THE VISUAL COMPLEX OF THE INSECT: RETINENE IN THE HOUSEFLY

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(Received March 1st, 1960)

SUMMARY

Housefly heads were extracted with acetone and the extract chromatographed on columns of alumina. One of the eluted fractions had an absorption maximum at 385 mμ; when reacted with antimony trichloride, a transient blue color, with a maximum absorption at 665 mμ was obtained. This fraction was identified as retinene$_1$. A rhabdomere fraction was isolated from the insect eyes by grinding the heads with buffered sucrose solution and fractionating by centrifugation. The rhabdomere fraction, when acetone extracted, also shows the presence of retinene$_1$. It appears that a visual complex retinene$_1$ isolated from the insect is similar to that of the retinal rods of vertebrates.

INTRODUCTION

The vertebrate photoreceptors are the retinal rods and cones. Each is composed of an inner segment and a rod or cone-shaped outer segment containing the photosensitive pigments, retinene$_1$ or retinene$_2$, (the aldehydes of vitamins A_1 and A_2) which are linked with a protein opsin to form pigment complexes, rhodopsin or porphyropsin for the rods, and iodopsin or cyanopsin for the cones[1]. The photoreceptors of the invertebrates, however, are more varied; they include eyespots, ocelli and compound eyes. The insect eye is a compound one, made up of ommatidia; in the housefly each ommatidium consists of a rhabdome of seven retinula cells. Each retinula cell has a differentiated structure, the rhabdomere, which is the photoreceptor and is similar in structure as well as in function to the vertebrate retinal rods[2-4]. However, little is known of the rhabdomere visual pigments as well as other associated pigments of the eye.

Recently GOLDSMITH[5,6] identified retinene$_1$ in the honeybee, and obtained evidence that the retinene is bound to a protein to form a photosensitive pigment complex with a maximum absorption at 440 mμ. BOWNESS AND WOLKEN[7] also had isolated from the housefly a photosensitive yellow pigment with a maximum absorption at 437 mμ. Since there were similarities in spectroscopic properties between this pigment and some retinene–protein complexes, it was thought that it might be the same type of complex as that found in the honeybee. Since then, we have also

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isolated retinene$_1$ from the heads of houseflies, and it now appears that a visual system in insects is chemically similar to that of the vertebrates.

METHODS AND MATERIALS

Materials

Houseflies (*Musca domestica*) were obtained from the Gulf Research and Development Co.*, and were packed in dry ice during transit. They were kept frozen in the freezer compartment of a refrigerator until they were used. The heads were detached and then separated from the residue by the freezing and sieving techniques of MOOREFIELD[8].

Fractionation and extraction

Three different extraction procedures were used, each of which ended with acetone extraction. Two of the methods follow those of GOLDSMITH[5,6]. Until the acetone extraction was completed, all operations were carried out under red dim light. Approximately 4,000 heads were used for each extraction.

1. The heads were ground with anhydrous sodium sulfate and acetone in a mortar and pestle, until no intact heads could be observed. The mixture was centrifuged at 3,000 rev./min, and the supernatant poured off and retained. The residue was re-extracted with acetone, and, after centrifuging, the supernatant was combined with the first extract. This procedure was continued until the extract appeared to be colorless.

2. The heads were ground with about 80 ml 0.2 *M* phosphate buffer, pH 6.5, and the mixture centrifuged at 12,000 rev./min for 20 min. The residue was extracted with acetone, while the supernatant was fractionated by saturation with ammonium sulfate at 45 % and 60 % (see ref. 5). The precipitates from the ammonium sulfate treatment were extracted with acetone.

3. The heads were ground with 20 ml of 50 % sucrose in *M*/15 phosphate buffer, pH 6.5, and the mixture centrifuged at 3,000 rev./min for 10 min. The residue was extracted with acetone, while the supernatant suspension was diluted with phosphate buffer to a sucrose concentration of 12.5 % and centrifuged at 14,000 rev./min for 20 min. The sedimented material was then extracted with acetone.

