J. Physiol. (1980), **309**, *pp.* 215–228
With 1 plate and 6 text figures
Printed in Great Britain

ELECTRICAL RESPONSES AND PHOTOPIGMENTS OF TWIN CONES IN THE RETINA OF THE WALLEYE

BY DWIGHT A. BURKHARDT*, GUIDO HASSIN*, JOSEPH S. LEVINE†‡ AND E. F. MACNICHOL, JR‡

From the Vision Laboratory, Elliott Hall, University of Minnesota, 75 East River Road, Minneapolis, Minnesota 55455, the Museum of Comparative Zoology,† Harvard University, Cambridge, Mass. 02138 and the Marine Biological Laboratory,‡ Woods Hole, Massachusetts 02543*

(*Received 24 January 1980*)

SUMMARY

1. The properties of twin and single cones in the retina of the walleye (*Stizostedion vitreum vitreum*) were studied by intracellular recording, dye injection and microspectrophotometry.

2. Twin cones generate hyperpolarizing responses to central illumination, can receive depolarizing influences (feed-back) from the receptive field surround, and show no detectable dye coupling when injected with Procion yellow. In seventeen of eighteen dye-injected cones, fluorescence was intense in the inner segment and undetectable or weak in the cone pedicle.

3. Both members of the twin cone contain the same photopigment in their outer segments. It absorbs maximally at about 605 nm.

4. A 533 nm green-sensitive photopigment was found in single cones. No blue-sensitive cones have been found.

5. With the exception of a modest discrepancy in the violet, the absorptance spectrum of the 605 nm photopigment of twin cones agrees closely with the action spectrum measured by intracellular recording.

6. The spectral properties established by the twin cone's photopigment are not detectably altered by the hyperpolarizing influences arising from nearby cones or by the depolarizing influences arising from the receptive field surround.

7. The twin cones of the walleye retina are thus 'identical twins', both photochemically and physiologically, and seem designed to function as long-wave, spectrally univariant receptor units for colour vision.

8. The available evidence suggests that identical twin cones differ functionally from double cones and non-identical twin cones.

9. Although they outnumber single cones by about three to one in adults, identifiable twin cones were rarely observed in the cone population of retinas examined 3–5 days after birth. If walleye twin cones develop by fusion of single cones this process apparently occurs only for cones containing the 605 nm photopigment.

0022-3751/80/2830-0759 $07.50

INTRODUCTION

Cone photoreceptors are found in virtually all vertebrate retinas and it is well established that they are the primary receptors for daylight vision. Since the turn of the century, three anatomical classes of cones have been recognized (Walls, 1942; Cohen, 1972). (1) Single cones which appear relatively isolated from their neighbours are found in all vertebrate classes. (2) Unequal double cones, consisting of two morphologically disparate cones in contact, are found in all vertebrate groups except placental mammals. (3) Twin cones, first observed and named *Zwillingszapfen* by Heinrich Müller in his remarkable anatomical work of 1857 (Müller, 1857), consist of two morphologically identical cones with apposed inner segments; they are widespread in teleost fish and often coexist with a population of single cones.

Over the years there have been various suggestions that these three anatomical classes of cones might differ in function but the physiological evidence is still fragmentary and largely indirect. In this paper we report some direct measurements in the walleye (*Stizostedion vitreum vitreum*), a freshwater teleost with exceptionally large twin cones. We have used intracellular recording and dye injection to provide the first unequivocal identification and description of the light-evoked responses of twin cones and have analysed the cone photopigments by microspectrophotometry. The two sets of independent measurements are in good agreement. They indicate that the twin cones in the walleye are 'identical twins' which contain the same 605 nm photopigment in both members. A green-sensitive photopigment was found in the walleye's single cones. From these and other findings, we consider the function of twin cones in colour vision, the development of twin cones, and possible functional differences between the anatomical classes of vertebrate cones.

METHODS

Intracellular recording and dye injection

Intracellular recordings were obtained by standard methods (Burkhardt, 1977) from fish (20–24 in long) which were netted in Minnesota lakes and rivers. The eye was removed, cut in half, and the posterior portion placed in a cooled, oxygenated chamber. For dye injection work, recordings were made with micropipettes filled with 7% M4RAN Procion yellow dye (electrode resistance: 600–900 MΩ). After recording the cell's response to precisely controlled light flashes, sinusoidal currents (3 Hz, 2 nA peak-to-peak) were applied for 4–10 min. The retina remained in the chamber for 1–2 h. It was then fixed in Bouin's solution overnight, dehydrated, and embedded in Spurr's medium. Sections were cut at 14 μm and inspected with a fluorescence microscope. For further details, see Hassin (1979).

To measure the action spectrum of cones, all recordings were made with micropippetes filled with 4 M-postassium acetate (electrode resistance: 200–400 MΩ) since these electrodes routinely yielded recordings whose signal/noise ratio and stability were much better than those obtained with Procion-filled pipettes. The action spectrum is the reciprocal of the relative number of photons required to evoke a criterion responses from the cone, using 0·5 sec flashes of 100 μm diameter centered in the receptive field. The criterion response amplitude was about one half of the cone's maximum response (8–15 mV, depending on the cone). Spectral lights were obtained from a grating monchromator (6·6 nm half-bandpass), and at each wave-length setting the intensity of the test flash was set at several levels to evoke responses whose amplitudes bracketed the criterion response. The criterion response amplitude was defined as that evoked by a 610 nm standard flash whose intensity remained fixed throughout the measurements. The standard flash was presented before and after obtaining the response series at each wave-length (see Fig. 4).

Thus, it was possible to allow for small shifts in the cell's absolute amplitude of response and to terminate measurements if marked changes occurred in the response to the standard flash. Responses were recorded on magnetic tape and written out later with an $X-Y$ plotter or penwriter. Spectral energy calibrations were made at the plane of the retina with a photomultiplier whose spectral sensitivity had been precisely determined with a thermopile. These measurements were checked with a calibrated photodiode (United Detector Technology, Model 122). The two sets of calibration measurements were virtually identical ($\pm 3\%$). The actual density of all 'neutral' wedges and filters in the optical system was calibrated at each wavelength setting.

