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Photoreceptor-specific efficiencies of β-carotene, zeaxanthin and lutein for photopigment formation deduced from receptor mutant *Drosophila melanogaster*

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Summary. *Drosophila* rearing media had only β-carotene, zeaxanthin or lutein as precursors for photopigment chromophores. Zeaxanthin and lutein are potentially optimum sources of the 3-hydroxylated retinoids of visual and accessory photopigments. Mutants made the electroretinogram in white (*w*) eyes selective for compound eye photoreceptors R1-6, R7 and R8: R1-6 domiantes *w*'s electroretinogram; R7/8 generates *w;ora*'s (*ora*=outer *r*habdomeres *a*bsent); R8 generates *w sev;ora*'s (*sev*=*sev*enless). Microspectrophotometry revealed R1-6's visual pigment. In *w*, all 3 carotenoids yielded monotonic dose-responses for sensitivity (Fig. 4) or visual pigment (Fig. 7). An ultraviolet sensitivity peak from R1-6's sensitizing pigment was present at high but not low doses (Fig. 1). In *w;ora*, all 3 carotenoids gave similar spectra dominated by R7's high ultraviolet sensitivity (Fig. 2). For *w sev;ora*, all spectra were the shape expected for R8, peaking around 510 nm (Fig. 3). The sensitivity dose-response was at its ceiling except for low doses in *w;ora* (Fig. 5) and zero supplementation in *w sev;ora* (Fig. 6). Hence, without R1-6, most of our dose range mediated maximal visual pigment formation. In *Drosophila*, β-carotene, zeaxanthin and lutein mediate the formation of all major photopigments in R1-6, R7 and R8.

Key words: Carotenoids – Xanthophylls – Rhodopsia – Visual pigment – Retinoids

Introduction

A major breakthrough in understanding visual pigments (rhodopsins) occurred with the discovery that some insects use 3-hydroxyretinal as the chromophore (Vogt 1984). The previous consensus was that only retinal (the aldehyde of vitamin A) or 3-dehydroretinal (from vitamin A2) served as the prosthetic group of photopigments. Since this initial discovery, 3-hydroxyretinal has been shown to be present in numerous invertebrate pigments, even as far ranging as the brain photoreceptor mediating circadian rhythms in the moth (Hasegawa and Shimizu 1988).

High pressure liquid chromatography (HPLC) has been used to show that 3-hydroxyretinal is the predominant retinoid extratable from the heads of *Drosophila melanogaster* and other dipteran fly genera (Goldsmith et al. 1986; Seki et al. 1986; Tanimura et al. 1986; Giovanucci and Stephenson 1987). This implies that the chromophore of fly rhodopsin is 3-hydroxyretinal. In the fly, there are carotenoids and retinoids in addition to the visual pigments. We will use the term visual pigment to mean the rhodopsin-metarhodopsin (sometimes called xanthopsin-metaxanthopsin) systems; we will use the more inclusive term photopigment to include, in addition to visual pigments, the vitamin A based accessory pigments which serve screening and sensitizing functions (but not the eye color screening pigments). An issue arises because there is a multiplicity of compound eye photopigments. For instance, there are 3 general classes of photoreceptor type in the *Drosophila* compound eye, namely R1-6, R7 and R8, each with different rhodopsins (Harris et al. 1976). It is not known whether R7's and R8's photopigments are based on 3-hydroxyretinoids (Kirschfeld et al. 1988). Further, since these early discoveries of photopigment multiplicity, it has been shown that there are several types of R7 and R8 with differing visual pigments (Hardie 1985) in *Musca* and *Calliphora*. Further two R7 opsins, RH3 and RH4, have been cloned in *Drosophila* (Montell et al. 1987; Zuker et al. 1987), though the correspondence between *Drosophila* and *Calliphora* and *Musca* is unclear (see Discussion). HPLC of head extracts would not be receptor specific or photopigment specific, but would be predominated by R1-6. An accessory pigment which sensitizes R1-6 (Kirschfeld

Abbreviations: *ERG* electroretinogram; *MSP* microspectrophotometry; *HPLC* high pressure liquid chromatography; *n.a.* numerical aperture; *w, sev, ora Drosophila* mutants; *y, p, r,* marg types of R7 and R8

et al. 1977) was shown to be a carotenoid (Stark et al. 1977a, b). Of particular interest is the mounting evidence that this pigment which sensitizes the R1-6 visual pigment to ultraviolet light is 3-hydroxyretinol (Kirschfeld 1986). UV sensitizing pigments are also present in R7r (r = red fluorescence), R7y (y = yellow), R8r and R8y in *Calliphora* and *Musca* (see also Discussion) (Hardie 1985); furthermore, zeaxanthin and lutein may serve as stable screening pigments in one class of R7 cell, namely R7y (y = yellow) (Hardie 1985).

