Butenandt, A., Schiedt, U., and Biekert, E. (1954), *Justus Liebigs Ann. Chem. 586*, 229.
Carr, E. H., and Price, E. A. (1926), *Biochem. J. 20*, 497.
Dyer, J. R. (1965), Applications of Absorption Spectroscopy, Englewood Cliffs, N. J., Prentice-Hall, p 29.
Fox, H. M., and Vevers, G. (1960), The Nature of Animal Colours, London, Sidgwick and Jackson, p 50.
Linzen, B. (1966), *Z. Naturforsch., B 21*, 1038.
Linzen, B. (1967), *Naturwissenschaften 21*, 259.
Long, C., Ed. (1968), *in* Biochemist's Handbook, Princeton, N. J., Van Nostrand, p 87.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Osanai, M., and Koga, N. (1966), *Z. Physiol. Chem. 12*, 1295.
Pfleiderer, W. (1963), *Angew. Chem. 75*, 993.
Riddiford, L. M. (1968), *Science 160*, 1461.
Shine, H. J., and Mach, E. E. (1965), *J. Org. Chem. 30*, 2130.
Thomson, R. H. (1962), *Comp. Biochem. 3*, 727.
Ziegler, I. (1961), *Advan. Genet. 10*, 139.

Purification and Characterization of an Ommochrome–Protein from the Eyes of Saturniid Moths*

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ABSTRACT: An ommochrome–protein has been extracted from the eyes of the silkmoth *Hyalophora cecropia* with the cationic detergent cetyltrimethylammonium bromide. The protein was purified by Sephadex G-200 and agarose gel filtration. It is homogeneous as judged by rechromatography and disc electrophoresis and has a molecular weight of 24,000 ± 1500. When oxidized by hydrogen peroxide, the pigment–protein shows absorption maxima at 440 and 470 nm and a shoulder at 510 nm. Upon reduction with ascorbic acid, a single broad peak at 510 nm results. Determination of the oxidation–reduction potential of the ommochrome–protein was accomplished by single point equivalency determinations; a value of 196 ± 7 at pH 7.0 was obtained. The chromophore, a substituted phenothiazine, appears to be covalently linked to its protein carrier and may be hydrolyzed by a variety of acidic or basic solvents. When free in solution at neutral pH, the chromophore chelates divalent cations, including Cu^{2+}, Fe^{2+}, and Ca^{2+}.

These results are discussed in relation to the biological significance of the ommochrome pigments which are found in the optic lobes, ocelli, and nerve cords of certain moths.

Although direct supporting evidence is scanty, the existence of polymeric or macromolecule-bound ommochromes has been suggested with regularity in reviews about the visual system of insects (Ziegler and Harmsen, 1969). Goldsmith (1964) reported that ommochromes in retinula cells "are usually bound to protein in discrete granules," and subsequently three electron microscopic studies have confirmed this opinion (Fuge, 1966; Schoup, 1966; Langer, 1967).

Attempts to identify the pigment–protein complexes by biochemical means have met with partial success. Bowness and Wolken (1959) claimed to have isolated a light-sensitive yellow pigment from housefly eyes, whose spectroscopic properties resembled those of xanthommatin and whose adsorptive properties on calcium phosphate columns indicated a protein. Osanai (1966) obtained an aqueous extract with redox properties from the integument of *Hestina japonica*, and he concluded that it was an ommochrome–protein of the xanthommatin type. In a more detailed study, Yoshida and Ohtsuki (1966) succeeded in partially characterizing, by extraction and gel filtration with cetyltrimethylammonium bromide,[1] a "photoactive" xanthommatin of large molecular size—presumably a protein-bound pigment—from the ocelli of the anthomedusan *Spirocodon saltatrix*.

In the preceding paper we identified an oxidation–reduction pigment from the eyes and nervous system of saturniid moths (Ajami and Riddiford, 1971). Our preliminary results showed that it was bound to a protein, and this paper discusses the isolation and characterization of such a protein from the eyes of *Hyalophora cecropia*.

Materials and Methods

1. Preparation of Tissues for Extraction. H. cecropia were obtained and treated as described previously (Ajami and Riddiford, 1971). Freshly emerged adult moths were decapitated and the compound eyes carefully dissected. These were washed twice with distilled water by homogenization, centrifugation (10,000*g* for 10 min), and decantation of the supernatant. The sediment was lyophilized and stored at −20° until used, but for no more than 48 hr.