Microspectrophotometry

Microspectrophotometric measurements were obtained using the Photon Counting Microspectrophotometer at the Marine Biological Laboratory (MacNichol, 1977, 1978). Eyecups were prepared and placed in low Ca^{2+} saline for 1 hr to facilitate dissociation. Small pieces of retina were placed in a drop of saline on a large cover slip, teased apart with forceps and a small quantity of material transferred to another cover-slip, ringed with silicone oil to prevent evaporation, and covered with an identical cover-slip (MacNichol, Kunz, Levine, Hárosi & Collins, 1978). Preparations of this type contain a large number of cones suitable for measurement which either protrude from the edge of small pieces of retina, or detach completely for microspectrophotometric measurements. All operations were carried out in total darkness using an infra-red image converter. The preparation was then placed between microscope objective lenses to project a small rectangular spot of monochromatic light of varying wave-length within the outer segment of a cone for viewing with infra-red T.V. and recording. All measurements were made transversely to the long axis of the receptors with light polarized perpendicular to this axis.

The spectrophotometer scanned the spectrum repetitively at 1 sec intervals, summing photoelectron counts from a photomultiplier (S20 surface) at corresponding wave-lengths in a computer memory. Usually fifty scans were made through a clear area of the specimen followed by ten to twenty scans through a receptor. A computer programme calculated the optical density, as O.D. = Log_{10} (average blank counts − average dark counts)/(average sample counts − average dark counts).

Histology and extracellular recording in walleye fry

Walleye fry (3–5 days old) were obtained from the St Paul Hatchery of the Minnesota Department of Natural Resources. Walleye fry survive only a few days in captivity. The fry studied were about 8 mm in total body length. The eyes were about 1 mm in diameter. For histology, the cornea was punctured and the whole eye was then fixed, dehydrated and embedded as described above. Tangential sections were cut at 6 μm and stained with methylene blue. To record the corneal electroretinogram, the fish was placed on moist paper in a cooled, oxygenated chamber. The retina was diffusely illuminated. A Ringer-soaked wick electrode was placed on the cornea and an Ag/AgCl indifferent electrode was placed near the tail. Because of base-line drift and the small amplitude of the responses (20–70 μV), all quantitative measurements were obtained with a 0·1–100 Hz recording bandpass.

RESULTS

Identification and analysis of responses of twin cones by dye injection

Intracellular recordings from cones were initially identified by physiological criteria of relative electrode depth and the finding that the receptive field centre was small: about 100 μm in diameter when mapped with a 100 μm spot (Burkhardt, 1977, Fig. 2). To allow enough time for subsequent dye injection, it was necessary to assess quickly the spatial and spectral organization by simple tests. Thus, after centering the test spot, the spatial organization was further assessed by comparing the responses evoked by test flashes of 0·25 and 2·2 mm diameter of constant illumin-

ance. Fig. 1*A* shows the response of a cone to flashes of 2·2 and 0·25 mm. The early parts of both responses are similar but the response to the 2·2 mm stimulus then falls to a considerably lower level for the remainder of the flash. Hence, the receptive field of this cone has an antagonistic centre–surround organization: illumination in the surround induces a delayed depolarizing influence which antagonizes the hyperpolarizing effect of light in the centre of the receptive field. The depolarization due

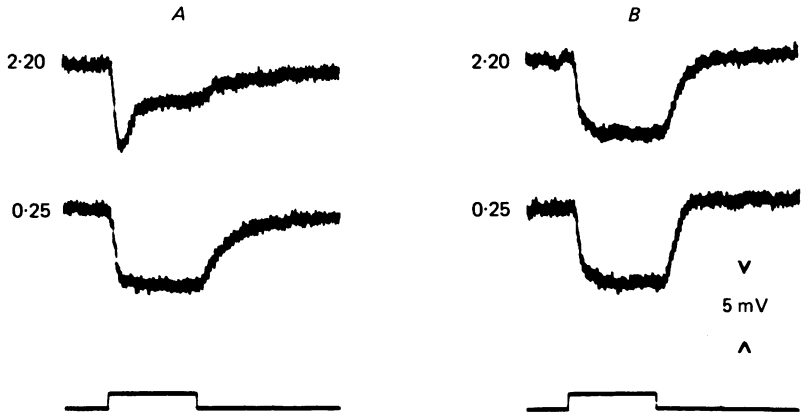


Fig. 1. A. responses of a cone evoked by circular test flashes of 2·2 and 0·25 mm diameter. The flashes were centred in the cone's receptive field, 0·50 sec in duration, 630 nm in wave-length, and of constant irradiance (equivalent to about 5×10^4 photons/μm^2.sec at the plane of the retina). Hyperpolarization is down. *B*, light-evoked responses of another cone to test flashes of 2·2 and 0·25 mm diameter. Other stimulus conditions as in *A*. Stimulus records are shown below. The voltage calibration applies to all responses.

to the surround has been studied previously in detail (Burkhardt, 1977), is in all major respects similar to that reported for turtle cones (Baylor, Fuortes & O'Bryan, 1970), and is believed to be due to feed-back from horizontal cells (Baylor *et al.* 1970; Cervetto & MacNichol, 1972; O'Bryan, 1973; Burkhardt, 1977). In both species, some cones fail to show the effect. In the walleye, it has been detected in about half of some 170 cones studied to date.

