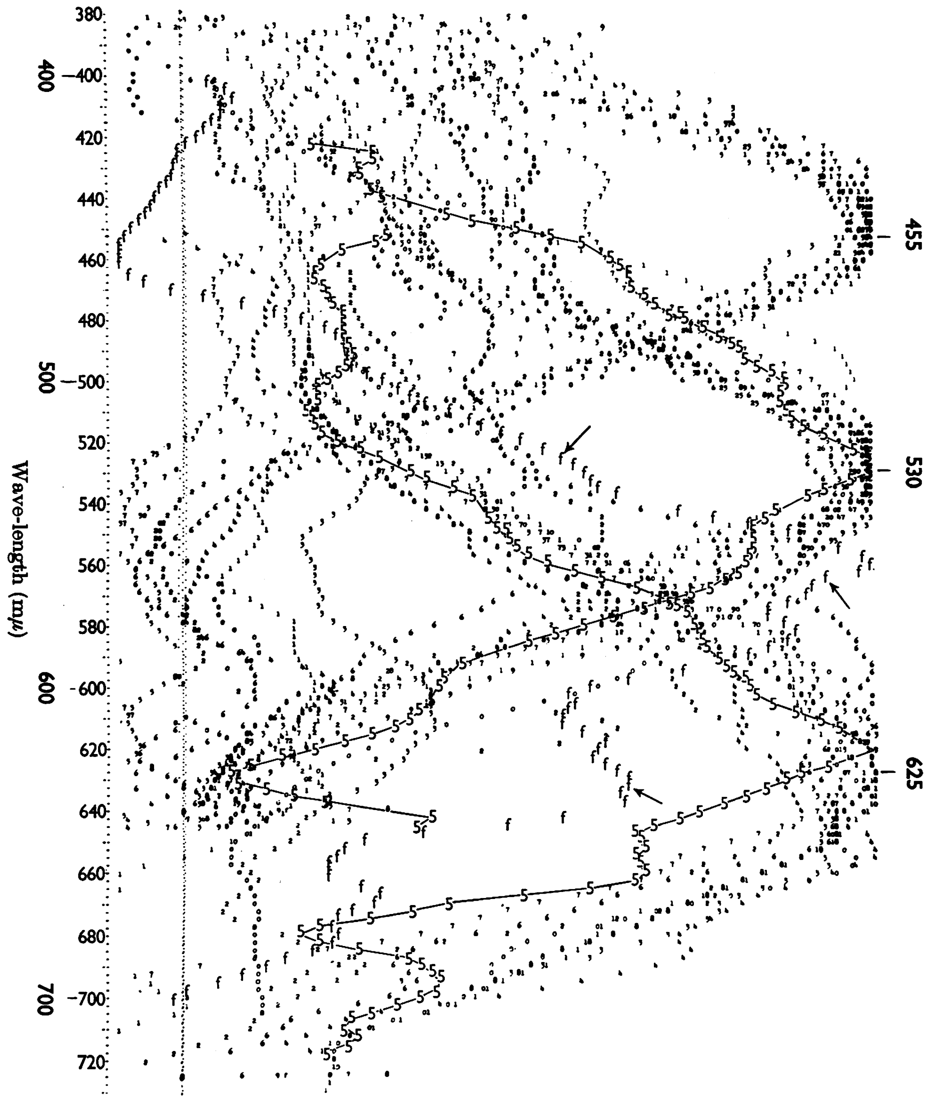
About half the cones in the goldfish retina are members of symmetrical, joined twin cones. Out of thirty pairs of twins examined, twenty-nine were red-green pairs; one was blue-green. The single cones occurred in the ratios red, green, blue, 2:4:1, approximately. These estimates are subject to the possibility of inadvertent selection of receptors and the contamination of the population of single cones by twin cones broken in two, and of the population of twin cones by single cones lying together.

Difference spectra of the visual pigments

The shapes of the difference spectra were distorted by progressive bleaching of the pigment and by photon noise fluctuations. The distortions caused by bleaching were largely removed by augmenting the absorption at each wave-length by an estimate of the fraction of pigment lost up to that wave-length (see Methods). To demonstrate the trimodal occurrence of the whole spectra, the twenty-eight experiments which gave the greatest change in absorption on bleaching were collected. The curves were all scaled to the same maximum and are plotted by the computer. They are shown in Text-fig. 4.

