SPECIAL ARTICLES

OVOVERDIN, A PIGMENT CHEMICALLY RELATED TO VISUAL PURPLE

VISUAL purple, according to the beautiful experiments of George Wald,[1] belongs to the class of carotenoid-proteins.[2] We have studied another representative of these widely distributed conjugated proteins, namely, the green pigment of the egg of the lobster (*Homarus americanus*). The carotenoid component of the pigment, which was first studied by Miss Newbigin[3] in 1897, has recently been shown to be an ester of astacene (4, 5, 4′, 5′-tetraketo-β-carotene) with an as yet unknown organic acid.[4,5] In the egg, this "ovoester" is linked to a protein of albuminoid character. The name *ovoverdin* is hereby proposed for this pigment. The free ovoester is orange-red and its crystals are practically insoluble in water; the chromoproteid ovoverdin is grass-green and readily water soluble.

Solutions of ovoverdin are obtained by crushing the green lobster eggs with sand and subsequent extraction with distilled water. The dark green, turbid solutions are treated with an equal volume of saturated ammonium sulfate solution. Filtration removes oil globules containing carotene and small amounts of globulins. Such partly purified preparations have been used for most of the present experiments because they contain no other pigment besides ovoverdin; and because, when stored in the dark and at low temperatures, they appear to be somewhat more stable than dialysed solutions or more highly purified preparations which may be obtained by repeated salting-out of the pigment by full saturation with ammonium sulfate.

Visual and photoelectric spectrophotometry reveals two absorption bands in the visible, centering around 6400 and 4700 A. The isoelectric point was found by cataphoresis to be in the vicinity of pH 6.7, close to the isoelectric point of hemoglobin. The rate of sedimentation in the field of the ultracentrifuge (see the following note by Dr. Wyckoff) indicates a molecular weight of the order of 300,000. According to rough calculations one molecule of the pigment appears to contain one molecule of astacene ester.

Alcohol, acetone, chloroform, pyridine, benzol, dioxane, but not petrol ether, liberate rapidly the orange ovoester from the green pigment. The protein is coagulated by this treatment. The pH-stability range of ovoverdin extends from about pH 4 to 8. Acids, *e.g.*, acetic acid, and alkali destroy the complex and liberate the orange-colored carotenoid. Ovoverdin is comparatively stable against dilute but not against concentrated ammonia. The pigment is much more light stable than visual purple. However, solutions of ovoverdin or films prepared with the aid of gelatin bleach to a straw yellow shade when exposed to diffuse daylight for one or two days at room temperature. Lactoflavin accelerates the bleaching process. The solutions of the ovoester in organic solvents fade even more quickly and yield colorless decomposition products which, in contrast to those of retinene, the carotenoid part of visual purple, give no positive Carr-Price test for vitamin A.

The most remarkable property of ovoverdin so far observed is that of *reversible thermal dissociation*. When the pigment solutions are rapidly heated to from 65° to 70° the color turns from grass green to bright orange-red. The absorption spectrum of the red form shows an increased extinction at 4800 A. and an almost complete abolition of the band of ovoverdin in the red, around 6400 A. Upon rapid cooling the green color reappears. This phenomenon is observed only in the presence of neutral salts. If the pigment is exposed to heat for longer periods the color change to red is no longer reversed by cooling. If the temperature is allowed to reach 80° to 100°, a turbidity appears and eventually an orange-pink protein coagulum is formed. These observations are interpreted as follows: The first step consists in a reversible dissociation of the chromoproteid into the ovoester and the free protein carrier. The ovoester may form a colloidal solution. In the second stage the protein is denatured by heat and thereby rendered incapable of recombining with the carotenoid upon cooling. The final stage consists in the coagulation of the denatured protein and adsorption of the ovoester by the precipitate.

The reversible and irreversible heat dissociation may also be observed with intact lobster eggs.

It has been suggested[1,6] that the bleaching of visual purple to visual yellow by light represents a disruption of the carotenoid-protein complex with liberation of the orange-colored carotenoid, retinene. It is assumed that this is due to a denaturation of the protein by light. However, when the energy of heat inactivation of visual purple of about 75,000 cal./mol. is compared with the energy content of the light at the effective wave-lengths, beginning at 5300 A. (53,000

[1] G. Wald, *Jour. Gen. Physiol.*, 19: 351, 1935–36.

[2] Literature in L. S. Palmer, "Carotenoids and Related Pigments," Chem. Catalog Co., New York, 1922.

[3] M. I. Newbigin, *Jour. Physiol.*, 21: 237, 1897.

[4] R. Kuhn and E. Lederer, *Ber. Deutsch. Chem. Ges.*, 66: 488, 1933.

[5] P. Karrer, L. Loewe and H. Huebner, *Helv. Chim. Acta*, 18: 96, 1935.