After injecting dye and processing the retina as described in Methods, the cell giving the responses of Fig. 1*A* was identified from the radial section reproduced in Pl. 1*A*. The inner segment of a cone shows strong fluorescence due to accumulation of dye. Dye is also visible in the distal part of the thin process which connects the inner segment to the cone pedicle. Dye is not apparent in the pedicle nor in the outer segment, although the latter is partially occluded by the surrounding pigment epithelium cells whose proximal tips contain discrete accumulations of black pigment

Pl. 1*B* shows the regular mosaic pattern of cones in the walleye retina. This is a tangential section at the level of the cone inner segments. Four twin cones surround each of the less numerous single cones. The straight region of apposition between the inner segments of the individual members of the twin cones is clearly demonstrated.

Fig. 1*B* shows responses from a cell which lacks a detectable depolarizing surround since the responses to flashes of 2·2 and 0·25 mm are similar. The origin of this recording was identified by the tangential section shown in Pl. 1*C*. One member

of a twin cone is clearly filled with dye. Pl. 1 *D* and *E* show two more examples of dye-injected twin cones in tangential section. The cone in *E* and five other dye-injected twin cones showed depolarizing influences due to illumination of the receptive field surround.

Because the inner segments of individual members of twin cones are significantly larger than those of single cones (Pl. 1 *B*), dye-injected twin cones could be unequivocally identified in either radial or tangential sections. Of a total of twenty-five dye-injected cones, eighteen were recovered. All were unequivocally identified as twin cones. In each case, only one member contained detectable dye. The reproductions in Pl. 1 are necessarily inferior to the appearance of this material when directly viewed in the microscope with the attendant advantages of colour contrast and increased definition of borders. When so viewed, none of our material showed detectable spread of yellow dye between twin cone members. In seventeen of the eighteen recovered twin cones, strong fluorescence was seen in the inner segment whereas the pedicle showed undetectable or weak fluorescence. In contrast, one recovered cone showed strong fluorescence in the pedicle and much weaker fluorescence in the inner segment. Depolarizing influences from the surround were not detectable in this recording.

Before dye injection, each cone recording was screened for spectral sensitivity by determining the relative sensitivity to red (630 nm) and green (530 nm) test flashes. As reported previously (Burkhardt & Hassin, 1978), the resulting 630/530 nm sensitiviy ratio clearly distinguishes the orange-sensitive cones from green-sensitive cones. The former have a 630/530 nm sensitivity ratio of about 1·62, and the latter about 0·16. In the present work, all the recovered dye-injected cones had sensitivity ratios significantly greater than one (mean: $1{\cdot}69 \pm 0{\cdot}22$ S.D.). Thus, these results indicate that all the seventeen recovered twin cones were the orange-sensitive type.

Microspectrophotometry

The results of the dye injection work suggest that both members of the twin cone contain the same orange-sensitive photopigment. A direct test of this proposition was obtained by microspectrophotometry by directly measuring the photopigment within each member of twin cones. Our measurements considerably extend those previously reported for a sample of three walleye cones (Ali, Ryder & Anctil, 1977).

Density spectra suitable for detailed analysis were obtained from both members of twenty-seven pairs of twin cones. All twenty-seven pairs showed maximum density around 605 nm. (Similar spectra were obtained from twelve other twin cones but were excluded from further consideration either because only one member of a pair was measured or because the records exhibited unsatisfactory signal/noise ratios.) The spectra for individual members of a given twin cone did not differ significantly from each other. The filled circles in Fig. 2 show the mean spectrophotometric density spectrum of the twin cone photopigment, based on ten cones giving the best signal/noise ratios. The small circles show the standard deviations. The average maximum absorbance (optical density) of the 605 nm photopigment was about 0·05 (Fig. 2). This corresponds to an absorptance (fraction of light absorbed) of 0·11. The average diameter of the portion of the outer segment measured was about 5 μm

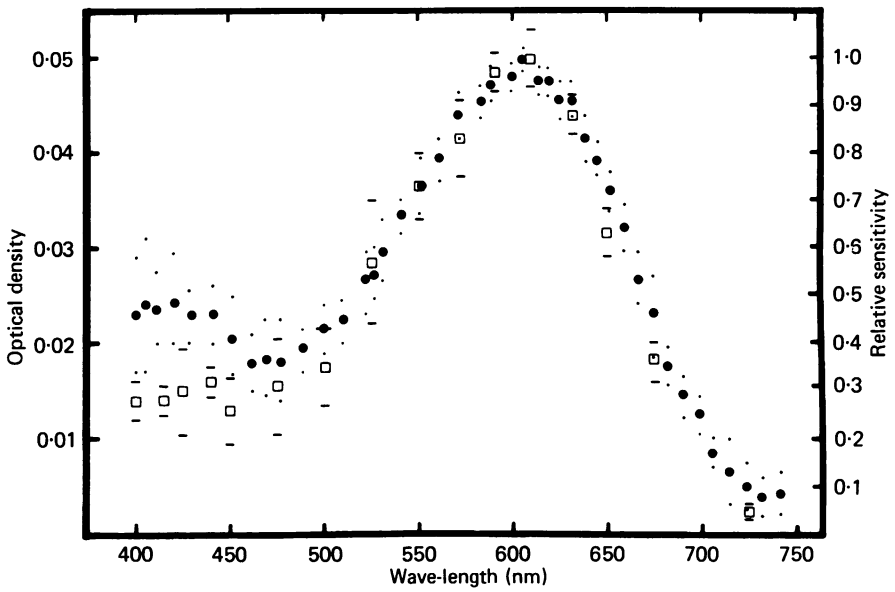


Fig. 2. Mean density spectrum of the photopigment found in the twin cones (filled circles) based on measurements from ten cones. The small circles show the standard deviations. The squares show the mean action spectrum based on intracellular recording from six orange-sensitive cones. The horizontal lines show the standard deviation. The action spectrum is plotted on a linear scale of relative quantum sensitivity (right ordinate).

