

# Microspectrophotometry of Arthropod Visual Screening Pigments

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**ABSTRACT** Absorption spectra of visual screening pigments obtained in vitro with a microspectrophotometer using frozen sections are given for the insects *Musca domestica*, *Phormia regina*, *Libellula luctuosa*, *Apis mellifera* (worker honeybee only), *Drosophila melanogaster* (wild type only) and the arachnids *Lycosa baltimoriana* and *Lycosa miami*. The spectral range covered is 260–700 nm for *Lycosa* and *Drosophila* and 310–700 nm for the remainder of the arthropods. A complete description of the instrumentation is given. For the flies, *Phormia* and *Musca*, light absorption by the yellow and red pigments is high from 310 to about 610 nm. This implies that for these insects there should be no wavelength shift in electroretinogram (ERG) results due to light leakage among neighboring ommatidia for this wavelength range. The same comment applies to *Calliphora erythrocephala*, which is known to have similar screening pigments. For some of the insects studied a close correspondence is noted between screening pigment absorption spectra and spectral sensitivity curves for individual photoreceptors, available in the literature. In some cases the screening pigment absorption spectra can be related to chemical extraction results, with the general observation that some of the in vitro absorption peaks are shifted to the red. The *Lycosa*, *Apis*, and *Libellula* dark red pigments absorb strongly over a wide spectral range and therefore prevent chemical identification.

## INTRODUCTION

Most discussions on insect color vision contain some references to the screening pigments present in many insect eyes. These pigments are located between the individual ommatidia and are responsible for the observed color of the eye for many types of flies and related insects. They are important for color vision studies because they can prevent light from spreading internally from one ommatidium to another. This was first demonstrated for the blowfly *Calliphora erythrocephala* (Autrum, 1955) by spectral sensitivity studies in the visible range on a white apricot mutant lacking some of the screening pigments as

compared to the wild type. Further studies (Langer and Hoffman, 1966) extended the investigations on *Calliphora* to a chalky white mutant lacking all screening pigments and covered the near ultraviolet wavelength regions. The results clearly indicated that for this insect it is possible to get an increase in the electroretinogram (ERG) response as the result of the spreading of light throughout the retina. This effect has also been recently demonstrated for the eyes of the drone honeybee and the locust *Schistocerca gregaria* (very little spreading of light), as compared to the crayfish which shows appreciable spreading (Shaw, 1969).

The question arises, however, whether light of all wavelengths is blocked by the screening pigments (Burkhardt, 1964). This question was first approached some years ago by extracting pigments from insect eyes and plotting their absorption spectra. More recent work (Langer, 1967) on *Calliphora* utilized direct absorption measurements made with a microspectrophotometer and demonstrated that the extraction techniques may alter the naturally occurring absorption spectra of the pigments.

In the present article absorption spectra of selected arthropod screening pigments obtained in vitro with a microspectrophotometer are presented. No staining or fixing of the eye sections is employed, and the data are believed by the writers to represent the naturally occurring absorption spectra of these pigments.

#### METHODS

*Instrumentation* The microspectrophotometer used for these studies is manually operated and of relatively simple design. A block diagram of the instrument is given in Fig. 1. The light source is a 150 w xenon arc with a DC power supply. The Bausch & Lomb 250 mm monochromator (Bausch & Lomb, Inc., Rochester, N.Y.) is equipped with a long focus quartz exit lens in order to image the exit slit at the microscope mirror. The light beam is chopped at approximately 50 cps in order to provide an ac

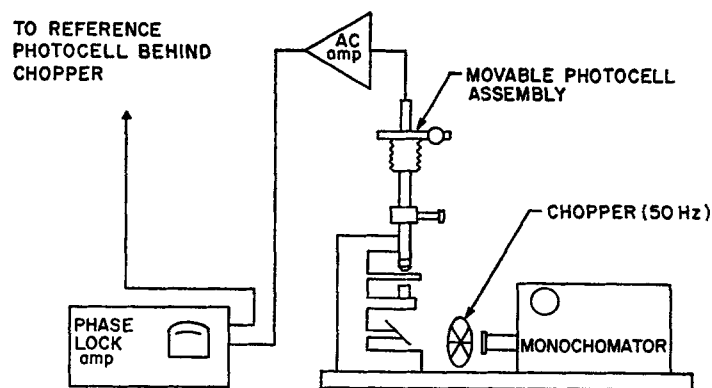


FIGURE 1. Block diagram of the microspectrophotometer. See text for discussion.

signal for the photodetector. The microscope is an American Optical Corp. (Southbridge, Mass.) Microstar Series 2 fitted with Zeiss Ultrafluar optics, including the UV condenser. Both microscope and monochromator are mounted on an optical bench to facilitate alignment.

Situated immediately above the microscope eyepiece is a telescope assembly with two small mirrors, one for viewing the specimen image as it appears on a reflecting screen 10 inches above the eyepiece. A small hole in the reflecting screen allows light to pass to a silicon photocell mounted along with the screen on a movable assembly, as shown in Fig. 1. The photodetector is a commercially available silicon junction device made by United Detector Technology, Santa Monica, Calif., Type UV-PIN-5. The spectral response ranges from 200 to 1100 nm, the sensitive area is 3 mm<sup>2</sup>, and the response time is of the order of microseconds. According to data supplied by the manufacturer the sensitivity is 0.22  $\mu\text{a}/\mu\text{w}$  at 4000 Å and the noise equivalent power (NEP) for 1 cycle bandwidth is  $5 \times 10^{-13}$  w/root Hz when operated in a conventional reverse bias mode. By comparison an RCA Type 1P21 photomultiplier tube has an NEP of  $5 \times 10^{-16}$  w/root Hz at the same wavelength. Operation in a conventional reverse bias mode was found to be too noisy at low wavelengths. However, operation in a photovoltaic mode provided very good signal-to-noise ratios (e.g. 4:1 at 240 nm, overall response). The two leads of the photocell are therefore connected directly across a 2 M $\Omega$  load resistor, with no bias battery in the circuit. This type of operation greatly reduces the dark current noise and negates the above-quoted NEP, which is for reversed bias circuitry. Because of this and because only signal ratios are utilized in the operation of the instrument, no attempt was made to measure the actual power level at the detector.

The 2 M $\Omega$  photocell load resistor serves as the AC signal source for a Tektronix Type 122 amplifier (Tektronix Inc., Beaverton, Ore.) operated in a differential mode with a 20 M $\Omega$  input impedance, a bandpass setting of 50 Hz, and a gain of 1000. This signal is further amplified and rectified to a low noise DC output by the phase lock amplifier, PAR Model JB-4 (Princeton Applied Research Corp., Princeton, N. J.). In this amplifier the AC signal from the photocell is fed into a balanced mixer along with a reference signal obtained from the same chopper using a separate photocell and light source. The mixer output is phase sensitive, and the over-all noise bandwidth can be reduced to less than 50 Hz. Variable gain settings up to  $9 \times 10^6$  times input voltage and response time settings up to 10 sec are available, along with a DC meter for readout.

In operation, the silicon photocell is positioned over the specimen area of interest, using the telescope and reflecting screen for viewing, and a meter reading at the chosen wavelength is observed. The photocell is then moved to a reference position off the area of interest and a reference reading is observed. The ratio of these readings allows one to calculate the optical density of that wavelength.