Thus, more selective measurement techniques are needed to begin to establish whether R7 and R8 visual pigment and accessory pigments are also based on 3-hydroxyretinoids. The purpose of this study was to begin to unravel the receptor specificity of *Drosophila* compound eye with respect to the resident carotenoids and retinoids. The electroretinogram, in combination with receptor mutants, becomes a powerful technique for separating R1-6, R7 and R8 sensitivities (Harris et al. 1976). We further used microspectrophotometry (MSP) to make correlated measurements of the R1-6 visual pigment levels. A short report of these findings has been published (Schilly et al. 1988).

Materials and methods

Animals. Stocks of white [*w*] mutant *Drosophila melanogaster* were used because visual receptor function could be deduced from the ERG and by MSP without the confounding spectral effects of eye color pigments. The *w* (otherwise wild type) stock was used since it had all compound eye receptor types (R1-6, R7 and R8) intact; measurements of compound eye function are dominated by R1-6 because it is the largest and most numerous receptor. The *w;ora* (white eyed outer rhabdomeres absent) stock was used to uncover the ERG responsivity of R7 and R8 since *ora* eliminates visual pigment and reduces the visual pigment containing organelles, the rhabdomeres, in R1-6 (the outer rhabdomeres) (e.g. Stark and Sapp 1987). The *w sev;ora* stock further eliminates R7 via the sevenless mutation, rendering an ERG from R8 alone. The *ora* and *sev* mutants were first characterized by Harris et al. (1976), and they have been used in numerous studies since. Flies were reared on a day/night cycle of fluorescent light of standard room lighting intensity.

Dietary manipulations. Flies were reared on Sang's medium (Doane 1967; Stark 1977a) prepared by Nutritional Biochemicals to which various doses of carotenoids were added. The diet was supplemented with *Drosophila* mold inhibitor (5 mg/ml, Carolina Biological) as well as with antibiotics penicillin (100 units/ml) and streptomycin (0.229 g/ml) to avert potential carotenoid metabolism by microorganisms. Adult flies were permitted to lay eggs on the medium. Then adults were cleared as well as any bodies of dead flies to avoid spurious carotenoid supplementation through cannibalism. Thus, flies were reared from egg to adult for one generation on the defined medium.

Carotenoid supplementation. Three carotenoids were used: (1) β-carotene (Nutritional Biochemicals), (2) zeaxanthin, and (3) lutein. The last two hydroxy carotenoids were isolated from corn and spinach leaves, respectively, by the following procedures. Warm methanol extracts were treated with KOH to saponify the leaf chlorophylls and corn oils. The carotenoids were separated by solvent partition using diethyl ether (Strain 1983). Purification and initial identification of the individual components were accomplished by thin layer chromatography following the methods of Hager and Meyer-Bertenrath (1966). For large scale work, these methods were adapted to liquid column chromatography. Identification of zeaxanthin and lutein was confirmed by mass spectroscopy, HPLC, UV-visible spectrometry and chemical derivatization. In all cases, agreement with literature data was obtained (Britton and Goodwin 1971; Strain 1938; Braumann and Grimme 1981; Heller and Milne 1978). A sample of zeaxanthin, a gift from Hoffmann LaRoche, was also used. The carotenoids were dispersed in 100% ethanol, and decreasing doses were prepared by successive dilutions. The medium (15 ml), cooled almost to room temperature at which it gels, was added to the ethanol/carotenoid (1 ml), and the mixture was thoroughly stirred before it solidified. Doses from -0.35 down to -5.25 $\log_{10}$ (mg/ml) were selected because these doses covered the dynamic range for dose responsivity for R1-6 visual receptor function (Stark et al. 1977) and R1-6 visual pigment levels (Stark and Johnson 1980).

Optics for electrophysiology. Electroretinograms were obtained according to this laboratory's standard techniques (e.g. Harris et al. 1976; Stark 1977a, b; Stark et al. 1977; Stark and Johnson 1980; Stark et al. 1985). The light source was a 150 W xenon arc (Hanovia 901C) which provided adequate light levels in the UV and visible portions of the spectrum. A Bausch and Lomb 500 mm monochromator was used to create 11 monochromatic stimuli from 350 to 600 nm. A UV blocking filter (Wratten #3) was used at 600 nm to block the second harmonic spectrum. Furthermore, a UV transmitting visible blocking filter (Corning) was added at 350 and 370 nm as an additional refinement, an innovation beyond this laboratory's previous publications. This visible blocker was important because the white light leakage of the monochromator becomes significant in the UV since a low amount of UV light is generated and transmitted. Inconnel on glass neutral density filters, calibrated on a spectrophotometer, were used to vary the intensity. Lenses and a 10× achromat microscope objective focussed the image of an iris onto the eye for ERG recording and the photodiode for calibrations.