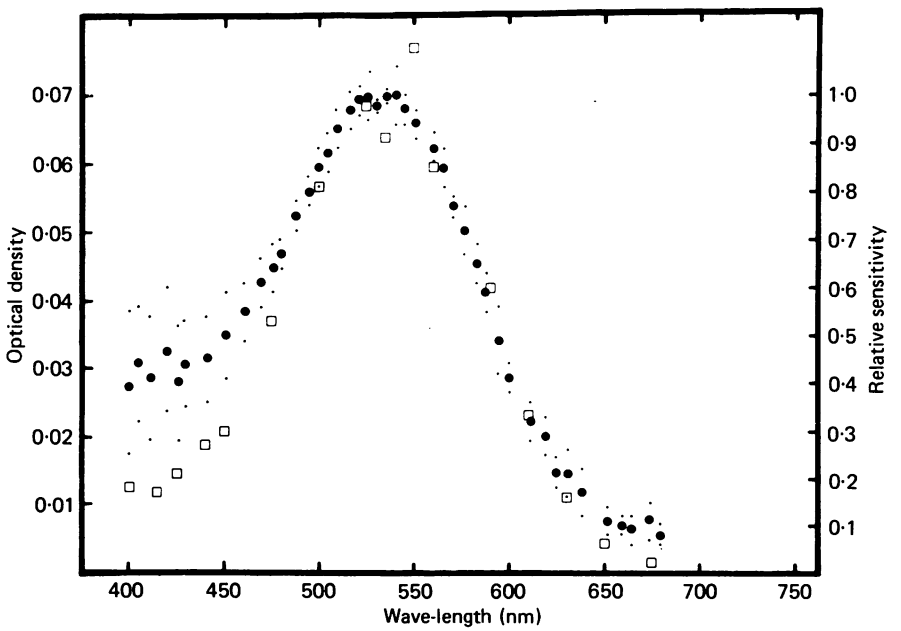


Fig. 3. Mean density spectrum of the photopigment found in single cones (filled circles) based on measurements from eight cones. The small circles show the standard deviations. The squares show the action spectrum (ordinate at right) obtained by intracellular recording from one green-sensitive cone.

which gives a specific absorbance of 0·010 per μm. This compares with the specific absorbances of 0·0136, 0·0132, and 0·0154 for the blue, green, and red absorbing cones of the goldfish (Hárosi, 1976).

Satisfactory microspectrophotometric measurements were also made on fourteen single cones. All these cones were green-sensitive, absorbing maximally at about 533 nm. The filled circles in Fig. 3 show the mean density spectrum, based on eight cones giving the best signal/noise ratios. The small circles show the standard deviations. Over the wave-length ranges of 450–630 and 500–675 nm, respectively, the 533 and 605 nm spectra of Figs. 3 and 2 agree very closely with the spectra predicted by the photopigment nomogram of Ebrey & Honig (1977). The nomogram spectra do not extend to shorter wave-lengths. At longer wave-lengths, the microspectrophotometric measurements are somewhat higher than the nomogram predictions.

Action spectra of walleye cones

The density spectrum of the 605 nm photopigment (Fig. 2) corresponds rather closely with the action spectrum previously reported for the orange-sensitive cones (Burkhardt & Hassin, 1978). To determine the degree of the correspondence more fully, we have used a refined method to measure the action spectrum for a new sample of cones and extended the measurements below 450 nm. It was essential to obtain relatively low noise recordings which were stable for 15–20 min. It therefore proved necessary to use micropipettes filled with potassium acetate rather than Procion yellow (see Methods). In four of the six cones sampled, it was possible to get two independent sensitivity determinations at all wave-lengths. Fig. 4 shows the sort of data from which the action spectra were derived. At the top of Fig. 4, amplitude–intensity series at 450 and 650 nm are shown interspersed between responses evoked by a standard, constant-intensity flash. Here and for the other five cones, centred stimuli of 0·10 mm diameter were used. With such stimulation, the hyperpolarizing response due to direct illumination is modestly enhanced by hyperpolarizing influences due to illumination of neighbouring cones (Witkovsky, Burkhardt & Nagy, 1979). The responses showed no apparent departures from the principle of univariance (Naka & Rushton, 1966). The mean amplitude of the standard responses preceding and following each amplitude–intensity series was taken as the criterion response for that series (see Methods). The data were then plotted as in the bottom of Fig. 4 and the relative number of photons required to evoke the criterion response was obtained by interpolation.

The open squares in Fig. 2 show the mean action spectra for the six cones sampled. The horizontal bars show the standard deviations. The action spectrum agrees closely with the photopigment density spectrum. The standard deviations of the two sets of measurements overlap throughout the spectrum with the exception of a slight but apparently systematic discrepancy in the violet.

The action spectrum of Fig. 2 should be exclusively due to the cone's central hyperpolarizing mechanism since the stimuli were too small to activate the surround mechanism of walleye cones (Burkhardt, 1977) and the particular cones of Fig. 2 displayed either very weak or undetectable depolarizing influences even when stimulated with 2·2 mm spots. Further spectral tests were performed on orange-sensitive cones which displayed clear depolarizing influences. These tests showed

that disparate spectral stimuli which were set at irradiances to evoke equivalent responses for small spots (0·10 mm) also yielded equivalent responses when the spots were enlarged to 2·2 mm to activate the depolarizing surround. Fig. 5 shows an example of such results. Since the spectral equivalence for small centred spots also holds closely for large spots, the surround mechanism must be primarily driven by cones which contain the 605 nm photopigment.

