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STUDIES ON THE INSECT VISUAL PIGMENT SENSITIVE TO ULTRAVIOLET LIGHT: RETINAL AS THE CHROMOPHORIC GROUP

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SUMMARY

1. The nature of the chromophoric group of the ultraviolet visual pigment of the neuropteran *Ascalaphus macaronius* Scop. was investigated. Analysis of the carotenoid content of the retina by the reaction with $SbCl_3$ indicated the presence of retinal.

2. Thin-layer chromatography of ethanol–ether extracts from retinae on silica gel demonstrated the presence of retinal isomers with R_F values corresponding to all-*trans*-retinal and 11-*cis*- and/or 13-*cis*-retinal. Other retinal isomers and retinol were not detected.

3. Thermal denaturation released retinal from the ultraviolet visual pigment A and its photoproduct B. Analysis of the compounds resulting from thermal denaturation indicated that all-*trans*-retinal was predominately released. Formation of cattle rhodopsin from cattle opsin and ultraviolet visual pigment A denatured by Ag^+ indicated that the stereoconfiguration of retinal in the native pigment is 11-*cis*.

4. As a consequence of the retinal nature of the chromophore, the ultraviolet visual pigment of *A. macaronius* was designated "rhodopsin 345", and photoproducts B and C correspond to acid and alkaline metarhodopsin, respectively. The stereoconfiguration of retinal in rhodopsin 345 and metarhodopsin, as well as the linkage of the chromophore in respect of the hypsochromic shift of rhodopsin 345, were discussed.

INTRODUCTION

While retinal has been identified as the chromophoric group of visual pigments of vertebrates, cephalopods and crustraceans[1], similar evidence for insect visual pigments is rare. Goldsmith[2] was the first to demonstrate the presence of retinal in extracts from honey bee heads. Similar results were reported for extracts from the heads of houseflies[3] and other insect species[4]. Extracts from tissue of the cockroach retina also contained retinal[5]. The participation of retinol (vitamin A_1) in the insect visual cycle is suggested from a comparison of the retinol and retinal content in light- and dark-adapted honeybees[6]. Furthermore, carotenoid deficiency in the diet of flies caused a decrease in photoreceptor sensitivity[7,8]. The role of retinal in insect vision is nonetheless speculative, since the nature of these pigments has, until recently, been virtually unknown.

The only insect visual pigment which has been photochemically well characterized is the ultraviolet-sensitive pigment from the compound eye of the neuropteran *Ascalaphus macaronius* Scop.[9-11]. Ultraviolet irradiation of the visual pigment A, extracted by digitonin, results in the formation of thermostable photoproducts B and C, which are in a pH-dependent equilibrium (pK 9.2). Microphotometric measurements have indicated that photoproduct B is predominately formed *in vivo*.

Evidence for the presence of retinal in this ultraviolet-sensitive pigment was first described by Schwemer *et al.*[11]. They obtained a compound having the λ_{max} of retinaldehyde oxime (360 nm), when photoproduct B undergoes thermal denaturation in the presence of hydroxylamine. If retinal is indeed the chromophoric group, this would be the first visual pigment absorbing maximally at shorter wavelength than the chromophore itself. In order to obtain a better understanding of this hypsochromic shift, the present study is concerned with the unequivocal identification of the chromophoric group and the possible stereoisomers involved in the photoconversions of this visual pigment.

MATERIALS AND METHODS

Preparation of the ultraviolet visual pigment

Origin and preparation of the retinae of *A. macaronius* were described by Schwemer *et al.*[11]. The method of visual pigment extraction was slightly modified. 160 retinae were washed in 40 ml of ice-cold 0.067 M sodium phosphate buffer (pH 7.0). The retinae were collected by centrifugation (at 40000 × g for 30 min; Sorvall RCB 2, rotor SS 34) and homogenized in 2 ml 0.067 M phosphate buffer (pH 7.7), using a Potter–Elvehjem homogenizer (5 ml) with a teflon pestle. The homogenate was diluted to 40 ml with 0.067 M phosphate buffer (pH 7.7) and allowed to stand for 2 h at 4 °C in order to extract soluble proteins and screening pigments. The insoluble material was sedimented by centrifugation (at 40000 × g for 30 min), and resuspended in phosphate buffer (pH 6.0). This was repeated five times, using alternatively buffer solutions of pH 7.7 and 6.0. The visual pigment was extracted from the pellet with 0.7 ml of 2% (w/v) digitonin (Merck, Darmstadt) in 0.067 M phosphate buffer (pH 6.0) over a 12-h period. The extracts had to be tested for the presence of visual pigment and photoproducts from photoreceptors (spectral sensitivity, maximally at about 520 nm) of the lateral eye. Therefore, all manipulations were carried out at 4 °C in dim red light.