A Zeiss  $\times 32$  Ultrafluar objective with a numerical aperture of 0.40 was used in combination with a Bausch & Lomb quartz  $\times 10$  eyepiece for all spectral runs. This corresponds to a specimen diameter of approximately 5  $\mu\text{m}$  at the 1.5 mm photocell opening, which is slightly less than the smallest chosen pigment cell areas under investigation.

The performance of the microspectrophotometer was checked for linearity of de-

tor output, wavelength accuracy, and light leakage around a small opaque object. The measured extinction of a series of Bausch & Lomb neutral density filters indicated good linearity of photocell output with light intensity up to 1.5 OD units. The check points were obtained with broad-band (20 nm) light centered at 500 nm. For spectral runs on pigmented cells the monochromator slits were set at 1.3 mm, corresponding to an entering half-band width of 8.5 nm.

Wavelength accuracy was checked with several Bausch & Lomb standard series second-order interference filters with maxima at 373, 485, and 684 nm. The transmission measurements agreed in all respects with the filter specifications, including the specified half-band widths.

In order to check the over-all optical system, the extinction of a small piece of graphite with a measured diameter of 5  $\mu\text{m}$  was determined to be 1.68 OD units. This corresponds to a 3% light leakage around the object. This measurement was made at  $\times 320$  without glycerol immersion.

For the graphite measurement and for all spectral runs the instrument was operated with Kohler illumination, using an image of the exit diaphragm of the monochromator lens to illuminate the specimen area. This prevents undesirable light concentration on the specimen and provides approximately even illumination over the field of view. With a xenon arc light source, even illumination is difficult to obtain. The use of a Schoeffel lamp housing (Schoeffel Instrument Corp., Westwood, N.J.) with a reflecting mirror greatly improved the situation, but a check on the field uniformity before and after each run was still necessary.

An entire spectral run takes about 20 min, during which the specimen is constantly illuminated. Successively repeated runs on a single preparation indicated that no observable bleaching occurred during this time for the arthropods described in this report. Some bleaching of eye screening pigments has been observed for mutant brown *Drosophila*, but it turned out to be ionic, not photodynamic, and has no effect on the wild-type eye where the brown pigment occurs in low concentration (Superdock, 1971).

For all wavelength settings below 400 nm, a Corning CS-7-54 filter (Corning Glass Works Science Products Div., Corning, N.Y.) with a peak transmission at 320 nm was used to cut off the stray white light present in all grating monochromators. Unfortunately, this filter also absorbs light in the region below about 240 nm, thus setting a low wavelength limit of 240 nm on the instrument as used here.

The above-described instrument, allowing for some minor changes, has been used routinely for various investigations and for graduate instruction in this laboratory for several years. Absorption spectra of freshly prepared intact human blood cells are obtained routinely as a student exercise. The data agree very well with the known oxyhemoglobin absorption spectrum (Lemberg and Legge, 1949). Feulgen-stained preparations have also been investigated using this instrument (Bloom et al., 1970). In addition, the lens of the housefly eye has been investigated in the ultraviolet region showing a single absorption peak near 280 nm, in good agreement with the results of Goldsmith and Fernandez (1968).

*Specimen Preparation and Experimental Procedure* All insects used in this study were killed with chloroform and immediately put in a freezing microtome set for 4–6- $\mu$  sections, depending on the size of the eye. The sections were placed on a quartz

microscope slide and the coverglass edges were sealed with Vaseline to retard drying. Only specimen areas clearly identifiable with eye structure were used for measurement. All data were taken immediately after specimen preparation, since storage even for a few days was found to change the observed absorption curves slightly.

Most of the data were obtained over the spectral range of 310–700 nm. Later addition of a new photocell extended the range to a lower limit at 250 nm, which was then utilized for data on fruit flies and wolf spider eye pigments.

The specimen areas actually used for investigation varied greatly in size depending upon the eye structure and the angle at which the section was cut. The smallest pigmented cells were approximately  $6 \times 10 \mu\text{m}$ ; the largest pigmented areas used were about  $7 \times 40 \mu\text{m}$  for a diagonal slice. In all cases the  $5 \mu\text{m}$  in diameter photocell opening was filled completely by the pigmented cells being studied. Reference areas were the nearest adjacent clear portions of the microscope slide.

#### RESULTS

The eyes of houseflies and blowflies are quite similar when sectioned on a freezing microtome. Yellow-pigmented cells appear near the top of the eye, but the red pigment separating the ommatidia predominates over most of the section (for a diagonal slice). The maximum extinction of a particular colored pigment was found to vary considerably depending upon which portion of the eye section was used for measurement, and it was evident from visual observation that the pigment concentration was nonuniform down through the eye. As an example, the maximum extinction of the red housefly screening pigment ranged from 0.51 to 0.89 OD unit for the five observations used in this report. The extinction data were therefore normalized to a value of 1.0 OD unit at the peak wavelength and the average of the normalized curves is shown in the figures, along with twice the standard (root mean square [rms]) error, calculated at 20-nm intervals.

Fig. 2 shows the relative absorption spectrum of the red screening pigment of the housefly *Musca domestica*. The data represent averaged absorption taken every 10 nm for five runs on four different flies, normalized at a wavelength of 530 nm. The monochromator slits were set at 1.3 mm, corresponding to an entering half-band width of 8.5 nm. The spectrum shows a broad maximum near 520–530 nm with a secondary peak in the region 380–390 nm. The observed averaged value of the 530 nm peak extinction was 0.7 OD unit; for the 385 nm peak the extinction was 0.38 OD unit.

The relative absorption spectrum of the yellow screening pigment of *Musca* is shown in Fig. 3. Unless otherwise stated, instrument settings and treatment of the data are the same as given above for these and the remainder of the results. The data shown in Fig. 3 represent six runs on four different flies, normalized at a wavelength of 440 nm. A single broad peak near 440 nm is evident; its observed extinction was 0.84 OD unit. Appreciable ultraviolet absorption is evident.

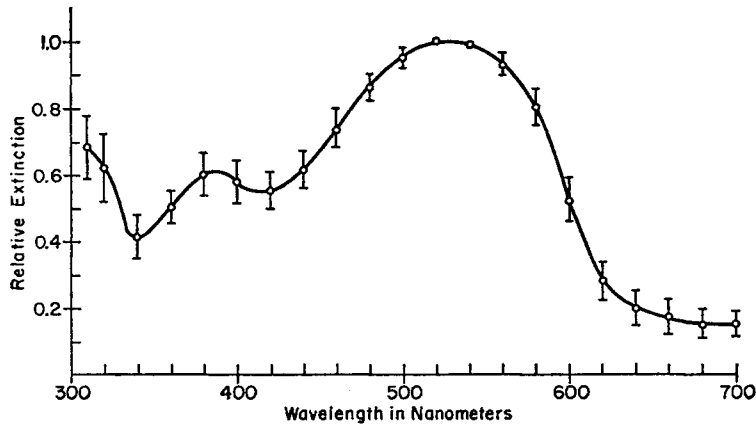


FIGURE 2. Absorption spectrum of red screening pigment of *Musca domestica*. The ordinate is relative extinction and the data represent averaged values for five runs on four different flies. Vertical bars represent  $2 \times$  rms deviation.