Electroretinography. ERG's were recorded with a subcorneally inserted glass micropipette filled with saturated NaCl. The pipettes were pulled from inner filament glass with a Narashige puller. The initial resistance of about 10 MΩ decreased to about 2, probably because electrodes broke slightly upon penetration of the cornea. Signals were amplified with a Getting (Model 5) amplifier and fed simultaneously into a Narco Physiograph (DMP 4B) and a Tektronic ocilloscope (5100 series). After carefully locating the fly eye at the focal plane of the light stimulus using 570 nm light, and, after a brief dark adaptation, responses to near threshold spectral stimuli repeated every 10 s were obtained. For *w*, whose ERG (after 570 nm adaptation then dark adaptation) is dominated by R1-6, a 3 mV criterion was sought: either a 3 mV response was obtained or it was interpolated by flanking stimuli differing in intensity by about 0.3 log units. For *w;ora* and *w sev;ora* (R7/8 and R8, respectively), the criterion was only 0.5 mV because of the lower responsivity. Experiments were counterbalanced in that about half of the preparations (each mutant at each dose of each carotenoid) were run from 600 to 350 nm while the other half were run in reverse.

Data analysis. Action spectra were calculated as the intensity required to obtain a criterion response, and interpolation was facilitated by a Basic program written for a TRS-80 pocket computer. Spectral sensitivities were averaged across preparations by first normalizing each preparation's entire curve to the mean sensitivity pooled across preparations. These analyses were facilitated with a spreadsheat (Visicalc) on a TRS-80 Model III computer. Dose-response functions for ERG based sensitivity were made by determining the levels of the spectral sensitivity curves (see Results for further details).

Microspectrophotometry. We used our time tested methods of MSP (e.g. Stark and Johnson 1980; Stark et al. 1985; Stark et al. 1988). We will summarize only the essentials here. A Leitz Dialux incident light fluorescence microscope with an MPV (1.1) photometer system, a Pacific photomultiplier system and Hamamatsu photomultiplier tube (R928) were built into a microspectrophotometer. The deep pseudopupil is an 80 μm diameter virtual image of the organelles which contain the visual pigment, the rhabdomeres. It is magnified and pooled from many ommatidia, and is situated about 150 μm deep beneath the cornea, hence the term deep pseudopupil. The number of ommatidia pooled depends on the numerical aperture (n.a.) of the microscope objective (in our case n.a. = 0.25, and n = about 25 ommatidia). We determined the relative concentrations of visual pigment from R1-6 which dominates this image based on the light induced interconversions of R1-6's 480 nm absorbing rhodopsin and its 580 nm absorbing metarhodopsin. Light at 579 nm (near metarhodopsin's peak) was shone antidromically through the head of a live fly fixed to a microscope slide to form the deep pseudopupil. Adaptation sufficient to establish the photo steady state at 450 nm was applied through the fluorescence epiluminator to maximize metarhodopsin while 620 nm was used to nearly maximize rhodopsin. After these actinic stimulations, transmission was measured through the deep pseudopupil at 579 nm, and the log of the ratio (after red/after blue) was calculated as the absorbance difference. It was this absorbance difference that reflected the relative amount of visual pigment.

Optical calibrations. The validity of our ERG sensitivity determinations depends greatly on the accuracy of our optical calibrations. We also calibrated the 620 and 450 nm beams in the MSP so that we could set these beams to maximize rhodopsin and metarhodopsin respectively without causing damage when applied for 10 s (Stark et al. 1985). Over the years, we have developed and modified procedures to get accurate readings of quantum flux (Stark et al. 1985), and only a summary will be presented here. We used a photodiode (EG&G HUV 4000B) which had been cross calibrated to numerous other calibrated devices. The focussed light spot in the ERG apparatus or actinic stimuli in the MSP setup was passed through a calibrated pinhole aperture in front of the diode for accurate area determinations. Calibrated neutral density filters were used to keep the diode's stimulation in the linear range. The readout voltage was calculated to the log quanta/cm^{2}s of the full balst beam (by factoring out whatever neutral density filters had to be used) with the help of a TRS-80 pocket computer and a Basic program.