2. Buffered Extraction. The eye powder was precipitated with acetone; then after centrifugation and decantation of the supernatant, we extracted for 1 hr at 25° the equivalent of two eyes (either consecutively or separately) with 1 ml of one of the following buffers: 0.05 M potassium chloride–hydrogen chloride (pH 2.0); 0.05 M sodium acetate–acetic acid (pH 5.0); 0.05 M sodium phosphate (pH 7.0); 0.05 M Tris-HCl (pH 7.0); 0.05 M Tris-HCl (pH 8.3); 0.05 M ammonium bicarbonate (pH 8.8); 0.05 M sodium carbonate (pH 10.0). The solutions

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: CTAB, cetyltrimethylammonium bromide.

were cleared routinely of particulate and colloidal material by centrifugation (30,000*g* for 30 min) and filtration through solvent-resistant filters (0.30-μ pore size). Their visible absorption spectra were then scanned in the Cary 14 spectrophotometer, both before and after dialyses against 100 volumes of the solubilizing buffer. We also lyophilized an aliquot of each of these solutions and treated the residue with 0.5% acidic methanol for thin-layer chromatography on silica gel with methanol–butanol–benzene–water–HCl (10:5:5:5:1, v/v) as developing solvent (Ajami and Riddiford, 1971).

3. CTAB Extraction. The frozen eye powder was suspended in CTAB solutions ranging from 0.01 M to 0.08 M in 0.05 M sodium phosphate buffer (pH 7.0) at room temperature. We found no appreciable difference in the solubilizing capacity of the surfactant solutions more concentrated than 0.03 M. Therefore, we chose 0.04 M as the desirable concentration for subsequent procedures since it had been used by Heller (1968) for the purification and characterization of bovine visual pigment.

Ommochrome–proteins were extracted from the lyophilized equivalent of 40 eyes in 5 ml of 0.04 M CTAB in 0.05 M sodium phosphate buffer (pH 7.0). The eye residue was dispersed in the detergent solution by vigorous homogenization and quick freezing and thawing followed by incubation at 25° for about 4 hr. Soluble pigment was separated from insoluble matter by centrifugation (30,000*g* for 30 min) and Millipore filtration. The pellet was reextracted in CTAB as indicated above, and the two extracts were pooled. The resulting reddish brown CTAB solution could be kept at room temperature for at least 8 hr and at 4° for several days without change in its visible absorption spectrum. Whenever a flaky sediment appeared after prolonged storage, the pigment solutions were discarded.

In certain cases the lyophilized eye powder was preextracted by thorough homogenization in distilled water in order to produce a suspension of ommatidial pigment granules. Low-speed centrifugation (3000*g* for 30 min) freed the suspension of cell debris, and the granules could be harvested either by precipitation with ascorbic acid at pH 3–4 or by ultracentrifugation (100,000*g* for 1 hr). We treated the resulting pellet with detergent as previously described.

4. G-200 Sephadex Fractionation. We equilibrated a Sephadex G-200 column (45 × 2.5 cm) with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.04 M CTAB, then layered 1- to 2-ml samples of the CTAB-solubilized eye pigment in 5% sucrose (w/v), and eluted with buffer–detergent at a rate of 45 ml/hr. We determined elution curves by measuring optical densities at 440 nm for each 3.0-ml fraction. Peak fractions were routinely dialyzed twice against 100 volumes of the pH 7.0 phosphate buffer, and then against buffered CTAB of the same concentration as used for extraction and gel filtration.

5. Molecular Weight Determination. GEL FILTRATION. Four-milliliter aliquots of eye pigment extract were applied to a column of G-200 Sephadex and eluted with CTAB–buffer. The reddish brown peak fractions were diluted with ethanol to a final concentration of 80% (v/v). After 5 hr at 25° denatured protein precipitated and was collected by centrifugation. We washed the sediment with distilled water, resuspended it in a minimal volume (3–5 ml) of CTAB–buffer by homogenization, and centrifuged (30,000*g* for 30 min). The resulting solution was dialyzed for 12 hr with one change against a 100-fold excess of surfactant solution.