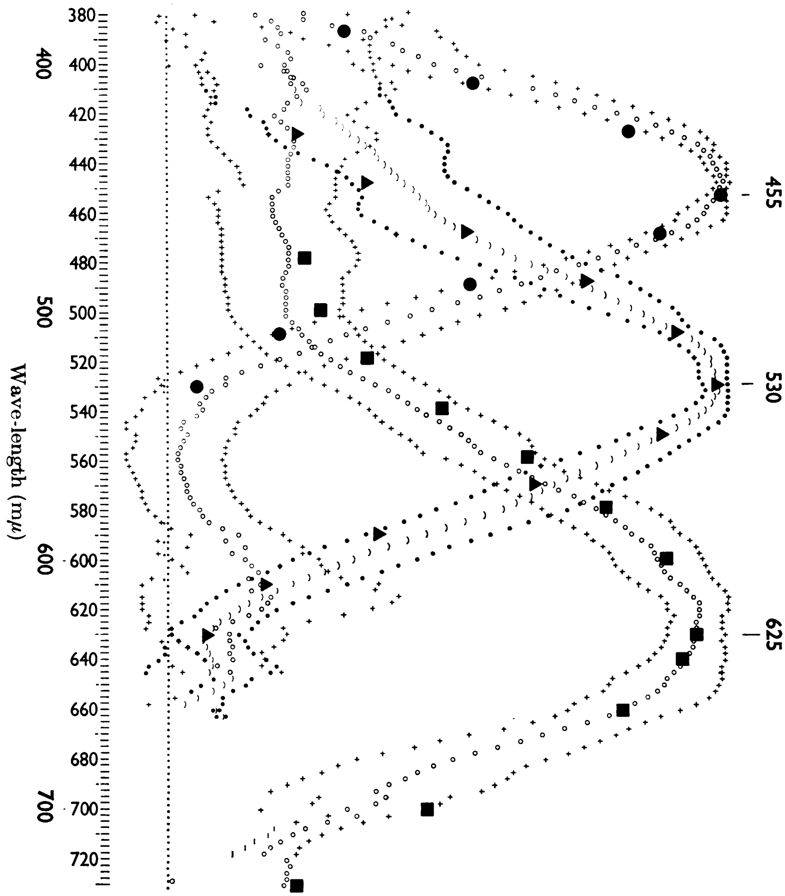
In addition to the noise of Text-fig. 2, the computed difference spectra of Text-fig. 4 contain the noise from the scan after bleaching used to compute the difference spectrum, though this could have been averaged out by repetitions of the 'bleached scan'. Also some of these curves contained 'ripples', interference fringes from reflexions within the cover-slips, visible in the cone record of Text-fig. 6. Errors in estimating S, the pigment attenuation per scan, discussed in the methods section also broadened the groups somewhat. The blue-sensitive spectra, unlike the others, were all scanned in one direction, from red to blue; they occupy the waveband of greatest photomultiplier efficiency. Hence, they have least scatter. The two spectra of Text-fig. 4 connected by lines were made at very low intensities so that correction for bleaching would be unnecessary. The most eccentric curve, '*f*', indicated by arrows, was a composite spectrum of the two pigments in a pair of touching twin cones. Since these spectra were not selected on the basis of wave-length, they suggest again that there are three types of cone in the goldfish.

