DERIVED PHOTOSENSITIVE PIGMENTS FROM INVERTEBRATE EYES

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Although most animals and plants are photosensitive, chemical data about visual pigments are restricted almost entirely to the visual purples of vertebrates. (For a recent review, see Hecht, 1942.) These substances are bleached by light and regenerate in the dark. Their absorption spectra correspond to the spectral luminosity function of the organisms (Koenig, 1894; Hecht, 1937; Wald, 1937).

Many invertebrates possess excellent eyes; some, like the squid, have eyes which contain all the important features of the vertebrate eye, including a retina composed of rod-like cells containing a clear red pigment and migratory black pigment granules. It is natural to extend the analogy of the vertebrate eye to the red pigment of the cephalopod retina by assuming it to be a light-sensitive pigment like the vertebrate visual purple.

Krukenberg (1882) challenged this analogy in his report that light has no effect on the squid pigment. His observation has been confirmed by Wald (1941) and others, but was rejected by Hess (1905) on the ground that the melanin in the intact retina masks the bleaching. This difficulty, according to Hess, could be avoided by inducing opacity of the retina with formalin.

Re-examination of this problem showed that both positions are correct. Following Hess' procedure, a dark-adapted squid retina was cut in half and immersed in 10 per cent formalin. No change occurred in the portion kept several hours in the dark; but it was astonishing to note that the part brought into the rays of the microscope lamp bleached in less than 5 minutes from bright red to dull gray. Obviously formalin had rendered the pigment photosensitive.

The red pigment may be brought into solution by means of saponin. Two retinas from a large squid are placed in 4 cc. of 4 per cent saponin solution. After 4 to 8 hours the insoluble residue is removed by centrifugation, and the brownish red supernatant removed for study. The solution behaves like the original retina. If it is illuminated for 1 hour by a 100 watt projection bulb at a distance of 6 inches through a water filter, no change in its color or photometric density can be detected. If, however, formalin is first added to make a concentration of 10 per cent, the same light causes an unmistakable bleaching.

Essentially the same results are obtained if another denaturant, like 10 per cent ethyl alcohol, is used instead of formalin. The solution decolors on exposure to light.

The quantitative changes which these solutions undergo in the visible spectrum have been studied by means of a photoelectric spectrophotometer. The apparatus includes a monochromator, the light from which passes through a 1 cm. double cell mounted on a shuttle, and impinges on a rubidium photocell. A one stage amplifier is controlled by a potentiometer reading directly in per