Results

Effects of β-carotene on sensitivity and visual pigment in w. The dark adapted spectral sensitivity of *w* should reflect R1-6's properties, and these data can be used as a basis for comparisons with other carotenoids and/or receptors. Figure 1a shows the spectral sensitivities of R1-6 in the compound eye at different doses of β-carotene as estimated from the dark adapted ERG of *w Drosophila*. In general, as doses decrease, the sensitivity decreases, especially in the UV. The high and low β-carotene sensitivities are as expected (Stark et al. 1976, 1977) with the added advantage of the present data being that it is collected at intermediate doses. Although the relative height of the UV peak has varied from study to study (cf. Stark 1975), the precautions in filtering the UV stimuli (cf. Methods) increase the reliability of these data.

A dose-response function for sensitivity level was constructed (Fig. 4a). To do this, we determined the level of the spectral sensitivity curve by averaging absolute sensitivity values at the wavelengths 420–600 nm. These visible wavelengths, with the UV wavelengths excluded, would reflect the rhodopsin level alone. This is because including the UV wavelengths would have confounded the effects of the UV sensitizing pigment with those of the visual pigment. The dose-response closely replicates the data collected at 470 nm alone (Stark et al. 1977) in terms of dose range which is effective in altering sensitivity. It is monotonic at the doses chosen. Further, it is located entirely at the dynamic range for dose-responsivity (i.e. between the floor and ceiling effects of the theoretical S-shaped curve). It spans 1.5 log units of sensitivity for a manipulation of doses of about 3.5 log units. A dose-response function for visual pigment level (Fig. 7a) shows that visual pigment, as measured directly from absorbance difference using MSP, has about the same dynamic range. Stark and Johnson (1980) did a similar experiment and concluded that ...the log of the ERG sensitivity correlates quite well linearly with the log of the absorbance difference (De)...obtained over a two log unit range...

Effects of zeaxanthin and lutein on R1-6. Figure 1b shows the spectral sensitivities at different doses of zeaxanthin; Fig. 4b gives the derived dose-response function for sensitivity; and Fig. 7b gives the dose-response for visual pigment (absorbance difference). The corresponding data for lutein are as follows: spectral sensitivity (Fig. 1c); ERG sensitivity dose-response (Fig. 4c); visual pigment dose-response (Fig. 7c). In general, all of the results are comparable with the data from β-carotene. For zeaxanthin, the dynamic range for visual pigment (Fig. 7b) is at a lower dose, implying that zeaxanthin might be more effective than the other carotenoids in visual pigment biosynthesis; furthermore, lutein was slightly less effective in mediating overall sensitivity (Fig. 4c) and UV sensitivity at higher doses (Fig. 1c) than the other carotenoids. However, these effects were minor, and in general the differences between the different carotenoids in mediating R1-6 function were not significant.

Sensitivities for R7/8 from w;ora. The data presented are as follows: spectral sensitivities (Fig. 2a–c) for β-carotene, zeaxanthin and lutein, respectively; dose-response functions for overall sensitivity (350–600 nm) (Fig. 5a–c) for the same three carotenoids respectively. [For *w;ora* as well as for *w sev;ora*, Figs. 3 and 6, we felt that it was legitimate to use the whole spectral sensitivity function, 350–600 nm, to estimate sensitivity since carotenoid deprivation had not been found to change the spectral sensitivity shape for R7 or R8 (Stark 1977b).] At first, we used the same dose range for *w;ora* flies as we did for *w* flies and used the ERG to assay spectral sensitivities and sensitivity levels. However, the clustering of spectral sensitivity functions (Fig. 2a–c), as well as the flatness of the dose-response functions

