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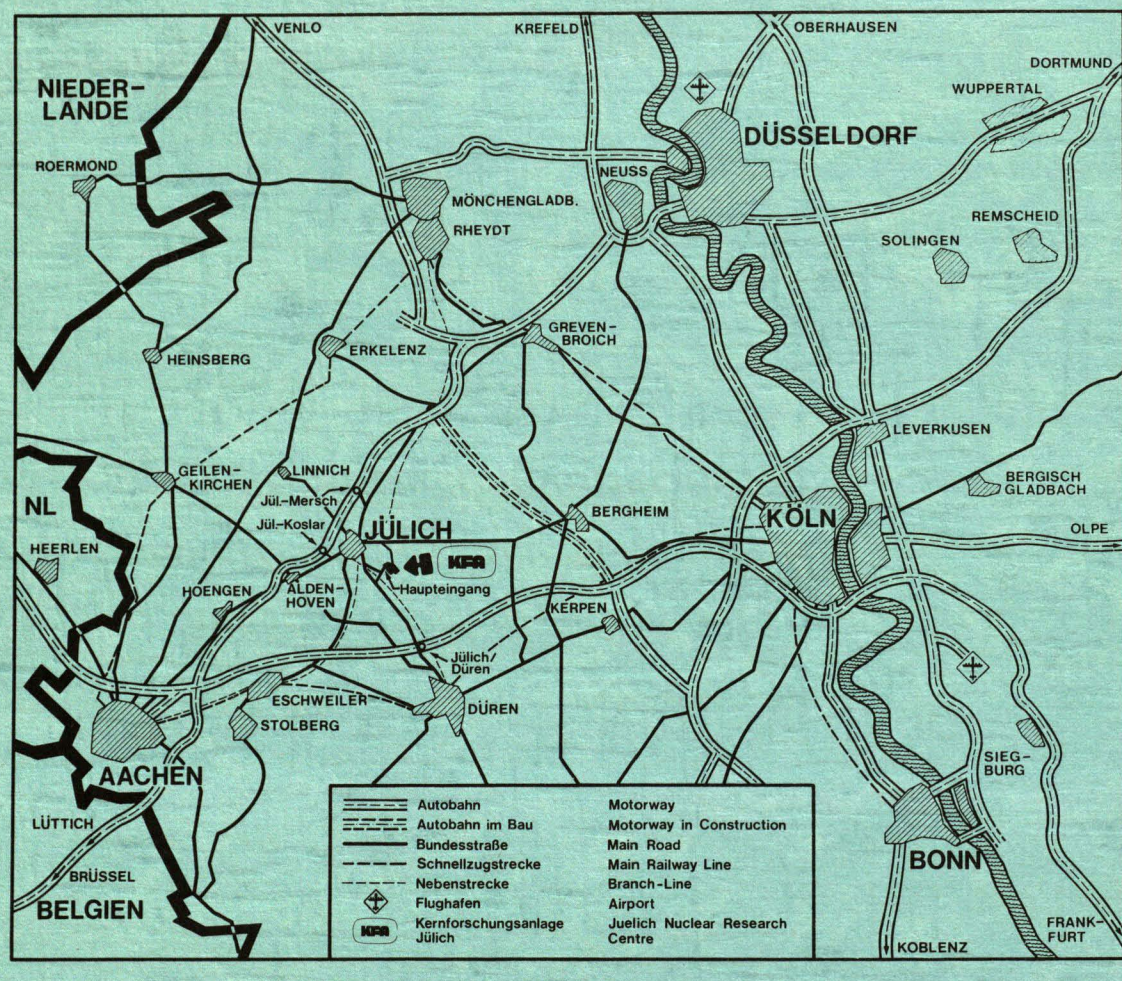
Jül - 1724
Juli 1981
ISSN 0366-0885



Als Manuskript gedruckt

Berichte der Kernforschungsanlage Jülich - Nr. 1724

Institut für Neurobiologie Jül - 1724

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Acknowledgements: We wish to thank K. Hamdorf and K. Hamacher for critical comments on the microspectrophotometric experiments, and E. Hildebrand for substantial help with the discussion of the electrophysiological measurements. This study is part of the SFB program 160 of the Deutsche Forschungsgemeinschaft. To T.H. Goldsmith we are indebted for critical comments.

Key words:
Crayfish, bistable rhodopsin and metarhodopsin system, microspectrophotometry, spectral sensitivity, monochromatic light adaptation.

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Abstract

Microspectrophotometric measurements of the spectral absorption of single rhabdoms of *Astacus leptodactylus* were carried out under various conditions of light adaptation and at different pH. The results were compared with electrophysiological measurements in *Astacus leptodactylus* and two other crayfish species (*Astacus fluviatilis and Procambarus (ortmannicus) acutus)*, obtained with selective adaptation by coloured light.

A bistable visual pigment system was found in the microspectrophotometric measurements. The absorption spectra for rhodopsin (λ_{max} ca. 553 nm), and metarhodopsin (λ_{max} ca. 495 nm) were calculated, on the basis of Ebrey-Honig nomograms, from typical difference spectra. In the electrophysiological measurements the maximal spectral sensitivity was recorded at 565 nm. No indication for a basic metarhodopsin could be found in the pH range from 4.5 to 10.5. After 2% glutaraldehyde treatment the bleaching product of rhodopsin was no longer photoreversible, probably due to decomposition of metarhodopsin into retinal and opsin. Our results do not indicate two different types of photoreceptor pigments in *Astacus* as have been demonstrated by Wald (1968) for *Orconectes* and *Procambarus*.

Introduction

In all invertebrate photoreceptors so far investigated, the light induced sequence of molecular reactions of the rhodopsin molecule terminates in a thermostable state of metarhodopsin. Under physiological conditions, this metarhodopsin is photoregenerated to some extent to the rhodopsin state.

In vertebrates, photoregeneration is not physiologically important because it can take place only in the short time before the separation of retinal from opsin.