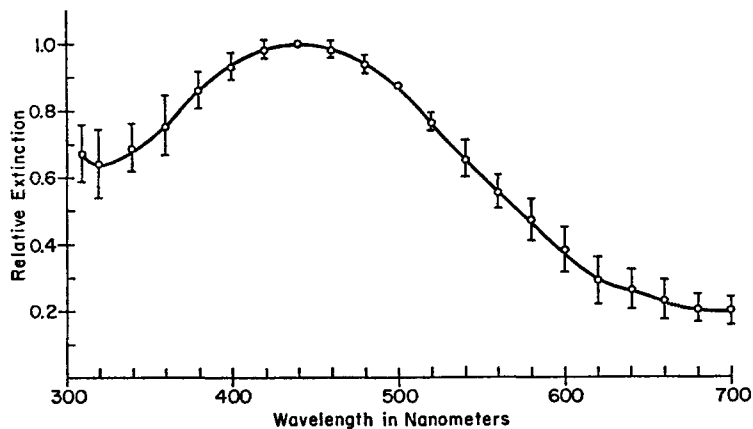


FIGURE 3. Absorption spectrum of yellow screening pigment of *Musca domestica*. The ordinate is relative extinction and the data represent averaged values for six runs on four different flies. Vertical bars represent  $2 \times$  rms deviation.

Fig. 4 shows the relative absorption of the red screening pigment from the eye of the blowfly *Phormia regina*. The data represent six runs on four different flies, normalized at 540 nm. The spectrum is quite similar to the *Musca* spectrum of Fig. 2. A primary broad absorption peak near 540 nm is evident, with a secondary peak at 385 nm. The observed extinction was 1.0 OD unit for the 540 nm peak and 0.57 OD unit for the 385 nm peak.

Fig. 5 shows the relative absorption of the yellow screening pigment from the eye of *Phormia regina*, normalized at 460 nm. The data represent five runs on four different flies. A single broad absorption peak in the region 460–470

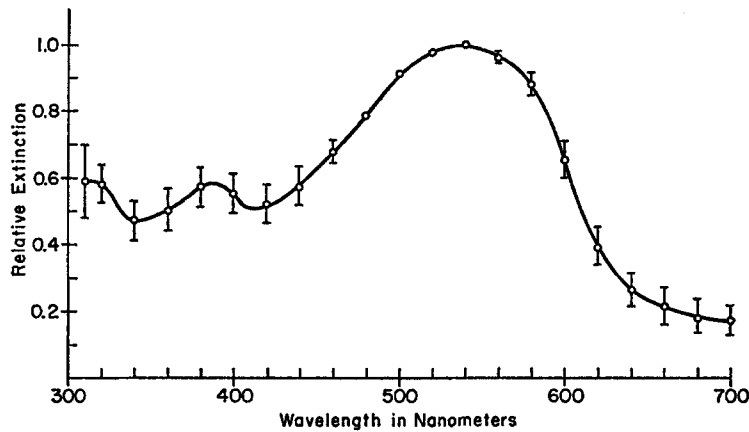


FIGURE 4. Absorption spectrum of red screening pigment of *Phormia regina*. The ordinate is relative extinction and the data represent averaged values for five runs on four different flies. Vertical bars are  $2 \times$  rms deviation.

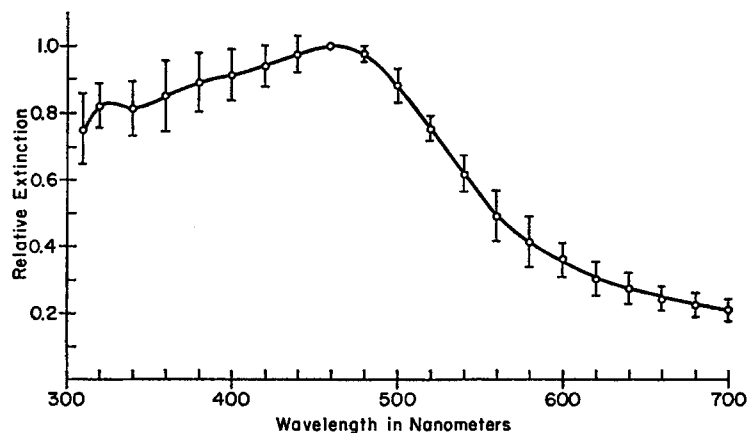


FIGURE 5. Absorption spectrum of yellow screening pigment of *Phormia regina*. The ordinate is relative extinction and the data represent averaged values for five runs on four different flies. Vertical bars represent  $2 \times$  rms deviation.

nm is evident, along with high absorption in the ultraviolet region. The observed value of the extinction at 460 nm was 0.6 OD unit.

Frozen sections of the eye of the dragonfly *Libellula luctuosa* reveal two quite distinct structures. The dorsal portion contains a light yellow screening pigment between the ommatidia and the ventral portion contains a dark red screening pigment, extending the full depth of the eye. The division of the eye is not obvious upon visual inspection.

Fig. 6 shows the relative absorption of the dark red screening pigment of the ventral portion of the eye of *Libellula luctuosa*. The data are normalized at

500 nm and represent four runs on three different flies. Although there is a broad peak near 500 nm, the results indicate high absorption throughout most of the visible and near ultraviolet region. The observed value of the extinction at 505 nm was 0.7 OD unit.

The yellow screening pigment of the dorsal part of the *Libellula* eye has the absorption spectrum shown in Fig. 7. The data represent four runs on three different flies and are normalized at 460 nm. A single broad peak at 460 nm is evident, with relatively high absorption throughout the ultraviolet region. The observed value of the extinction at 460 nm was 0.64 OD unit.

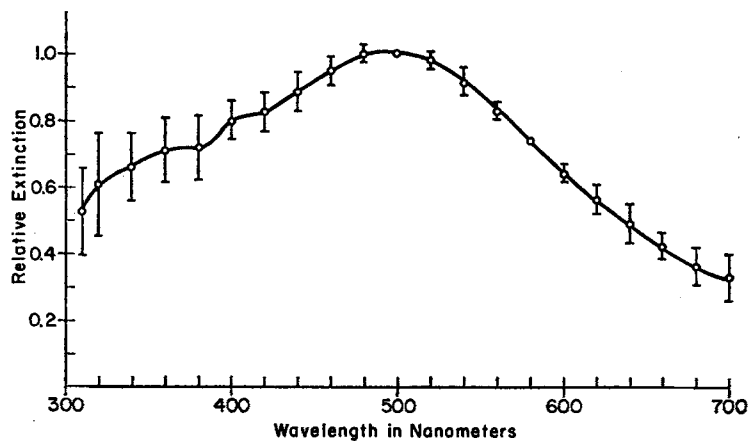


FIGURE 6. Absorption spectrum of dark red screening pigment of *Libellula luctuosa*. The ordinate is relative extinction and the data represent averaged values for four runs on three different dragonflies. Vertical bars represent  $2 \times$  rms deviation.

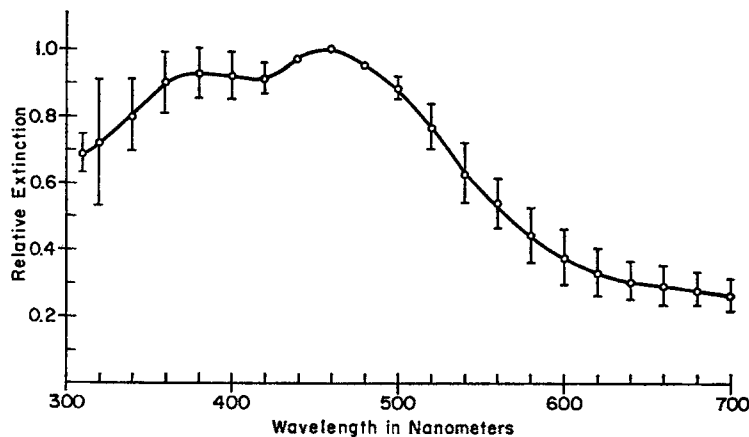


FIGURE 7. Absorption spectrum of yellow screening pigment of *Libellula luctuosa*. The ordinate is relative extinction and the data represent averaged values for four runs on four different dragonflies. Vertical bars represent  $2 \times$  rms deviation.