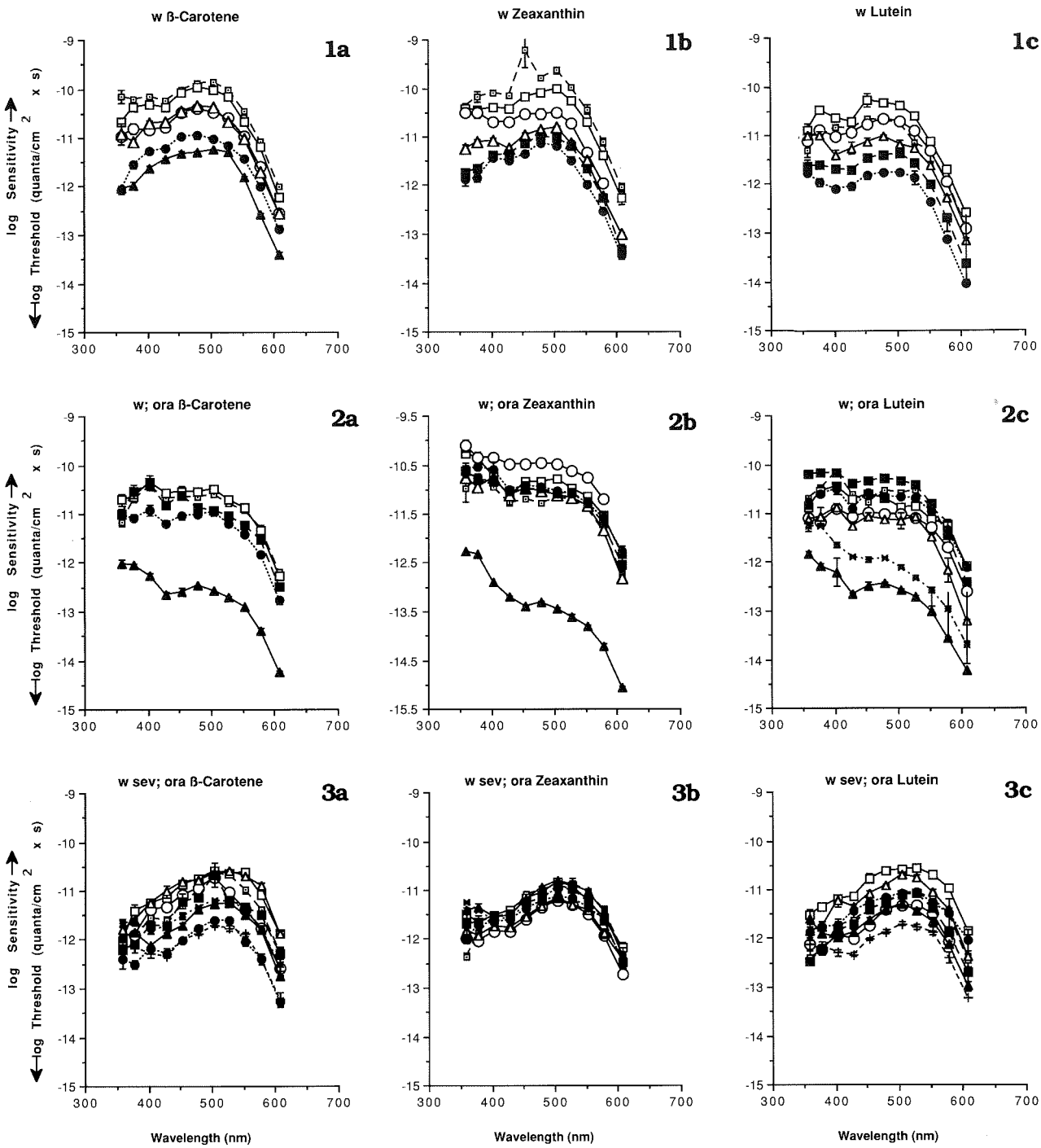


Fig. 1a–c. Spectral sensitivity of white-eyed (*w*) *Drosophila's* R1-6 receptors recorded from dark adapted animals. Ordinate log sensitivity (upward) or inverse log threshold (downward, in units of log quanta/cm^2 × s) as a function of wavelength in nm. Each graph is based on data from animals raised on different doses of carotenoid supplementation, and the key shows the different data points used for Figs. 1–3. (Note that zero dose is used only in Fig. 3.) Curves averaged (see Methods) with SEM shown as error bar when larger than the data point. Numbers of animals averaged for each curve presented in the corresponding data point in Fig. 4. **a** Supplementation with β-carotene. **b** Supplementation with zeaxanthin. **c** Supplementation with lutein

Fig. 2a–c. Spectral sensitivity for R7 plus R8 determined by using dark adapted *w;ora* (white-eyed outer rhabdomeres absent) flies. All analysis and **a–c** as in Fig. 1. The *n*'s see Fig. 5

Fig. 3a–c. Spectral sensitivity for R8 alone from dark adapted *w sev;ora* (white eyed, sevenless, outer rhabdomeres absent) flies. Analyses and **a–c** as in Figs. 1 and 2. The *n*'s see Fig. 6

(Fig. 5a–c) at the same higher doses used for the R1-6 study, prompted us to conclude that we might have a ceiling effect; hence lower doses were tried. It was clear that with extremely low doses, sensitivity could be lowered (Figs. 2 and 5). The spectral sensitivities are all the shape expected for R7 plus R8 regardless of carotenoid or dose (Fig. 3).