Invertebrate rhodopsins have been investigated in cephalopods (Hubbard and St. George, 1958; Hamdorf et al., 1973), in several insects (Hamdorf et al., 1979) and in various crustacea (Wald, 1967, 1968; Goldsmith and Fernandes, 1968; Waterman and Fernandes, 1970; Kong and Goldsmith, 1977; Goldsmith and Wehner, 1977; Goldsmith, 1978 a,b). Two visual pigments have been extracted from *Orconectes* (Wald 1967) with λ_{max} at 562 and 510 nm, resp. This is in line with electrophysiological experiments (Wald 1968) which show a shift in the peak of spectral sensitivity after blue and red adaptation. These results were confirmed by spectrophotometric measurements of Waterman et al. (1969) in *Orconectes*.

Olivo and Chrismer (1979) demonstrated that in *Procambarus* the sensitivity spectrum of pigment migration agrees with the sensitivity spectrum (without selective adaptation) of the electroretinogram measured by Wald (1968) for the same species.

The physiological relevance of the photoregeneration of metarhodopsin has been demonstrated in insects (Hamdorf et al. 1973). Corresponding experiments by long term selective adaptation with light of 409, 501, and 611 nm neither proved nor excluded photoreversibility in *Astacus* (Stieve et al., 1973).

The present microspectrophotometric measurements were intended to give data on the spectral sensitivity of isolated rhabdoms

of *Astacus* (which so far are not available as far as we know), and to analyse the rhodopsin system of *Astacus* and the possible influence of salines and pH on its reactions. In order to study crayfish photoreceptors with a view to the possible existence of receptor types with different spectral sensitivity we performed a set of electrophysiological experiments using selective adaptation. The experiments were carried out in three crayfish species (*Astacus leptodactylus, Astacus fluviatilis, Procambarus (ortmannicus) acutus*)* under similar conditions as used by Wald (1968).

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* *Procambarus (ortmannicus) acutus* was imported from the U.S.A. We are indebted to Prof. G. Hartmann, Zoologisches Staatsinstitut Hamburg, who determined the species.

Material and methods of the microspectrophotometric measurements*

Isolated rhabdoms of *Astacus leptodactylus* Eschholz were obtained according to a procedure described by Waterman et al. (1969). The preparation was carried out under red light (Osram 4563) at room temperature after at least 2 hs dark adaptation. The retina of one eyestalk was removed, and about 100-300 isolated rhabdoms (Fig. 1) were obtained by shaking the retina in a mixer in ca. 0.2 ml physiological saline. The saline contained (after v. Harreveld, 1936) 211 mmol/l NaCl, 5 mmol/l KCl, 3 mmol/l $MgCl_2$, 2.3 mmol/l $NaHCO_3$, 10 mmol/l $CaCl_2$, 4.3 mmol/l Na_2HPO_4, 0.7 mmol/l NaH_2PO_4. The pH of this saline was 7.5. For measurements at different pH, the pH of this saline was changed by citric acid or NaOH. In one set of experiments 2% glutaraldehyde was added to the saline.

Measuring technique

The measurements were carried out with a dual-beam microspectrophotometer as described by Schlecht et al. (1978). For these measurements the rhabdoms were contained in a drop of physiological saline which was kept between two cover glasses and sealed by silicon grease. The measuring light beam was 5 µm in diameter and passed through the thickest part of a rhabdom (ca. 30 µm).

The instrument scanned the spectrum from 700 to 300 nm within 4 s, measuring 200 light intensity values which were sampled and analysed by an on-line PDP 11/10 computer. For the calculation of an extinction spectrum of the rhabdom two different scans are necessary, a first scan through the solution nearby the rhabdom, which is used as basis, and a second scan through the rhabdom, representing the actual measurement. In order to demonstrate the spectral changes brought about by an adapting illumination the two scans recorded before and after the adapting illumination have to be compared. In this way a difference spectrum is obtained.

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* The microspectrophotometric measurements were carried out at the Institut für Tierphysiologie der Ruhr-Universität Bochum