The ten curves in the green group except '*f*', the eight curves of the blue group, and the nine of the red group, were each averaged together. The



Text-fig. 4. Twenty-eight spectra such as those described in Text-fig. 3 selected only for signal-to-noise ratio, corrected for bleaching by calculating the rate of loss of pigment, normalized to the same height, and plotted by the computer. The dotted line represents zero difference on bleaching. Curve *f*, indicated by arrows, was the composite spectrum of a pair of touching twin cones. The two curves connected by solid lines were made at intensities that bleach less than 5% per scan, and hence, though noisier, required no correction for bleaching. Their similarity to the other spectra suggests that photolabile photoproducts do not greatly distort the spectra. A number of the spectra that have peaks in the blue become negative near the centre of the spectrum, then go through a submaximum in the red region. The spectra that have peaks in the red and green show little tendency to become negative. The scatter of the spectra was caused by photon noise fluctuations of the original and bleached scans, inaccuracies in estimating the amount of bleaching per scan (S), and interference fringes due to reflexions within the cover-slips.

three average curves are shown in Text-fig. 5 bracketed by curves that show the standard deviation of the data. The large solid symbols show the shape of absorption spectra of hypothetical pigments derived from the Dartnall nomogram with maxima at 455, 530 and 625 mμ. When plotted versus frequency rather than wave-length, such curves have the shape of the absorption spectrum of rhodopsin. This is a property of most known visual pigments (Dartnall, 1953, 1962). The difference spectrum of the green-sensitive pigment shown in Text-fig. 5 fits this shape rather well,



Text-fig. 5. Averages of the three groups of difference spectra shown in Text-fig. 4, excluding curve *f*. The bracketing curves show the standard deviation of the data. N = 8, 10, 9 from blue to red. The large points show the shapes of Dartnall (1953) nomogram pigments with peak wave-lengths of 455, 530, and 625 mμ. The deviations of the spectra from these expected shapes may have been caused by the change in focal length of the condenser with wave-length (2 μ through the spectrum), or by photoproducts. Note the hump at about 615 mμ of the spectrum with the peak in the blue.

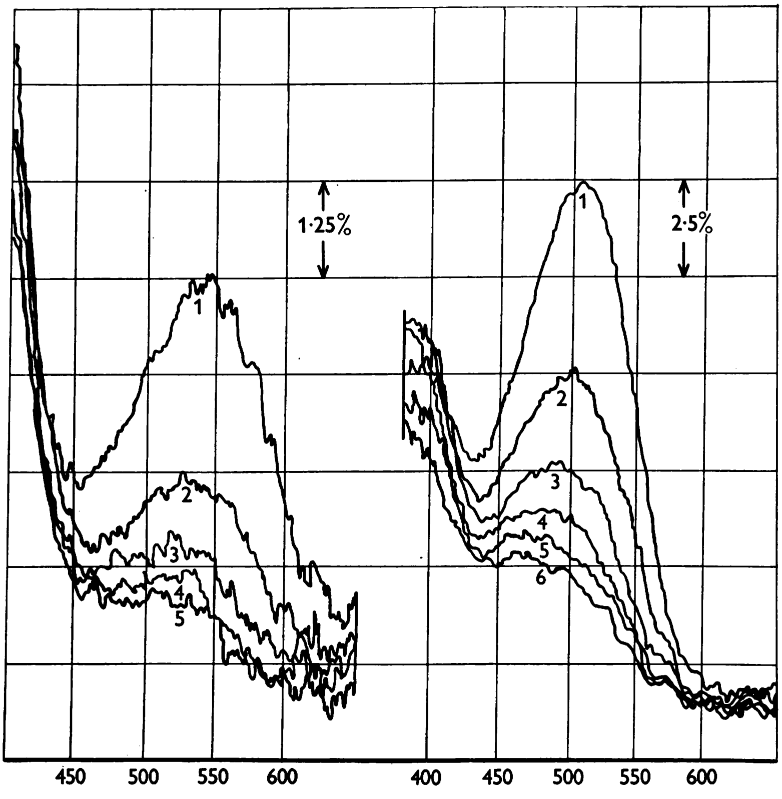
but the spectrum of the red-sensitive pigment is somewhat narrower. This may have been caused by the narrowing tendency of the method used to correct for bleaching, defocusing of the measuring beam in the far red, or by a photoproduct. The spectrum with the peak in the blue is a little wider than the nomogram pigment, and has an auxiliary hump at about 600 mμ. This hump may be part of the main pigment spectrum or the spectrum of an admixture of another pigment. It also becomes negative on occasion, showing a portion of the spectrum of a photoproduct. Suggestions of this appear in Text-fig. 4 as negative-going portions of the blue-sensitive spectra near 550 mμ. Another source of error in these spectra may be small changes with wave-length in the focal length of the apochromatic condenser.