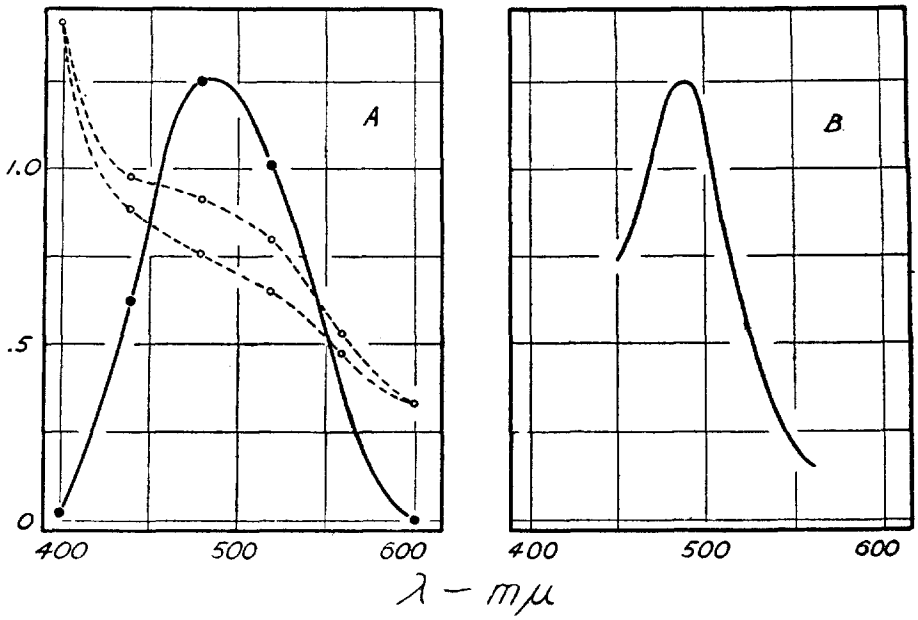

FIG. 1. Spectral sensitivity of squid pigment. In A the broken lines show the density spectra before and after bleaching of an extract of a squid retina in 2 cc. 4 per cent saponin brought to 10 per cent ethanol, while the continuous line shows the 10 times density difference spectrum due to illumination of the same solution. In B is given Piper's curve for spectral sensitivity of *Eledone*, recalculated for equal energy spectrum and equal response.

cent transmission. Results are expressed as photometric density according to the equation $D = \log (I_{\text{incident}}/I_{\text{transmitted}})$.

Measurements are made in accordance with either of two procedures. If the density of the experimental solution is desired, it is placed in one side of the cell, and the solvent in the other. If the effect of light is to be determined, the blank contains the same solution as the experimental. This method has the virtue of cancelling out changes not due to light, and permits accurate measurement of changes in regions of high density.

Fig. 1A shows the absorption spectrum of one such saponin solution containing 10 per cent alcohol before and after its exposure to light, as well as the

difference between the two due to the bleaching action of the light. The difference spectrum is an approximately symmetrical curve whose maximum is at 480 mμ. The same maximum occurs with formalin-treated solutions.

It is significant that the absorption curve of the artificially rendered photosensitive material is similar to the spectral sensitivity of the squid's eye. The best available data are those of Piper (1904) who measured the magnitude of the retinal electric response given by *Eledone moschata* in the spectrum of a Nernst lamp. Data of this type do not constitute a sensitivity curve because of unequal energy distribution of the Nernst lamp spectrum, and the lack of knowledge of the relation between response and energy. However, a rough approximation may be made first by correcting the data in terms of Pflüger's (1902) measurements of the spectral energy curve of a Nernst lamp; and second, by using Chaffee and Hampson's (1924) formulation of the relation of the frog retinal response to energy at low levels of response. This states that $R \sim (I)^{1/2}a$, where I is the relative energy of the Nernst lamp at a given wavelength, and a is the spectral sensitivity. The spectral sensitivity curve computed in this way is in Fig. 1B and shows a rough agreement with the bleaching spectrum in Fig. 1A. Closer agreement should not be looked for until the spectral sensitivity of the squid eye is measured in terms of the energy required for equal response in the spectrum.

The squid is not unique in the possession of a pigment which becomes light-sensitive after formalin treatment. So far I have examined only the blue soft shelled crab, *Callinectes hastatus*, and the horseshoe crab, *Limulus*; in spite of their taxonomic distance from the squid they contain similar pigments which become light-sensitive on treatment with formalin. The data are shown in Fig. 2.

The response of an extract depends somewhat on the treatment. Thus, the prolonged bleaching of the formolized pigment of the crab results in a secondary maximum at about 590 mμ, as shown in Fig. 3. Another anomaly is shown by extracts of dried squid retinas, in which bleaching is maximal at 455 mμ. One extract of fresh squid eyes showed an interesting resemblance to vertebrate visual purple in that after bleaching it regenerated in the dark to within 50 per cent of its original concentration.

These formalin-sensitized light-sensitive pigments should not be confused with the melanin-like pigments found in insects and also in the squid. Thus the red screening pigment in the retinal sheath cells of *Drosophila*, when treated with formalin, does not bleach in light and neither does the melanoid pigment from the squid eye.