Gel filtration chromatography for molecular weight determination was performed as follows: a column (2.5 × 40 cm) of either Sephadex G-200 or agarose (Bio-Gel A 1.5 m) was equilibrated as before with 0.04 M CTAB in 0.05 M sodium phosphate buffer (pH 7.0), at 25°. Bed volumes were approximately 200 ml. The columns were operated with downward flow of eluent at a constant rate of 30 ml/hr under a water head pressure of 15 cm. Samples of purified pigment containing 5% sucrose (w/v) were applied in 1-ml volumes; 3-ml fractions were collected from the time the sample penetrated the gel surface, and column effluents were monitored by their absorbancy at 280 and 470 nm. In order to standardize the columns, 0.25% solutions of proteins of known molecular weight [bovine serum albumin (monomer and dimer), ovalbumin, carbonic anhydrase, chymotrypsinogen, myoglobin, lysozyme, horse heart cytochrome *c*, insulin, glucagon, and bacitracin] were eluted, both before and after the experiment with the eye extract. We also used Blue Dextran 2000, molecular weight 2×10^6, to identify the void volume.

In order to identify chromophores, the peak fractions were treated in the same way as the buffered extracts (section 2).

6. Molecular Weight Determination. ELECTROPHORESIS. Polyacrylamide gel electrophoresis in 10% gels containing sodium dodecyl sulfate was performed according to Weber and Osborn (1969). Lyophilized eye powder was extracted into 0.01 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. After incubation for 2 hr at 37° and centrifugation to remove suspended material, the protein solution was dialyzed 4 hr against the extraction buffer. Then 10–50 μl of this preparation, adjusted to a protein concentration of approximately 0.02 mg/ml with appropriate sample buffers, was layered onto gels. Ethanolic precipitates of peak fractions from gel filtration were treated in a similar manner. We calibrated the system with the marker proteins used in section 5. These were applied in concentrations of 0.01 mg/ml.

7. Measurements of Oxidation–Reduction Potential. Redox potentials were determined at 25° by single potentiometric readings at the equivalence point between oxidized and reduced ommin–protein (Volke, 1963), as defined by the following form of the Nernst equation: $E_h = E_m' + RT/NF \ln$ ([oxidized pigment]/[reduced pigment]), where R is the gas constant; T, the absolute temperature; F, the faraday constant; N, the number of reducing equivalents; and E_m', the midpoint potential at a specified pH value.[2] E_h, the corrected potential with reference to the hydrogen half-cell, was determined with a Fisher Model 210 pH meter fitted with platinum and saturated calomel electrodes. The equivalence point, where [oxidized ommin–protein]/[reduced ommin–protein] equals 1, was calculated by spectrophotometric titration in the Zeiss PMQ II spectrophotometer with hydrogen peroxide (20% Superoxol) as oxidant and ascorbic acid (20%) as reductant. These reagents were added to the partially purified ommin–protein solution in increments of 10 or 25 μl. Reduction was followed by the decrease in absorbancy at 440 nm, and oxidation by an increase in absorbancy ratio at 470/510 nm. We also investigated the changes in E_m' from pH 4 to pH 8.

The accuracy of this single point equivalence determination method was checked against horse heart cytochrome *c* as standard. The E_0' of the hemoprotein was calculated both in 0.05 M phosphate buffer (pH 7.0) and in the buffer containing 0.04 M CTAB.

[2] The notation is that recommended by Clark (1960). The term E_m', rather than E_0', is used to denote the midpoint potential of a system for which N is not known.

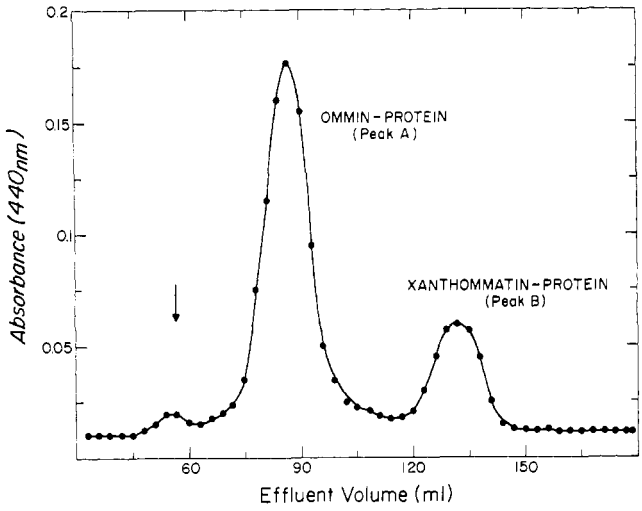

FIGURE 1: Gel filtration of Cecropia eye extract on Sephadex G-200 in buffered CTAB at pH 7.0. Column conditions were as described in Materials and Methods, part 4. The arrow indicates the elution volume of pigmented contaminants.