Comparison with rhodopsin

Liebman (1962) measured the absorption spectrum of rhodopsin in single frog rods by a microspectrophotometric method similar to that employed here, but with a larger measuring beam that bleached a very small fraction of the pigment. A 17-fold brighter measuring beam, however, converted all the rhodopsin into a photolabile photoproduct during the early part of the first scan. On the subsequent scans the absorption spectra were those of the photoproduct alone, which, as it hydrolysed away, produced curves suspiciously similar to these cone spectra.

In order to exclude the possibility that the intensities used here were in this range and these pigments were simply photoproducts, an experiment was done under the usual conditions on a frog rod. To compare the properties of rods and cones a green-sensitive fish cone was subjected to the same experiment. The series of transmission spectra of the rod is shown in Text-fig. 6 on the right. That for the cone is on the left. In each series the wave-lengths were scanned from long to short, and in each the diameter of the measuring beam was 3·8 μ and its flux $(1 \pm 0{\cdot}2) \times 10^8$ photons/sec polarized perpendicularly to the axis of the receptor. The thickness of the rod was about 7 μ and that of the cone 4 μ. The absorption maximum of the difference spectrum of the frog rod was at 510 mμ, but it was calculated by computing the rate of loss of pigment that the maximum in the absence of bleaching by the measuring beam would have been at 504 mμ, in agreement with experiments done at intensities that did not bleach. Wald *et al.* (1963) also find the absorption maximum of single frog rods at 504 mμ, and for suspensions of frog rods Dartnall (1961) finds 505 mμ. Besides confirming the correction for bleaching, this agreement suggests that at these intensities photoproducts do not greatly distort the spectrum of rhodopsin.

If fish cone pigments were quite different from frog rhodopsin, these conclusions might not apply to the cone spectra. But a comparison of the curves in Text-fig. 6 suggests that these pigments are quite similar. Both fit the Dartnall nomogram with an error in breadth of about 5 mμ when corrected for bleaching. The recorded peak changes in absorption, 10·6 % for the rod and 4·3 % for the cone, become 13·6 % and 7·04 % when corrected for initial bleaching. The thickness of the rod and cone were about 7 and 4 μ; hence their pigments each absorb about $1{\cdot}8 \pm 0{\cdot}3$ % of the incident light per μ thickness. Furthermore a fish cone and a frog rod irradiated with equal quantum fluxes of the wave-length they absorb best had similar time constants of disappearance of absorption: $4{\cdot}5 \pm 1$ min.



Text-fig. 6. Experiments performed under identical conditions on a fish cone (left) and a frog rod (right). For each the beam diameter was 3·8 μ and the flux was $(1 \pm 0{\cdot}2)\ 10^8$ photons/sec. When corrected for progressive loss of pigment, the spectra of the cone and rod are maximum at 525 and 504 mμ, and fit the Dartnall (1953) nomogram with about 5 mμ error in width. Bleaching correction increased the peak absorption of the cone from 4·3 to 7 %, and of the rod from 10 to 13·6 %. The diameter of the cone was about 4 μ, and that of the rod about 7 μ. Hence, the change in absorption on bleaching for each was about 1·8 % per μ thickness. The rod pigment bleaches away somewhat more slowly, probably because there is more of it in the fringes of the beam where the intensity is lower.

Since the photosensitivity determines this time constant (Dartnall, Goodeve & Lythgoe, 1936), the green-sensitive fish cone pigment must have a photosensitivity similar to that of rhodopsin. Finally, preliminary experiments with polarized light suggest that the dichroic ratio of the visual pigments in the fish cones is similar to that in rods, so that there too the molecules are oriented to catch light polarized perpendicularly to the axis of the outer segment.

The actual pigment concentration within the receptor outer segment may be measured by the light absorption per unit thickness, the extinction of the pigment, and the orientation of the pigment molecules. The pigment molecules in rods and cones seem to be similarly oriented, the absorption per unit thickness across these rods and cones is about the same, and the equality of photosensitivity implies that the extinctions are approximately equal. Hence, the concentrations of pigment molecules in the outer segments of goldfish cones and frog rods must be about the same. This concentration is about 2·5 mM (Liebman, 1962).