Escher-Desrivieres, Lederer, and Verrier (1938) have purified such a red pigment extraced from squid retinas by dilute alkali. Examination of retinas treated in this manner shows clearly that the great mass of the pigment they studied is composed of dissolved melanin granules. Its properties are dis-

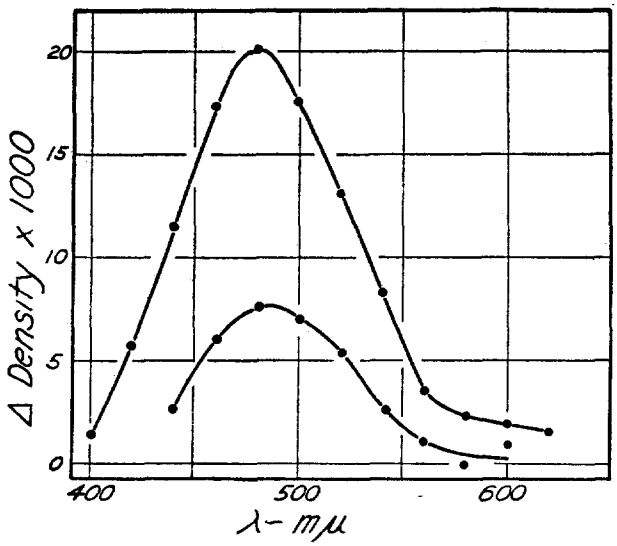

FIG. 2. Absorption spectra of derived retinal pigments. The upper curve is the difference spectrum for *Callinectes* derived from one retina dissolved in 1 cc. 4 per cent saponin, and brought to 10 per cent formalin. The lower curve is 2 times the difference spectrum for *Limulus* derived from 15 retinas of 4 cm. wide animals, dissolved in 3 cc. 2 per cent alkaline digitalin, and brought to 10 per cent formalin.

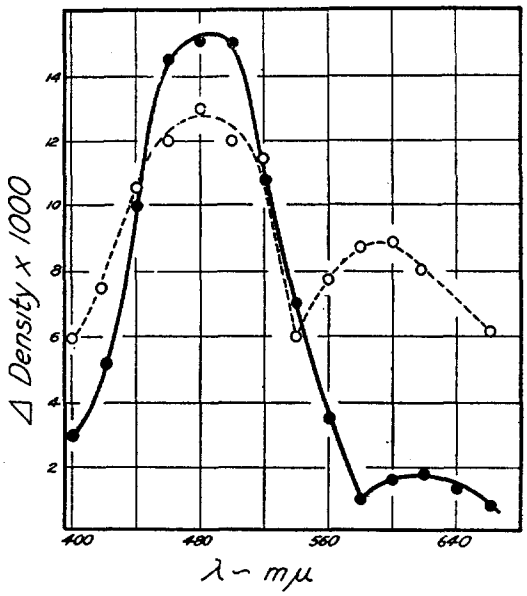

FIG. 3. Prolonged bleaching of *Callinectes* pigment. The continuous curve gives the difference spectrum due to the 1st hour of bleaching, while the broken curve gives the difference spectrum due to the 2nd hour of bleaching.

tinctly different from those of the homogeneously distributed red pigment of the cephalopod rods. The granular pigment is stable in the presence of acid and alcohol, and is insoluble in detergents; the homogeneously distributed pigment is destroyed in the dark by acid, and in the light by alcohol and formaldehyde, and is extractable by detergents like saponin and digitonin, used to dissolve visual purple.

If the residue from a saponin extraction of the squid's eye is treated with dilute alkali, a magenta pigment is obtained whose absorption spectrum shows

