Sensitivity of Visual Receptors of Carotenoid-Depleted Flies: A Vitamin A Deficiency in an Invertebrate

Abstract. *House flies* (Musca domestica) *raised under sterile conditions on a diet lacking carotenoids and retinol (vitamin A) have visual receptor sensitivities —as assessed electroretinographically—which average more than 2 log units below normal, both in the near ultraviolet (340 mμ) and visible (500 mμ) regions of the spectrum. Loss of sensitivity can be prevented by the addition of β-carotene to the larval food. Flies reared for several generations on a carotenoid-free diet, but under conditions where the adults are not kept sterile, do not show a further loss of sensitivity. It is suggested that carotenoid stored in the egg prevents complete blindness in the first generation, and that microorganisms can supply small amounts of carotenoid and thereby prevent complete blindness in the second and successive generations.*

Vitamin A plays two roles in the lives of vertebrates. First it is necessary for proper growth, for its absence leads to lesions of the mucous membranes, loss of weight, and eventually death (*1*). Very little is known of the means by which vitamin A exerts its necessary effect in a healthy organism.

The second role of vitamin A—or retinol as it is now called (*2*)—is in vision. Retinal (formerly retinene or vitamin A aldehyde) is the chromophore of the visual pigments. In the dark, 11-*cis* retinal combines with proteins in the visual cells (called opsins) to form light-sensitive substances, of which rhodopsin, the pigment of the rods, is the most familiar example. In the light, the 11-*cis* chromophore isomerizes to the all-*trans* configuration; the retinal then no longer fits its site on the protein; and after changes in the structure of the protein the retinal is removed by hydrolysis (*3*). Free retinal is then reduced to retinol (*4*). The cycle is completed by reoxidation of the alcohol to the aldehyde (*5*) and, through an incompletely understood mechanism, by the reisomerization of the *trans* to the hindered *cis* configuration (*6*).

With variations, this role of retinal in vision is played in the invertebrate phyla as well, for the visual pigments of cephalopod molluscs (*7*) and arthropods (*8*) also are formed of retinal that is conjugated to protein. After irradiation, in the lobster, the retinal leaves its site on the protein only very slowly, and consequently retinol may not participate in the visual cycle (*9*). Nevertheless, the eyestalks of lobsters contain large storage depots of 11-*cis* retinol (*10*). Honey-bees, however, present a different story. Retinol forms from retinal during light adaptation, but there is no reserve of retinol of significant size, and in the dark it is virtually all reoxidized to the aldehyde (*11*).

In most instances the question of whether retinol or its derivatives are required by invertebrates for growth cannot be answered, largely because the appropriate experiments have not been done. The nutritional requirements of insects, however, have been studied extensively, and it is generally found that a dietary source of retinol or carotenoids is not required for growth (*12*). The participation of retinol and retinal in vision, though, suggests that insects reared in the absence of retinol or its provitamins should be unable to synthesize the normal amount of visual pigment and should exhibit a fall in visual sensitivity. To test this prediction, we have performed the following experiments.

A stock culture of house flies (*Musca domestica*) was maintained on the C.S.M.A. (*13*) medium, a fermenting mixture of alfalfa meal, brewer's grain, wheat bran, and yeast. These flies served both as controls and as an initial source of eggs for experimental animals. The carotenoid-free diet was that of Monroe (*14*), consisting of vitamin-free casein, celluflour, sodium oleate, cholesterol, ribose nucleic acid, agar, inorganic salts, and a mixture of B vitamins. The diet was placed in erlenmeyer flasks and autoclaved. Eggs were washed repeatedly in distilled water and the surfaces were sterilized by washing for 15 minutes in 0.1 percent hypochlorite ("Chlorox"). After being rinsed with sterile distilled water, the eggs were placed on the food under aseptic conditions.

Flies were maintained for 15 successive generations on the carotenoid-free diet. Adults were kept in open cages—not under sterile conditions—and were fed Monroe's adult diet (*14*). Eggs were collected on moist cotton, treated as already described, and placed on fresh medium (*15*).