Chromatography

The initial acetone extract from each of the above preparations (1, 2, 3) was evaporated to dryness, and the residue was dissolved in petroleum ether. The petroleum ether extract was then dried over anhydrous sodium sulfate, evaporated to dryness, redissolved in petroleum ether and chromatographed on alumina (Fisher Adsorption Alumina, 80–200 mesh, obtained from Fisher Scientific Co., Pittsburgh, Pa.). The alumina was "weakened" by exposing it to a water-saturated atmosphere and allowing it to adsorb 5 g water/100 g alumina. The best results were obtained by chromatographing each extract twice. The first column was eluted with 40 ml of 40 % acetone in petroleum ether (v/v). The eluate was evaporated to dryness, redissolved in petroleum ether, and applied to a second column. This was developed, and fractions were eluted with some or all of the following solutions: 4 %, 10 %,

* Thanks to Dr. A. MALLIS Gulf Research Laboratories for supplying us with houseflies.

17.5 %, 25 % and 40 % acetone in petroleum ether (v/v). The effluent fractions were evaporated to dryness, then stored in a vacuum desiccator in the dark.

Examination of fractions

The dried fractions from the chromatograms were dissolved in 0.6 ml chloroform. The antimony trichloride test was performed by placing 0.25 ml of the chloroform solution in a quartz microcuvette and then adding 0.02 ml acetic anhydride and 0.5 ml antimony trichloride reagent. After mixing, the spectrum of the test solution was read against a blank containing chloroform, acetic anhydride and antimony trichloride, with a Beckman DK-1 recording spectrophotometer. The acetone extracts of the precipitate from the ammonium sulfate fractionation (procedure 2) and the extract of the material which sedimented in 12.5 % sucrose solution (procedure 3) were evaporated to dryness and dissolved in chloroform in order to perform the antimony trichloride test as above.

RESULTS

The result of a typical chromatogram of an acetone extract of the housefly heads is shown in Table I. This table in fact represents an average of several chromatograms;

TABLE I

FRACTIONS IN ORDER OF ELUTION FROM THE CHROMATOGRAM OF AN ACETONE EXTRACT

Fraction	*Acetone content of eluting fluid (% v/v)*	*Antimony trichloride tests*	
		Maximum absorption mμ	$E_{max}-E_{750}$
1	4	660–690	0.05
2	10	660–690	0.58
3	10	—	—
4	25	625–630	0.80
		690–695	0.27
5	25	665	0.90
6	40	650–660	0.07
7	40	—	—

individual chromatograms showed a better separation of one or more of the carotenoids present. Of the various carotenoids obtained, only retinene$_1$ can be identified with certainty. The fraction eluted with 25 % acetone in petroleum ether had an absorption maximum at 385 mμ in chloroform (Fig. 1) and, when reacted with antimony trichloride, a transient blue color with a maximum absorption at 665 mμ was observed (Fig. 2).

When the heads were extracted with phosphate buffer, the amount of carotenoid material remaining in the heads (as determined by the intensity of the blue color produced in the antimony trichloride reaction) was slightly less than the amount of carotenoid present in the untreated heads. However, when the heads were treated with the sucrose solution, this decrease was considerably greater. For the material identified as retinene$_1$, the value $E_{max}-E_{750}$ in the antimony trichloride reaction was: 0.90 for the untreated heads, 0.79 when the heads were extracted with phosphate buffer before acetone extraction, and 0.20 when they were treated with sucrose solution before acetone extraction.

Examination of the material removed by the sucrose and phosphate buffer treatments shows a similar result (see Table II). An acetone extract of the material precipitated from the phosphate buffer extract by 45 % saturation with ammonium sulfate showed a maximum absorption peak in the 635–645 mμ range, and an inflection at about 690 mμ when treated with antimony trichloride. Similarly, when

