Is the Fly Visual Pigment a Rhodopsin?

Klaus Vogt

Max-Planck-Institut für biologische Kybernetik, Spemannstraße 38, D-7400 Tübingen, Bundesrepublik Deutschland

Z. Naturforsch. **38 c**, 329–333 (1983); received January 20, 1983

Rhodopsin, Insect Visual Pigment, Retinal, Thin Layer Chromatography, Xanthophyll

Thin layer chromatography of retina extracts from *Calliphora* yielded, instead of retinal (or retinaloximes after NH_2OH treatment) a considerably more polar aldehyde or its corresponding oximes. That these compounds originate from the chromophoric group of the visual pigment was shown by the occurrence of different stereoisomers in the extracts depending on the colour with which the intact visual pigment complex was previously illuminated. The absorbance maximum of the oxime found after blue illumination is the same as that of all-trans-retinal-oxime (356 nm). This spectral accordance means that previous spectral evidence for retinal as the chromophoric group of fly visual pigment is not conclusive. Flies raised on a carotenoid deficient diet enriched with the hydroxy-xanthophyll lutein possess a high visual pigment content. This points to a hydroxy-xanthophyll as a possible precursor of the chromophoric group.

Introduction

The visual pigment of muscoid flies is believed to be a rhodospin, *i.e.* to have retinal as the chromophoric group. This was first claimed by Wolken *et al.* [1] who found in acetone extracts of Musca heads a compound whose $SbCl_3$ product has an absorbance peak (665 nm) fitting to the $SbCl_3$ product of retinal. Bleaching of the visual pigment of *Calliphora* (digitonin extract, room temperature) results in a difference spectrum peaking at about 380 nm [2] as expected if retinal is liberated. Further the reaction of the visual pigment of *Drosophila* with hydroxylamine yields a compound with absorption peak near 360 nm [3] in accordance with the spectrum of retinaloxime. Besides this spectral evidence the results of Schwemer [2], who injected 11-*cis*-retinal into the eyes of carotenoid deprived *Calliphora* and found an increase of the visual pigment content, also points to retinal as the chromophoric group.

However, in this study evidence is presented that the chromophoric group is not retinal but rather a considerably more polar compound. Considering its unknown nature this compound is designated in the following as X-aldehyde and the hydroxylamine derivative as X-oxime.

Materials and Methods

Animals

White eyed *Calliphora* "chalky" were used preferentially. Flies were either raised on bovine liver in order to promote high visual pigment content or on lean horse skeletal muscle to get animals with low visual pigment content. In addition some flies were raised on lutein (preparation see below) or carotene (β carotene with 10 to 20% α carotene, Sigma) enriched diets. In both cases 1.8 mg of each compound was added to 80 g of horse meat together with about 100 fly eggs. The adults were fed with sugar and water only.

Reprint requests to Dr. K. Vogt.

0341-0382/83/0300-0329 $ 01.30/0

Preparation of retina extracts

In the standard procedure fly heads were frozen in liquid nitrogen and freeze dried for several hours. The retinae (together with Semper- and pigment cells) were separated from the optical ganglia and 20 to 100 were placed in a cooled centrifugal glass with 40 to 200 µl of the solvent. Solvents were methanol or acetone (with 0 to 3% water) or, for the preparation of oximes 0.2 M NH_2OH in methanol (pH 6.2). After 5 min in a cooled ultrasonic bath and centrifugation at $12{,}000 \times g$ for 15 min the supernatant was applied to the chromatographic plate. The preparation was performed in dim red light and whenever possible under nitrogen.

Variations yielding qualitatively the same results as the above method were: Addition of the antioxidant BHT to the solvents; grounding of fresh eye calottes with Na_2SO_4; Saponification of the retinae (10% methanolic KOH, 15 h at 4 °C) and, after neutralisation, extraction with petroleum ether. In order to demonstrate the different stereoisomers of the chromophore two methods were applied. 1. Batches of 50 retinae were prepared after lyophilization and

placed in a glass homogenizer with 50 µl phosphate-buffer (1/15 M, pH 6.8). After homogenizing each mixture was illuminated (Schott, KL 1500) with either red (OG 590) or blue (linefilter 460 nm) light for 45 min at 2 °C. 0.4 ml of 0.2 M NH_2OH in methanol (pH 6.2) was added in the dark. After freeze drying the acetone extracts were applied to the plate. 2. 100 fresh eye calottes were excised from the heads with a razor blade, treated and illuminated as above, and subsequently heated at 90 °C for 5 min to denature the opsin. After adding 0.4 ml of 0.2 M NH_2OH in phosphate buffer the same procedure as above was followed. Both methods yielded the same result (Fig. 1).