Preparation of cattle opsin

Opsin was extracted by 2% (w/v) digitonin (pH 6.4) from rod outer segments of cattle eyes, prepared in daylight by a modified flotation technique[12,13]. Capacity of opsin to regenerate rhodopsin was tested with 11-*cis*-retinal.

Detection of retinal with $SbCl_3$

Retinae from 80 dark-adapted eyes of *A. macaronius* were homogenized in 0.067 M sodium phosphate buffer (pH 7.0) and centrifuged at 29000 × g for 40 min. The sediment was ground with anhydrous sodium sulfate and extracted with chloroform–methanol (2:1, v/v) at 25 °C and 70 °C, respectively. The chloroform–methanol extract was taken to dryness *in vacuo*, and the residue redissolved in light petroleum.

This extract was chromatographed on a column (13 cm × 1 cm) of basic aluminium oxide (Merck, Darmstadt; activity III). Elution was carried out with 20-ml aliquots of 4 %, 10 % and 30 % (v/v) acetone in light petroleum (b.p. 40–60 °C). 5-ml fractions of the eluate were collected and the solvent evaporated. Each residue was taken up in 0.2 ml chloroform, and the spectral absorbance of this solution recorded. 0.5 ml of chloroform, saturated with $SbCl_3$, containing 5 μl acetic anhydride, was then added and the spectral absorbance recorded immediately. All manipulations were performed in dim red light and, whenever practicable, under N_2.

Thin-layer chromatography of retinol and retinal isomers

All-*trans*-retinal and all-*trans*-retinol were a gift from Hoffmann-La Roche, Basel. As reference compounds, 9-*cis*- and 13-*cis*-retinal were purchased from Sigma, St. Louis. In general, *cis*-isomers of retinal were produced from the all-*trans*-configuration by irradiation of 0.3 % (w/v) all-*trans*-retinal in ethanol with white light (Xenon arc; 150 W) at 0 °C for 30 min. The siomers were separated in the dark by ascending thin layer chromatography on silica gel with light petroleum (b.p. 40–60 °C)–acetone–pyridine (94:5:1; by vol.) as developing solvent. This method allows for the separation of retinal isomers from retinol siomers. In Fig. 2a, F3–F5 represent the retinal isomers produced by photoisomerization of all *trans*-retinal. F3 contains 9-*cis*-retinal as indicated by co-chromatography and by the reaction with cattle opsin to form isorhodopsin. F4 contains two stereoisomers. The presence of 13-*cis* was indicated by co-chromatography, while the presence of 11-*cis*-retinal was demonstrated by reaction of eluates from F4 with cattle opsin to form rhodopsin. Identity of stereoisomer(s) in F5 was not achieved, although there is evidence that 11,13-di-*cis*-retinal is present[14]. Stereoisomers of retinal were visualized as deep orange spots with 4 % (w/v) rhodanin (2-thion-4-oxothiazolidine) in ethanol[15] (Reagent I). When a saturated solution of $SbCl_3$ in chloroform (Reagent II) was used afterwards as an overspray, both retinol and retinal were stained. Chromatography time on 20 cm plates at 4 °C was 90 min. The preparation of retinal from retinae of *A. macaronius* or cattle rod outer segments followed the method described for the retinal detection with $SbCl_3$, with the exception of the retinal extraction. Instead of chloroform–methanol, the extraction of retinal from retinae homogenates was carried out with ethanol–ether (3:1, v/v) at room temperature.

Spectrophotometric measurements were made with the Hitachi recording spectrophotometer Model 356 or a modified Zeiss PM QII described elsewhere[16].

