Microspectrophotometric Evidence for Two Photointerconvertible States of Visual Pigment in the Barnacle Lateral Eye

BARUCH MINKE and KUNO KIRSCHFELD

From the Department of Physiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel, and the Max-Planck-Institut für Biologische Kybernetik, 7400 Tübingen, West Germany

ABSTRACT Microspectrophotometrically derived difference spectra from the barnacles Balanus amphitrite and B. eburneus show that a blue illumination after an orange illumination causes a decrease in absorption in the blue region and an increase in absorption in the green-yellow region, with an isosbestic point around 535 nm. Orange-following-blue illumination causes the reverse changes. The dark time between the adapting and measuring lights has no influence on the data. The results confirm previously reported ERP measurements which indicate that the barnacle visual pigment has two photointerconvertible dark-stable states. If one assumes a Dartnall nomogram shape for the two absorption spectra, a best fit to the observed difference spectra is obtained with nomograms peaking at 492 nm and 532 nm, with a peak absorbance ratio around 1.6:1. These two nomograms fit very well the ERP action spectra of metarhodopsin and rhodopsin, respectively, thus indicating that the ERP is a reliable measure of visual-pigment changes in the barnacle. The existence of a photostable blue pigment is demonstrated in B. eburneus and in some of B. amphitrite receptors, and the possible influence of this photostable pigment on the various action spectra measured in the barnacle is discussed.

INTRODUCTION

The barnacle visual pigment has become an important model bistable visual-pigment system, in which both stable states, rhodopsin and metarhodopsin, are physiologically active (Hillman et al., 1972; Hochstein et al., 1973; Minke et al., 1973a,b; Minke et al., 1974). The correlation between the activation of the stable states of the barnacle visual pigment and a physiological effect has been established on the basis of pigment spectra deduced from measurements of the early receptor potential (ERP) (Hochstein et al., 1973; Minke et al., 1973a; Minke et al., 1974). The ERP action spectra measured in Balanus amphitrite indicate that the rhodopsin absorption has a peak at 532 nm and can be photointerconverted to a dark-stable metarhodopsin with a peak at 495 nm. Shifting the pigment from rhodopsin to metarhodopsin with a red light after blue adaptation induces an excitatory process that manifests itself in a prolonged depolarizing afterpotential (PDA) which far outlasts the stimulus. Photoconvert-
ing the pigment back from metarhodopsin to rhodopsin with blue light induces an inhibitory process and results in depression or prevention of the PDA (Hochstein et al., 1973; Minke et al., 1974). Hochstein et al. (1973) also found: (a) that there is a good fit between the ERP action spectrum of rhodopsin and the action spectra of the late receptor potential (LRP) and PDA induction; and (b) that the ERP action spectrum of metarhodopsin fits the action spectrum of PDA depression.

Brown and Cornwall (1975) recently presented a study of the barnacle B. eburneus lateral ocelli in which they failed to demonstrate by direct photometric measurements the photointerconvertibility of the visual pigment responsible for PDA induction and depression. They also found by microspectrophotometric measurements in an intact photoreceptor preparation that illumination with wavelengths longer than 540 nm induced an absorbance decrease between 550 and 420 nm with maximum change between 480 and 510 nm, and an absorbance increase at wavelengths shorter than 420 nm with an isosbestic point at 420 nm. They obtained similar results by photometric measurements of ocelli extracts. In contrast to Hochstein et al. (1973), they did not find a correlation between pigment changes and the various physiological effects: (a) their light-induced current action spectrum has a maximum at 540 nm, but they could not find a pigment with equivalent absorption; (b) their PDA depression action spectrum has a maximum at 510-520 nm, also without a good correlation to their microspectrophotometric results. Therefore, they concluded that the correlation between a photointerconvertible pigment system and the phenomena of PDA induction and depression has not been established.

We present here microspectrophotometric measurements indicating (a) that there exist two photointerconvertible dark stable states of pigment in the barnacle lateral ocelli; (b) that the microspectrophotometrically derived difference spectrum agrees very well with a difference spectrum calculated from two Dartnall nomograms peaking at 492 nm and 532 nm, which fit very well the ERP action spectra of metarhodopsin and rhodopsin, respectively, measured by Minke et al. (1973a).