Frozen sections of the eye of the worker honeybee *Apis mellifera* indicate that only a dark red screening pigment is present. Fig. 8 shows the relative absorption spectrum of this pigment. The data represent six runs on four different bees, normalized at 500 nm. No strong absorption maximum is present, but the absorption declines slowly at wavelengths longer than 520 nm. The observed value of the extinction at 490 nm was 0.80 OD unit.

Fig. 9 shows the relative absorption of the red screening pigment found in the eye of the wild-type *Drosophila melanogaster*. The data represent five runs on five different flies taken over the range 260–700 nm, normalized at 520 nm.

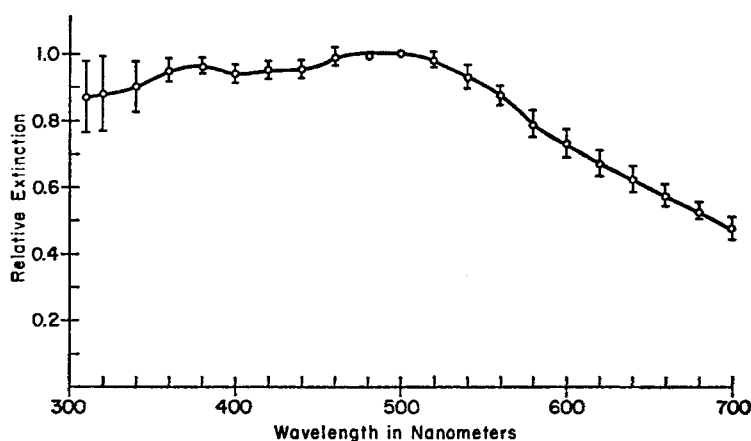


FIGURE 8. Absorption spectrum of dark red screening pigment of the worker honeybee, *Apis mellifera*. The ordinate is relative extinction and the data represent averaged values for six runs on five different bees. Vertical bars represent  $2 \times$  rms deviation.

The major absorption peak occurs at 520 nm, with a strong secondary peak near 290 nm. The flies ranged from 1 to 7 days old. The observed extinction at 520 nm was 0.63 OD unit; the corresponding figure for the 290 nm peak was 0.59 OD unit.

A recent investigation of the spectral sensitivity of wolf spider eyes (DeVoe et al., 1969) indicated that the absorption of screening pigments in these eyes would be of some interest. The heads of these arthropods contain eight eyes consisting of two main types, the anterior and the posterior. The posterior eyes contain a dark purple screening pigment between the rhabdomes. In the anterior eyes the pigment is situated below the rhabdomes. The spectral sensitivity curves were obtained on *Lycosa baltimoriana* and *Lycosa miami* by R. D. DeVoe, who kindly supplied the same species for the screening pigment data.

Fig. 10 (dashed line) shows the absorption of the screening pigment in the anterior median eye of *Lycosa* over the range 260–700 nm. Absorption is high

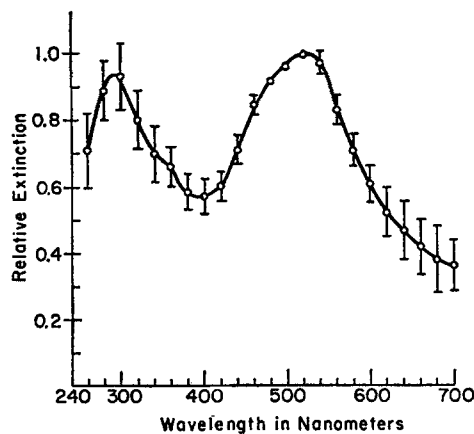


FIGURE 9

FIGURE 9. Absorption spectrum of red screening pigment of wild-type *Drosophila melanogaster* over the range 260–700 nm. The ordinate is relative extinction and the data represent averaged values for five runs on five different flies. Vertical bars represent  $2 \times$  rms deviation.

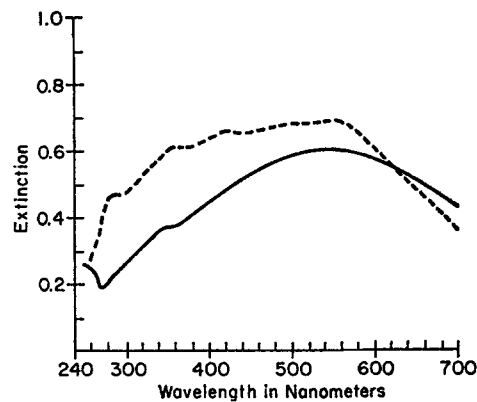


FIGURE 10

FIGURE 10. Absorption spectra of *Lycosa* dark purple screening pigments over the range of 260–700 nm. Dashed line: anterior median eye of *Lycosa miami*; solid line: posterior median eye of *Lycosa baltimoriana*. Each curve represents averaged data from two runs on one spider.

TABLE I  
COMPARISON OF SCREENING PIGMENT ABSORPTION  
MAXIMA WITH SPECTRAL SENSITIVITY MAXIMA

Arthropod pigment	Absorption maxima <i>nm</i>	Spectral sensitivity maxima <i>nm</i>	Reference for spectral sensitivity
<i>Musca</i>			
Red	385, 530	350, 500	Goldsmith and Fernandez (1968)
Yellow	440		
<i>Calliphora</i>			
Red	520–540 (broad) 385	350, 493, 515	Burkhardt (1962)
Yellow	460–480 (broad)	475	
<i>Libellula</i>			
Dark red	505 (broad)	520 (broad)	Horridge (1969)
Yellow	360–465 (broad)	350–470 (broad)	
Worker honeybee			
Red	490 (broad)	345–535	Goldsmith (1960)
<i>Drosophila</i>			
Red	290, 520	365, 485	Bertholf (1932)
<i>Lycosa</i>			
Purple	550, 545	370, 513 510	DeVoe et al. (1969)
<i>Phormia</i>			
Red	385, 540		
Yellow	460 (broad)		

throughout the range 280–560 nm. The solid line in Fig. 10 shows the absorption of the screening pigment in the posterior median eye of *Lycosa*. Absorption is greater in the long wavelength region than in the short, with a broad maximum at about 540 nm. Each curve represents averaged data from two runs on one insect. The spectral sensitivity results obtained by DeVoe using a mass response (ERG) technique gave peaks near 370 and 510 nm; thus no correlation with the screening pigment absorption is evident.

The absorption spectra described above and shown in Figs. 2–10 are summarized in Table I.

#### DISCUSSION

The screening pigment absorption spectra shown in the Results section will be discussed first from a chemical viewpoint and later from a physiological viewpoint.