Thin layer chromatography

Silica gel 60 HPTLC plates with concentrating zone (10×10 cm, Merck) were used. Solvent systems were: 1. dichloromethane + ethyl acetate, 7 + 3; 2. diethylether + petroleum ether + acetone, 6 + 2.5 + 1.5; 3. petroleum ether + methylheptenon, 11 + 2. After development the plates were sprayed with $SbCl_3$ in chloroform or single bands were scraped off and redissolved (in ethanol or acetone) for repeated chromatography or spectroscopic measurements.

Chemicals

All-trans-retinal, retinol and β-carotene were obtained from Sigma. Retinal oximes were prepared according to Oesterhelt *et al.* [4] by mixing retinal solutions in ethanol with an equal volume of 2 M NH_2OH in 50% aqueous ethanol for 30 min and subsequently reextracting the oximes with petroleum ether. The different stereoisomers of retinal and its oximes were prepared by illuminating an *all-trans*-retinal solution for 15 min with a xenon lamp (75 W). The presence of all-*trans*, 9 *cis*, 11 *cis*, and 13 *cis* retinal oximes was ascertained by the accordance of the chromatograms (petroleum ether + methylheptenon, 11 + 2) with those of Oesterhelt *et al.* [4]. Vitamine A_2-aldehyde (3-dehydroretinal) was prepared by extraction of A_2-alcohol from saponified pike liver, chromatographical purification and oxidation with MnO_2. Lutein was prepared by repeated chromatography (PLC plates silica gel 60, Merck) of acetone extracts from lyophilized carrots or spinach, and zeaxanthin in the same way from maize grits. The solvents (with the exception of petroleum ether and methylheptenon) were of spectroscopic grade (Uvasol).

Spectroscopy

Measurements were performed with a Hitachi 100–80 spectrophotometer. Solutions of bands scraped from the chromatographic plates were measured in microcuvettes (10 to 200 µl, stainless steel or teflon with quartz windows). The measurements of the visual pigment content of eyes were performed according to Schwemer [2] with eye calottes placed on a 0.8 mm diaphragm in a wet chamber. The absorbance change at 580 nm resulting after saturating illumination with red (OG 590) and blue (line-filter 460 nm) light respectively was taken as a relative measure of the visual pigment content.

Electrophysiology

Intracellular recordings were performed using standard methods [5]. The spectral sensitivity of previously red adapted receptor cells Rl-6 of wild type *Calliphora* was measured by flashes (40 msec) with a pointsource (1,5 ° visual angle) on the optical axis. The sensitivity (criterion response 20 mV) at 580 nm – where selfscreening is negligible – was taken as a measure of visual pigment content.

Results

Bands related to the carotenoid metabolism were identified by their absence or faint appearance in the chromatograms of carotenoid deprived flies.

Two of them (L, Z, Fig. 1) were identified by their position, their spectra and by the ability to form methylether as the hydroxy-xanthophylls lutein (L, Fig. 1) and zeaxanthin (Z, Fig. 1) whereby zeaxanthin is abundant (Vogt and Kirschfeld, in prep.). A faint spot of a less polar carotenoid was sometimes seen, probably a monohydroxy compound, but β carotene was never detected. Two white fluorescent (blue color after $SbCl_3$ spraying) bands (Fig. 1, S_1, S_2) behind the zeaxanthin were observed which could be promising candidates for sensitizing pigment (sensitizing pigment see [6–8]).