RESULTS

Identification of chloroform-extracted retinal by reaction with $SbCl_3$

The column chromatography described for detection of retinal from retinae of *A. macaronius* with $SbCl_3$ yielded two fractions (elution with 4 % acetone in light petroleum) with measurable absorbance in the near ultraviolet. These fractions. combined in a chloroform solution, contained material which reacted with $SbCl_3$ (Fig. 1). The maximum of absorbance was at 664 nm, which is characteristic of retinal. Goldsmith[17] reported that retinal bound to a soluble protein can be extracted from heads of honeybees. Proteins which were extracted by 0.067 M phosphate buffer (pH 7.0) from retinae of *A. macaronius* and precipitated by 70 % saturation with

ammonium sulfate did not contain measurable amounts of $SbCl_3$-reactive substances. Therefore, most of the retinal is bound, prior to extraction with chloroform–methanol,

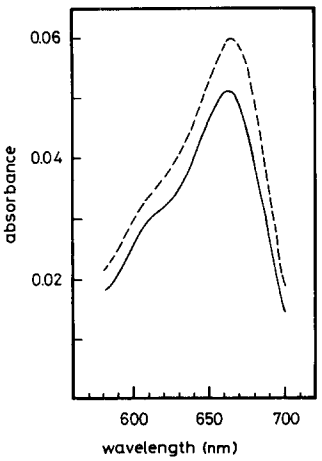

Fig. 1. Identification of retinal by reaction with $SbCl_3$. Spectral absorbance of an extract from the retinae after adding $SbCl_3$ (—); for reference, reaction of $SbCl_3$ with a standard solution of 0.5 μg all-*trans*-retinal (– – – – –).

to particles and membranes of the retinae, sedimented by centrifugation. Furthermore, it can be concluded that the retina of *A. macaronius* does not contain measurable amounts of retinol. With the retinae available it was not possible to determine the loss of retinal during tissue preparation, extraction procedures and column chromatography. Nevertheless, the retinal content, as estimated by the $SbCl_3$ reaction, must be higher than $1.5 \cdot 10^{-6}$ mg per retina. These results support the hypothesis that retinal is the chromophore of the ultraviolet visual pigment.

Analysis of retinae extracts by thin-layer chromatography

Thin-layer chromatography was used as an additional method to reveal retinal or retinol in retina homogenates and furthermore, to test the stereo-configuration of retinal in light- and dark-adapted retinae. The applicability of thin-layer chromatography for the analysis of retinal stereo-isomers in retinae of *A. macaronius* was tested by extracting retinal from dark-adapted and bleached rod outer segments of cattle and frog. As expected, the extracts from dark-adapted and bleached rod outer segments differed in the content of isomers found to be present in F2 (all-*trans*) and F4 (11-*cis*). This is in agreement with results published recently by Rotmans *et al.*[18]. Aspecific isomerization during development was not detected in F2–F5 (Fig. 2b), when two-dimensional thin-layer chromatography was carried out with a pyridine-containing solvent at low temperature in the dark. This two-dimensional thin-layer chromatography shows that F6 is a non-polar product of retinal isomers.

The thin-layer chromatography of extracts from retinae of *A. macaronius* revealed the presence of compounds with R_F values corresponding to all-*trans*-retinal (F2) and 11-*cis* (13-*cis*)-retinal (F4), as shown in Figs 2c and 2d. Distinct differences in the results obtained from light- and dark-adapted retinae could not be observed.