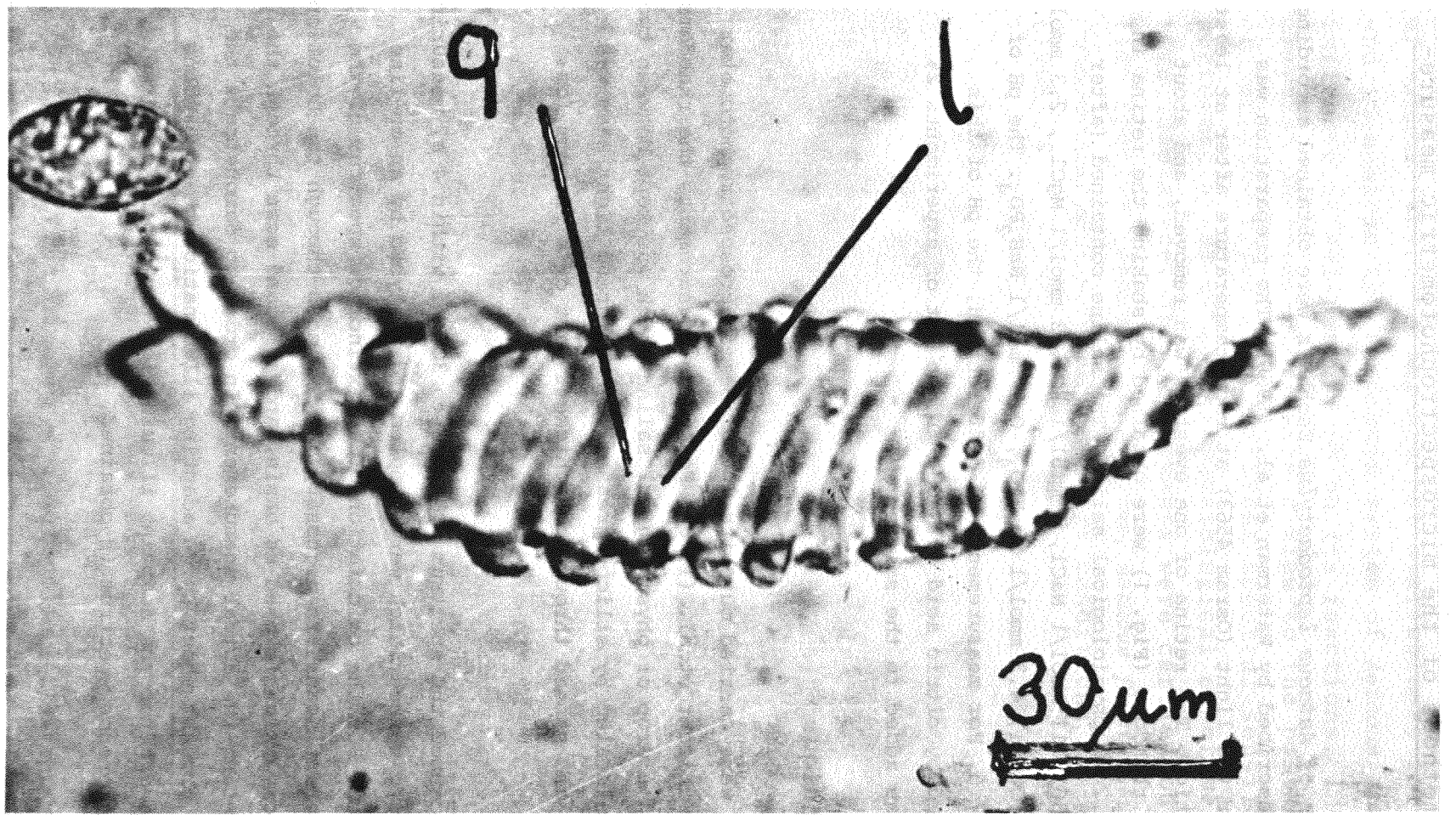

Fig. : Isolated rhabdom of *Astacus leptodactylus* in physiological saline (v. Harreveld, 1936). Layers of microvilli parallel (p) and perpendicular (s) to plane of figure (Photograph by E. Uebags, unpublished)

As monochromatic adapting light the measuring light beam originating from a Xenon 450 W lamp was used. Its intensity was measured with an optometer (UDT 40 A) and found to be $0.29 \cdot 10^{-9}$ W (corresponding to approximately 10^9 photons/s at 610 nm). The number of rhodopsin molecules in the illuminated area was estimated as $4 \cdot 10^8$; taking into account the extinction of the preparation at λ_{max} (0.05, corresponding to about 10% absorption), every rhodopsin molecule will be hit once within 5 s of illumination. Thus, an exposure time of 1 min should be sufficient to reach the photosteady state between rhodopsin and metarhodopsin. For white light adaptation the incandescent microscope lamp was used. Its intensity was approximately 100 times larger than that of the monochromatic measuring beam. The microscope lamp was also used for red light adaptation in connection with an edge filter Schott RG 645. This red light was also higher in intensity than the monochromatic light. The experiments were carried out at room temperature. For details of the experimental procedure see Wilms (1977).

Material and methods of the electrophysiological measurements

Receptor potentials were recorded extracellularly from retina slices (cut perpendicular to the eye surface) of *Astacus leptodactylus* placed in a test vessel and superfused with physiological saline (v. Harreveld, 1936). A Xenon high pressure lamp (XBO 900 W) was used for light adaptation and stimulation. The maximal light intensity was measured with an optometer (UDT; calibration against thermopile) and found to be ca. 25000 lx at the preparation (corresponding to ca. $1.03 \cdot 10^{16}$ photons cm^{-2} s^{-1} at 550 nm). The light intensity was adjusted by neutral density filters (Schott).

For red adaptation a wide bandpass filter (Schott, RG 610 nm, 90% transmission for $\lambda > 610$ nm) and for blue adaptation a filter combination (Schott, GG 375, and Balzers, DT-blue; band width 380 to 480 nm, max. 442 nm, 75% transmission) was used. The adapting light was adjusted to evoke equally high receptor potentials (RePs).

Procedure

The experiments were carried out at ca. 6°C. The duration of one experiment was up to ca. 4 hs. After a pre-period (60 min) the intensity of a white reference stimulus was adjusted to evoke RePs of ca. 150 µV. Reference measurements were made every 40 min and at the beginning and the end of each adaptation period. The chronological order of the measurements was: 10 min dark adaptation; 30 min light adaptation (red or blue), measurement of the spectral sensitivity (see below) with the adapting light as background illumination; 60 min dark adaptation; 30 min light adaptation (blue or red); again measurement of the spectral sensitivity with the adapting light as background illumination.

The spectral sensitivity was recorded by the use of 50 ms light stimuli at intervals of 7.5 s. Sixteen monochromatic filters in the range between 382 and 724 nm were used in a sequence alternating between high and low wavelengths.

Evaluation

The spectral sensitivity was plotted as the reciprocal relative number of quanta ($\frac{1}{Q}$) needed to produce responses of equal height.

The number of quanta of the white reference stimulus was calculated from the light intensity (lx) according to Stieve (1963). This number of quanta was used as 100% value for the measurements of the spectral sensitivity.

Because of the different sensitivity of the preparations the light stimuli were adjusted to evoke approximately equal responses. At each wavelength therefore eight stimuli were applied at each of three different light sensitivities, evoking responses near 50 µV. (This value of 50 µV is about 5% of the saturation value of ca. 1 mV, i.e. it is in the low energy range of the response-energy function of the preparation.) The intensity necessary for a response of exactly 50 µV was obtained by graphic interpolation (or extrapolation if the measured values were located only at one side of the desired value).

For each wavelength, the light intensity was calculated as the required number of quanta and normalized with respect to the white reference stimulus. Each point in the plots of sensitivity ($\frac{1}{Q}$) versus wavelength (λ) was averaged from the eight successive measurements (see description above) by a transient averager (Biomac 1000).

Results of the microspectrophotometric measurements

Absorption spectra were recorded under various experimental conditions in 76 rhabdoms from 17 eyes of *Astacus leptodactylus*. (We measured through the thickest part of the rhabdom; measurements at various other parts of the rhabdom gave the same results.)