It should be clearly understood that no positive chemical identification can be expected from the data presented in this report. Chemical extractions of arthropod eye screening pigments are well known to yield pterins and ommochromes. Many varieties of these two major pigment structures may exist in the natural state. In particular, it is possible for more than one type of pigment to coexist in an arthropod eye (Visconti et al., 1957), even though the pigment looks homogeneous. In addition, the screening pigment may be complexed with a protein. All of this adds up to the fact that an absorption spectrum taken in vitro may represent either a very simple or a very complex chemical entity. If a single pigment is present, chemical identification may be possible. For the complex case there is little chance of separating the two or more possibilities.

When a compound eye is frozen and sectioned it returns to its unfrozen state immediately upon being placed on the microscope slide. If there are two pigments present, some mixing of these is possible when the coverglass is pressed down. However, only slide preparations in which the eye structure was visible were used in these studies, and clearly separated pigmented areas were used for observation and measurement. The reference areas used were clear portions of the microscope slide immediately adjacent to the pigmented cells. Due to the water matrix surrounding the specimen the reference areas were assumed to be essentially lacking in cytoplasmic proteins absorbing maximally at 280 nm.

Microspectrophotometry of insect screening pigments was performed on the blowfly *Calliphora erythrocephala* by H. Langer (1967) who also compared his data with reduced and oxidized xanthommatin spectra. For the red pigment Langer obtained a spectrum essentially identical to the *Phormia* results shown in Fig. 4, with major and minor peaks near 540 and 390 nm, respectively. Data taken in this laboratory on *Calliphora* using the microspectrophotometer

are in good agreement, with peaks near 540 and 385 nm (average values for four runs on two flies). The results therefore indicate that the red pigments of *Calliphora* and *Phormia regina* are the same, which is believed to be reduced xanthommatin complexed with a protein, since the extracted xanthommatin has a long-wavelength peak at 496 nm (Langer, 1967). The red screening pigment of *Musca* shown in Fig. 2 is obviously quite similar to the blowfly red pigments but the long wavelength peak is shifted by about 10 nm towards the blue.

For the yellow *Calliphora* screening pigment, results obtained in this laboratory are in good agreement with the spectrum obtained by Langer (1967) showing a single broad maximum near 440 nm, with high absorption in the ultraviolet region (average of four runs on two flies). The absorption spectrum shown in Fig. 5 for *Phormia regina* is essentially identical to the *Calliphora* spectrum, indicating that the blowfly yellow pigments are the same, believed to be a mixture of oxidized and reduced xanthommatin (Langer, 1967). The *Libellula* yellow pigment spectrum shown in Fig. 5 is quite similar to the *Phormia* spectrum although a broad shoulder appears near 380 nm. Within the limits of error shown on the graphs the results indicate that the dragonfly yellow pigment is the same as the blowfly yellow pigment. The yellow pigment of *Musca* shown in Fig. 3 has a single absorption maximum near 440 nm, which agrees with the spectrum of oxidized xanthommatin in neutral solution (Langer, 1967). However, the width of the peak is such as to indicate that other pigments or other forms of xanthommatin may be present.

The dark red pigments of *Libellula* and *Apis* shown in Figs. 6 and 8, respectively, have a single absorption maximum near 500 nm, which is in agreement with the spectrum of reduced xanthommatin in neutral solution (Langer, 1967). However, ommin has been extracted from the eyes of *Apis mellifera* and Odonata (*Aeschna juncea*) with a maximum absorption at 520 nm in buffered solution at pH 7.5 (Butenandt et al., 1958). It is quite possible, therefore, that the broad absorption shown in Fig. 4 is due to ommin and/or xanthommatin.

The eye of the wild-type *Drosophila* is known to contain a brown pigment and a red pigment. Early extractions of these pigments revealed single absorption peaks in the visible region at 480 nm for the red component and 444 nm for the brown component (Nolte, 1952). The region below 400 nm was not investigated and the concentration ratio was 10:1, red:brown. Subsequent work indicates that the brown pigment is an ommochrome and the red pigment is a mixture of three pterins, two of which peak near 500 nm in the visible and have secondary peaks in the ultraviolet at 265 nm (Visconti et al., 1957). Fig. 9 shows the ultraviolet peak of the *in situ* pigment to be at 290 nm, corresponding to a shift towards the red of 25 nm as compared to the extracted pigment. The visible peak of one of the pterins present, namely

drosopterin, occurs at 510 nm in 0.1 N NaOH solution, in comparison to a value of 520 nm for the *in situ* measurement shown in Fig. 9. The results therefore indicate that a mixture of pterins and ommachromes occurs in the wild-type fruit fly eye, in agreement with the extraction method analysis (Visconti et al., 1957).

Screening pigment absorption spectra are very important from a physiological standpoint. In this regard, the absolute values of the screening pigment absorption spectra are of some interest. The data were taken on samples sectioned at about 6  $\mu\text{m}$  on a freezing microtome and pressed flat with a coverglass. The highest extinction recorded was about 1 OD unit, corresponding to 90% absorption. Many curves had maxima lower than this. In the eye, the light path of an oblique ray might be incident upon a pigment layer much deeper than that used here. For *Apis* and *Calliphora* the interommatidial angle is of the order of 2°–4° (Goldsmith, 1964) and the pigment sleeve may be estimated at about 3  $\mu\text{m}$  thickness. For this geometry the path length of an incident oblique ray through a pigment sleeve would be a maximum of 29  $\mu\text{m}$  for a 10° angle and 4.6  $\mu\text{m}$  for a 45° angle of incidence with a single frontal ommatidium. Since the measured extinctions correspond to a path length of approximately 6  $\mu\text{m}$  it is evident that for wavelengths for which the pigment extinction is relatively high the screening effect is very efficient. Thus any extinction scaled off these curves should be very low before one can assume that appreciable light leakage occurs at that wavelength. For instance, light leakage through the red *Musca* screening pigment is known to occur at about 620–630 nm (Goldsmith, 1965), which corresponds to an extinction of 0.16 as measured from the original data.

Insect spectral response studies aimed at revealing attributes of color vision have been extensive. These studies are often based upon electrophysiological measurements, either a mass response (ERG) or single cell measurements; however, behavioral responses to color stimuli have been extensively investigated for many years. The data of Fig. 9 on the fruit fly can only be compared on this basis, since no electrophysiological investigations are available at present. Previous investigations on the behavioral color response of this insect (Bertholf, 1932; Wolken et al., 1957) indicate response peaks near 500 and 360 nm, with the ultraviolet peak much stronger than the visible peak. It is therefore possible that the screening pigment absorption matches a photopigment spectrum in the visible, since the spectra agree at 500 nm; however, the ultraviolet sensitivity peak occurs at a minimum in the screening pigment spectrum, as shown by Fig. 9, so the analogy is incomplete.

Comparison of the *in vitro* absorption spectra shown in Figs. 2–10 with available spectral sensitivity data obtained by observing the mass response of whole eyes (ERG) shows very little correlation. The spectral sensitivity of *Musca domestica* peaks at 500 and 350 nm (Goldsmith and Fernandez, 1968);

for the worker honeybee the peaks are at 535 and 350 nm (Goldsmith, 1960). For the dragonfly *Libellula luctuosa* the peaks are at 420 and 510 nm (ultra-violet range not investigated) (Ruck, 1965). None of the above-listed ERG curves (see Table I) show any obvious relationship to the spectral data of Figs. 2-10.