To test further the possibility that these cone spectra were merely those of photoproducts, difference spectra were measured using intensities about one tenth those of Text-fig. 6. The resulting spectra could not be distinguished from those obtained at higher intensities except that they were noisier. Two of them are shown in Text-fig. 4, connected by solid lines. These lower intensities would bleach only about one twentieth of the rhodopsin in a rod during a scan, so, if the cone visual pigment bleached away so quickly as to escape notice, it must be much more than twenty times as sensitive as rhodopsin. This is very unlikely since rhodopsin is one of the most photosensitive substances known (Dartnall, 1957).

DISCUSSION

It seems unlikely that these different types of cone all contain a single visual pigment, their spectra being modified differently in the wavelengths allowed to strike this pigment (Enoch, 1963, reviews this and similar possibilities). For such a pigment would need an extraordinarily broad, high absorption spectrum, and the similarity in shape of the spectra actually recorded to that of rhodopsin would then be a remarkable coincidence. The photoreceptor could not rely on the gross light distribution within the outer segment to achieve this, for experiments performed on both ends of the same outer segment give identical spectra. The qualitative and quantitative similarity of the difference spectra recorded from the fish cone and frog rod suggest that the correct interpretation for these results is that in the cone, as in the rod, photons with equal access to the pigment bleach the pigment and that this accounts wholly for the decrease in absorption.

The major errors in difference spectra are usually caused by labile photoproducts, which distort the initial and intermediate scans by absorbing light according to their own spectra, and stable photoproducts, which remain at the end and distort the final scan. Spectra recorded with negligible bleaching (the two in Text-fig. 4 and others), when photolabile photoproducts are absent, have unchanged shape. Dartnall (1962) finds that stable photoproducts shift the difference spectrum of carp rod suspensions 10 mμ towards longer wave-lengths. However, the spectra he reports reveal the presence of photoproducts by becoming negative below 440 mμ, whereas the red- and green-sensitive spectra reported here, trustworthy above about 410 mμ, are everywhere positive. Hence, photoproducts probably shift these spectra less than 10 mμ. The same was said of the calculation to correct for bleaching. Hence, the red- and green-sensitive difference spectra of Text-fig. 5 are probably within about 5 mμ the same as the absorption spectra of the cone visual pigments. The red-sensitive pigment is very similar to the 618 mμ pigment cyanopsin (Wald, Brown & Smith, 1953). The green- and blue-sensitive pigments may be the same as the 533 and 467 mμ pigments of the tench (Dartnall, 1952).

Finally, it should be noted that the finding of Wald, Brown & Smith (1955), that in the chicken each rod outer segment contains about 13 times as much pigment as is contained in each cone, is in agreement with the hypothesis that the pigment concentration and distribution in all cones is approximately equal to that in rods. For the ratio of the volumes of the outer segments of peripheral chicken rods and cones, according to the illustrations of Walls (1942), is also about 13.

SUMMARY

1. By measuring the spectral absorption of single photoreceptors, microspectrophotometry can test the hypothesis that colour vision depends on the presence in the retina of a variety of visual pigments segregated at the cellular level.

2. The necessity of using small amounts of light when recording the spectra of single cones introduces statistical uncertainties into the spectra. A microspectrophotometer is described that makes maximum use of the limited number of photons available, reducing these uncertainties to nearly the theoretical limit. The fluctuations can be further reduced by measuring the spectra with a beam that bleaches some pigment, and then correcting the spectra for bleaching.

3. Goldfish retinas contain three kinds of cones, each containing a single kind of visual pigment with an absorption maximum at 455 ± 15, 530 ± 5 or 625 ± 5 mμ, except that the blue receptor may contain some red-labile pigment.

4. There are five to ten times as many red and green receptors as blue receptors in the retina. The constituents of the twin cones are almost always a red receptor and a green receptor.