Quantitative measurements of visual

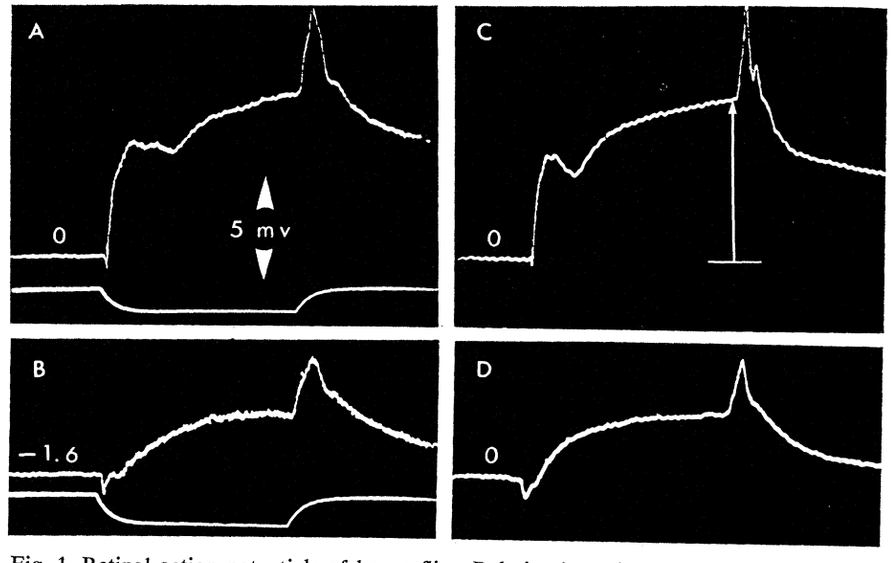

Fig. 1. Retinal action potentials of house flies. Relative intensity of the stimulus is given in logarithmic units at the beginning of each trace. *A*, *B*, control diet; *C*, Monroe's diet plus β-carotene; *D*, Monroe's diet (carotenoid-free). The animal raised in the absence of retinol or its provitamins (*D*) is only 2.5 percent as sensitive as the control (*A*, *B*), but normal sensitivity is maintained by β-carotene (*C*). Most of the carotenoid-deficient flies were even less sensitive than the example in *D*. The photocell response (lower trace, *A* and *B*) is 0.5 second. Stimulating light was 500 mμ. Log I = 0 corresponds to an energy flux at the cornea of 5×10^3 ergs sec^{-1} cm^{-2}. Negativity of the illuminated eye is indicated by an upward deflection; the 5 mv voltage calibration in *A* applies to all frames. The arrow in *C* indicates the component of the response on which determinations of sensitivity were based. This sustained negativity arises in the layer of sense cells.

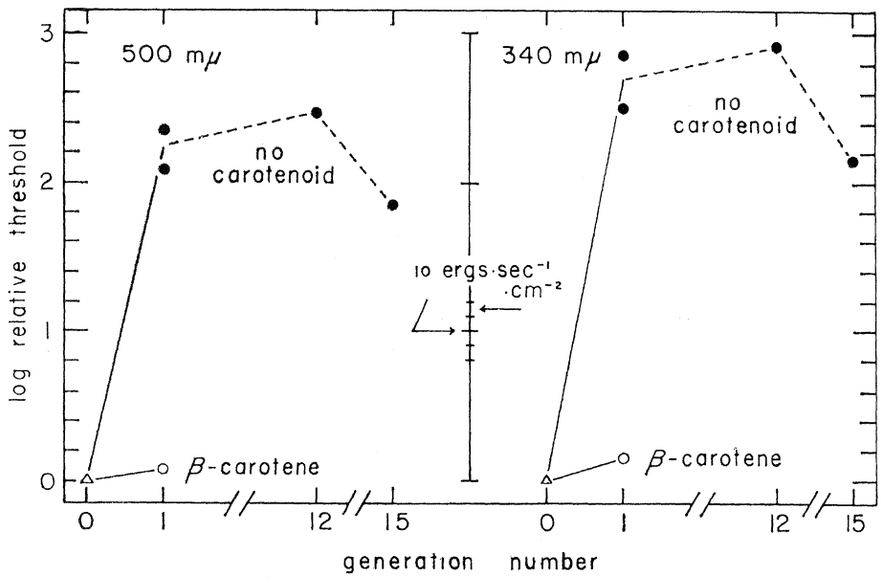

Fig. 2. Log relative threshold of flies reared on the C.S.M.A. diet (open triangles), Monroe's diet with no carotenoid (filled circles), and Monroe's diet supplemented with β-carotene (open circles). The relative energies necessary for a response of about 300 μv to a 0.5-second flash of light (Fig. 1) are given on the ordinate. In the case of the 500 mμ test light, the energy flux (log erg sec^{-1} cm^{-2}) required for the criterion response can be read directly along the left margin of the figure. The C.S.M.A.-grown flies were about 40 percent more sensitive to ultraviolet than to green light, however, and, in order that the curves on each side of the figure might start at zero on the ordinate, the scale for the 340 mμ test light has been shifted up 0.15 log unit, as shown in the center of the figure. Each point represents an average of about six animals.