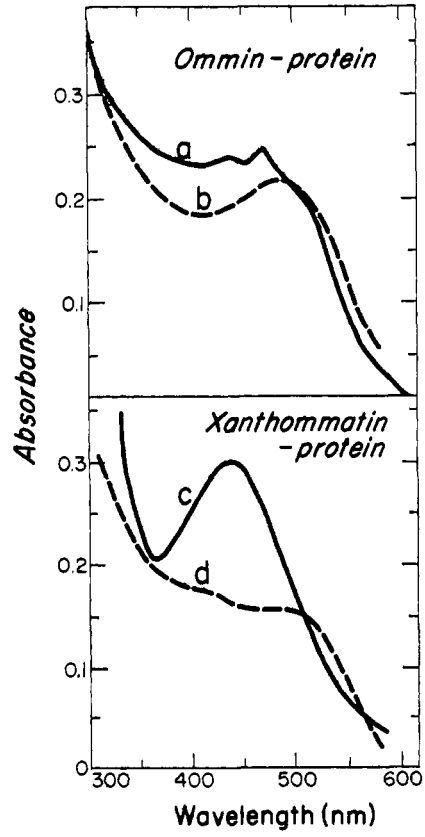

FIGURE 2: Visible absorption spectra of Cecropia ommin-protein and xanthommatin-protein in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.04 M CTAB after gel filtration on Sephadex G-200. Ommin-protein: a, oxidized with molecular oxygen; b, reduced with ascorbic acid added as solid. Xanthommatin-protein: c, oxidized with molecular oxygen; d, reduced with ascorbic acid added as solid.

8. Salt Binding. Ommin was extracted from ommin-protein with acidic methanol according to the procedure described in the preceding paper (Ajami and Riddiford, 1971). The binding of mono- and divalent cations to the pigment obtained in this manner was examined by the method of Butenandt *et al.* (1958). We dissolved ommin in dilute NaOH and brought the solution to neutrality by careful titration with either dilute hydrochloric or nitric acid. Metal ions were added as saturated solutions of their chloride or nitrate salts, depending on the acid used to titrate the ommin solution. Precipitates were collected by centrifugation (30,000*g* for 20 min) or filtration through a solvent-resistant filter.

9. Chemical Assays. Protein concentrations were determined by the Lowry reaction (Lowry *et al.*, 1951). Proteins and peptides were also identified as described by Rydon and Smith (1952). Ammonium sulfate precipitations were performed by direct addition of salt and trichloroacetic acid precipitations by addition of a 50% aqueous solution.

10. Materials. We used only A grade reagents. CTAB, B grade, purchased from Fisher Scientific, was twice recrystallized from ethanol-water. Sephadex G-200 (medium) was from Pharmacia; agarose Bio-Gel A 1.5 m from Bio-Rad. We obtained the protein molecular weight standards from Mann Research and Solvinert solvent-resistant filters from Millipore.

Results

1. Extraction into Buffers. This series of experiments was designed to test the nature of the attachment of the pigment to the carrier granules in the compound eye. When extracted with buffers of pH 5 and with distilled water at pH 6.5, homogenates of Cecropia eyes yielded a yellow pigment with absorption maxima at 250, 345, and 450 nm. The pigment did not display the redox properties of xanthommatin or of other ommochromes, and it chromatographed in methanol–butanol–benzene–water–HCl (10:5:5:5:1, v/v) with an R_F of 0.26. These data suggest that it is either a yellow pteridine or more likely a breakdown product of ommochrome since it shares many of the characteristics of xanthurenic acid.

At pH 7.0 the xanthommatin became solubilized, as judged by its oxidation–reduction behavior in solution. Occasionally, we detected traces of ommin in neutral buffers but only when the high-speed centrifugation or Millipore-filtration steps had been omitted.

In the buffers at pH 8.3 and 8.8 both xanthommatin and ommin passed into solution. It is of interest to note that the two pigments could be separated either with trichloroacetic acid (20% v/v), or with ammonium sulfate (33–50% w/v) saturation). A pigment identified as ommin precipitated, and yellow xanthommatin remained dissolved.

At pH 10, buffered extracts of eyes assumed a deep red coloration due almost exclusively to the presence of ommin, since xanthommatin is alkali unstable.

2. CTAB Extraction and Gel Filtration. Upon treatment with CTAB–buffer at neutral pH, Cecropia eye powder yielded a deep reddish brown solution. Figure 1 illustrates the elution profile of eye extracts on Sephadex G-200. When we eluted the sample with phosphate buffer containing 0.04 M CTAB, two major peaks were present: one at an effluent volume of 87 ml (peak A) and the other at 132 ml (peak B). Acidified methanol extraction followed by thin-layer cochromatography with pigment standards as previously described (Ajami and Riddiford, 1971) showed peak A to contain ommin and peak B xanthommatin. The visible absorption spectra of the oxidized and reduced forms of the ommochrome proteins obtained from each peak are presented in Figure 2. These will be discussed in detail in a subsequent publication.