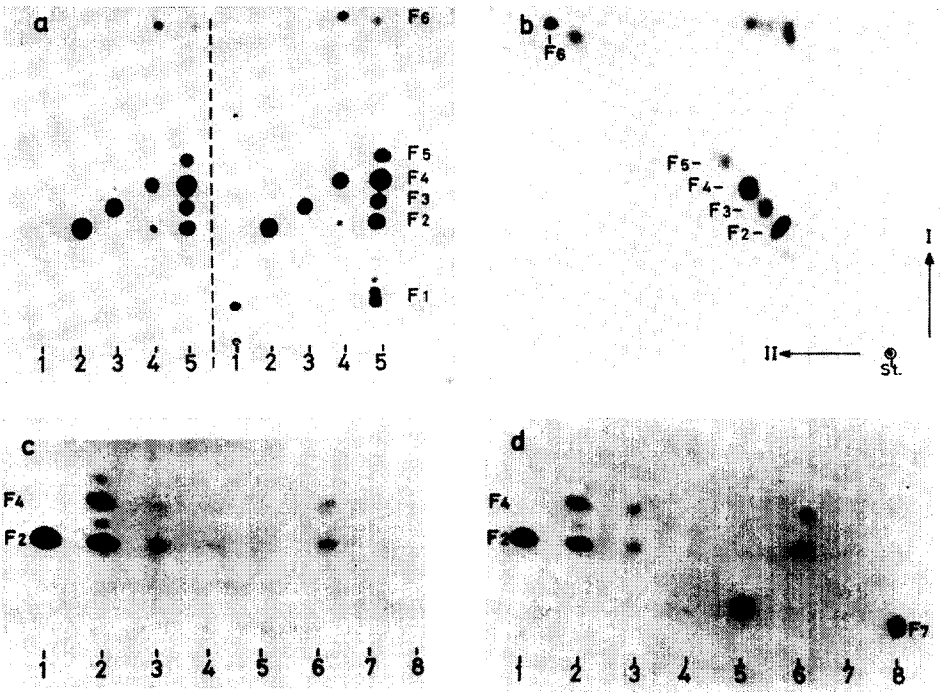

Fig. 2. Thin-layer chromatography of retinal isomers on silica gel (developing solvent: light petroleum–acetone–pyridine, 94:5:1, by vol.). (a) all-*trans*-retinol (1); retinal isomers: all-*trans* (2; F2), 9-*cis* (3; F3)13-*cis* (4; F4), 5 μg of each; mixture of retinol isomers (5; F1) and retinal isomers (5; F2–F6), produced by irradiation of the all-*trans* form. F5 and F6 were not further identified. Detection: rhodanin (left), $SbCl_3$ (right). (b) Two-dimensional separation of retinal isomers. Same developing solvent for direction I and II; F2–F6 as described under a. St., starting point. Detection: rhodanin. (c): all-*trans*-retinal (1; F2); mixture of retinal isomers (2): all-*trans* (2; F2) and 11-*cis*/13-*cis* (2; F4); fractions from column chromatography of light- (3–5) and dark-adapted (6–8) retinae. Detection: rhodanin. (d): As in c after spraying $SbCl_3$; cholesterol is revealed (5; F7 and 8; F7).

The absence of marked differences in the amount of stereoisomers of retinal extracted from dark- and ultraviolet light-adapted retinae may be due to aspecific isomerization during extraction and isolation of retinal at room temperature. Light-independent isomerization of retinal is reported for extraction of 11-*cis*-retinal from cattle rhodopsin at higher temperatures[18]. Milder extraction conditions reduce aspecific isomerization to less than 25 %[18], but extraction with ethanol for 10 min at 0 °C did not release detectable amounts of retinal from the available retinae. Furthermore, ultraviolet irradiation produces a steady state between ultraviolet visual pigment A and photoproduct B[11]. In this way the presence of more than one isomer in the light-adapted retina could be explained. Carotenoid derivatives other than retinal isomers were not detected. but spraying with Reagent II revealed the presence of cholesterol (Fig. 2d, F7).

Products of denaturation of ultraviolet visual pigment A and its photoproduct B

In order to establish that retinal is present in visual pigment A and its photoproduct, the pigments were thermally denatured and the resulting products investi-

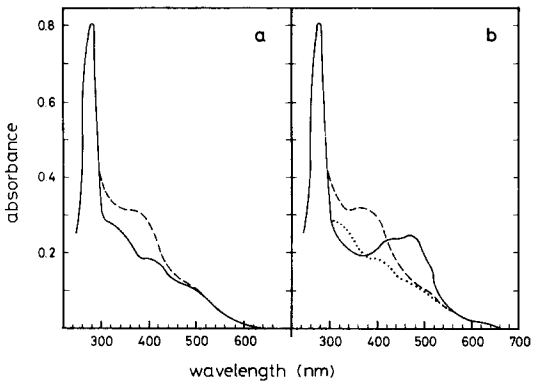

Fig. 3. Thermal denaturation at 70 °C of the visual pigment A and the photoproduct B. (a) Spectral absorbance of the ultraviolet visual pigment A (—) and of the products, arising from thermal denaturation (– – – –). (b) Spectral absorbance of the photoproduct B (—) and of the products, arising from thermal denaturation (– – – –); for comparison, the absorbance of the ultraviolet pigment is shown as dotted curve.