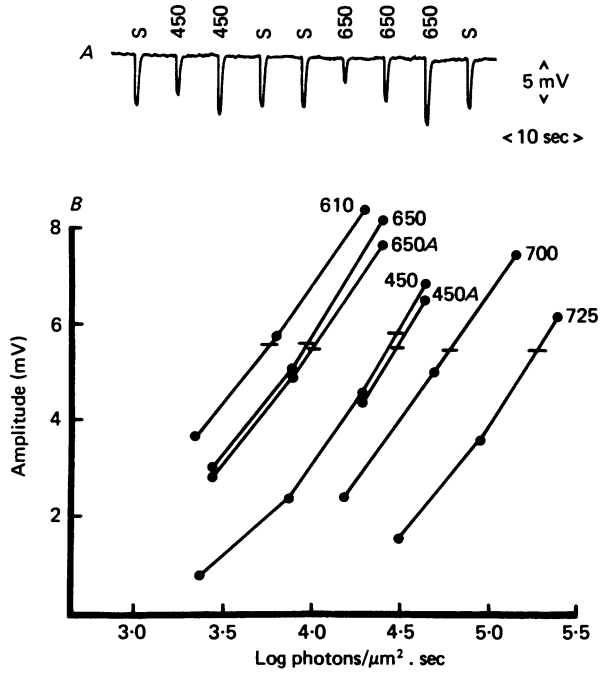


Fig. 4. *A*, responses of a walleye cone to flashes of 610, 450 and 650 nm. A standard, constant-intensity flash of 610 nm is presented at S. Between these flashes, amplitude–intensity series at 450 or 650 nm are generated by increasing the test flash in approximately 0·5 log unit steps. *B*, a plot of peak amplitude of the cone response *vs.* flash intensity. The numbers by each curve indicate the stimulus wave-length. The short horizontal lines show the criterion response based on the average of the responses evoked by the standard flash immediately before and after each series (see text). The intersection of these lines with the appropriate curve defines the photon levels necessary to evoke the criterion response. The curves marked 450*A* and 650*A* are based on the records shown above in *A*. About 7 min later, the response to the standard flash was slightly larger and a second series was taken at 450 and 650 nm. These data, shown by the curves marked 450 and 650, yield sensitivity measurements within 0·04 log unit of those obtained from the earlier series. The criterion response is about half of the cone's maximum response (12 mV).

It has been very difficult to get stable intracellular recordings from green-sensitive cones (Burkhardt & Hassin, 1978), probably because these cones are considerably smaller and less numerous than the orange-sensitive cones. However, we have recently obtained one unusually stable recording. The resulting action spectrum measurements (squares, Fig. 3) agree relatively well with the 533 nm photopigment spectrum. Depolarizing influences were undetectable in this cone. Due to limitations in other recordings the spectral properties of the surround mechanism of green-sensitive

cones are as yet uncertain. In the microspectrophotometric sample and extensive electrophysiological recordings at all levels of the walleye retina, no evidence for blue-sensitive cones has been found. Thus, the walleye retina seems functionally dichromatic.

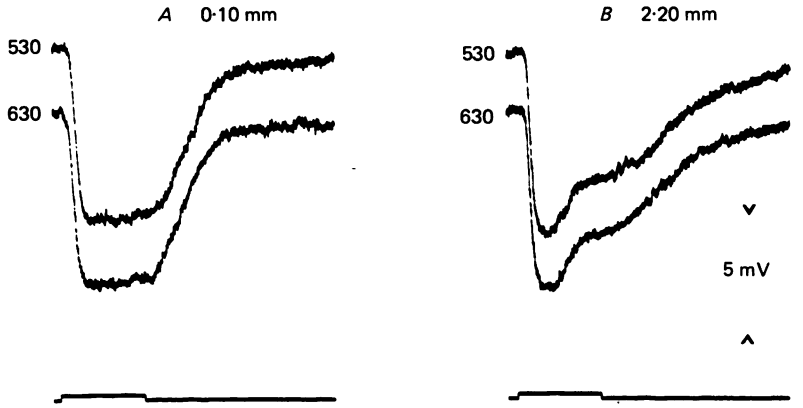


Fig. 5. Spectral equivalence of responses of an orange-sensitive cone for 530 and 630 nm flashes of 0·10 nm (*A*) and 2·2 mm diameter (*B*). In *A* the intensity levels of the 530 and 630 nm flashes were set at approximately $8{\cdot}2 \times 10^4$ and $4{\cdot}6 \times 10^4$ photons/μm^2.sec, respectively, to evoke equivalent responses from the cone. In *B*, the intensities were unchanged. Although the delayed depolarizing influence of the surround is now clearly evident, the responses to 530 and 630 nm remain equivalent. The maximum response of this cone was about 9 mV. Spectral equivalence also held for responses of smaller amplitude when the 530 and 630 nm stimuli were reduced by equal amounts. The bottom records show the timing and duration (0·5 sec) of the flashes.

Anatomical and physiological observations on walleye fry

The receptor mosaic was examined in ten walleye fry (3–5 days old). In radial sections, all the receptors observed had an inner and outer segment morphology which was clearly characteristic of cones. Rods were not evident. Rods evidently appear somewhat later than cones, as has been reported for other teleosts. Fig. 6 shows camera lucida drawings of representative sections at the level of the cone inner segments. The orderly array of the adult mosaic is not evident. Neighbouring inner segments are separated. The cones appear relatively uniform in both size and shape. However, some cones have semicircular profiles and a few of these are oriented with their straight regions facing each other (arrowheads, Fig. 6). These elements, although rare and not yet in contact, may be precursors of adult twin cones.

The corneal electroretinogram was recorded in an attempt to assess the spectral properties of the receptors. The light-evoked response consisted of an initial negative wave followed by a positive wave. The amplitude from the negative to positive peak was measured. The responses were small ($\leqslant 70\ \mu V$), relatively noisy and insensitive, so it was only feasible to use relatively intense lights from interference filters to estimate the 630/530 nm sensitivity ratio. The data from five fry yielded a mean 630/530 nm sensitivity ratio of 0·79 (range: 0·48–1·02). This value lies about halfway between the characteristic values for orange- and green-sensitive cones (see above), so these results suggest that both orange- and green-sensitive cones are present in the fry retina.