Curve a of Fig. 2 shows the extinction spectrum of a dark adapted rhabdom with maximal extinction at 516 nm. After illumination of the rhabdom with red light for 15 min the extinction spectrum is altered, as shown by curve b. In the spectral range from 400 to 530 nm the extinction increases whereas between 530 and 650 nm it decreases and the peak of absorption shifts to 500 nm. Subtraction of curve a from curve b gives the difference spectrum of curve c which shows the changes in extinction caused by red light adaptation.

A similar effect can be obtained after white light adaptation (15 s) as shown by the experimental curve of Fig. 3. The difference spectra seem to be independent of the colour of the adapting light and to be invariant with respect to the maximum at about 480 nm, the minimum at 570 nm, and the isosbestic point at about 530 nm. Also, the duration of the white light adaptation (up to 10 min) causes no significant change of the difference spectrum.

In order to test whether the extinction spectrum changes during a dark period following illumination we measured the spectrum 20 min after a 10 min white light adaptation and compared it with the spectrum recorded immediately after the dark period. But also this experimental procedure does not significantly affect the difference spectum. Thus, the photoproduct created by the red or white adapting light must be thermostable.

To test whether the light induced spectral changes are photoreversible, we exposed, in a series of measurements, the rhabdom to successive illumination with adapting light of 610 and 460 nm. The exposure time was 1 min, which is sufficient to

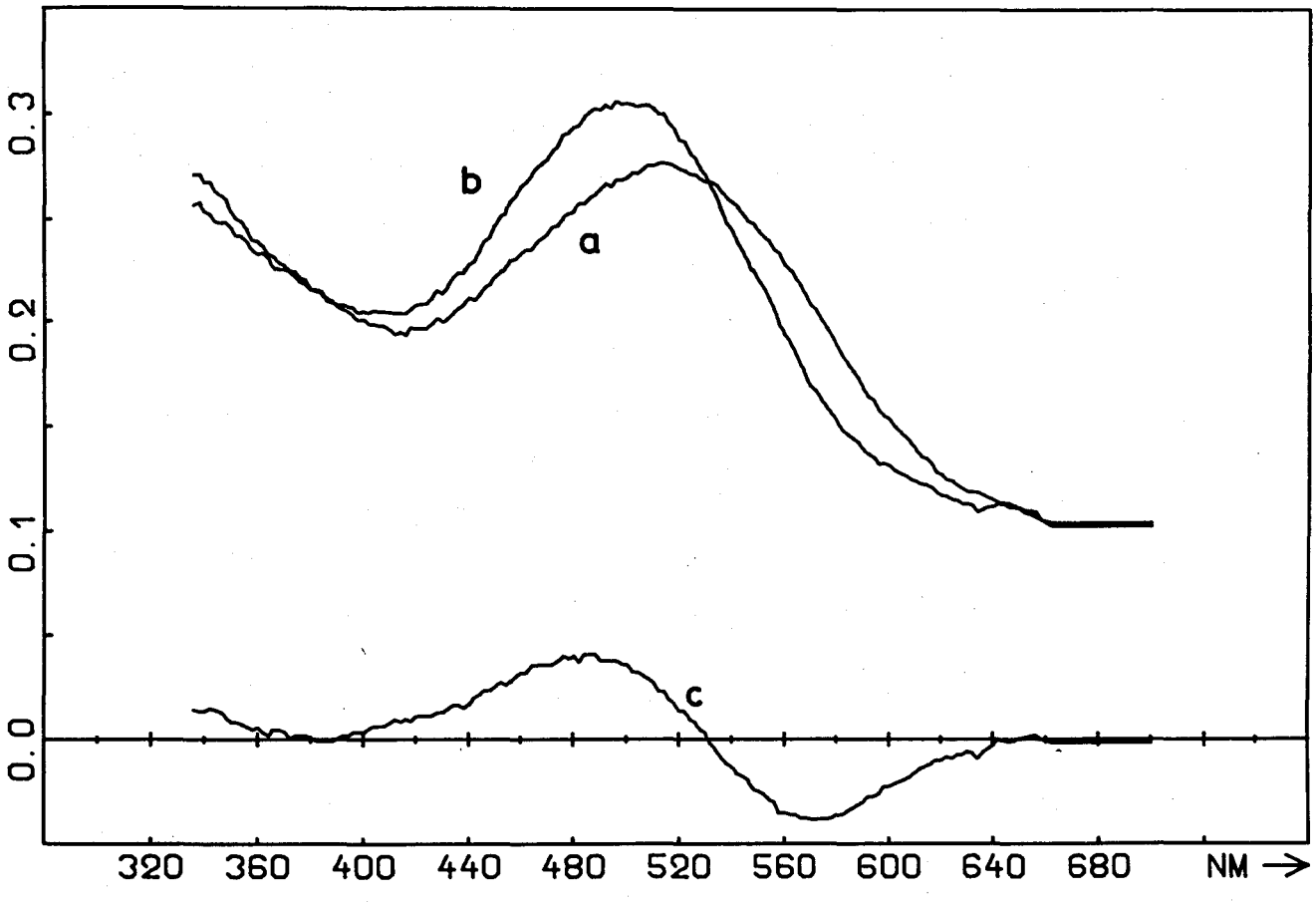

Fig. 2: Extinction spectra of a single rhabdom of *Astacus leptodactylus*: curve a: extinction spectrum of a dark adapted rhabdom; curve b: extinction spectrum of the rhabdom after 15 min red light adaptation; curve c: difference spectrum (difference between curve b and a).

reach the photosteady state as shown in Fig. 4. Curve a shows the spectral changes induced by a 460 nm illumination to a rhabdom previously adapted to 610 nm light, curve b shows the changes induced by 610 nm light to a rhabdom previously adapted to 460 nm light. Such difference spectra could be repeated at least 10 times without a decrease in amplitude. This shows that the visual pigment system in *Astacus* is completely photoreversible.