gated. This procedure was formerly used to characterize the stereo-isomers of retinal in squid rhodopsin and metarhodopsin[19]. The compounds arising from thermal denaturation of ultraviolet pigment A and photoproduct B exhibit the same spectral properties (Fig. 3). After thermal denaturation of ultraviolet visual pigment A and photoproduct B the absorbance increases at about 380 nm, the peak absorbance of retinal (Figs 4a and 4b). While thermal denaturation decreases the absorbance of photoproduct B at 480 nm (Fig. 4b), the loss of absorbance due to denaturation of ultraviolet visual pigment A produces a minimum at about 345 nm in the difference

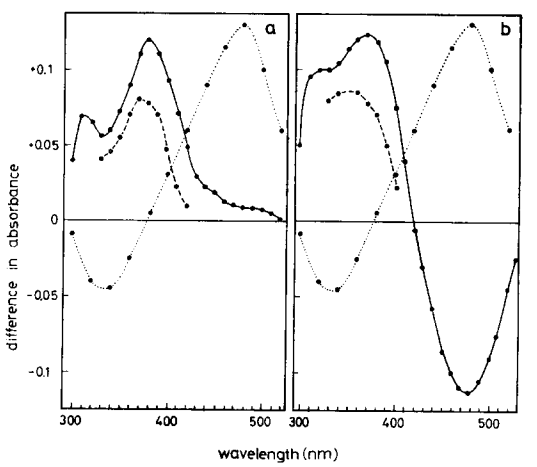

Fig. 4. Release of retinal by thermal denaturation of the ultraviolet visual pigment A and the photoproduct B. (a) Spectral differences between ultraviolet visual pigment A and the product formed by thermal denaturation (●—●), between ultraviolet visual pigment A and the thermally denatured pigment after irradiation with white light (●- - - - -●). (b) Spectral differences between photoproduct B and the product formed by thermal denaturation (●—●), between photoproduct B and the thermally denatured pigment after irradiation with white light (●- - - - -●). For comparison, the spectral difference between the ultraviolet visual pigment A and the photoproduct B is shown as ●·····●.

spectrum (Fig. 4a; solid curve), corresponding to the decrease in absorbance after ultraviolet irradiation of the ultraviolet visual pigment A (Fig. 4a; dotted curve). The presence of 11-*cis*-retinal was tested by adding native cattle opsin in excess, which should combine with this isomer to yield rhodopsin. However, differences in the formation of cattle rhodopsin from ultraviolet visual pigment A and photoproduct B, both denatured thermally, were not observed.

The stereoconfiguration of the retinal released was further investigated by irradiating the thermally denatured pigments with white light (Xenon arc; 150 W). The irradiation decreases the absorbance at 380 nm, and causes a shift of λ_{max} to shorter wavelengths (Fig. 4). When hydroxylamine was added, the λ_{max} shifted to 360 nm with a concomitant increase in absorbance, which is characteristic for the formation of retinaldehyde oxime. The results indicate that thermal denaturation of both ultraviolet pigment and photoproduct, under the conditions employed, primarily yielded all-*trans*-retinal.

It is likely that the absence of differences in the stereoconfiguration of the chromophore, released from the native pigment and photoproduct B, is due to the denaturation procedure. 11-*cis*-Retinal, released by thermal denaturation, isomerizes at the temperatures required for the denaturation of rhodopsin and is sterically labile in the presence of denatured opsin[19,20]. Therefore, as an alternative procedure, which excludes stereoisomerization of the released chromophore, denaturation with Ag^+ was used[21]. After addition of native cattle opsin in excess to ultraviolet visual pigment A or photoproduct B denatured by Ag^+, cattle rhodopsin was formed (Fig. 5). The differences in the amount of regenerated cattle rhodopsin indicate that

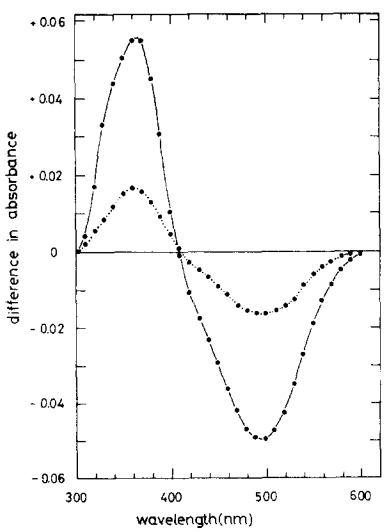

Fig. 5. Formation of rhodopsin from cattle opsin and Ag^+-denatured ultraviolet visual pigment A (●—●) and photoproduct B (● · · · · ●). Ultraviolet visual pigment A (0.2 ml in aqueous digitonin) and photoproduct B, produced by ultraviolet irradiation (353 nm) from ultraviolet visual pigment A were denatured by addition of $AgNO_3$. Excess Ag^+ was removed. After incubation with cattle opsin in excess (2 h; 25 °C; pH 6.4) in the dark NH_2OH was added. The difference spectra represent changes in absorbance after irradiating with light of 514 nm.