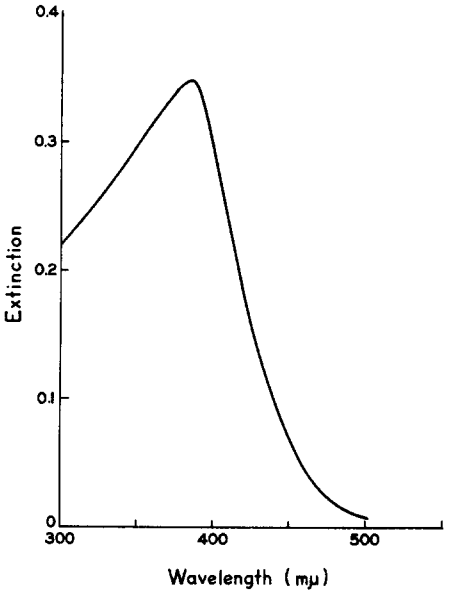

Fig. 1. Absorption spectrum of the retinene-containing fraction in chloroform. (Tracing of Beckman DK-1 spectrophotometer recording.)

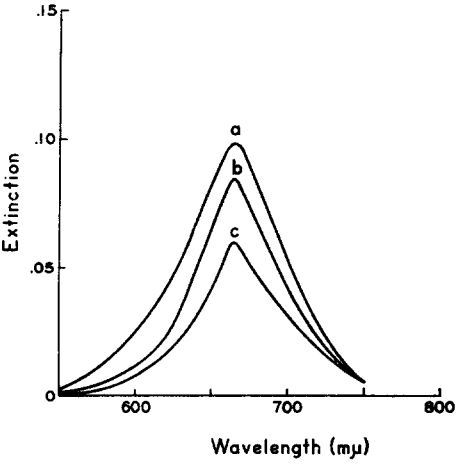

Fig. 2. Absorption spectrum of antimony trichloride reaction of the retinene-containing fraction: a, immediately after mixing; b, approximately 1 min after mixing; c, approximately 2 min after mixing. (Tracing of Beckman DK-1 spectrophotometer recording.)

TABLE II

PHOSPHATE BUFFER AND SUCROSE-IN-PHOSPHATE BUFFER EXTRACTS OF HOUSEFLY HEADS

Material	*Antimony trichloride test of acetone extract*	
	Maximum absorption mμ	$E_{max} - E_{750}$
Procedure 2		
1. Precipitate obtained by 45 % $(NH_4)_2SO_4$ saturation	635–645	0.32
	690	0.20
2. Precipitate obtained by 60 % $(NH_4)_2SO_4$ saturation	630–640	0.11
	690	0.05
Procedure 3		
1. Precipitate obtained on dilution of 50 % sucrose extract	650–660	0.64
	690	0.36

the concentration of ammonium sulfate was increased to 60 %, a maximum in the 630–640 mμ range and an inflection at about 690 mμ were found when the precipitate was extracted with acetone and reacted with antimony trichloride. However, when the sucrose extract was diluted with 3 volumes of phosphate buffer, and the resulting precipitate extracted with acetone, the antimony trichloride reaction gave a maximum

between 650 and 660 mμ—the only case in which the maximum approached the retinene peak of 665 mμ.

The amount of retinene present in the housefly heads may be estimated by comparison with the known $E^{1\%}_{1\,cm}$ value for retinene in the antimony trichloride reaction[9]. A figure of 0.31 μg of retinene/g fresh weight of heads was obtained, as compared with 0.22 μg of retinene/g fresh weight of honeybee heads reported by GOLDSMITH[5]. Using the average value of 24,500 rhabdomeres per eye[2], this corresponds to $3.7 \cdot 10^7$ molecules of retinene per rhabdomere, a figure within the range of visual pigment molecules per vertebrate retinal rod ($1 \cdot 10^6$–$1 \cdot 10^9$) for the vertebrates so far studied.