sensitivity were made by an electrophysiological procedure 0 to 3 days after emergence. Flies were immobilized in soft wax and placed with one eye at the focus of a stimulating light. The electrical response to illumination, the retinal action potential, was recorded with silver–silver chloride electrodes connected to a high impedance, direct coupled amplifier and an oscilloscope. One electrode was a Ringer-filled pipette with a tip diameter of about 25 μ placed in a hole in the illuminated cornea; the reference electrode was a cotton wick situated on the opposite side of the head and shielded from the stimulating beam by a piece of aluminum foil.

The stimulating lights were narrow bands of wavelengths (10 mμ half band width) drawn from the spectrum of a high-pressure xenon arc lamp with a Bausch and Lomb grating monochromator. Duration of stimulus was controlled with a photographic shutter; intensity, with a pair of optical wedges.

Photographic records of retinal action potentials from flies on normal and experimental diets are shown in Fig. 1. For the purposes of this work, the transients at "on" and "off" were ignored, and all conclusions were based on the magnitude of the sustained negativity at a point just prior to the off effect. The response which was measured is indicated by the arrow in Fig. 1*C*. This component of the retinal action potential is known to arise in the layer of sense cells and reflects a depolarization of the retinular cell membranes (*16*).

Figure 1 shows that flies deprived of carotenoid have smaller retinal action potentials than normal. Their responses, however, can be matched in normal flies simply by altering the energy of the stimulating light (compare Fig. 1, *B* and *D*). The amount by which the intensity has to be changed in order to obtain identical responses is the difference in sensitivity. In these experiments sensitivity was based on the energy required to produce a response of about 300 μv; however, the selection of this particular criterion response, although arbitrary, is immaterial. Graphs of height-of-response as a function of log intensity for deficient animals were parallel to corresponding curves for controls (*17*). The horizontal displacement, in units of energy, is the decrease in sensitivity; and because the response-energy curves are parallel, the decrease in sensitivity is independent of the magnitude of the criterion response. Consequently, decrease in sensitivity is the same as increase in threshold; the two terms are equivalent. In determining sensitivity, height-of-response was routinely measured over a range of intensities.

Because the spectral-sensitivity function of flies exhibits maxima in both the near ultraviolet blue-green (*18*), each animal was tested at both 340 and 500 mμ. The responses to ultraviolet light were similar to those at 500 mμ, examples of which appear in Fig. 1.

Figure 1*C* shows that the decreased sensitivity of flies reared on the carotenoid-free diet was prevented by the addition of β-carotene (a provitamin A) to the larval food. The increase in threshold of animals fed the Monroe diet can therefore be attributed specifically to the absence of carotenoid and not some other difference between the C.S.M.A. and Monroe media.

The results of several experiments are summarized in Fig. 2. There are two major points to be made. First, in flies grown on the carotenoid-free diet the thresholds at both 340 and 500 mμ were raised. Furthermore, the presence of β-carotene prevented loss of sensitivity at both test wave lengths. This is very strong evidence that vision in the ultraviolet is mediated by a retinaldehyde chromophore. Interestingly, the rise in threshold observed in the absence of carotenoid was somewhat greater in the ultraviolet than in the visible. The explanation of this is not clear.

The second point is that the flies did not become completely blind, even after 15 generations on Monroe's diet. The threshold rose in the first generation and thereafter remained about 2.4 (1.85 to 2.92) log units (averages in different experiments) above normal. There appears to be a greater variance in a group of deficient animals than among controls, and the sensitivities in the 12th and 15th generations were not significantly different from each other or from the deficient animals in the F_1. With each individual deficient fly, however, the sensitivity was invariably less at 340 mμ than at 500 mμ, whereas in the control group the reverse relationship prevailed. This observation is the basis for the statement that the deficiency produces a greater effect in the ultraviolet than in the visible.

Carotenoid stored in the egg might prevent complete blindness in the first generation, but such an endogenous supply should be diluted rapidly in suc-

cessive generations. That the F_{12} and F_{15} were not much different in sensitivity from the F_1 suggests that trace amounts of carotenoid were made available to the later generations.