Our results suggest that the difference spectrum of curve c in Fig. 2 is due to the phototransition between rhodopsin and metarhodopsin of a single visual pigment. For this case, using Ebrey-Honig nomograms to obtain the shape of the rhodopsin and metarhodopsin spectrum, the difference spectrum has been analysed by a computer programmed fitting procedure (see Schlecht et al., 1978) to yield the wavelengths of maximal absorption. The results are shown in Fig. 3. For rhodopsin, λ_{max} is at 547 nm, for metarhodopsin 495 nm, the maximal absorption of metarhodopsin being larger by a factor of 1.28 than that of rhodopsin. From the analysis of four similar spectra we obtained the following mean values and variations: λ_{max} of rhodopsin 553±8 nm, of metarhodopsin 495±8 nm, relative height factor 1.3±0.2.

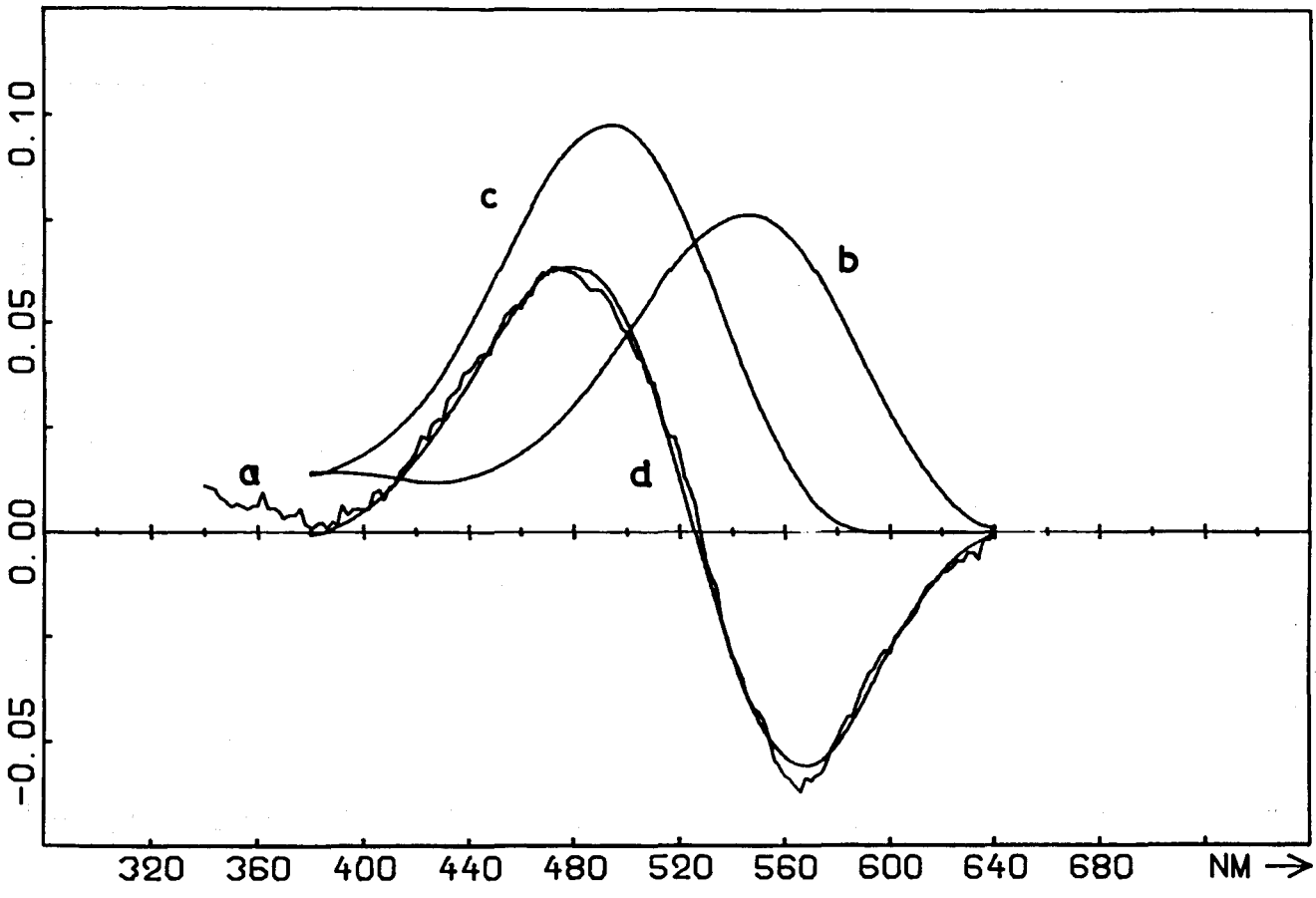

Fig. 3: Computer analysis of a difference spectrum:
curve a: difference spectrum, experimental curve, obtained after 15 s white light adaptation of a dark adapted rhabdom;
curve b and c: absorption spectra of rhodopsin (λ_{max} = 547 nm) and metarhodopsin (λ_{max} = 495 nm) obtained from Ebrey-Honig nomograms; the absorption maximum of metarhodopsin is higher by a factor of 1.28 than that of rhodopsin;
curve d: difference between curve b and c.