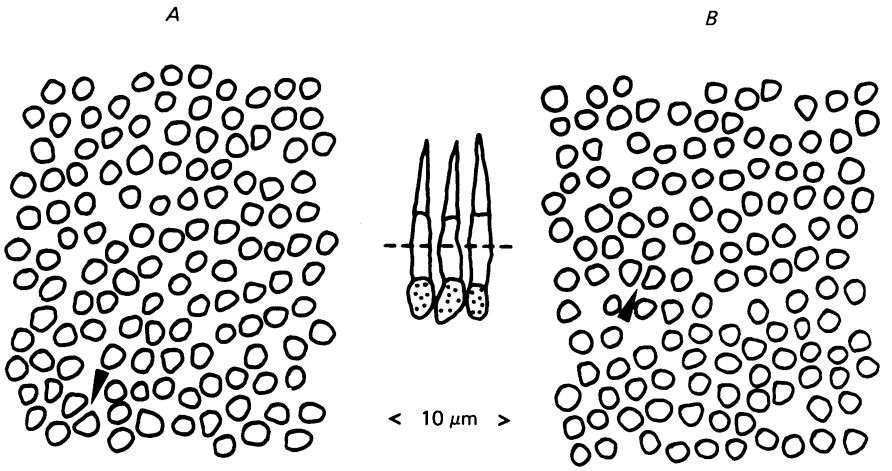


Fig. 6. Camera lucida drawings of the cone mosaic observed in walleye fry, 3–5 days old. *A* and *B* show tangential sections at the level of the inner segments for two fish. The insert in the middle shows a typical radial view of the distal part of fry cones. The dashed line indicates the approximate level of the sections shown in *A* and *B*. The arrowheads point to presumed precursors of twin cones (see text).

DISCUSSION

Dye injection, coupling and depolarizing influences in cones

We have repeatedly found strong fluorescence in the inner segment of one member of the twin cone but no detectable fluorescence in the apposed member. Thus, if twin cones are electrically coupled at the apposed inner segments, the coupling channels must be too small to allow effective passage of Procion yellow molecules (Simpson, Rose & Lowenstein, 1977). In contrast, Procion yellow seems to pass easily between the members of double cones (Richter & Simon, 1974). In addition to possible coupling at the inner segment, the paired members of the twin cone could be functionally coupled with each other and with neighbouring cones via the gap functions observed between pedicles and their basal processes in the walleye retina (Witkovsky *et al.* 1979).

The generality and functional consequences of depolarizing influences (feed-back) in vertebrate cones are, as yet, unsettled (Burkhardt, 1977; Burkhardt & Hassin, 1978). To date, such influences have only been clearly demonstrated in turtle, tiger salamander (J. Skrzypek & F. S. Werblin, unpublished) and walleye, and even in these retinas, not all cones show the effect. Thus, if the depolarizing surround is to be accepted as a general property of vertebrate cones, it seems necessary to assume that experimental limitations account for the reported failures to detect the effect in various species. One possibility is that the depolarizing potential generated in the pedicle could be drastically attenuated and hence, undetectable at the level of the inner segment (Byzov, 1979). On anatomical grounds, the walleye twin cone seems a prime candidate for such attenuation since the process connecting the inner segment is very long (about 30 μm) and slender (about 1 μm in diameter). Our dye-injection results indicate that the majority of intracellular recordings from walleye cones are

obtained from the inner segment and that depolarizing potentials are detectable in such recordings. Hence, the reported absence of depolarizing potentials (feed-back) in cones seems more likely due to (1) selective deterioration or (2) a true absence of the feed-back mechanism. There is evidence for the first possibility (for summary, see Burkhardt, 1977) but until feed-back connexions can be independently identified by morphological or neurochemical criteria, the second possibility cannot be excluded.

Correspondence between cone photopigments and cone action spectra

To the best of our knowledge, the agreement between photopigment and action spectra in Fig. 2 is the closest yet reported for vertebrate cones. These data therefore merit further scrutiny. For rigorous comparison of physiological and photopigment spectra, the photon-based action spectrum must be compared to the absorptance spectrum of the photopigment and the latter must be based on the optical density pertinent to the physiological measurements (Darnall, 1962). The microspectrophotometric measurements of Fig. 2 were made transversely across outer segments which measured about 5 μm in diameter and show a maximum density (absorbance) of 0·05, so the specific absorbance of the photopigment is 0·01/μm. Since the action spectrum was measured with light passing axially through outer segments about 14 μm long, the total absorbance of the cone would be 0·14. Thus, for comparison with the physiological measurements, the measured density spectrum of Fig. 2 should be scaled up to have a maximum density of 0·14 and the values then transformed to absorptance (fraction of light absorbed). When this was done, the resulting absorptance spectrum was a little broader than the density spectrum, but the differences were very small. For example, the filled circles plotted in Fig. 2 at 400, 566 and 740 nm fall, respectively, at 46, 83 and 8·4 % of the 605 nm peak. The corresponding values for the absorptance spectrum were 50, 85 and 9·5 % of the 605 nm peak. Thus, the close agreement evident in Fig. 2 is not materially altered when the photopigment data are adjusted for the physiologically pertinent optical density and expressed as absorptance.

At short wave-lengths, comparisons of physiological and microspectrophotometric spectra are susceptible to secondary distortions due to pre-receptor media, scatter, photoproducts, focusing and residual calibration errors. We can neither eliminate or confirm the possibility that one or some combination of these factors may underlie the modest discrepancy found in Fig. 2 below 450 nm, in the region of the photopigment's cis-band. In sum, throughout the main band of the photopigment, our results clearly support the hypothesis that the photopigment absorptance fully accounts for the cone's action spectrum. Whether this correspondence extends to the cis-band is still uncertain and a question of some consequence for colour discrimination in the violet.