We believe that Monroe's diet is free of carotenoid and that the most likely source of carotenoid was microorganisms which either survived efforts to sterilize the eggs or reinfected the adults in each generation. In the latter case, carotenoid supplied to adults by the synthetic activity of microorganisms might then be passed to the next generation in the eggs. These possibilities are being studied.

Alternative explanations of the residual sensitivity can also be proposed. On the basis of present evidence, however, it seems unlikely that the fly has either a visual pigment with a totally different chemistry or the capacity for synthesis of carotenoid *de novo*. There is no precedent for either in animal tissues, and, moreover, should the fly synthesize carotenoid we might reasonably expect to find more than mere traces.

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15. House flies have recently been reared on these diets in the same manner (C. F. Cohen and R. J. Barker, *J. Cell. Comp, Physiol.* **62**, 43, 1963). No chemical evidence for retinol or retinal was obtained with flies grown on the carotenoid-free diet, but qualitative behavioral tests indicated the animals were still sensitive to light. Eggs from F_1 flies were extracted and analyzed for retinol, which was not found. The sensitivity of the test was reported to be 10^{-12} g per egg. A simple calculation indicates that the flies grown from such eggs might have as many as 10^6 molecules of retinal in each ommatidium, which is probably enough to account for our observations reported here.
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Specific Inhibition of Antibody Formation by Passively Administered 19S and 7S Antibody

Abstract. *Intravenously administered 7S antibody is more effective than 19S antibody in inhibiting the formation of antibody to bacteriophage φX174. Since considerable amounts of 7S antibody are needed for inhibition, serum antibody formation may act as a "feedback" mechanism to prevent hyperimmunization.*

Primary antibody formation can be prevented by mixing antigen with excess antibody before injection (*1*). Since passively administered diphtheria antitoxin can inhibit completely the primary antitoxin response when injected as long as 5 days after toxoid immunization (*2*) and since it can partially inhibit the secondary antitoxin response when injected prior to secondary immunization (*3*), we suggested that antibody formation may act as a "feedback" mechanism. These experiments (*2, 3*) were performed, however, with large amounts of hyperimmune serum, so that it was not clear whether this proposed mechanism acted only during hyperimmunization. We now report the capacity of antibody obtained at various intervals after immunization to inhibit antibody formation. Guinea pigs were injected intravenously with bacteriophage φX174 and homologous antiserum to this phage. This immunization system appeared advantageous since trace amounts of φX without adjuvants regularly stimulate antibody formation, and the kinetics of the primary 19*S*, primary 7*S*, and secondary 7*S* antibody responses are known and predictable (*4*).

Two preparations of bacteriophage φX174 (*5*) were used. Immunized animals were bled at 1 week and 3 to 4 weeks after primary immunization or 1 week after secondary immunization in order to obtain peak titers of the primary 19*S*, primary 7*S*, and secondary 7*S* responses, respectively. Serum antibody to φX (k) was determined by the phage neutralization assay (*6*); k is the velocity constant with the dimension minute^{-1} of the inactivation of phage by a particular antiserum, and it is a convenient measure of the concentration of neutralizing antibody. Thus, $k \times$ ml is a measure of the quantity of neutralizing antibody. Antibody inactivated by 0.1*M* 2-mercaptoethanol (2-ME) was considered to be 19*S* (γ_{1M-}); antibody not inactivated by 2-ME was considered to be 7*S* (*4*). Actively formed antibody was distinguished from passively administered antibody by also injecting the antiserums used into nonimmunized animals (*7*).

The antiserum to φX used for passive administration was prepared by immunizing guinea pigs with 10^{11} PFU (plaque forming units) of φX intravenously and bleeding at intervals afterwards as follows: for obtaining 19*S* antibody, at 6 or 7 days; for primary 7*S*, at 2 weeks; for secondary 7*S*, at 8 to 10 days after reimmunization. The 19*S* pool had a k value of 15, and less than 1 percent of the k value was due to 7*S* antibody. The primary 7*S* pool had a k of 20 and there was no detectable 19*S* antibody. Three antiserums having secondary 7*S* antibody were used: two with k values of 140 and 500, respectively (designated secondary 7*S* and secondary 7*S*-A, respectively), were each obtained from a guinea pig primarily immunized 1 month earlier; the third serum with a k of 500 (designated secondary 7*S*-B)