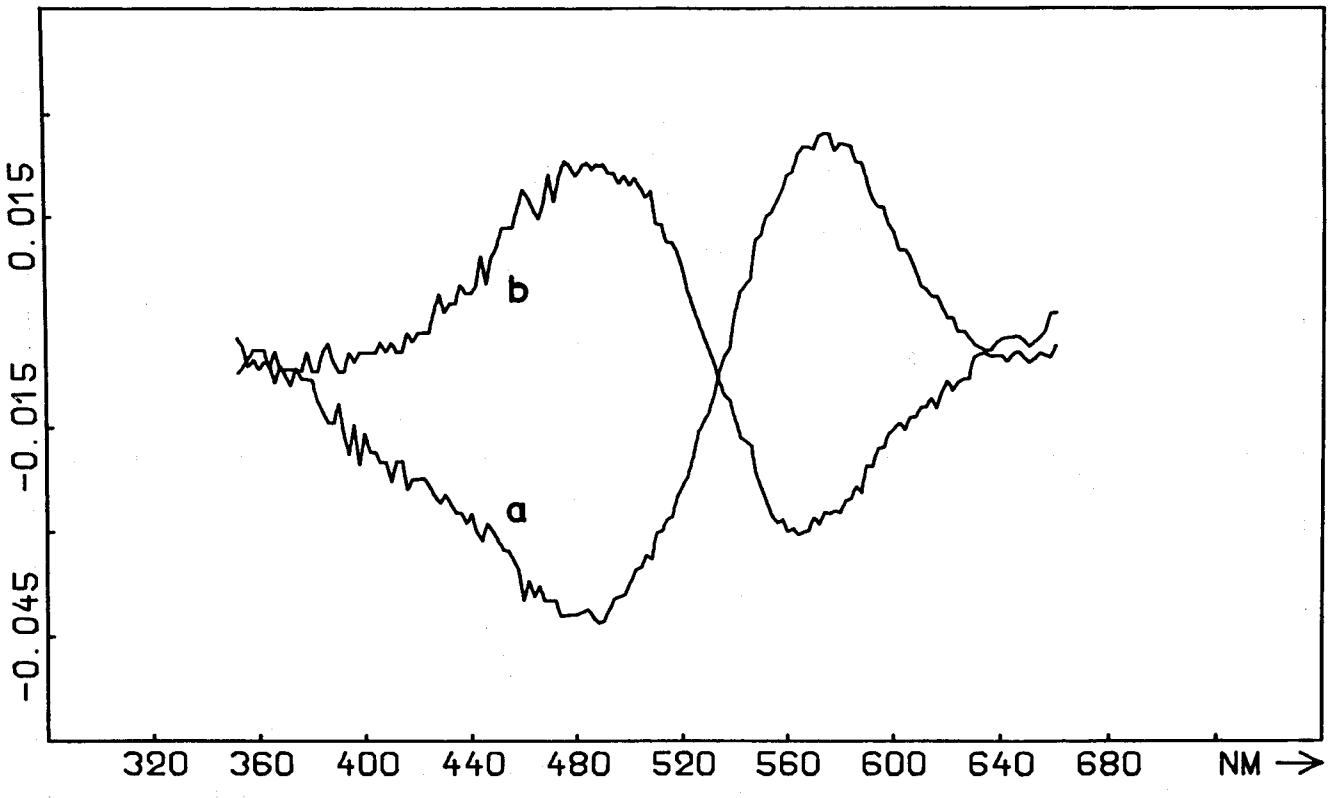

Fig. 4: Example of difference spectra obtained in a rhabdom of *Astacus leptodactylus* during a series of alternating periods of 1 min illuminations with light of 460 nm (curve a) and 610 nm (curve b).

Influence of glutaraldehyde on the light reaction

Glutaraldehyde is known to stabilize the structure of crustacean rhabdoms (Hays and Goldsmith, 1969; Goldsmith and Bruno, 1973). To test the effect of glutaraldehyde under our experimental conditions we added 2% glutaraldehyde to the rhabdom suspension of 2 eyes 30 min prior to the measurement.

We recorded the spectral changes which occurred after 1 min illumination with white light. These results are shown in Fig. 5. This difference spectrum has a minimum at 560 nm. A maximum, which would indicate the formation of metarhodopsin, is missing. Instead, the increase in extinction from 440 to 380 nm indicates the release of retinal. This result means that the photoreversibility is not preserved in the presence of glutaraldehyde, probably due to the decomposition of metarhodopsin.