The effects of light leakage through screening pigments at specific wavelengths include a possible shift of the ERG spectral sensitivity peaks, due to an increase in the number of excited ommatidia. This effect has been documented in *Calliphora* (Hoffman and Langer, 1961) and in *Musca* (Goldsmith, 1965), both of which show a spectral sensitivity peak at 620 nm in the wild type which does not appear when chalky white mutants lacking the screening pigments are investigated. Some concern for this effect at shorter wavelengths has been expressed by Burkhardt (1964). However, as previously discussed, light leakage is not likely to occur except at low pigment extinction values. For insects with both yellow and red screening pigments in the same part of the eye, the combined absorption indicates that there should be no spectral shifts in the ERG peaks due to light leakage over the range of 310 to about 610 nm. This would apply to *Phormia*, *Calliphora*, and *Musca*. In a similar fashion the absorption of the *Apis mellifera* screening pigment is broad enough to cover the same range. For the wild-type *Drosophila* eye and the dorsal *Libellula* eye, the absorption is not high over the entire spectrum and some shifting of ERG peaks might be anticipated, when the data become available.

In contrast to the ERG data, a comparison between screening pigment absorption and the spectral response of individual retinal neuroelements obtained by intracellular recording proves to be more interesting. In this type of recording the incident light is adjusted until the single cell response is a maximum, corresponding to direct stimulation down the optic axis of one ommatidium. This eliminates the influence of possible light leakage through the screening pigments at specific wavelengths (Burkhardt, 1964).

The visual system of the dragonfly *Libellula needhami* has been investigated by Horridge (1969) using single cell electrophysiology. Fig. 11 shows the spectral response of a blue-sensitive cell (curve B) replotted from Horridge's data as compared to the *L. luctuosa* yellow screening pigment absorption spectrum (curve A) obtained by the writers. Both curves are relatively flat from about 350 to 470 nm and decrease rapidly at longer wavelengths. The results indicate that the yellow pigment would be very effective in preventing light leakage between ommatidia containing a visual pigment with the absorption spectrum of curve B.

Fig. 12 shows the spectral response of the most common green-sensitive cell replotted from the same report (curve B) as compared to the *L. luctuosa* dark red screening pigment absorption spectrum obtained in this laboratory

(curve A). Although the agreement in shape of the two curves is not as good as for those of Fig. 11, it is evident that the red pigment would still very effectively perform a screening function for cells with visual pigments char-

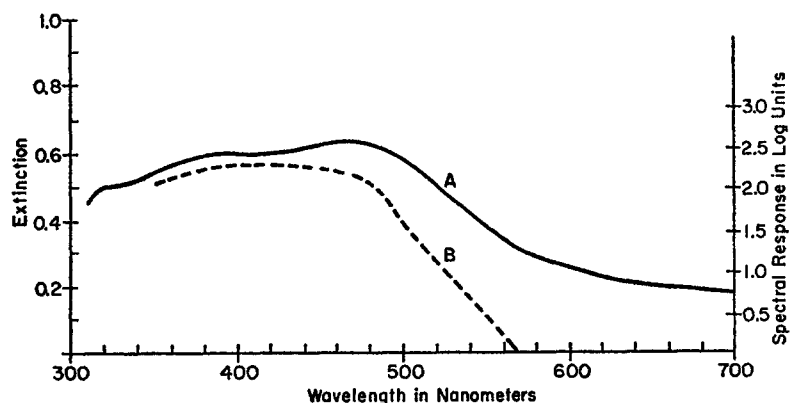


FIGURE 11. Absorption spectrum of yellow screening pigment of *Libellula luctuosa* (curve A, averaged data, left ordinate) as compared to the spectral response of a blue-sensitive cell of *Libellula needhami* (curve B, right ordinate) replotted from the data of Horridge (1969). See text for discussion.

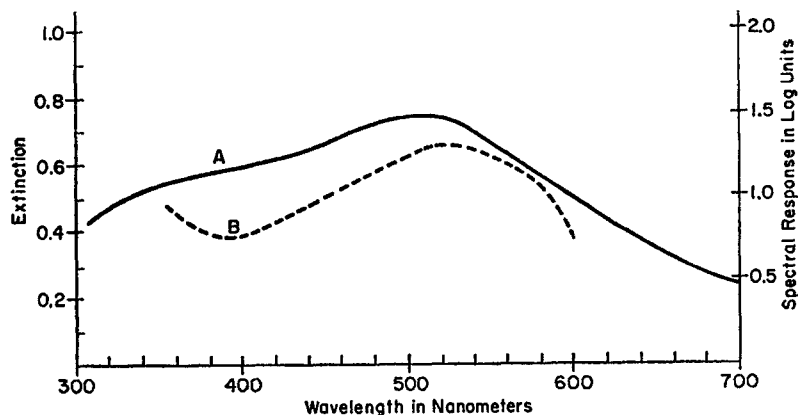


FIGURE 12. Absorption spectrum of dark red screening pigment of *Libellula luctuosa* (curve A, averaged data, left ordinate) as compared to the spectral response of a green-sensitive cell of *Libellula needhami* (curve B, right ordinate) replotted from the data of Horridge (1969). See text for discussion.

acterized by curve B. It is interesting to note that many of the green-sensitive cells observed by Horridge had a much flatter spectral response than is shown in curve B, and would fit closely under curve A. Due to the variability of his data, Horridge's most typical curve was used here for comparison. For both

the yellow and the red pigments the anatomical location is suitable for performance of the screening function.

Red and yellow screening pigments from the eye of the blowfly *Calliphora erythrocephala* (wild type) have been investigated by microspectrophotometry in this laboratory. The absorption spectra agree with the data published by Langer (1967) using the same technique and are therefore not illustrated (except for comparison purposes as discussed in the next paragraph).

It would be of some interest to compare absorption spectra of insect visual pigments with spectra of the screening pigments. At present the only directly observed insect visual pigment data available are the results of Langer and Thorell (1966) on the blowfly *Calliphora* obtained with a recording microspectrophotometer. Of the seven rhabdomeres in the ommatidium of this insect, six of them showed the double-peaked absorption spectrum illustrated in Fig. 13, curve B, replotted from their data. Curve A of Fig. 13 shows the absorption spectrum of the red screening pigment of *Calliphora erythrocephala* (averaged from three runs on two flies) obtained in this laboratory. The general shape of the two curves is similar and suggests that the red screening pigment may be shielding this particular photoreceptor. The seventh rhabdomere of the *Calliphora* eye showed the absorption spectrum illustrated in curve B, Fig. 14, also replotted from the data of Langer and Thorell (1966). For comparison, the absorption spectrum of the yellow screening pigment of *Calliphora* (averaged from four runs on two flies) is shown as curve A of Fig. 14. Again, the general shape of the curves is similar enough to indicate that the yellow screening pigment may be shielding this particular photoreceptor.