Sensitivities from R8 from w sev;ora. Figure 3a–c gives spectral sensitivities for β-carotene, zeaxanthin and lutein respectively. Figure 6a–c gives the respective dose-response functions for sensitivity (pooled from 350–600 nm). As was the case with *w;ora*, there was a clustering of spectral sensitivity curves (Fig. 3) and a likely ceiling effect in the dose-response curves. In this case, a modest lowering of sensitivity could only be achieved by zero dose (the best carotenoid deprivation we could achieve in one generation of deprivation). All spectral sensitivity curves are the shape expected for R8 regardless of carotenoid or dose.

Discussion

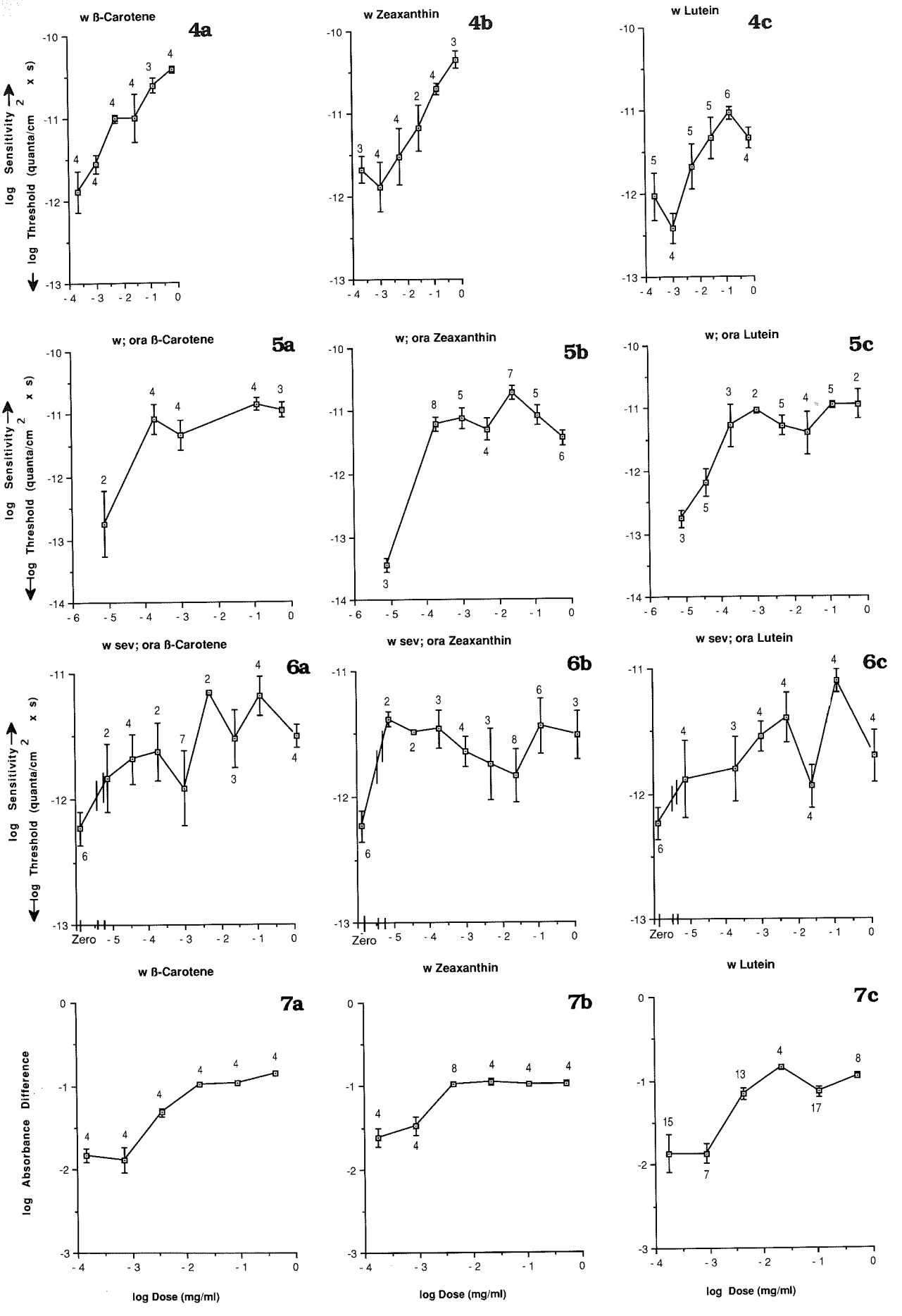
That β-carotene could serve as a precursor for photopigments in the fly had been known for a long time. For instance, Goldsmith and Fernandez (1966) had used β-carotene in carotenoid deprivation and supplementation experiments with successful results. The usefulness of β-carotene for visual pigment formation in the moth *Manduca sexta* is also time tested (Carlson et al. 1967; Bennett and White 1989). Later, Stark et al. (1977) showed that carotenoids were not only important in visual pigment formation, but also in the deployment of the UV sensitizing pigment of R1-6, and, in so doing, implicated β-carotene as its precursor. With the initial discovery of the importance of 3-hydroxyretinoids (Vogt 1984) came certain implications about the metabolism of carotenoid precursors in the development and deployment of visual pigments. An interesting note concerning this is that yellow cornmeal, whose vitamin A precursor is largely zeaxanthin, has often been a favored component of *Drosophila* media and that recipes using white cornmeal caused the flies to develop a carotenoid deprived phenotype (Stark 1977a).