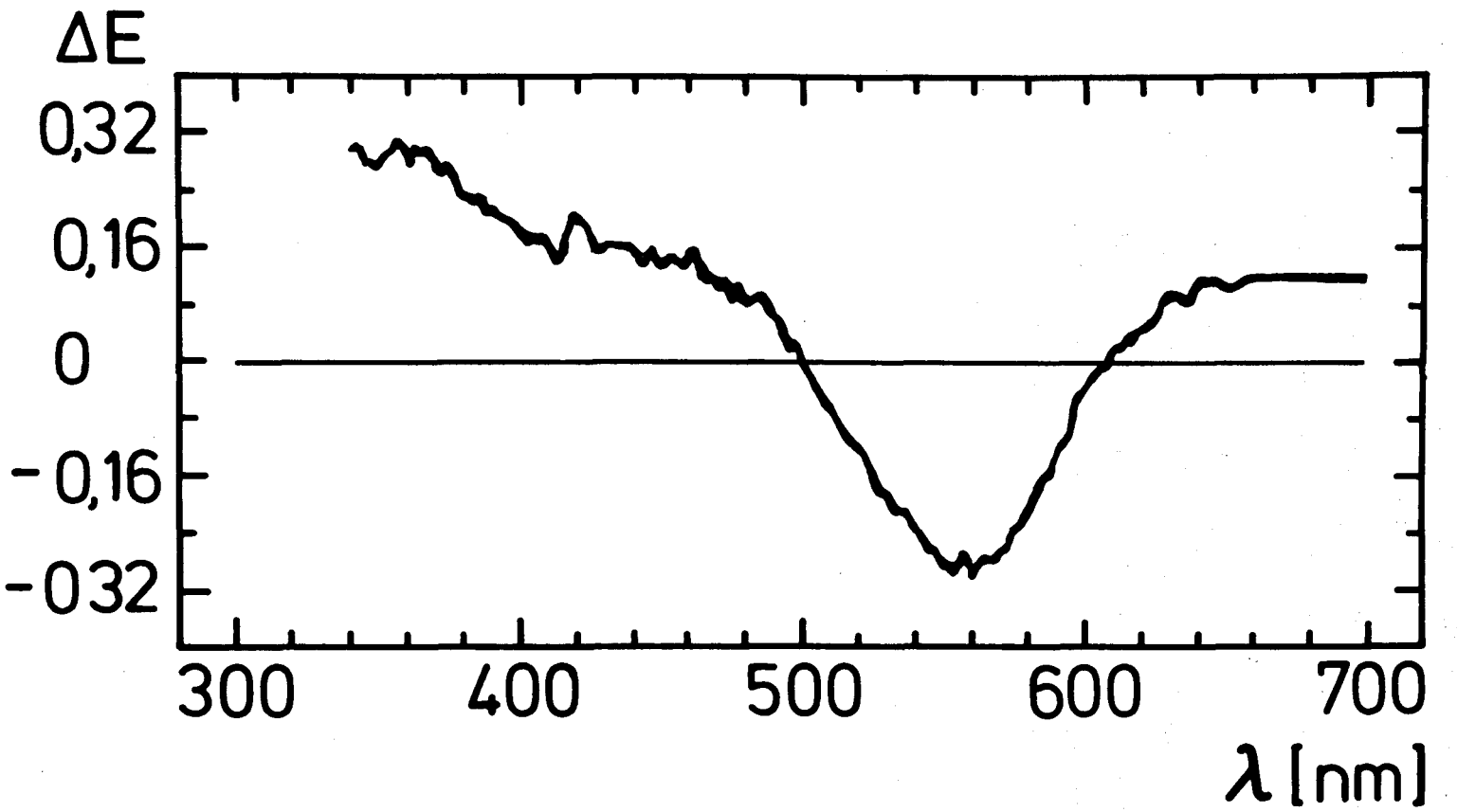

Fig. 5: Difference spectrum of a rhabdom of *Astacus leptodactylus* in saline containing 2% glutaraldehyde, obtained by 1 min white light adaptation of a dark adapted rhabdom.

Influence of pH

In order to test the possible occurrence of basic metarhodopsin we percormed measurements at different pH values in the range from pH 4.5 to 10.5. A basic metarhodopsin, with maximal absorption at 380 nm, in addition to the normal, acid metarhodopsin is found in many invertebrates as in *Loligo* by Hubbard and St. George (1958) and in *Ascalaphus* by Hamdorf et al. (1973). The pK value for the transition between acid and basic metarhodopsin was found to be 7.7 in *Loligo* and 9.2 in *Ascalaphus*.

If basic metarhodopsin is present one has to expect an increase of absorption at 380 nm resulting in a shift of the maximum of the difference spectrum to shorter wavelengths and a corresponding shift, of the isosbestic point. We made five experiments at different pH values. At every pH 10 dark adapted rhabdoms (20 for pH 7.5) were illuminated for 1 min by light of 610 nm and the difference spectra recorded. At the different pH values we obtained the following values for the mean position of the maximum and its standard deviation:
pH 4.5: 478 $\pm$ 5.5 nm; pH 5.4: 476 $\pm$ 3.7nm; pH 7.5: 478.0 $\pm$ 4.3 nm; pH 9.2: 477.8 $\pm$ 4.2 nm; pH 10.5: 477.7 $\pm$ 7.1 nm.

So we do not find a shift of the position of the maximum and do not obtain any indication for the presence of basic metarhodopsin in the pH range between 4.5 and 10.5. Therefore the pK of the basic metarhodopsin should be even more alkaline than in *Ascalaphus*, or the pH-sensitive site of the pigment molecule is not accessible in the rhabdom.

It seems probable that in *Astacus*, similar to the conditions found in *Ascalaphus*, basic metarhodopsin does not play a role under physiological conditions. Our results are not in accordance with those of Goldsmith (1978 a) who finds a considerable absorption shift to shorter wavelengths by exposure to acid pH in *Procambarus* and *Orconectes*.

Results of the electrophysiological experiments

In 5 experiments the spectral sensitivity was measured in the range of 382 to 724 nm by recording RePs from retina slices of *Astacus leptodactylus* in the state of dark adaptation and after adaptation by red and blue light. The results of these experiments were not significantly different from each other; the position of the maximal sensitivity was almost identical. A single experiment is shown in Fig. 6. In the dark-adapted state (upper curve) the maximal sensitivity is at ca. 565 nm. The maximum is shifted neither for the state of red adaptation (open circles) nor for blue adaptation (squares). The curves do not indicate the existence of blue-sensitive cells in *Astacus leptodactylus*.

Comparison with two other crayfish species

Under similar conditions as those described for *Astacus leptodactylus* the influence of long-term adaptation with different chromatic light was tested in *Astacus fluviatilis* (two experiments) and *Procambarus (ortmannicus) acutus* (two experiments).

In both species the spectral sensitivity curves after long-term adaptation with blue light (380 to 500 nm) and red light (610) are not significantly different from the spectral sensitivity curve of the dark-adapted eye, which is characterized by a broad maximum at 540, 565, 584 nm. This means that in neither of the species *Astacus leptodactylus*, *Astacus fluviatilis* and *Procambarus (ortmannicus) acutus* a significant shift of λ_{max} of the spectral sensitivity curve due to chromatic adaptation was observed. Such a shift would indicate the existence of different red- and blue-sensitive photoreceptors in the retina.

The experiments with *Astacus fluviatilis* and *Procambarus* were carried out in winter. According to Nosaki (1969) the spectral sensitivity of the eyes of *Procambarus* has a maximal value at 560 nm in most cases in winter, and in summer at over 600 nm in 90% of the cases (in 10% at 460 nm); Nosaki ascribes this difference to changes of the screening pigment.