In acetone extracts (without previous treatment with NH_2OH) a UV absorbing spot could be found in a position between the spot X_1 and the cholesterin (C) spot in Fig. 1. This spot is the presumptive chromophore (X-aldehyde) which reacts with NH_2OH to give the oximes (X_1, X_1', X_2, X_2') shown in Fig. 1. Retinal travels much further (lying slightly above R

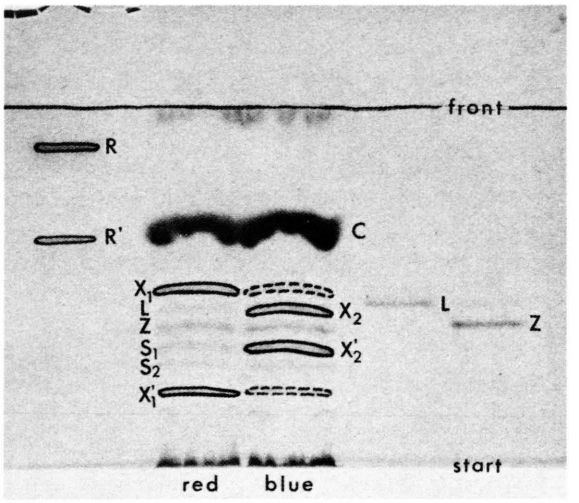

Fig. 1. Thin layer chromatogram (dichloromethane + ethyl acetate, 7+3) of extracts from NH_2OH treated retinae previously adapted with red (OG 590) or blue (460 nm) light. Retina extracts: C, cholesterine (colour after spraying with $SbCl_3$ first red then blue); L, lutein; Z, zeaxanthin; S_1, S_2 white fluorescing (long wavelength UV excitation) compounds which appear blue after spraying with $SbCl_3$; X_1, X_1', X-oximes after red adaptation; X_2, X_2', distinct and X_1, X_1', fainter X-oxime bands after blue adaptation. Like the retinaloximes the X-oximes exhibit a reddish fluorescence (long wavelength UV excitation) and a orange-yellow colour after $SbCl_3$ spraying. Reference substances: R, R' *all-trans*-retinal-oximes; L, lutein; Z, zeaxanthin.

in Fig. 1) showing that the X-aldehyde is considerably more polar.

For the following reasons in most cases the chromatography was not performed with this aldehyde directly but with its oximes: 1. especially in methanol extracts most of the chromophore aldehyde was bound to phosphatidylethanolamine (like retinylidene PE) in a protonated Schiff-base linkage (as indicated by the orange colour) and, in the solvent systems chosen, remained on the startline. 2. the oximes are more stable than the aldehyde; 3. the stereoisomeres of the oximes are easy to separate; 4. the oximes are easier to detect by their reddish fluorescence that the non fluorescing aldehyde.

Since it is known that oximes can exist in two stable isomeric forms, the *syn* and the *anti* form [9], the occurrence of pairs of spots (R, R' or X_1, X_1' or X_2, X_2'; Fig. 1) in the chromatograms is caused by the syn and anti forms of one and the same *cis*- or *trans*-compound (see [4]).

All observed stereoisomers of the X-oximes are considerably more polar than the oximes of the different stereoisomers of retinal (*all-trans*, 9 *cis*, 11 *cis*, 13 *cis*) or of vitamine A_2-aldehyde (stereoisomers unidentified). However the X-oximes exhibit the same reddish fluorescence and the same orange-yellow colour after spraying with $SbCl_3$ as do the retinaloximes. The absorbance spectra of both a X-oxime (X_2, Fig. 1) and *all-trans*-retinal oxime (R, Fig. 1) peak at 356 nm (Fig. 2).

The X-oximes' origin from the chromophoric group of the visual pigment can be shown by the definite occurrence of different stereoisomers (Fig. 1) when the intact visual pigment is illuminated with blue (460 nm) or red (OG 590) light respectively and subsequently denatured by heat or methanol. Since the free chromophore absorbs in the UV the isomerisation has to take place in the visual pigment complex which is known to be thermostable and to reach an equilibrium between a "rhodopsin" (490) and a "metarhodopsin" (570) for each adapting wavelength (rev. Hamdorf [10]). The equilibria