Also, we removed the detergent by precipitation of the

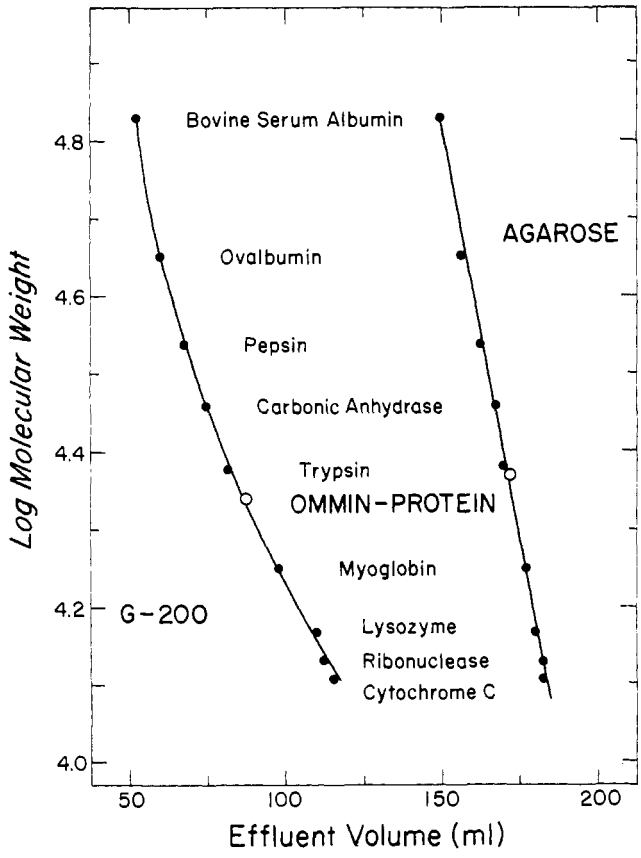

FIGURE 3: A plot of log molecular weight as a function of elution volume after gel filtration on two different columns of various calibrating proteins and purified ommin-protein. The columns were packed with either Sephadex G-200 or agarose. Conditions were as described in Materials and Methods, part 5.

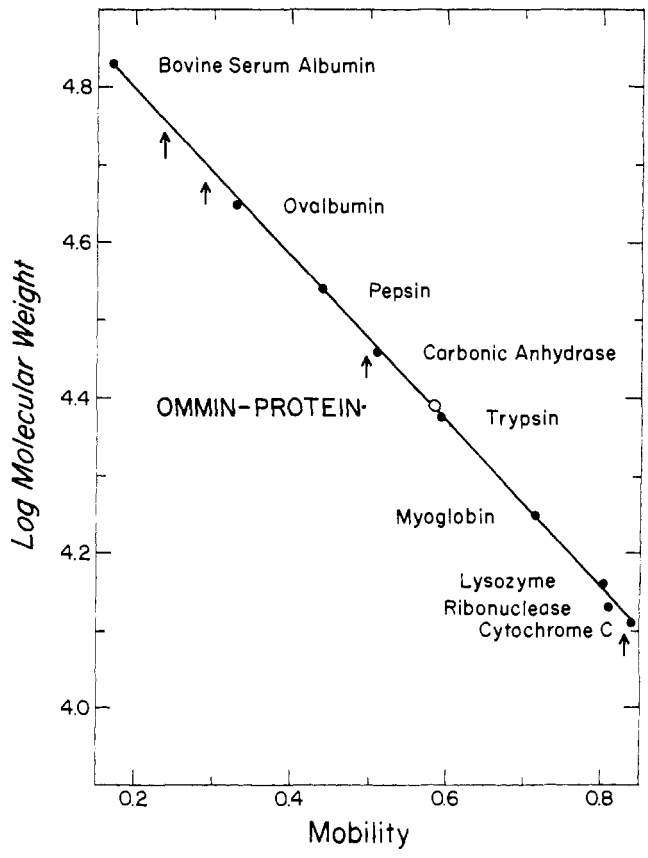

FIGURE 4: A plot of log molecular weight as a function of electrophoretic mobility. Purified ommin-protein and various calibrating proteins were electrophoresed on 10% sodium dodecyl sulfate gels as described in Materials and Methods, part 6. Arrows indicate the R_F values of additional protein bands obtained in electrophoretic runs of crude Cecropia eye extracts.

purified pigments with ethanol and tested the sediment for the presence of protein by the Lowry reaction and also by the displacement of labile N–Cl bonds with iodide as described by Rydon and Smith (1952). Protein was indicated by both tests.

