Drosophila melanogaster Lacks Eye-Pigment Binding Proteins†

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ABSTRACT: *Drosophila melanogaster* contains no detectable eye-pigment binding proteins, and the previous evidence for the presence of such proteins in the cecropia moth is probably not valid. The major brown pigment of *Drosophila* (and of *Cecropia*), dihydroxanthommatin, behaves as a high molecular weight compound in Sephadex chromatography, thus leading to false conclusions.

Mutations at a large number of loci in *Drosophila* are known to affect eye pigmentation, and the distinction between the two types of eye pigments (drosopterins and ommochromes) was originally made by use of eye color mutants. Subsequent chemical and biochemical investigations have increased our information on the pigments and their origins, but the relationships between them (as exemplified by the absence of both types in the white mutant) remain essentially unknown, as are the functions of many of the loci affecting either brown or red pigment synthesis and deposition.

The classical theory linking drosopterins and ommochromes was that they were related through a common site for the final (oxidative) steps in their synthesis. At least two types of pigment granules, red and brown, have been distinguished using the electron microscope and appropriate mutants (Shoup, 1966; Fuge, 1967).

It seems reasonable (although not essential) to the "common site" theory (most recently reviewed by Phillips & Forrest, 1973) that a pigment-binding protein might be a common constituent of the two pigment granules and, therefore, might provide the link between the pigments. We, therefore, examined head extracts of *Drosophila* for a pigment-binding protein, encouraged by the results of Ajami & Riddiford (1971) who described an ommochrome-binding protein in the cecropia moth, *Hyalophora cecropia*. Although superficial evidence suggested the presence of such a protein in *Drosophila*, we show in this paper that it does not in fact exist. Furthermore, no evidence could be adduced for the presence of a drosopterin-binding protein.

Materials and Methods

Organisms. *Drosophila melanogaster Oregon-R* (*Ore-R*) was used as the wild-type fly. The eye color mutants used were white (*w*; 1–1.5), white apricot (w^a; 1–1.5), vermilion (*v*; 1–33.0), cinnabar (*cn*; 2–57.5), brown (*bw*; 2–104.5), sepia (*se*; 3–26.0), and scarlet (*st*; 3–44.0). The flies were grown in glass milk bottles with the standard cornmeal agar medium sprayed with yeast. Only young adult flies (0–6-days old) were used and their heads were collected using the method of Oliver & Phillips (1970). Either freshly collected heads or heads frozen for no longer than two months were used.

Chemicals. Cetyltrimethylammonium bromide (CTAB)[1] was purchased from Sigma. Actinomycin D and 3-hydroxykynurenine were purchased from Calbiochem. Proteinase K was purchased from EM Biochemicals. Dihydroxanthommatin was extracted from *bw* fly heads according to the method of Ryall & Howells (1974). After extraction into the butanol phase, water (2 mL) was added and then anhydrous ether (10:1 ether–butanol, v/v). The dihydroxanthommatin was displaced into the aqueous phase as a red precipitate. Dihydroxanthommatin was also synthesized from 3-hydroxykynurenine by the method of Butenandt et al. (1954).

Preparation of Tissue Samples. Fly heads were homogenized in 0.05 M sodium phosphate buffer, pH 7.0, containing 4×10^{-5} M phenylthiocarbamate, at a concentration of 0.5 g of heads/5 mL of buffer. All homogenizations were carried out using a motor driven Teflon homogenizer. The homogenate was strained through a nylon mesh and centrifuged at 500*g* for 10 min to remove nuclei and other debris. The supernatant was then centrifuged at 22000*g* for 20 min. The resulting pellet was mainly composed of mitochondria and pigment granules. The supernatant from this fraction was used for some of the experiments and is referred to as the fly head supernatant. The pellet was detergent treated with either 0.04 M CTAB, 1% $NaDodSO_4$, 2% Triton X-100, or 0.6% digitonin in phosphate buffer and centrifuged at 30000*g* for 10 min. The resulting supernatant, referred to as pigment-granule extract, was then used for some of the experiments.

Sephadex G-75 Fractionation. Two Sephadex G-75 (fine) columns were used; a 1.6 × 55 cm column was used for molecular weight estimation and a 1.5 × 20 cm column was used for the other experiments. Both columns were equilibrated with the homogenizing buffer and the various detergents and reducing agents. One-milliliter samples of either fly head supernatant or pigment-granule extract were applied to the columns which were then developed with the appropriate buffer (homogenizing buffer with or without detergent and reducing agents); 1.5-mL fractions were collected and analyzed on a Cary 14 spectrophotometer at 280 nm for protein and at 440 and 500 nm for xanthommatin and dihydroxanthommatin.