The color responses of *Calliphora* have also been investigated using single

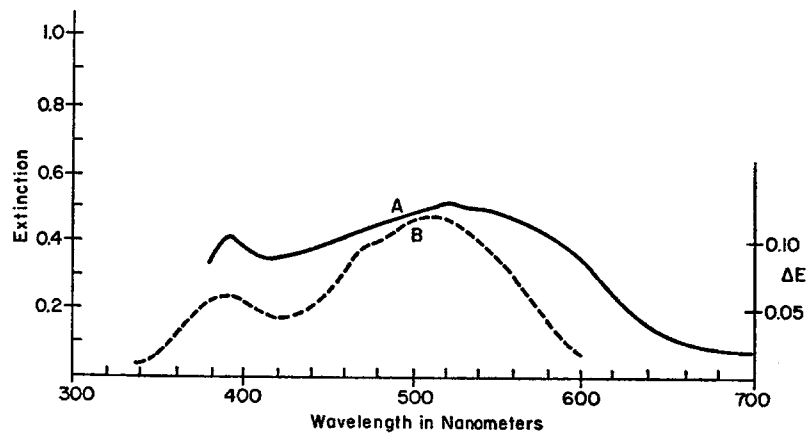


FIGURE 13. Absorption spectrum of red screening pigment of *Calliphora erythrocephala* (curve A, averaged data, left ordinate) as compared to spectrum of green receptor of *Calliphora* replotted from data of Langer and Thorell (1966) (curve B, dashed line, right ordinate). See text for discussion.



cell electrophysiology (Burkhardt, 1962). Three types of receptors are indicated, each with a strong response at 350 nm. The visible wavelength peaks are located at 475, 493, and 515 nm for each type, respectively. The shapes of the spectral response curves closely resemble the directly observed data of Langer and Thorell (1966); for the wavelengths listed above the red and yellow pigments would very effectively shield these receptors.

In contrast to the above, the worker honeybee data of Fig. 8 indicate a very broad absorption spectrum. Since this insect is known to have color vision, light is probably being screened for several different types of receptors

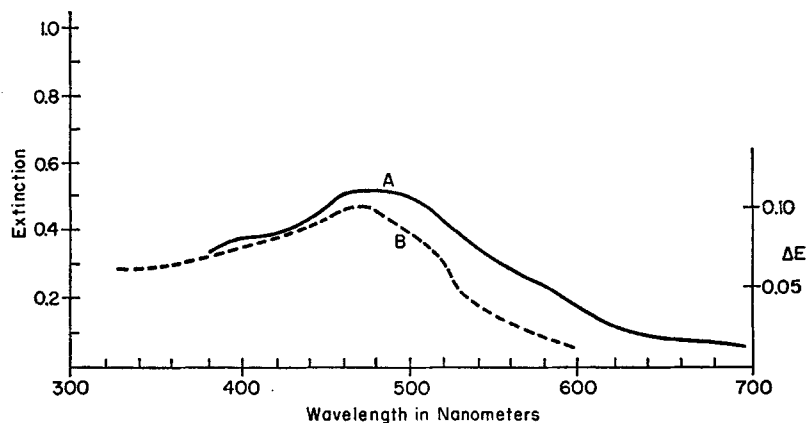


FIGURE 14. Absorption spectrum of yellow screening pigment of *Calliphora* (curve A, averaged data, left ordinate) as compared to spectrum of blue receptor of *Calliphora erythrocephala* replotted from data of Langer and Thorell (1966) (curve B, dashed line, right ordinate). See text for discussion.

(Autrum, 1965). Preliminary data (Casella, 1969) indicate that the eye of the drone honeybee contains two separate screening pigments, but this work is incomplete.

The absorption of the wolf spider screening pigments shown in Fig. 10 is characterized by a lack of any sharp spectral peaks and precludes any attempt at chemical identification of these pigments. Although the screening pigments look identical in the microscope, Fig. 10 shows that they are different for each type of eye.

It is of some interest to note that an investigation of the screening pigments of another arachnid, the scorpion (Machan, 1968), indicates that the lateral and median eyes have different screening pigments. Light absorption by these pigments is high in the visible and near ultraviolet range, but the absorption spectra do not closely resemble those shown in Fig. 10 for the *Lycosa* eyes.

During his spectral sensitivity investigation, DeVoe noted some secondary

ERG peaks in the posterior median eyes; the peaks appeared only at longer wavelengths, namely those greater than 580 nm. Using narrow band adapting lights he obtained a spectral sensitivity curve for these secondary peaks. By working on the assumption that these responses were due to light leakage through the screening pigment, an unsuccessful attempt was made to correlate the data with *Musca* screening pigment absorption in this wavelength region previously published (Strother, 1966). When the *in vitro* absorption of *Lycosa* screening pigment is used, however, a good match between it and the secondary peak ERG response is obtained. The results are shown in Fig. 15.

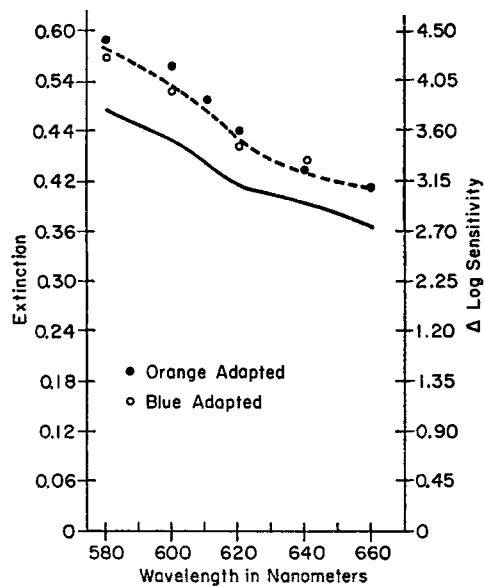


FIGURE 15. Absorption spectrum of *Lycosa* posterior median eye screening pigment over the range 580–660 nm (solid line, left ordinate) as compared to the change in secondary peak spectral response replotted from the data of DeVoe (1969) (dashed line through experimental points, right ordinate).

The dashed line is the difference between the secondary peak spectral response and the dark-adapted spectral response (right ordinate) replotted from DeVoe's data. The solid line is the absorption of *Lycosa* posterior median eye screening pigment (obtained from five runs on two spiders) representing the light leakage assumed to be producing the secondary response. Note that no spectral sensitivity maximum is illustrated; only the leakage at longer wavelengths is of concern. Since the slopes of these graphs are similar, these results substantiate DeVoe's hypothesis that the long wavelength ERG responses are due to light leakage through the screening pigment rather than a separate type of photoreceptor (see DeVoe et al., 1969, Fig. 6, for more details).

It is somewhat disappointing that the screening pigment data obtained to date show no obvious correlation with the 350 nm sensitivity peak exhibited by all insects. The light red pigments of *Musca*, *Phormia*, and *Drosophila* have a minimum at 350 nm, but the yellow pigments do not exhibit this minimum. Thus for these insects some 350 nm light may be scattered among the green receptors, but not so for the blue receptors. It is also of interest to note that the yellow pigment location near the top of the eye for *Musca* and *Phormia* indicates that the "blue" receptors should be similarly located, with the green receptors further down. Thus far, of course, the photoreceptor locations have not been well documented except for the work on *Calliphora* (Langer and Thorell, 1966).

It is possible that where only one screening pigment is present in an eye, the contrast sensitivity may be wavelength sensitive. Although visual acuity is probably not directly related to screening pigment absorption (Goldsmith, 1964), experiments on *Drosophila* (Wehner et al., 1969) indicate that contrast sensitivity is a function of the screening pigment absorption. According to the absorption data presented here, this wavelength effect should be observable in the dorsal part of the *Libellula* eye, in the *Drosophila* eye, and in the *Lycosa* eye at very short wavelengths. The dark red pigments of the worker honeybee and the ventral *Libellula* eye absorb appreciably throughout the physiological spectrum and should not show this effect except possibly beyond 600 nm.

A comparison of screening pigment absorption maxima with spectral sensitivity maxima is given in Table I.