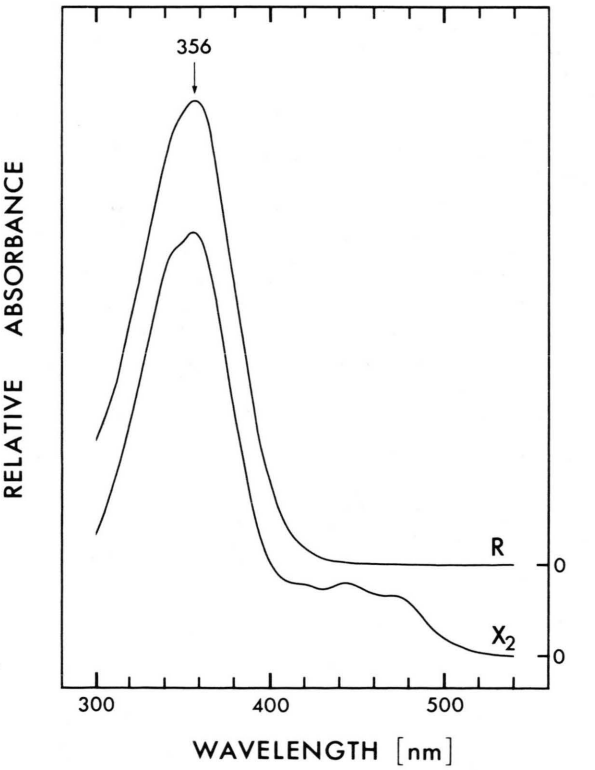

Fig. 2. Absorbance spectra of *all-trans*-retinaloxime (R, Fig. 1) and the X-oxime (X_2, Fig. 1) in ethanol. In the blue range the X-oxime spectrum is contaminated by traces of lutein which overlaps on the chromatogram.

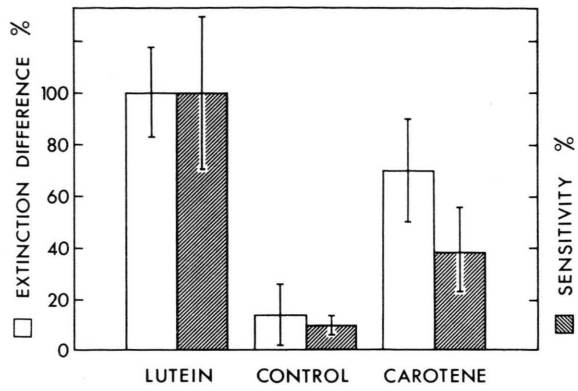

Fig. 3. Relative visual pigment content in flies raised on a carotenoid deficient diet (horse meat) enriched with lutein or carotene (β-carotene with 10% to 20% α-carotene); control: pure horse meat diet. Measuring methods: 1. Mean extinction difference at 580 nm in eyes of white eyed flies (n=8 flies in each case). 100% corresponds to an extinction difference of 0.056. 2. Mean sensitivity at 580 nm in photoreceptors (R1–6) of wild type flies (in each case average of 5 flies with 2 to 3 cells measured). 100% correspond to 5.3×10^{11} quanta/cm^2 · sec (20 mV criterion, 40 msec flash). Bars denote standard deviations.

expected for the adapting lights used are 100% "rhodopsin" (red) and about 80% "metarhodopsin" (blue). Accordingly the chromatography following red adaptation yields two X-oxime spots (X_1, X_1') – which means only a single *cis* or *trans* stereoisomer if the *syn* and *anti* form of the oximes is taken into account – however, following blue adaptation two distinct oxime spots (X_2, X_2') in new positions are seen together with the same two spots (X_1, X_1'), now much fainter. A decision as to which stereoisomer is created by the red and blue adaptation respectively is not possible from these results*.

The exclusive occurrence of hydroxy-xanthophylls and the complete absence of β-carotene respectively suggests the possibility that in contrast to the vertebrates a hydroxyxanthophyll could be a precursor of the chromophore of the visual pigment. Therefore flies were raised on carotenoid deficient diets (horse skeletal muscle) enriched with either lutein or carotene; control: pure horse meat. The relative visual pigment concentration in the eyes of the adults, tested by spectroscopical and electrophysiological methods was as follows: Lutein-diet: 100%; carotene-diet: 70% and 39%; control: 14% and 10% respectively (Fig. 3). A single extraction experiment with flies raised on carotene yielded the same X-oximes and C_{40}-carotenoids *i.e.* exclusively zeaxanthin and lutein without any trace of β-carotene.