3. Molecular Weight by Gel Filtration. Crude extracts were purified once by gel filtration on Sephadex G-200 with detergent. When peak A fractions were concentrated by ethanol precipitation and rechromatographed on a calibrated G-200 column, only one symmetrical peak was obtained at an effluent volume of 87 ml, indicating a molecular weight of 22,000 ± 1000 (Figure 3). The absorbancy ratio at 280/500 nm was used as a criterion for the degree of purity in the effluent fractions. It reached a constant value of 3.8 ± 0.10 across the peak fractions. When the same fraction was rechromatographed on a calibrated agarose column, we found a symmetrical peak at an effluent volume of 170 ml, indicating a molecular weight of 24,000 ± 1200 (Figure 3).

4. Molecular Weight by Electrophoresis. Like CTAB solutions, buffered sodium dodecyl sulfate extracted a deep red-brown color from the lyophilized Cecropia eye powder. The results obtained from disc electrophoresis of this solution are shown in Figure 4. Three bands moving toward the anode were seen in the crude pigment extract at R_F values corresponding to molecular weights of 12,000, 24,500, and 30,000 (±5% respectively). At least four bands could be distinguished near the top of the gels corresponding to molecular weights greater than 50,000. Reddish purple particles remained at the gel–buffer interface. A single protein band of molecular weight 24,700 ± 5% was obtained with samples of pigment extract purified by G-200 or agarose gel filtration.

5. Redox Potential of Ommin–Protein in CTAB Solution. Of the many reagents capable of reducing ommochromes (Ajami and Riddiford, 1971), ascorbic acid was found to be the most convenient for use with ommin–protein solutions in CTAB since, unlike sodium dithionite and sodium hydrosulfite, it did not react with the detergent to form a precipitate. Hydrogen peroxide was used as the preferred oxidant for the same reason.

Application of single point equivalence determinations gave reproducible results, which were not noticeably affected by variations in the total concentration of ommin–protein. These are summarized in Table I. At pH 7.0, the redox potential representing an average of five trials was found to be 196 ± 7 mV. The change in E_m' with pH was investigated only from pH 4 to 8, since above pH 8 ommin dissociates from its apoprotein and below pH 4 the pigment undergoes rapid autoxidation, rendering accurate titrations impossible. Ommin–protein solutions remained stable between these limits for at least 1 hr, as judged by the constant absorbance values obtained with the spectrophotometer. The E_m' of the pigment decreased by 0.79 ± 4 mV per unit increase in pH.

As a check on the reliability of the single point equivalence determination, we examined the E_0' of horse heart cytochrome *c*. The average of these determinations gave a redox potential

in 0.05 M phosphate buffer, pH 7.0, of 257 ± 8 mV and of 244 ± 6 mV in buffer containing 0.04 M CTAB.

6. Binding of Metal Cations. Ommin, extracted from ommin-protein, was dissolved in dilute sodium hydroxide, and the solution was titrated with acid to pH 7. Addition of the following metal cations caused flocculent precipitates: Ag^{+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and Ca^{2+}. The precipitates were dark red in all cases except the ones formed by Co^{2+}, which were deep purple.

Discussion

1. Solubility Properties. Attachment of a pigment to a macromolecule may be possible through a variety of interactions which include ionic, hydrogen, hydrophobic, and covalent bonding (Bellin, 1965). Each possibility is reflected in the solubility properties of the chromophore. In general, pigments that are bound by a noncovalent linkage partition into organic solvents or dissolve in aqueous buffers at either high or low pH values which cause structural changes in the macromolecule. Covalent linkages in most cases can only be destroyed through hydrolysis by strong acids or bases at elevated temperatures.

Although xanthommatin and ommin cannot be extracted into organic solvents (Ajami and Riddiford, 1971), they can be extracted into aqueous buffers between pH 8 and 10. Xanthommatin appeared to be free in solution, but extracted ommin remained bound to a protein as it was precipitated when 20% trichloroacetic acid or 50% ammonium sulfate was added. Therefore, xanthommatin appears to be noncovalently bound, probably to groups with pK_a values of about 8. Ommin is more likely covalently bound, although its linkage is hydrolyzed at pH values greater than 10.0.