DISCUSSION

The presence of retinene$_1$ in the extract indicates that vitamin A should also be present, since, in the visual cycle, retinene$_1$ is reduced to vitamin A_1 (see ref. 1). In several chromatograms fractions were obtained whose antimony trichloride tests gave maxima near 620 mμ and 696 mμ, the maxima for vitamins A_1 and A_2; in some cases these were found in the same fraction (Fig. 3). However, these compounds cannot be positively identified. In all chromatograms where such fractions were obtained, they were eluted from the column before the retinene fraction rather than after it, as would be expected by their structure[10]. Thus, positive identification could not be made. A possible explanation for the absence of vitamin A is that the vitamin may be oxidized to retinene (or changed in some other way) during the procedure. The identification of vitamin A_2 is particularly crucial, since it is so far known to occur only in fresh-water fishes and in some amphibians; this would be the first instance of its presence outside of these vertebrate groups.

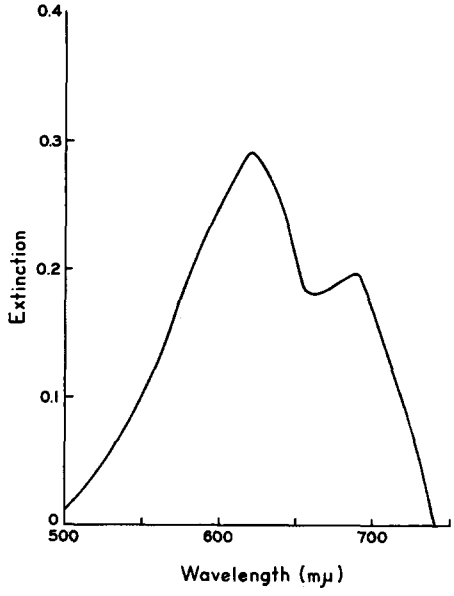

Fig. 3. Absorption spectrum of antimony trichloride reaction of fraction eluted with 17.5% acetone in petroleum ether (v/v) showing maxima at 623 mμ and 693 mμ. (Tracing of Beckman DK-1 spectrophotometer recording.)

GOLDSMITH[5,6] found that 80 % of the retinene-protein material in honeybee heads was extracted with phosphate buffer, and that most of this material could be precipitated by 60 % saturation with ammonium sulfate. The possibility cannot be excluded that a small amount of retinene was extracted with phosphate buffer from the housefly heads, but it was certainly much less than 80 % of the total. Whatever retinene was extracted with phosphate buffer must have been precipitated by 45 % saturation with ammonium sulfate, as there was no trace of the retinene band in the antimony trichloride test of the material precipitated by 60 % saturation (see Table II). Although the phosphate buffer does extract a photosensitive pigment complex, this complex may not be a typical rhodopsin. However, the presence of retinene is indicative of a rhodopsin.

Since the amount of retinene extracted from the housefly heads with phosphate buffer is so small, the light-sensitive yellow pigment previously isolated from the housefly[7] is probably not a rhodopsin. Observations since made on this pigment indicate that it may be related to the xanthommatins described by BUTENANDT, SCHIEDT AND BIEKERT[11], although none of the ommochrome class of pigments has previously been reported to be sensitive to light[12]. Also, there are similarities in spectroscopic properties between this housefly pigment and the photolabile yellow pteridine isolated from *Drosophila* by FORREST AND MITCHELL[13]. However, there is still the possibility that it may contain a retinene–protein complex. Less is known of the protein than of the chromophore, retinene. It has previously been indicated that such proteins would vary in different species[1,14]. A protein soluble in phosphate buffer would indicate a different protein from that of the vertebrate rods.

Of the other fractions eluted from the column, two contained light-stable carotenoids. The fraction eluted with 4 % acetone in petroleum ether was yellow in color and its absorption spectrum in chloroform showed maxima at 486, 455 and 426 mμ. These maxima correspond closely with those of lutein (xanthophyll) (see ref. 15). Another fraction, eluted with 25 % acetone after the retinene fraction, showed absorption maxima at 477, 450 and 427 mμ. Although this material cannot be definitely identified, the position of these absorption bands appears to be closest to "canary-xanthophyll" which has been found in some organisms together with esterified lutein[16].

ACKNOWLEDGEMENTS

This investigation was aided in part by grants from U.S.P.H.S. (B-397-C5 and C6). One of us (J.M.B.) was a Stoner-Mudge Foundation Fellow. I.J.S. was aided by a Fellowship Lions International-14B.

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