[6] A. E. Mirsky, *Proc. Nat. Acad. Sci.* (Washington), 22: 147, 1936.

cal. per quantum) there remains a deficit between the energy required for denaturation and the energy actually available.

The present observations would suggest that the primary step in the bleaching process of visual purple is not a denaturation of the protein carrier but perhaps a photodissociation of the type of the reversible thermal dissociation of ovoverdin. Such a conception would account for the rapidity of regeneration of visual purple under physiological conditions; at the same time, it would help to understand why the visual cycle proceeds at an energy level below that of protein denaturation.

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THE SEDIMENTATION CONSTANT OF OVOVERDIN

THROUGH the courtesy of Dr. K. G. Stern and Dr. K. Salomon and with their help, preliminary ultracentrifugal studies have been made of rates of molecular sedimentation in ovoverdin solutions prepared from lobster eggs according to the method described in the preceding note.[1] Several points of interest concerning the degree of purity of such solutions and the order of molecular size and homogeneity of the pigmented protein they contain are brought out in this way.

These studies of sedimentation rates are based on series of photographs made with an analytical air-driven ultracentrifuge[2] arranged for measurements according to the original absorption method of Svedberg. Exploratory examination of a solution of the protein in half-saturated ammonium sulfate indicated the presence of a homogeneous protein so light that it should be sedimented from a solvent of lower density. All subsequent measurements were accordingly made, using as starting material a solution freshly dialyzed to remove all but a trace of the ammonium sulfate.

The pigmented solutions of ovoverdin are dark green in color. They show[1] a very strong light absorption in the blue and violet and an appreciable absorption in the yellow; besides this they have the usual protein ultra-violet absorption of wave-lengths below ca 2800A. Series of sedimentation photographs have been made with blue light and with ultra-violet light shorter than 2700A; simultaneous visual obser-

[1] K. G. Stern and K. Salomon, SCIENCE, current issue.
[2] J. Biscoe, E. G. Pickels and R. W. G. Wyckoff, *Jour. Exp. Med.*, 64: 39, 1936; R. W. G. Wyckoff and J. B. Lagsdin, *Rev. Sci. Instr.*, 8: 74, 1937.

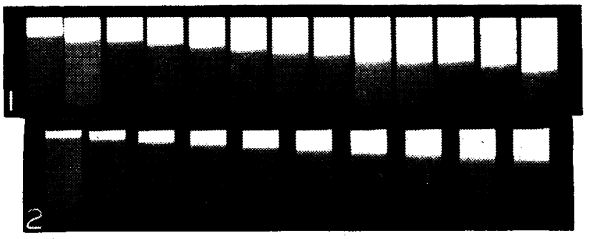

FIG. 1. Sedimentation photographs of ovoverdin using blue light. Source—high pressure mercury arc. Filter—Jena BG 12. Photographic film—Agfa Process. Centrifugal field—ca 90,000 g. Interval between photographs—5 minutes. Exposure—ca ½ second. Fig. 2. A series of sedimentation photographs of ovoverdin, using ultra-violet light. Source—high pressure mercury arc. Filter—chlorine plus bromine cells. Centrifugal field—ca 90,000 g. Interval between photographs—5 minutes. Exposure 1.5 seconds.

vations were made with yellow light. A photograph with blue light is reproduced in Fig. 1; the second figure is one of the ultra-violet series. In order to have sufficient absorption in the blue, a four millimeter thick cell was used with a solution containing ca 22 milligrams of protein per cc. The sedimentation constant calculated from the diagram of such a solution (Fig. 1) was $s_{20°} = 10.3 \times 10^{-13}$ cm sec^{-1} dynes^{-1}. This solution is far too concentrated to give optimum results with ultra-violet illumination, but a series of photographs made with prolonged exposures showed boundaries yielding the same sedimentation constant—$s_{20°} = 10.4$. It is thus apparent that the blue-absorbing molecules sediment at the same rate as those that absorb in the extreme ultra-violet; from the visual observations it is clear that the substance responsible for the absorption in the yellow comes down at substantially the same rate. These results suggest that the yellow and blue absorptions are due to the same pigment and that this pigment forms part of the protein which presumably is responsible for the ultra-violet boundary.

Besides this pigmented substance furnishing the boundary with $s_{20°} = 10.3$, these solutions contain an appreciable amount of "unsedimentable" material transparent to visible light but showing a marked ultra-violet absorption. This impurity is not sedimented in the centrifugal fields that have been used. It must therefore be lighter than egg albumin. If it is a protein the viscosity of its solution could be great enough to retard the sedimentation of the large pigmented molecules. This possibility was tested by making sedimentation pictures of the original protein solution after dilution with two parts of water and with eight parts of physiological saline. The slightly higher values of $s_{20°}$ thus obtained—ca 10.8—are probably very near the true sedimentation constant for ovo-