5. Goldfish cone pigments resemble the frog rod pigment in spectral shape, photosensitivity, and concentration within the outer segment.

I thank especially Edward F. MacNichol, Jr. for suggesting this problem and originating the components and many of the concepts of the instrument; Warner E. Love for computer programming aid; William H. Dobelle for assistance with the experiments; and W. A. H. Rushton for his helpful criticism of the manuscript. Text-figs. 2, 3, 4 and 5 are reproduced from the article 'Retinal Mechanisms of Color Vision', by E. F. MacNichol Jr., in *Vision Research*, **4**, No. 1/2 by permission of the Editors. Research was supported by grants from N.S.F. and N.I.H.

REFERENCES

BOLL, F. (1877). Zur Anatomie und Physiologie der Retina. *Arch. Anat. Physiol., Lpz.*, pp. 4–36.

BROWN, P. K. (1961). A system for microspectrophotometry employing a commercial spectrophotometer. *J. opt. Soc. Amer.* **51**, 1000–1008.

BROWN, P. K. & WALD, G. (1964). Visual pigments in single rods and cones of the human retina. *Science*, **144**, 45–51.

DARTNALL, H. J. A. (1952). Visual pigment 467, a photosensitive pigment present in tench retinae. *J. Physiol.* **116**, 257–289.

DARTNALL, H. J. A. (1953). The interpretation of spectral sensitivity curves. *Brit. med. Bull.* **9**, 24–30.

DARTNALL, H. J. A. (1957). *The Visual Pigments*, p. 82. London: Methuen; New York: Wiley.

DARTNALL, H. J. A. (1960). Visual pigment from a pure cone retina. *Nature, Lond.*, **188**, 475–479.

DARTNALL, H. J. A. (1961). Visual pigments before and after extraction from visual cells. *Proc. Roy. Soc.* B, **194**, 250–266.

DARTNALL, H. J. A. (1962). The photobiology of the visual process. In *The Eye*, vol. 2, ed. DAVSON, H. London, New York: Academic Press.

DARTNALL, H. J. A., GOODEVE, C. F. & LYTHGOE, R. J. (1936). The quantitative analysis of the photochemical bleaching of visual purple solutions in monochromatic light. *Proc. Roy. Soc.* A, **156**, 158–170.

DENTON, E. J. (1954*a*). A method of easily observing the dichroism of visual rods. *J. Physiol.* **124**, 16*P*.

DENTON, E. J. (1954*b*). On the orientation of molecules in the visual rods of *Salamandra maculosa*. *J. Physiol.* **124**, 17*P*.

DENTON, E. J. (1955*a*). Une nouvelle méthode pour déterminer la courbe d'absorption du pourpre rétinien. *C.R. Acad. Sci., Paris*, **239**, 1315–1316.

DENTON, E. J. (1955*b*). On the bleaching of the photosensitive substance in the retinal rods of *Xenopus laevis*. *J. Physiol.* **116**, 32–33*P*.

DENTON, E. J. & WYLLIE, J. H. (1955). Study of the photosensitive pigments in the pink and green rods of the frog. *J. Physiol.* **127**, 81–89.

ENOCH, J. M. (1963). Optical properties of the retinal photoreceptors. *J. opt. Soc. Amer.* **53**, 71–85.

HANAOKA, T. & FUJIMOTO, K. (1957). Absorption spectra of a single cone in carp retina. *Jap. J. Physiol.* **7**, 276–285.

KÜHNE, W. (1878). *On the Photochemistry of the Retina and on Visual Purple*. Trans. FOSTER, M. London: MacMillan.

LIEBMAN, P. A. (1961). Spectral changes upon rhodopsin photolysis in outer segments of single intact retinal rods. *Biophys. Soc. Abstr.* TD2.

LIEBMAN, P. A. (1962). In situ microspectrophotometric studies on the pigments of single retinal rods. *Biophys. J.* **2**, 161–178.

MacNichol, Jr., E. F. & Marks, W. B. (1965). *Applied Optics*, to be published.

Marks, W. B. (1963). *Difference spectra of the visual pigments in single goldfish cones.* Dissertation, Johns Hopkins University, Baltimore, Maryland.