It is useful to consider the structures of the carotenoids we used (Fig. 8). Zeaxanthin is the xanthophyll which is essentially a dimer of 3-hydroxyretinol, and it should therefore be ideal as a precursor to the visual pigments (as well as to other photopigments, see Introduction). Lutein is another xanthophyll which is derived from vegetables and is a major component in spinach leaves for instance. Lutein has one 3-hydroxy β ring and one 3-hydroxy ε ring. There are several generalizations concerning the metabolism of these xanthophylls by animal systems (Goodwin 1986) which are important in our work. First, the reduction of the 3-hydroxyl groups is only clearly established in certain fish, suggesting that these xanthophylls would not be efficient supplements should the photopigments be based on retinal or retinol. Secondly, α-carotene, which possesses the same α and ε rings as lutein (without the 3,3′-hydroxyl groups) is only half as active biologically as a precursor of vitamin A (Bauernfind 1981), suggesting that only half of the lutein molecule could be a source of known chromophores in fly visual pigments.

If the first assumption is true, then it is suggested that all the photopigments, visual and accessory pigments, of all receptors, R1-6, R7 and R8, as pooled in the ERG are 3-hydroxylated. This is because the zeaxanthin and lutein gave the same spectra as β-carotene even though vitamin A1 could not, according to the first assumption, be biosynthesized. Further tests of this assumption in *Drosophila* would certainly be warranted. The second generalization suggests that lutein should be half as efficient as zeaxanthin as a dietary precursor of photopigments. While some of our data suggest that zeaxanthin works better than lutein (compare Figs. 4b and c, 6b and c and 7b and c), the effect is small and inconsistent. Thus, we are forced to conclude that the methods used are not sensitive enough to firmly establish this conclusion. We find no consistent difference in β-carotene and zeaxanthin, suggesting that the fly has no difficulty in performing the 3-hydroxylation reaction when β-carotene is in the diet.

In addition to the importance of 3-hydroxycarotenoids in fly photopigments, there have been other recent important discoveries about the metabolism of the chromophores. One of the most important is that the fly uses light rather than an isomerase in the *trans* to *cis* isomeration needed for the formation of visual pigment (Schwemer 1983, 1984). Thus, light at blue wavelengths in particular is needed for flies to develop functional rhodopsin if they are supplied with all *trans* retinoids. Isono et al. (1988) have shown that β-carotene can form visual pigment in *Drosophila* reared in the dark, suggesting that an enzymatic isomerization could be present when R1-6 rhodopsin is formed from carotenoids rather than from retinoids. However, it is alternatively possible that the carotenoids they used were not fully *trans*. A second important line of research concerning the metabolism of vitamin A in the fly eye is the discovery that the *ninaD* mutants of *Drosophila* affect vitamin A metabolism (Giovanucci and Stephenson 1986, 1987, 1988). It is likely that *Drosophila* mutants will help to dissect the critical steps in carotenoid metabolism in the visual system.

Photopigment multiplicity in the fly compound eye has served as a source of continued interest since the initial discovery, based on the ERG (Harris et al. 1976), that R1-6, R7 and R8 had different visual pigments, and that R7 was a UV receptor. Our present understanding, based largely on single cell approaches in *Calliphora* and *Musca*, has recently been summarized (Hardie 1985). There are several classes of R7 receptor: R7y which has yellow fluorescence, a 430 nm rhodopsin, a UV sensitizing pigment and lutein and zeaxanthin screening pigments, R7p with pale fluorescence, a 335 nm rhodopsin and no accessory photopigments, R7r with red fluorescence and photopigments like R1-6's and R7 marg which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w Zeaxanthin
4b
w Lutein
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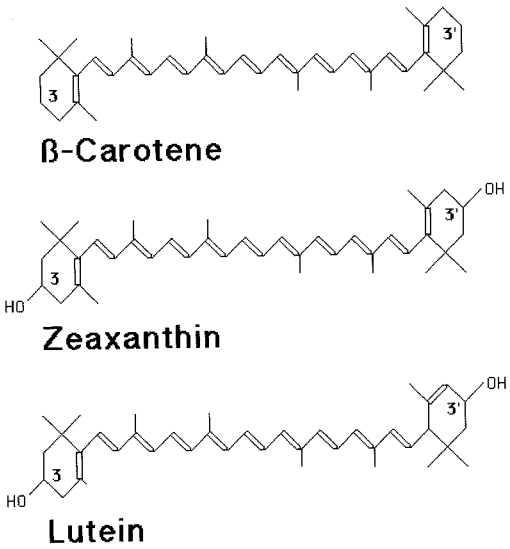