This report should therefore help to clarify the conflicting findings of Hochstein et al. (1973) and Minke et al. (1973a) on the one hand, and those of Brown and Cornwall (1975) on the other.

**MATERIALS AND METHODS**

The measurements were carried out on excised ocelli of B. amphitrite and B. eburneus which were obtained from Eilat, Israel, and Woods Hole, Mass., respectively. The reflecting tapetum was removed under white illumination and the eye was mounted in a closed quartz chamber containing artificial seawater on a Peltier element for controlling temperature. All measurements were carried out at a temperature of approximately 6°C which was necessary to get stable recordings. The single beam microspectrophotometer was composed of a Leitz (D-6330 Wetzlar) UV-microscope photometer UVMP, equipped with Zeiss (D-7082 Oberkochen) ultrafluar condensor and ultrafluar x32 objective and UV projective, selected for minimal chromatic aberration. The photomultiplier was EM 9558 Q (EMI Electronics, Hays, Middlesex, England) selected for low dark current. For the difference spectra (Fig. 1), transmission of the photoreceptor was measured directly, at a fixed wavelength, after an adaptive illumination, which was switched off during the
measurement. The diameter of the measuring beam was 0.2-0.1 of the diameter of the photoreceptor cell. Fig. 1 inset shows that the measuring intensity was weak enough to prevent measurable shifting of the pigment, and also that the measuring light was stable over the time of measurement. This was checked at regular intervals at specific wavelengths. The intensity of the monochromatic adapting light (Fig. 1) was strong enough to shift all the shiftable visual pigment molecules in approximately 5 s. For the absolute extinction spectrum (Fig. 3), first a run through the spectrum without a photoreceptor in the beam-path \( J_o(\lambda) \) was measured. Then the photoreceptor was inserted (by means of a Servosystem) into the beam path, and a second run through the spectrum was recorded \( J_t(\lambda) \). Finally, the receptor was withdrawn and \( J_o(\lambda) \) was measured once more. The extinction spectrum \( E(\lambda) \) was calculated according to \( E(\lambda) = \log(J_o/ J_t) + K \). \( J_o \) and \( J_t \) control did not differ by more than \(-8\%\), so no corrections for intensity drifts were necessary. Since there is always other (photostable) tissue in the path of the measuring beam besides the photoreceptor itself, \( K \) (the zero line in Fig. 3) is not determined.

For the derivation of extinction spectra from difference spectra a standard computer program of K. Hamdorf and P. Schlecht was used. The program calculated the difference of two Dartnall nomograms (Fig. 2 A) to give the best least-squares fit to a given microspectrophotometrically measured difference spectrum. The wavelengths of maximum extinction of the two nomograms and their peak-absorption ratio were free parameters in the calculations.

**RESULTS**

Fig. 1 shows the microspectrophotometrically derived difference spectra from a single photoreceptor of the barnacle lateral eye measured in the species *B. eburneus*. Fig. 1 inset gives an example of the absorption measurements: the absorption at 495 nm is smaller after saturating (more light did not affect the results) blue than after saturating orange adaptation, and the reverse is true for absorption at 570 nm.