Function of identical twin cones

Recent measurements of photopigments in a variety of teleosts indicate that there are actually two types of twin cones: 'non-identical twins' whose members contain spectrally distinct photopigments and 'identical twins' whose members contain the same photopigment (Loew & Lythgoe, 1978; Levine, MacNichol, Kraft & Collins, 1979). Our results show that the twin cones in the walleye are 'identical twins', both photochemically and physiologically. The spectral sensitivity established by

the photopigment is substantially preserved by subsequent events, including summative interactions with neighbouring cones and depolarizing influences from the receptive field surround, which intervene between light absorption and the receptor potential. Thus, these identical twin cones seem designed to function as orange-sensitive, spectrally univariant receptor units. From the combined evidence of this and previous work (Hassin, 1979; Witkovsky *et al.* 1979), it is now clear that the output of twin cones is routed to both luminosity-type and chromatic-type horizontal cells. In this way, walleye twin cones evidently contribute to both brightness and colour discrimination.

Functional differences between twin cones and double cones

The spectral organization of identical twin cones revealed here contrasts with evidence now available on the function of double cones. The double cones in turtle seem to consist of functionally coupled red- and green-sensitive members whose responses show strong departures from univariance (Richter & Simon, 1974; Baylor & Fettiplace, 1975) and thus seem designed to promote chromatic interactions at the receptor level. Such function may be characteristic of vertebrate double cones since studies in goldfish, rudd, frog and birds all show that the associated members contain spectrally dissimilar photopigments or photopigment–oil droplet combinations (Marks, 1965; Hárosi & MacNichol, 1974; Scholes, 1975; Stell & Hárosi, 1976; Bowmaker, 1977; Mariani & Leure-dePree, 1978). The members of non-identical twin cones are, likewise, spectrally dissimilar (Loew & Lythgoe, 1978; Levine *et al* 1979). Thus, of the three cone types, double cones and non-identical twin cones seem functionally analogous, while the identical twin cone, with its simple spectral organization, stands apart. Perhaps identical twin cones have complex or highly specialized functions in other aspects of vision. For this, however, we can as yet offer no compelling evidence from our measurements on twin cones and the comparable measurements now available on cones in other vertebrates.

Development of twin cones and the cone mosaic

The development of twin cones in the walleye seems fundamentally like that described for it's European counterpart, the pikeperch *Lucioperca lucioperca*, and for the common perch (*Perca fluviatilis*). In both these species, twin cones are abundant in adults but only single cones seem evident in fry (Ahlbert, 1970, 1973). From these findings and depth-dependent changes in rockfish, it has been suggested that twin cones develop via fusion of single cones (Ahlbert, 1973; Boehlert, 1978). To form the receptor mosaic reported here for the mature walleye, such fusion would have to be restricted to the orange-sensitive cones. Furthermore, if the neonatal population of single cones is composed of both green- and orange-sensitive members, the fusion mechanism must be highly selective to promote the formation of orange-sensitive twin cones while impeding the formation of green-green and orange-green combinations.

Supported in part by grants from the National Eye Institute, U.S. Public Health Service, to D. A. Burkhardt (EY 00406) and E. F. MacNichol, Jr. (EY 02399) and grants from the Rowland Foundation and other non-governmental sources to E. F. MacNichol, Jr. We thank Douglas Shamp, Jon Gottesman, Sharon Grundthner and Barbara Ann Collins for technical assistance

and Messrs W. Diley, E. Feiler, J. Hanay, R. Ruths, D. Schodeen, W. Scidmore, E. Tews, R. Weller and other personnel of the Minnesota Department of Natural Resources and Dr John L. Forney of Cornell University for supplying the fish used in this research.

REFERENCES

AHLBERT, I.-B. (1969). The organization of cone cells in the retinae of four teleosts with different feeding habits (*Perca fluviatilis*, L., *Lucioperca lucioperca* L., *Acerina cernus* L., aud *Coregonus albula* L.). *Ark. Zool.* **22**, 445–481.

AHLBERT, I.-B. (1973). Ontogeny of double cones in the retina of perch fry (*Perca fluviatilis*, Teleostei). *Acta zool., Stockh.* **54**, 241–254.

ALI, M. A., RYDER, R. A. & ANCTIL, M. (1977). Photoreceptors and visual pigments as related to behavioral responses and preferred habitats of perches (*Perca* spp.) and pikeperches (*Stizostedion* spp.) *J. Fish Res. Bd Can.* **34**, 1475–1480.

BAYLOR, D. A., FUORTES, M. G. F. & O'BRYAN, P. M. (1971). Receptive fields of cones in the retina of the turtle. *J. Physiol.* **214**, 265–294.

BAYLOR, D. A. & FETTIPLACE, R. (1975). Light path and photon capture in turtle photoreceptors. *J. Physiol.* **248**, 433–464.

BOEHLERT, G. W. (1978). Intraspecific evidence on the function of single and double cones in the teleost retina. *Science, N.Y.* **202**, 309–311.

BOWMAKER, J. K. (1977). The visual pigments, oil droplets and spectral sensitivity of the pigeon. *Vision Res.* **17**, 1129–1138.

BURKHARDT, D. A. (1977). Responses and receptive field organization of cones in perch retinas. *J. Neurophysiol.* **40**, 53–62.

BURKHARDT, D. A. & HASSIN, G. (1978). Influences of cones upon chromatic- and luminosity-type horizontal cells in pikeperch retinas. *J. Physiol.* **281**, 125–137.

BYZOV, A. L. (1979). Origin of non-linearity of voltage–current relationships of turtle cones. *Vision Res.* **19**, 469–478.

CERVETTO, L. & MACNICHOL, E. F., Jr. (1972). Inactivation of horizontal cells in turtle retina by glutamate and aspartate. *Science, N.Y.* **178**, 767–768.

COHEN, A. I. (1972). Rods and cones. In *Handbook of Sensory Physiology*, vol. II/2, ed. FUORTES, M.G.F., pp. 63–110. Berlin: Springer-Verlag.