2. CTAB Extraction and Purification. CTAB proved to be the best solubilizing agent for removing the pigments from their protein carriers. The same cationic detergent had been used extensively by Yoshida *et al.* (1967) to extract ommochrome from anthomedusan ocelli, by Heller (1968) to purify bovine rhodopsin, and by Massini and Voorn (1968) to examine the photochemical properties of chlorophyll. CTAB forms stable clear solutions with no absorption at 280 nm and minimal absorption at 230 nm; it can also be easily removed from proteins by precipitation of the proteins with ethanol. The detergent forms spheroidal micelles in aqueous solution as long as the concentration is above 10^{-3} M; thus we used at least a 20-fold excess.[3]

Although two distinct peaks (Figure 1) were always found in gel filtration (Sephadex G-200) of these CTAB extracts, the best separation was obtained when the elution buffer contained detergent. Peak A contained the nondialysable ommin-protein and peak B the dialysable xanthommatin-peptide. By analogy with the use of CTAB to extract membrane-bound pigments as rhodopsin (Heller, 1968) and chlorophyll (Massini and Voorn, 1968), the ommochrome-proteins must be membrane bound. These data correlate with the findings of Schoup (1966) and Langer (1967) that the accessory pigment granules are associated with the endoplasmic reticulum and the Golgi apparatus.

3. Molecular Weight of Ommin-Protein. As illustrated by Figure 3, for agarose gel filtration, the plot of the molecular weight of globular protein standards *vs.* the effluent volume is linear, so it may be used as a valid calibration curve. A similar linear relationship was obtained when the electrophoretic mobility of the same protein markers in sodium dodecyl sulfate gels was plotted against their molecular weight. On the assumption that ommin-protein (peak A) from Cecropia eyes adheres to the relationship shown by globular proteins, these two experimental lines determine its molecular weight to be about 24,000 ± 1500. Heller (1968) found the molecular weight of cattle rhodopsin to range between 27,000 and 30,000. Assuming that insect visual pigment is in the same molecular weight range, any contamination would obscure the true molecular weight of the ommin-protein. The preextraction step for ommochrome granules, followed by G-200 gel filtration, which is known to exclude cattle rhodopsin (Heller, 1968), minimized this possibility. Also, no carotenoid pigment was present (Ajami and Riddiford, 1971).

TABLE I: The Redox Potential of Ommin-Protein.

pH	Ommin-Protein Midpoint Potentials (mV)[a] 0.1%	0.5%
4	430 ± 5	429 ± 5
5	356 ± 6	355 ± 4
6	275 ± 6	274 ± 6
7	196 ± 7	195 ± 7
8	114 ± 4	115 ± 5

[a] Values reported represent the average of five trials.

4. Redox Potential of Ommin-Protein. The single equivalence point determination permits one to obtain an approximate redox potential of a pigment directly without a titration. The E_0' of 257 ± 8 mV for cytochrome *c* in phosphate buffer (pH 7.0) obtained by this method compares favorably with the accepted potential of 254 mV. The decrease in E_0' in buffered CTAB to 244 ± 6 mV may be attributed to denaturation caused by the detergent. Denaturation is usually accompanied by a lowering of the normal redox potential toward more negative values (Margoliash and Schejter, 1966).

On the assumption that there is also a 5% decrease in the potential of the pigment when measured in CTAB, the native midpoint potential for ommin-protein can be extrapolated to 205 mV. Therefore, it is not surprising that ascorbic acid (E_0', 80 mV at pH 6.4) reduces ommin-protein and that 2,6-dichlorophenol indophenol (E_0', 217 mV at pH 7.0) oxidizes the reduced pigment (Yoshida *et al.*, 1967).

5. Functional Significance of the Ommin-Protein. The biological significance of the ommin-protein which may be extraced from Cecropia ocelli, optic lobes, and ganglia as well as the eyes is not clearly understood. Bückmann (1965) proposed that these ommochrome pigments were merely storage compounds for nitrogenous waste products, particularly tryptophan. Yet the eye ommochromes appear essential as screening and reflecting pigments (Fox and Vevers, 1960).

A pink pigment has been implicated in the photoperiodic response of aphids (Lees, 1968) and of the silkmoth pupae *Antheraea pernyi* (Williams and Adkisson, 1964) and *H. cecropia* (Williams, 1969). A similar pigment is also believed to be involved in the egg hatching rhythm of *Pectinophora gossypiella* (Bruce and Minis, 1969) and of *A. pernyi* (Riddiford and Johnson, 1968). It is of interest to note that the absorption

[3] Czerniawski (1966) determined the critical micelle concentration to be 9.2×10^{-4} M at 25° in aqueous solutions and Massini and Voorn (1968) found it to be 1.7×10^{-4} M in 0.05 M Tris buffer (pH 7.8).

spectra of ommochromes—ommin–protein in particular—are strikingly similar to the action spectra and spectral response curves obtained in the studies on insect photoperiodism just mentioned.