ultraviolet visual pigment A contains 11-*cis*-retinal. From the solution, containing denaturated photoproduct B, cattle rhodopsin was formed to a much lower extent (Fig. 5, dotted curve). The amount of 11-*cis*-retinal corresponding to the cattle rhodopsin formed in this case is in good agreement with the percentage of ultraviolet visual pigment A, estimated for a photo-equilibrium between ultraviolet visual pigment A and photoproduct B after prolonged ultraviolet irradiation (353 nm) of the native pigment[11].

DISCUSSION

As a result of the various methods employed, retinal was identified as the chromophoric group of the ultraviolet visual pigment present in the retina of the neuropteran *A. macaronius*. Following the nomenclature that differentiates between visual pigments of the rhodopsin type, with retinal as chromophoric group, and the porphyropsin type, containing 3-dehydroretinal as chromophore, the ultraviolet visual pigment can be classed among the rhodopsins. Thus, the ultraviolet visual pigment A is henceforth designated "rhodopsin 345". Photoproducts B (λ_{max} 480 nm) and C (λ_{max} 375 nm) correspond to acid and alkaline metarhodopsin, respectively.

When the chemical properties of the metarhodopsins of *A. macaronius* are compared with those of metarhodopsins from other arthropods, differences in thermal stability are apparent. Whereas acid metarhodopsin of *A. macaronius* exhibits a thermal stability similar to that of cephalopod metarhodopsin[20,22-24], a different thermal stability is reported for the photoproducts of crustacean visual pigments[25-28]. Irradiation of visual pigment preparations from other insects apparently releases the chromophore[2,5,29], as in the case of vertebrate rhodopsin. While there are indications that under physiological conditions the metarhodopsins of insects are thermostable in the photoreceptor membrane, the thermal stability of some metarhodopsins is lowered after extraction by digitonin, resulting in the release of the chromophore at ambient temperature (Schwemer, J. and Paulsen, R., unpublished). Goldmith and Warner[6] conclude from their results that the visual pigment in the honeybee is regenerated from retinol by an enzymatical, and not photochemical reaction. In *A. macaronius*, however, the stability of metarhodopsin and the absence (or low concentration) of retinol in the retina further supports the concept of the photoreversal of rhodopsin 345 *in vivo*[30].

11-*cis* stereoconfiguration of retinal in rhodopsin 345 is indicated by thin-layer chromatography and the formation of cattle rhodopsin from Ag^+-denatured rhodopsin 345 and cattle opsin. Acid metarhodopsin assumed to contain all-*trans*-retinal (λ_{max} 480 nm) has about 1.7 times the relative maximal absorbance of rhodopsin 345 with 11-*cis*-retinal as chromophore. Similar relations were previously reported for cephalopod rhodopsin and metarhodopsin[19,22-24].

Rhodopsin 345 is the first visual pigment shown to absorb maximally light at a wavelength shorter than 380 nm, the λ_{max} of the free chromophore. In all other known rhodopsins a bathochromic shift is observed. The spectral properties of the visual pigments are influenced by the kind of linkage between chromophore and lipoprotein part. In metarhodopsin II of vertebrates and rhodopsin and metarhodopsin of cephalopods, the chromophore has been considered to be bound as a Schiff base to an ε-amino group of lysine[31-33]. The indicator properties of the metarhodopsins of

A. macaronius agree with characteristics of Schiff bases of retinal[34-37]. Therefore, in agreement with proposals of Kropf and Hubbard[38], it is assumed that in acid metarhodopsin of *A. macaronius* the all-*trans*-retinal is attached to the lipoprotein moiety by a protonated aldimine linkage. Since unprotonated retinylidene imines show maximal absorbance about 20 nm shorter than free retinal[34], such a linkage of the 11-*cis*-retinal may contribute to the hypsochromic shift as found for rhodopsin 345. Experiments to analyse the linkage of retinal in rhodopsin 345 and acid metarhodopsin are in progress.

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