Fig. 8. Chemical structures of β-carotene, zeaxanthin and lutein

is on the margin of the eye but is otherwise much like R7p. In *Drosophila*, 2 R7 rhodopsins have been cloned and sequenced (Montell et al. 1987; Zuker et al. 1987). The two cloned *Drosophila* rhodopsins, RH3 and RH4, are both UV rhodopsins (Feiler, Kirschfeld and Zuker, personal communication). Thus, the situations for *Drosophila, Calliphora* and *Musca* may be different, and the mass ERG may be actually quite good for determining R7 function in *Drosophila*. R7 is a UV receptor with 2 different UV rhodopsins in *Drosophila*, while, curiously, in *Calliphora* and *Musca*, R7y and R7p arrive at that UV sensitivity by completely different mechanisms (Kirschfeld et al. 1988). For R8, there is also multiplicity (Hardie 1985): R8r has a 495 nm rhodopsin and a UV sensitizing photopigment; R8p has only a 460 nm rhodopsin; and R8y has a 520 nm rhodopsin and a UV sensitizing pigment. It should also be noted that screening from the different R7's distal to R8 and in tandem can affect R8. With respect to this information, it should be noted that the ERG can only be used to infer sensitivities of R7 and R8 pooled from all classes of R7 and R8, respectively. Further, in the white eye, screening functions such as that of R7 upon R8 are minimized since much of the light is able to enter the receptors obliquely.

It took extreme deprivation to lower sensitivity in flies in which only a small fraction of the compound eye receptors competed for the dietary supplement (Fig. 5, *w;ora*, R7 and R8, also Fig. 6, *w sev;ora*, R8). When just a few cells compete for a limited carotenoid supply, the small amount from low doses, the egg, dietary components or microorganisms may suffice. This finding is not, with hindsight, surprising, but it has interesting implications. For example, Zimmerman and Goldsmith (1971) showed that vitamin A deprivation did not affect the brain's circadian receptor but it did lower the compound eye's sensitivity. On this basis, it was concluded that the circadian receptor was not a rhodopsin. However, it is possible that the circadian receptor, if it has a small volume and developed before the compound eyes or had a preferential affinity for the limited carotenoid supply, could appear normal even if its chromophore were carotenoid based.

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Fig. 4a–c. Dose-response functions for ERG sensitivities of R1-6 as a function of level of carotenoid supplementation. Each data point is in log sensitivity as in Fig. 1 and is averaged from the level of the spectral sensitivity function of *w* in Fig. 1 which corresponds to that dose. For *w*, only spectral sensitivity data from 420–600 nm were averaged since it was considered that the spectral sensitivity at visible wavelengths was determined largely by the level of R1-6 rhodopsin while sensitivity at UV wavelengths was confounded by the sensitizing pigment. Numbers next to the data points show numbers of animals averaged for that data point as well as for the corresponding entire spectral sensitivity curve in Fig. 1. Error bars are SE's. **a** β-carotene; **b** zeaxanthin; **c** lutein

Fig. 5a–c. Sensitivity dose-response functions for R7 plus R8 from *w;ora* corresponding to Fig. 2. All analyses and carotenoids **a–c** as in Fig. 4 except that the level of the entire spectral sensitivity function (from 350–600 nm) was used to calculate the log sensitivity

Fig. 6a–c. Sensitivity dose-response functions for R8 from *wsev;ora* corresponding to spectral sensitivities of Fig. 3. All analyses, *n*'s and carotenoids **a–c** as in Fig. 5. Note that since zero carotenoid supplementation cannot be quantified on the log dose axis, it is set apart by broken axes and curves

Fig. 7a–c. Dose-response functions for R1-6 visual pigment level based on microspectrophotometry of the deep pseudopupil of *w*. Ordinate log absorbance difference, data are averaged from the *n* drawn next to each data point with SE's shown when larger than data points. **a** β-carotene; **b** zeaxanthin; **c** lutein

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