Our preliminary evidence suggests that the ommin–protein complex is photoactive and undergoes a slow photoreduction under anaerobic conditions in the absence of external reducing agents. Similar results have been obtained by Yoshida and his collaborators, who have postulated the involvement of an ommochrome in photoprocesses leading to the reflex pulsation and contraction movements of *Spirocodon* (Yoshida and Ohtsuki, 1966; Yoshida *et al.*, 1967).

In the present report we have shown an ommochrome from the eyes of Cecropia to be attached, probably covalently, to a membrane-bound protein. Investigations still in progress indicate that the same ommin–protein with nearly identical oxidation–reduction properties can be found in the optic lobes, ocelli, and ganglia of this silkworm. The significance of a related pigment system also localized in nervous tissue has been discussed already by Chalazonitis (1964). He demonstrated in *Aplysia* that illumination of pigmented nerve cells or of nerve fibers vitally stained with several dyes[4] can generate photocurrents and decreases in membrane resistance by a mechanism involving photoreduction with a membrane protein as possible hydrogen donor.

The parallels between results of our investigations and those from other laboratories lend support to the proposition that ommochromes may play some role in a photoprocess vital to insect development. Such a proposition is made more attractive by two additional results of physiological interest: ommin–protein shows an oxidation–reduction potential at an intermediate value between the potentials of cytochromes *b* and *c*, and its chromophore can chelate divalent cations.

Acknowledgments

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References

Ajami, A. M., and Riddiford, L. M. (1971), *Biochemistry 10*, 1451.

Bellin, J. S. (1965), *Photochem. Photobiol. 4*, 33.

Bowness, J. M., and Wolken, J. J. (1959), *J. Gen. Physiol. 42*, 779.

Bruce, V. G., and Minis, D. H. (1969), *Science 163*, 583.

Bückmann, D. (1965), *J. Insect Physiol. 11*, 1427.

Butenandt, A., Biekert, E., and Linzen, B. (1958), *Z. Physiol. Chem. 312*, 227.

Chalazonitis, N. (1964), *Photochem. Photobiol. 3*, 539.

Clark, W. M. (1960), Oxidation–reduction Potentials of Organic Molecules, Baltimore, Williams and Wilkins, p 91.

Czerniawski, M. (1966), *Rocz. Chem. 40*, 1935.

Fox, H. M., and Vevers, G. (1960), The Nature of Animal Colours, London, Sidgwick and Jackson, p 50.

Fuge, H. (1966), *Naturwissenschaften 53*, 136.

Goldsmith, T. H. (1964), *in* The Physiology of Insecta, Vol. I, Rockstein, M., Ed., New York, N. Y., Academic Press, p 397.

Heller, J. (1968), *Biochemistry 7*, 2906.

Langer, H. (1967), *Z. Vergl. Physiol. 55*, 354.

Lees, A. D. (1968), *in* Photophysiology, Vol. IV, Giese, A. C., Ed., New York, N. Y., Academic Press, p 47.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 114.

Massini, P., and Voorn, G. (1968), *Biochim. Biophys. Acta 153*, 589.

Osanai, M. (1966), *J. Insect Physiol. 12*, 1295.

Riddiford, L. M., and Johnson, L. K. (1968), *Proc. Int. Congr. Entomol., 13th, 1968* (in press).

Rydon, H. M., and Smith, P. W. G. (1952), *Nature (London) 169*, 922.

Schoup, J. R. (1966), *J. Biophys. Biochem. Cytol. 29*, 233.

Volke, J. (1963), *Phys. Methods Heterocycl. Chem. 1*, 727.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Williams, C. M. (1969), *Symp. Soc. Exp. Biol. 23*, 285.

Williams, C. M., and Adkisson, P. L. (1964), *Biol. Bull. 127*, 511.

Yoshida, M., and Ohtsuki, H. (1966), *Photochem. Photobiol. 5*, 99.

Yoshida, M., Ohtsuki, H., and Suguri, S. (1967), *Photochem. Photobiol. 6*, 875.

Ziegler, I., and Harmsen, R. (1969), *Advan. Insect Physiol. 9*, 139.

[4] These included methylene blue and thionine, which have the same phenothiazine ring system as ommins.