Discussion

The gravest objection to the described result is the possibility of an artificial compound which could be formed from retinal during the extraction procedure or the chromatography. To test this possibility we prepared rod outer segment suspensions from fresh cattle eyes which were lyophilized, extracted with methanol / NH_2OH and run together with a methanol / NH_2OH extract from fly eyes. For the cattle eye extracts these control chromatograms yielded exclusively retinaloxime and retinol without any trace of the more polar X-oximes. A 5,6-epoxide as a possible artifact [12] can be excluded since the X-oximes absorption spectrum is not shifted the shorter wavelengths compared with the retinaloxime (Fig. 2). Also the identical results with a variety of methods and the ability to isolate different stereoisomeres are not in favour of an artifact. The spectral accordance of the X-oxime with retinaloxime also means that the spectral evidence for retinal, cited in the introduction, is not in contradiction to the result of this study.

In order to determine the chemical identity of the chromophore further investigations such as infrared spectroscopy and mass spectroscopy are called for. However chromophores with an additional oxygen, particularly 3-hydroxy-retinal, are possible candidates, this being suggested from the polarity of the compound and from the ability of the flies to synthesize the chromophore from a hydroxy-xanthophyll like lutein (3,3′ hydroxy-α-carotene). If this is true the visual pigment of flies should no longer be regarded as a rhodopsin and a name derived from the xanthophyll precursors could be "xanthopsin". No statement can be made as yet on the chromophoric group of the visual pigments of the different R7 and R8 cells since their chromophore content may be be-

* In principle an alternative explanation for the definite isomerisation could be an isomerisation on a retinochrome-like pigment which is known to exist in cephalopod eyes (ref. [11]) and is presumed in fly eyes [2]. But since a considerable contribution of a blue absorbing retinochrome [2] should result in more than one *cis-trans*-stereo isomer even in the case of red adaptation, this possibility is not very likely.

low the resolution of the method. The possible occurrence of the described chromophore in other insect orders has to be judged reservedly since *e.g.* Goldsmith [13] found retinal in bee heads and in the neuropter *Ascalaphus* there is good evidence – spectral as well as chromatographical – for retinal as the chromophoric group of the UV visual pigment [14].

Acknowledgement

I thank Ms. Gonnert and Mr. Ettig for technical assistance; Prof. K. Kirschfeld and Dr. R. Hardie for discussions and critical reading of the manuscript; Dr. Stransky for a discussion and reference substances.

[1] J. J. Wolken, J. M. Bowness, and I. J. Scheer, Biophys. Acta **43,** 531–537 (1960)
[2] J. Schwemer, Habilitationsschrift, Ruhr Universität Bochum 1979.
[3] S. E. Ostroy, J. Gen. Physiol. **72,** 717–732 (1978).
[4] D. Oesterhelt, M. Meentzen, and L. Schuhmann, Eur. J. Biochem. **40,** 453–463 (1973).
[5] R. C. Hardie, J. Comp. Physiol. **129,** 19–33 (1979).
[6] K. Kirschfeld, N. Franceschini, and B. Minke, Nature (Lond.) **269,** 386–390 (1977).
[7] K. Vogt and K. Kirschfeld, Biophys. Struct. Mech. in press (1983).
[8] K. Kirschfeld, R. Feiler, R. Hardie, K. Vogt, and N. Franceschini, Biophys. Struct. Mech. in press (1983).
[9] C. G. McCarty, in: (S. Patai, ed.), Chemistry of the Carbon-Nitrogen Double Bond, 383–392, Interscience Publishers, London 1970.
[10] K. Hamdorf, in: (H. Autrum, ed.), Handbook of Sensory Physiology VII/6A, 145–224, Springer, Berlin-Heidelberg-New York 1979.
[11] T. Hara and R. Hara, in: (H. Autrum, ed.), Handbook of Sensory Physiology VII/I, 720–746, Springer, Berlin-Heidelberg-New York 1972.
[12] R. Hubbard, P. K. Brown, and D. Bownds, in: (S. P. Colowick and N. O. Kaplan, eds.), Methods in Enzymology, 615–653 (1971).
[13] T. H. Goldsmith, Proc. Natl. Acad. Sci. USA **44,** 123–126 (1958).
[14] R. Paulsen and J. Schwemer, Biochim. Biophys. Acta **283,** 520–529 (1972).