Marks, W. B. & MacNichol, Jr., E. F. (1962). Bleaching spectra of single goldfish cones. *Biophys. Soc. Abstr.* TE 2.

Marks, W. B. & MacNichol, Jr., E. F. (1963). Difference spectra of single goldfish cones. *Fed. Proc.* **22**, 519, abstract 2143.

Marks, W. B., Dobelle, W. H. & MacNichol, Jr., E. F. (1964). Visual pigments of single primate cones. *Science*, **143**, 1181–1183.

Schmidt, W. J. (1938). Polarizationsoptische Analyse eines Eiweiss-Lipoid-Systems, erläutert am Aussenglied der Sehzellen. *Kolloidzschr.* **85**, 137–148.

Wald, G., Brown, P. K. & Gibbons (1963). The problem of visual excitation. *J. opt. Soc. Amer.* **53**, 20–35.

Wald, G., Brown, P. K. & Smith, P. H. (1953). Cyanopsin, a new pigment of cone vision. *Science*, **118**, 505–508.

Wald, G., Brown, P. K. & Smith, P. H. (1955). Iodopsin. *J. gen. Physiol.* **38**, 628–681.

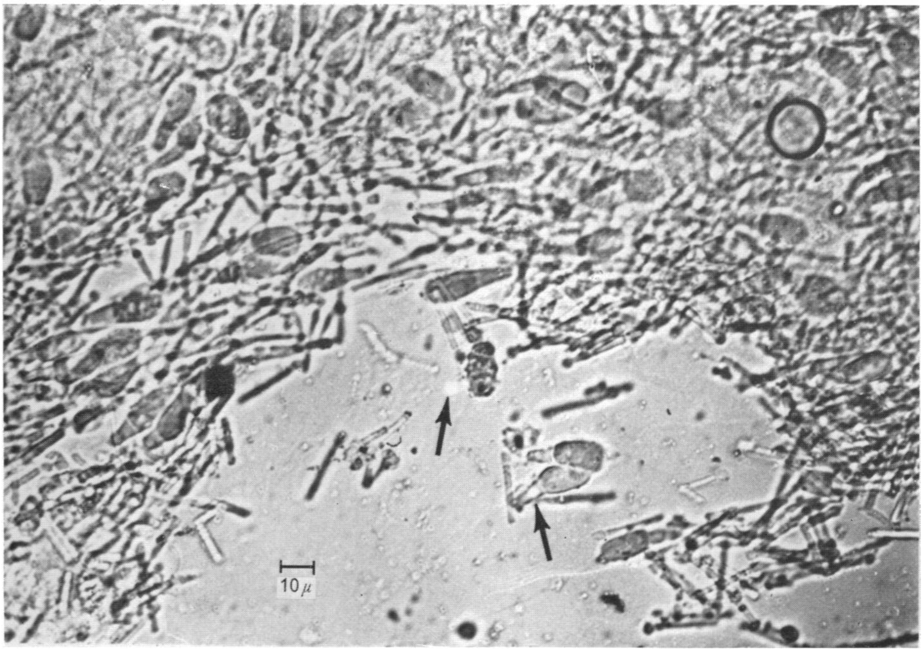
Walls, G. L. (1942). *The Vertebrate Eye and Its Adaptive Radiation.* Bloomfield, Michigan: Cranbrook Institute of Science.

Wolken, J. J. (1963). Structure and molecular organization of retinal photoreceptors. *J. opt. Soc. Amer.* **53**, 1–19.

Young, T. (1802). On the theory of light and colours. *Phil. Trans.*, pp. 12–48.

EXPLANATION OF PLATE

Microphotograph of a piece of goldfish retina squeezed between two cover-slips, and in place on the stage of the microspectrophotometer. The measuring and reference beams (dia.: 3 μ) appear as white spots. One of them passes through the outer segment of one of a pair of twin cones, the other passes through a clear area. The cone outer segments are 3–5 μ wide at the base, and 7–12 μ long. Other twin cones, single cones, and 1–2 μ wide rods are scattered among the retinal debris.



W. B. MARKS

(*Facing p.* 32)