The results presented in this report cover only a small fraction of the insects that deserve study by these methods. Studies on *Drosophila* mutants are in progress even though spectral sensitivity data are lacking for this insect. The drone honeybee is also under investigation, and much work remains to be done on other bees, flies, and related insects.

In summary, the results of in vitro measurements of selected insect screening pigment absorption indicate the following:

(a) In some cases the absorption spectra are very broad over the wavelength range 310–700 nm. In other cases the data indicate characteristic absorption over limited regions of the spectrum which correlate well with known single cell spectral sensitivity curves. There is no obvious correlation with the mass response (ERG) of the eyes, except where the single-cell and ERG data are in agreement. This indicates that there exist specific screening pigments for specific photoreceptor types, which is particularly evident for the dragonfly *Libellula*. This specificity is not observable for the worker honeybee or the wolf spiders, and it is possible that one pigment may be screening for several different closely spaced photopigments in these two animals.

(b) The data presented are quite useful for correlating ERG data at long wavelengths with light leakage through the screening pigments at these wave-

lengths. A case in point is made here for the wolf spider *Lycosa*. The same remarks should also apply for a wavelength-dependent contrast sensitivity, since it is a function of light leakage through the eyes of many insects.

(c) For the insects studied, light screening by red and yellow pigments taken together provides complete coverage from 310 to about 610 nm. This means that there should be no effect on ERG data in this wavelength range. When only one pigment is present, the ERG data may be affected in regions of low absorption. This might apply here to wild-type *Drosophila melanogaster* and to the dorsal *Libellula* eye.

(d) Although two species of blowfly and the housefly have quite similar red pigments, the housefly yellow pigment is different from the other two yellow pigments. Thus there appears to be a variety of screening pigments among the insects studied even though the colors appear similar.

(e) No obvious correlation appeared between screening pigment absorption at 350 nm and the enhanced visual sensitivity at this wavelength for all insects.

(f) Some of the in vitro absorption spectra obtained from the arthropods studied show clearly defined peaks which can be correlated with chemical extraction results available in the literature. For these cases there is usually some shift to the red for the absorption peaks, indicating possible combination with protein. For other cases the spectra show broad absorption maxima not easily interpreted from a chemical standpoint.

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#### REFERENCES

- AUTRUM, H. 1955. Die spektrale Empfindlichkeit der Augen mutation white-apricot von *Calliphora erythrocephala*. *Biol. Zentralbl.* 74:515.
- AUTRUM, H. 1965. The physiological basis of color vision in honeybees. In *Color Vision*. A. V. S. DeReuck and J. Knight, editors. Little, Brown and Company, Boston, Mass. 288.
- BERTHOLF, L. M. 1932. The extent of the spectrum for *Drosophila* and the distribution of stimulative efficiency in it. *Z. Vergl. Physiol.* 18:32.
- BLOOM, S. E., E. G. BUSS, and G. K. STROTHER. 1970. Cytological and cytophotometric analysis of binucleated red blood cell mutants (bn) in turkeys (*Meleagris gallopavo*). *Genetics.* 65:51.
- BURKHARDT, D. 1962. Spectral sensitivity and other response characteristics of single visual cells in the arthropod eye. *Symp. Soc. Exp. Biol.* 16:86.
- BURKHARDT, D. 1964. Color discrimination in insects. *Advan. Insect Physiol.* 2:131.
- BUTENANDT, A., E. BIEKERT, and B. LINZEN. 1958. Über Ommochrome. XIV. Zur verbreitung der Ommine im Tierreich. *Hoppe-Seyler's Z. Physiol. Chem.* 313:251.
- CASELLA, A. J. 1969. The design and construction of a recording microspectrophotometer and its use in investigations on insect visual systems. Ph.D. Thesis. The Pennsylvania State University, University Park, Pa.

- DEVOE, R. D., R. J. W. SMALL, and J. E. ZVARGULIS. 1969. Spectral sensitivities of wolf spider eyes. *J. Gen. Physiol.* **54**:1.
- GOLDSMITH, T. H. 1960. The nature of the retinal action potential and the spectral sensitivities of ultraviolet and green receptor systems of the compound eye of the worker honeybee. *J. Gen. Physiol.* **43**:775.
- GOLDSMITH, T. H. 1964. The visual system of insects. In *The Physiology of Insecta*. M. Rockstein, editor. Academic Press, Inc., New York. 1:397.
- GOLDSMITH, T. H. 1965. Do flies have a red receptor? *J. Gen. Physiol.* **49**:265.
- GOLDSMITH, T. H., and H. R. FERNANDEZ. 1968. The sensitivity of housefly photoreceptors in the mid-ultraviolet and the limits of the visible spectrum. *J. Exp. Biol.* **49**:669.
- HOFFMAN, C., and H. LANGER. 1961. Die spectrale Augereupfindlichkeit der Mutant "chalky" von *Calliphora erythrocephala*. *Naturwissenschaften*. **48**:605.
- HORRIDGE, G. 1969. Unit studies on the retina of dragonflies. *Z. Vergl. Physiol.* **62**:1.
- LANGER, H. 1967. Über die Pigmentgranula im Facettenauge von *Calliphora erythrocephala*. *Z. Vergl. Physiol.* **55**:354.
- LANGER, H., and C. HOFFMAN. 1966. Elektro- und Stoffwechselfysiologische Untersuchungen über den Einfluss von Ommochromen und Pteridinen auf die Funktion des facetten Auges von *Calliphora erythrocephala*. *J. Insect Physiol.* **12**:357.
- LANGER, H., and B. THORELL. 1966. Microspectrophotometry of single rhabdomes in the insect eye. *Exp. Cell Res.* **41**:673.
- LEMBERG, R., and J. W. LEGGE. 1949. Hematin Compounds and Bile Pigments. Interscience Publishers, Inc., New York. 228.
- MACHAN, L. 1968. Spectral sensitivity of scorpion eyes and the possible role of shielding pigment effect. *J. Exp. Biol.* **49**:95.
- NOLTE, D. J. 1952. The eye-pigmentary system of *Drosophila*. III. The action of eye-colour genes. *J. Genet.* **51**:142.
- RUCK, P. 1965. The components of the visual system of a dragonfly. *J. Gen. Physiol.* **49**:289.
- SHAW, S. R. 1969. Optics of the compound eye. *Science (Washington)*. **165**:88.
- STROTHER, G. K. 1966. Absorption of *Musca domestica* screening pigment. *J. Gen. Physiol.* **49**:1087.
- SUPERDOCK, D. A. 1971. In situ microspectrophotometric determination of screening pigment absorption spectra of *Drosophila melanogaster* eye color mutants. D.Ed. Thesis. The Pennsylvania State University, University Park, Pa.
- VISCONTI, M., E. HADORN, and P. KARRER. 1957. Fluoreszierende Stoffe aus *Drosophila melanogaster* die roten Augenfardstoffe. *Helv. Chim. Acta.* **40**:579.
- WEHNER, W., G. GARTENMANN, and T. JUNGLI. 1969. Contrast perception in eye color mutants of *Drosophila melanogaster* and *Drosophila subobscura*. *J. Insect Physiol.* **15**:815.
- WOLKEN, J. J., A. D. MELLON, and G. CONTIS. 1957. Photoreceptor structures. II. *Drosophila melanogaster*. *J. Exp. Zool.* **134**:383.