The points of the difference spectrum were calculated from the absorption differences measured at various wavelengths after blue and orange saturating adaptation. In all the measurements it was possible to shift the pigment “forward” to a green-absorbing pigment with a difference spectrum peak located around 570 nm and “backward” to a blue-absorbing pigment with difference spectrum peak located around 485 nm. Between measurements (in the spectral range from 380 to 650 nm), the preparation was illuminated alternately with saturating blue (495 or 442 nm) and orange (596 nm) lights, which shift the pigment to either the green-absorbing or the blue-absorbing states of the pigment, respectively. We confirmed that the difference-spectrum changes only in amplitude and not in shape when other wavelengths are used for shifting the pigment from one state to the other. The magnitude of the difference-spectrum peaks varied among individual animals, as indicated by the two sets of points (○, ●) which were measured in two different animals. It was possible to shift the pigment forward and backward many times over a period of hours without any accumulation of a UV pigment absorbing maximally at 380 nm, as reported by Brown and Cornwall (1975). The isosbestic point of the various difference-spectrum measurements was always in the range of 530-540 nm. Occasionally, with the same positions of the maximum,
minimum, and isosbestic point, the relative size of the minimum at 570 nm was smaller compared to the maximum at 485 nm. We tested the stability of the green- and blue-absorbing states of pigment by waiting in the dark up to 30 min between the orange and blue adapting lights and the measuring light, and found no influence of the dark time on the results. We also measured the difference spectra in *B. amphitrite*. The dissection of this preparation was more difficult because the animals were smaller and the tapetum was more adherent to the cells. Accordingly, we did not have the complete spectra, but did succeed in showing that the peaks and isosbestic point fall at the same wavelengths as for *B. eburneus*. The results show directly that the photoreceptors of both species have a pigment with two dark-stable photointerconvertible states.

Fig. 2A shows two Dartnall nomograms peaking at 492 nm and 532 nm with a ratio of the peaks of 1.6:1, respectively. A calculated difference spectrum from these curves gives the best fit to the results of Fig. 1 (○). We used the data of Fig. 1 (○) for the curve fitting (Fig. 2B) since they are from the preparation that gave the optimum signal-to-noise ratio and the best long-time stability (see measurements at 495 and 570 nm in Fig. 1). The calculated difference spectrum is demonstrated in Fig. 2B (smooth curve), together with a replot of one set of measurements presented in Fig. 1. The curves of Fig. 2A also fit very well the action spectra of metarhodopsin and rhodopsin that were deduced from ERP data (Minke et al., 1973a).

The second difference spectrum (Fig. 1, ●) by means of the same calculation, yields two Dartnall nomograms peaking at 494 nm and 533 nm with a ratio of the peaks of 2.1:1, respectively.

There are differences among the peak wavelengths and the shape of the various action spectra of the barnacle photoreceptors reported in the literature (Stratten and Ogden, 1971; Shaw, 1972; Hochstein et al., 1973; Brown and
MINKE AND KIRSCHFELD Two Photointerconvertible States of Visual Pigment

Cornwall, 1975). These differences may arise from photostable pigments which, in addition to the visual pigments, have been reported to exist in several invertebrate photoreceptors (Kirschfeld and Franceschini, 1977; Kirschfeld et al., 1977), and can also be demonstrated in the barnacle.

The photoreceptors of B. eburneus show a strong yellow color, while those of B. amphitrite are usually pale, without color, but sometimes yellow. Fig. 3 presents extinction spectra as measured in B. amphitrite yellow (□) and B. amphitrite pale (●). The extinction spectrum of the yellow receptor has a peak close to 450 nm with a shape typical of carotene. A very similar absorption spectrum was measured by us in B. eburneus (unpublished data) and was seen in the rhabdomere of the central receptor (no. 7) of the fly (Kirschfeld and Franceschini, 1977).

DISCUSSION

Two Photointerconvertible States of Visual Pigment

The agreement between the photometrically derived difference spectra and that calculated from two Dartnall nomograms, peaking at 492 and 532 nm, is

1 Kirschfeld, K., R. Feiler, and N. Franceschini. Manuscript in preparation.
very good. The 492 and 532 nm Dartnall nomograms of Fig. 2A also fit the ERP action spectra of metarhodopsin and rhodopsin, respectively (Minke et al., 1973a). To see how good the fit is between the actual ERP measurements and the microspectrophotometric results, we calculated a difference spectrum from curves that give the best fit to the ERP data of Minke et al. (1973a, Fig. 5), using a metarhodopsin-to-rhodopsin peak-absorption ratio around 1.6:1, respectively, and found a very good fit to the data of Fig. 1. The ratio of the peak absorption of metarhodopsin to rhodopsin of around 1.6:1 agrees with similar data derived from other invertebrates (Hamdorf and Schwemer, 1975). This ratio was measured in the barnacle by Minke et al. (1974) in two independent ways with the ERP. The ratio found in one way was 4:1 and in the other, 1.6:1. The reason for this difference might possibly arise from an effect of a photostable pigment (see below).