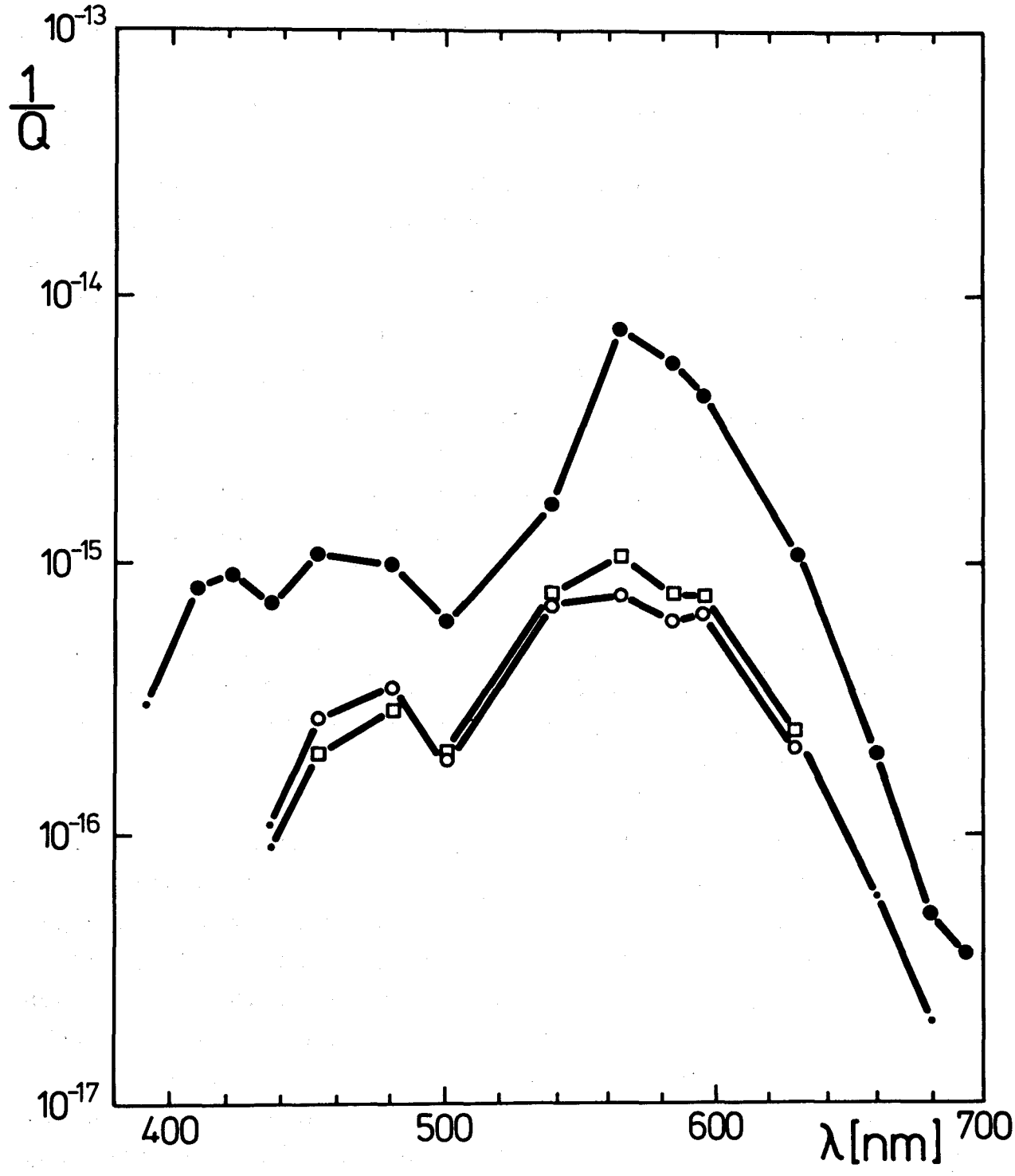

Fig. 6: Spectral sensitivity ($\frac{1}{Q}$) obtained from retina slices of *Astacus leptodactylus* in physiological saline in the state of dark adaptation (solid circles), after adaptation with red light (open circles), and adaptation with blue light (squares). The small black dots are extrapolated values (see Procedure).

Discussion

Our microspectrophotometric results show a single visual pigment with two thermostable states (rhodopsin and metarhodopsin) in the photoreceptors of *Astacus*. The absorption spectra for both rhodopsin and metarhodopsin were obtained by an analysis of the difference spectra. Maximal absorption was found for rhodopsin around 553 nm and for metarhodopsin around 495 nm. The maxima are thus at similar wavelengths as in the barnacle lateral eye (Minke and Kirschfeld, 1978). According to the overlapping of the absorption spectra (see Fig. 3) illumination with light of 610 nm causes an almost quantitative transition of rhodopsin to metarhodopsin, while rhodopsin is regenerated to ca. 65% of its maximal quantity by illumination with light of 460 nm.

Obviously metabolic regeneration does not occur in isolated rhabdoms (shown by the fact that rhodopsin was not regenerated from metarhodopsin within 20 min in the dark at room temperature). Such a metabolic regeneration can, however, not be excluded for the living animal.

The electrophysiological experiments show maximal sensitivity around 565 nm. This suggests, for the analysis of microspectrophotometric difference spectra, that the long wavelength pigment is indeed rhodopsin and the other pigment with λ_{max} at 495 nm is metarhodopsin. But the comparison of the peak of sensitivity with the pigment absorption peak shows a difference of about 10 to 20 nm. This difference has been tentatively assigned by Nosaki (1969) to the effect of screening pigments. One could also attribute this difference to the self-screening effect of metarhodopsin. This effect has been postulated by Hamdorf (1979) for the blow fly photoreceptor at blue adaptation. In flies, maximal absorption of metarhodopsin is at longer wavelangths than rhodopsin absorption. Higher percentages of metarhodopsin are obtained only by blue adaptation. Under such conditions the self-screening effect causes a shift of spectral sensitivity to lower wavelengths. In *Astacus*, con-

ditions are inverse. Metarhodopsin absorption is at lower wavelengths than rhodopsin absorption. Metarhodopsin is present under any light adaptation conditions to an amount of at least 35%; here the self-screening effect should lead to a shift of maximal sensitivity to longer wavelengths and should be present in any case, so that a coincidence of pigment absorption and spectral sensitivity cannot be expected. The magnitude of the shift depends on the total extinction of the rhabdom; a shift of 10 to 20 nm is quite possible. At red light adaptation, the percentage of rhodopsin is considerably higher. Therefore, the spectral sensitivity maximum should shift to even longer wavelengths.

Our electrophysiological experiments with blue and red light adaptation did not show a marked shift of the peak of spectral sensitivity so that no indications for a blue-sensitive receptor could be found in *Astacus* as well as in the two other crayfish species.

So the electrophysiological experiments do not suggest two receptor types in *Astacus*, in contrast to the results of Wald (1968) in *Orconectes* and *Procambarus*. This result is also in accord with the microspectrophotometric findings which show that the rhabdom is homogenous with no indication for more than a single bistable visual pigment.

If, nevertheless, a blue-sensitive receptor shold be present it must exist in much smaller amounts than the dominant receptor with maximal sensitivity at 565 nm.

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