DARTNALL, H. J. A. (1962). The photobiology of visual processes. In *The Eye*, vol. 2, pp. 323–536, New York: Academic Press.

EBREY, T. G. & HONIG, B. (1977). New wavelength dependent visual pigment nomograms. *Vision Res.* **17**, 147–151.

HÁROSI, F. I. (1976). Spectral relations of cone pigments in goldfish. *J. gen. Physiol.* **68**, 65–80.

HÁROSI, F. I. & MACNICHOL, E. F., Jr. (1974). Dichroic microspectrophotometer: a computer-assisted, rapid, wavelength-scanning photometer for measuring linear dichroism in single cells. *J. opt. Soc. Am.* **64**, 903–918.

HÁROSI, F. I. & MACNICHOL, E. F., Jr. (1974). Visual pigments of goldfish cones. *J. gen. Physiol.* **63**, 279–304.

HASSIN, G. (1979). Pikeperch horizontal cells identified by intracellular straining. *J. comp. Neurol.* **186**, 529–540.

LEVINE, J. S., MACNICHOL, E. F., Jr., KRAFT, T. & COLLINS, B. A. (1979). Intraretinal distribution of cone pigments in certain teleosts. *Science, N.Y.* **204**, 523–526.

LOEW, E. R. & LYTHGOE, J. N. (1978). The ecology of cone pigments in teleost fish. *Vision Res.* **18**, 715–722.

MACNICHOL, E. F., Jr. (1977). A photon counting microspectrophotometer for single receptor cells: design features and preliminary results. *Invest. Ophthal. Suppl.* **118**.

MACNICHOL, E. F., Jr. (1978). A photon counting microspectrophotometer for the study of single vertebrate photoreceptor cells. In *Frontiers in Visual Science*, ed. COOL, S. J. & SMITH, E. L., pp. 194–208. New York: Springer Verlag.

MACNICHOL, E. F., Jr., KUNZ, Y. W., LEVINE, J. S., HÁROSI, F. I. & COLLINS, B. A. (1978). Ellipsosomes: organelles containing a cytochrome-like pigment in retinal cones of certain fishes. *Science, N.Y.* **200**, 549–552.

MARIANI, P. & LEURE-DUPREE (1978). Photoreceptors and oil droplet colors in the red area of the pigeon retina. *J. comp. Neurol.* **182**, 821–838.

MARKS, W. B. (1965). Visual pigments of single goldfish cones. *J. Physiol.* **178**, 14–32.

MÜLLER, H. (1857). Anatomisch-physiologische Untersuchungen über die Retina bei Menschen und Wirbelthieren. *Z. wiss. Zool.* **8**, 1–122.

NAKA, K. I. & RUSHTON, W. A. H. (1966). S potentials from colour units in the retina of fish (*Cyprinidae*). *J. Physiol.* **185**, 536–55.

O'BRYAN, P. M. (1973). Properties of the depolarizing synaptic potential evoked by peripheral illumination in cones of the turtle retina. *J. Physiol.* **235**, 207–223.

RICHTER, A. & SIMON, E. J. (1974). Electrical responses of double cones in turtle retina. *J. Physiol.* **242**, 673–683.

SCHOLES, J. H. (1975). Colour receptors, and their synaptic connexions, in the retina of a cyprinid fish. *Phil. Trans. R. Soc. B* **270**, 61–118.

STELL, W. K. & HÁROSI, F. I. (1976). Cone structure and visual pigment content in the retina of the goldfish. *Vision Res.* **16**, 647–657.

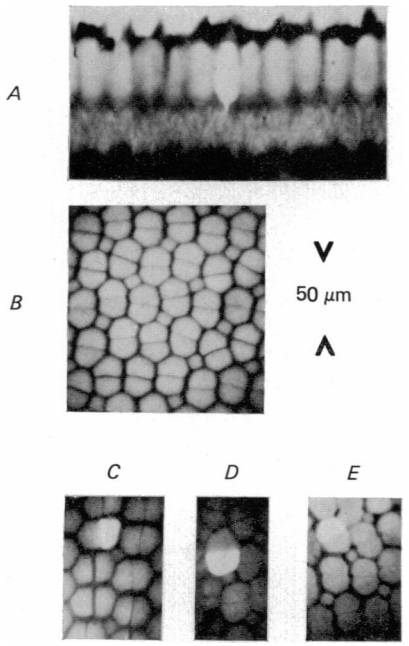
SIMPSON, I., ROSE, B. & LOWENSTEIN, W. R. (1977). Size limit of molecules permeating the functional membrane channels. *Science, N.Y.* **195**, 294–296.

WALLS, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiations.* Bloomfield Hills: Cranbrook.

WITKOVSKY, P., BURKHARDT, D. A. & NAGY, A. R. (1979). Synaptic connections linking cones and horizontal cells in the retina of the pikeperch (*Stizostedion vitreum*). *J. comp. Neurol.* **186**, 541–560.

EXPLANATION OF PLATE 1

A. Radial section of the distal retina of the walleye. The inner segment of a twin cone is filled with Procion yellow dye. The responses shown in Fig. 1*A* were recorded before dye was injected. Dye is also seen in the initial part of the thin process which connects the inner segment to the cone pedicle. Dye is not apparent in the cone's outer segment, although the latter is partially occluded by the surrounding pigment epithelium cells whose proximal tips contain discrete accumulations of black pigment. The pigment epithelium cells, cone inner segments and the more proximal outer nuclear layer all show considerable autofluorescence. *B*, tangential section at the level of the cone inner segments. The smaller and less numerous single cones are surrounded by four twin cones. The straight region of apposition between the inner segments of the individual members of the twin cones is clearly evident in this 14 μm section. *C*, tangential section shows one member of a twin cone injected with dye. The responses in Fig. 1*B* were recorded from this cone. *D* and *E*, two other examples of dye-injected twin cones in tangential section.


A
B
50 μm
C
D
E