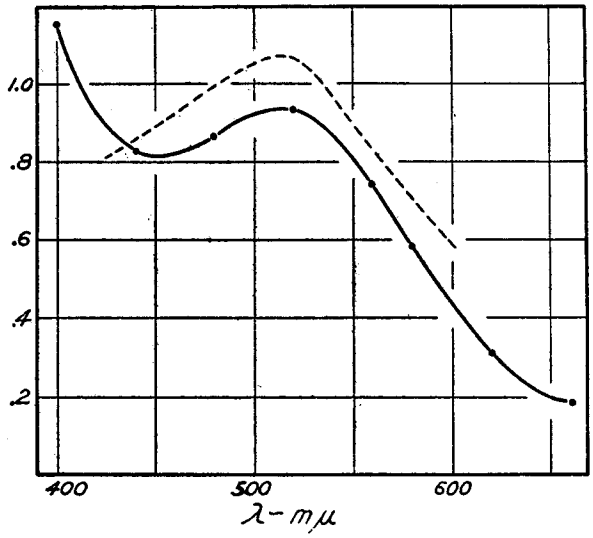

FIG. 4. Absorption spectra of non-light sensitive pigments. The continuous curve is the absorption spectrum of the residue from a saponin extract of squid retina, dissolved in 0.1N NaOH. The broken curve is the absorption spectrum of the "prosthetic group" from squid retinal chromoprotein, according to Escher-Desrivieres, Lederer, and Verrier.

a maximum at 510 mμ. An example is in Fig. 4. This pigment may be related to the "prosthetic" group of Escher-Desrivieres, Lederer and Verrier, obtained by hot acid hydrolysis from their 95 per cent phenol-soluble "chromoprotein." However, it is unaffected by light in the presence of formalin.

In its bleaching by light, the formalin-sensitized pigment shows some chemical similarity to visual purple because it liberates retinene. Retinene was first found in the squid eye by Wald (1941) who showed that, although the total potential concentration of retinene in the fresh squid retina is high (which I can confirm), only about 10 per cent of it is released by exposure to 45 minutes of daylight. In the present experiments retinas first treated with formalin release large quantities of retinene on illumination.

Retinas from freshly decapitated animals were removed immediately in daylight to filter paper and dried in a vacuum desiccator. They were then shaken for 30 minutes with petroleum ether to remove free carotenoids. A pair of weighed retinas was soaked 5 minutes in water and placed in 10 per cent formalin for 15 minutes. One retina was illuminated 15 minutes by a 100 watt projection bulb at 6 inches distance through 3 inches of water and a yellow filter (Corning 368 half thickness) to protect the liberated retinene from photic destruction. The second retina served as a dark control, but otherwise received similar chemical treatment. After washing for 5 minutes and then drying, the retinas were again shaken with petroleum ether for 30 minutes.

TABLE I

Retinene Production in the Retina of the Squid

The figures give the photometric density of retinene in $CHCl_3$, at 390 mμ, per mg. dry weight of retina.

Treatment	Extraction	Retinene concentration	
		Retina in light	Retina in dark
First illumination, 15 min.	1	0.0060	0.0031
	2	0.0051	0.0016
	3	0.0027	0.0017
	4	0.0019	0.0008
	5	0.0007	—
	Total	0.0164	0.0072
Second illumination, 15 min.	1	0.0008	0.0031

Each solution was evaporated to dryness and the residue taken up in 2 cc. of chloroform for spectrophotometric analysis. Table I gives the retinene content of six successive petroleum ether extractions of the same pair of retinas. After all the retinene was extracted, the retinas were again treated with formalin and extracted. It is evident that considerable retinene is released by the formol treatment itself, but that an illumination of 15 minutes releases nearly all of the remaining retinene.

Just how related all this is to the vision of the squid, it is hard to say. It may merely be that the normal squid photopigment is relatively light-stable, and that the formalin treatment renders it light unstable. In that case serious consideration must be given to the possibility that the bleaching of vertebrate visual purple is a specialization and that the absence of bleaching, as in photosynthesis and photodynamic action, may have no direct bearing on the efficiency of a visual pigment.

SUMMARY

The red pigment in the eyes of the squid, blue crab, and horseshoe crab becomes photosensitive when treated with formalin, and bleaches in the light. The resulting change in density is approximately symmetrical around a maximum at 480 mμ in the blue green. This difference absorption spectrum is in rough agreement with the spectral sensitivity of the cephalopod eye and differs only slightly from the difference absorption spectrum of vertebrate visual purple.

The formalin-sensitized pigment is not melanoid. Its bleaching in squid retinas releases large quantities of retinene.

It is suggested that the light sensitivity of the normal squid photopigment may be independent of its light stability.

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