![Figure 3](image)

**Figure 3.** Absolute absorption spectra measured from photoreceptors of the lateral eyes of *B. amphitrite* yellow (△) and pale (■) receptors. The spectrum with a peak close to 450 nm is very similar to that of a carotene.

The good fit between the photometrically derived difference spectra and the spectrum calculated from the difference of the two Dartnall nomograms and from the ERP action spectra strongly suggests that the same visual-pigment system is measured by both techniques and that the ERP is a reliable measure of the visual-pigment changes in the barnacle.

When we compare our microspectrophotometric results with those of Brown and Cornwall (1975), we note that the major difference arises from their inability to convert the blue-absorbing state of pigment (metarhodopsin) to the green-absorbing state (rhodopsin). Instead, they found an irreversible accumulation of pigment in the UV. This finding might be expected in an extract of photoreceptors, since in other crustacea there is evidence for a reduction in the stability of metarhodopsin in extracts, which causes an irreversible increase in a UV-absorbing photoproduct, probably retinaldehyde (Goldsmith, 1972). The
fact that they found similar difference spectra in both extracts and intact photoreceptors-preparations suggests that their intact preparation was damaged during their experimental procedures, possibly due to long strong-adapting lights. In our experiments we used much shorter adapting lights (seconds instead of minutes) and, in addition, we lowered the temperature to 6°C in order to get stable recordings. Since the previously reported ERP action spectra (Hillman et al., 1972) did not show consistent differences between measurements at low (8°C) and at room temperature, we assume that the low temperature during our microspectrophotometric experiments did not affect the measured spectra.

Possible Effects of the Photostable Blue Pigment

The microspectrophotometrically derived absorption spectrum of rhodopsin fits very well the action spectra of the LRP and PDA induction, that of metarhodopsin fits the action spectrum of the PDA depression, as reported by Hochstein et al. (1973).

The PDA depression action spectrum and the action spectrum of light-induced current, reported by Brown and Cornwall (1975), have maxima at 510–520 nm and 540 nm, respectively. A difference spectrum of two Dartnall nomograms with the same peaks does not fit the data of Fig. 1 as well as does the spectrum presented in Fig. 2B.

The question arises of why there is a difference between the peak of the action spectrum of PDA depression (510–520 nm) as reported by Brown and Cornwall (1975) in *B. eburneus* and the absorption peak of metarhodopsin (492 nm), since we expect that the transition from metarhodopsin to rhodopsin induces PDA depression in *B. amphitrite* (Hochstein et al., 1973) as well as in *B. eburneus*.

The existence of a photostable blue pigment in the photoreceptors of *B. eburneus*, and its usual absence in *B. amphitrite* might possibly explain the discrepancy between the action spectra of PDA depression reported by Brown and Cornwall (1975) in *B. eburneus* and by Hochstein et al. (1973) in *B. amphitrite*.

The function of such photostable pigments can be manifold (Kirschfeld and Franceschini, 1977): a) They may act as a screening pigment which in the case of the barnacle could induce a red shift in the action spectrum derived from activation of metarhodopsin, so that it will better fit the action spectrum of PDA depression reported by Brown and Cornwall (1975). b) They may also act as "sensitizing-pigments", that is as photostable pigments that absorb light and transfer the energy to a photochemically active pigment. Such an "antenna"-function of photostable pigments, (e.g. of carotenes) is well known in photosynthesis (Govindjee, 1975), but has recently also been demonstrated in photoreceptors of the fly (Kirschfeld et al., 1977). However, assuming a resonance Förster-type energy transfer from donor to acceptor (Förster, 1966), we expect a blue shift in the action spectrum of metarhodopsin, if the blue pigment in the barnacle acts as an antenna pigment. In our present state of knowledge it is difficult to know which mechanism is more effective in the yellow receptors of the barnacle.

In contrast to the action spectrum which can be influenced by a photostable
pigment, the shape of the photometrically derived difference spectrum should be unaffected by activation of a photostable (i.e. unbleachable) pigment. This may explain why we have agreement between the photometrically derived difference spectrum (Fig. 1) from the yellow receptors of B. eburneus and the calculated difference spectrum from ERP data measured in receptors of B. amphitrite, which usually do not have the photostable pigment.

A detailed investigation of the role of the photostable pigment in the yellow receptors of B. eburneus seems highly desirable.

We are very grateful to Prof. P. Hillman and Dr. S. Hochstein for their very helpful comments and discussions. We thank Mr. R. Feiler for his help in the microspectrophotometric measurements, Prof. K. Hamdorf and Dr. P. Schlecht for the use of their standard computer program to calculate pigment spectra from difference spectra, Prof. H. Stieve and P. Hillman for the barnacles, and Dr. P. McIntyre for reading the English manuscript.

This research was supported in part by a grant from the United States-Israel Binational Science Foundation (B.S.F.) Jerusalem, Israel.

Received for publication 1 June 1977.

REFERENCES

BROWN, H. M., and M. C. CORNWALL. 1975. Spectral correlates of a quasi-stable depolarization in barnacle photoreceptors following red light. J. Physiol. (Lond.). 248: 555-578.

GOLDSMITH, T. H. 1972. The natural history of invertebrates visual pigment. In Handbook of Sensory Physiology. Vol. VII/1. Photochemistry of Vision. H. J. A. Dartnall, editor. Springer-Verlag, Berlin. 685-719.

GOVINDEE, editor. 1975. In Bioenergetics of Photosynthesis. Academic Press, Inc., New York.

FÖRSTER, T. 1966. In Modern Quantum Chemistry. Istanbul lectures. O. Sinanoglu, editor). Academic Press, Inc., New York. Sect. III B. 93.

HAMDORF, K., and J. SCHWEMER. 1975. Photoregeneration and the adaptation process in insect photoreceptors. In Photoreceptor Optics. A. W. Snyder, and R. Menzel, editors. Springer-Verlag, Berlin, 263-289.

HILLMAN, P., S. HOCHSTEIN, and B. MINKE. 1972. A visual pigment with two physiologically active stable states. Science (Wash. D.C.). 175: 1486-1488.

HOCHSTEIN, S., B. MINKE, and P. HILLMAN. 1973. Antagonistic components of the late receptor potential in the barnacle photoreceptor arising from different stages of the pigment process. J. Gen. Physiol. 62: 105-128.

KIRSCHFELD, K., and N. FRANCESCHINI. 1977. Photostable pigments within the membrane of photoreceptors and their possible role. Biophys. Struct. Mech. 3: 191-194.

KIRSCHFELD, K., N. FRANCESCHINI, and B. MINKE. 1977. Evidence for a sensitising pigment in fly photoreceptors. Nature (Lond.). 269: 386-390.

MINKE, B., S. HOCHSTEIN, and P. HILLMAN. 1973a. Early receptor potential evidence for the existence of two thermally stable states in the barnacle visual pigment. J. Gen. Physiol. 62: 87-105.

MINKE, B., S. HOCHSTEIN, and P. HILLMAN. 1973b. Antagonistic process as source of visible-light suppression of afterpotential in Limulus UV photoreceptors. J. Gen. Physiol. 62: 787-791.
MINKE AND KIRSCHFELD  Two Photointerconvertible States of Visual Pigment

MINKE, B., S. HOCHSTEIN, and P. HILLMAN. 1974. Derivation of a quantitative kinetic model for a visual pigment from observations of early receptor potential. Biophys. J. 14: 490-512.

SHAW, S. R. 1972. Decremental conduction of the visual signal in barnacle lateral eye. J. Physiol. (Lond.). 220: 145-175.

STRATTEN, W. P., and T. E. OGDEN. 1971. Spectral sensitivity of the barnacle Balanus amphitrite. J. Gen. Physiol. 57: 435-447.