Protein Estimation. The amount of protein in fractions from the column was estimated using the technique of Lowry et al. (1951). As CTAB interfered with Folin's reagent, all fractions from experiments involving this detergent were treated with 95% ethanol (3 mL), centrifuged, rewashed with 95% ethanol (1 mL), and recentrifuged. In control experiments, this procedure was shown to precipitate bovine serum albumen (M_r 66 500) and cytochrome *c* (M_r 12 384). The resulting pellet was resuspended in 1 N NaOH overnight and then protein estimated according to Lowry et al.

Proteinase Digestion. One-milliliter samples of fly head supernatant without detergent were treated with 100 μg of proteinase K and incubated at 37 °C overnight. The proteinase K was shown to be active by observing the change in elution pattern from a Sephadex G-75 column after treating a head

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[1] Abbreviations used: CTAB, cetyltrimethylammonium bromide; $NaDodSO_4$, sodium dodecyl sulfate; BME, β-mercaptoethanol; BSA, bovine serum albumin.

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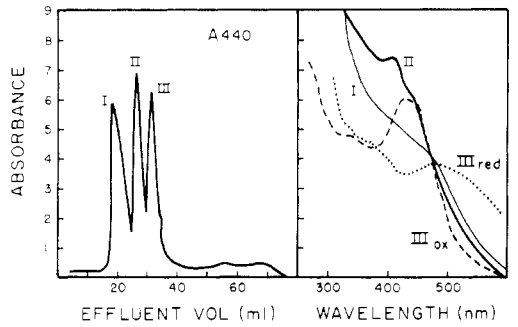

FIGURE 1: Extract from *bw* pigment granules chromatographed on a Sephadex G-75 column. The effluent shows three peaks of material absorbing at 440 nm. On the right are the absorption spectra of these peaks. The reduced and oxidized forms of the substance in peak III are given.

extract. No 280-nm absorbing material remained in the high molecular weight fractions.

Sucrose Gradients. Fly head supernatant from *bw* flies was layered on top of a 13-mL, 0.2–0.45 M sucrose gradient (containing 0.05 M sodium phosphate buffer, pH 7.0, and 4 $\times 10^{-5}$ M phenylthiocarbamate) and centrifuged in an SW41 rotor in a Beckman L2-65B centrifuge at 140000*g* for 18 h.

Results

The pigment-granule pellet was isolated from various mutants and treated with the cationic detergent CTAB and β-mercaptoethanol (BME), as described by Ajami & Riddiford (1971). After a 30-min incubation, the homogenate was centrifuged at 30000*g* and the supernatant was layered on a Sephadex G-75 (fine) column. In all flies having the brown pigment (i.e., *bw*, *Ore-R*, *se*), three pigment bands were observed in the high molecular weight fractions from the column (Figure 1). The absorption spectra of these three bands (Figure 1) allowed us to determine that the third band probably contained xanthommatin, by comparison with the spectrum of pure xanthommatin. As expected, this pigment on treatment with sodium metabisulfite underwent the spectral changes characteristic of xanthommatin reduction to dihydroxanthommatin (Figure 1). Characterization of the pigments in the first two bands was not certain because they did not have spectra similar to any easily identifiable pigments. The solubilities of the three pigments were examined by first lyophilizing the fractions from the column and then treating with various solvents. First, 95% ethanol was added to each lyophilized sample and then acidified methanol, which is known to extract brown pigment from whole eyes (Ephrussi & Herold, 1944). Pigment from the first band was not soluble in either of the solvents, pigment from the second band dissolved immediately in ethanol, while pigment from the third band was insoluble in ethanol but dissolved readily in acidified methanol. This latter observation was another indication that the third peak contained xanthommatin since its solubility in acidified methanol is a characteristic feature of this pigment. The pigment in the first peak is related to xanthommatin because in extracts from the mutants *st*, *v*, and w^a, which lack the ability to synthesize ommochromes, very little pigment occurred in that region of the column. This pigmented material probably belongs to the ommin class. In all of the mutants lacking brown pigment a slightly pigmented band did occur in the region of band II (Figure 2). (The A_{440} absorbance in the band I region is due to end absorption.) The solubility properties of the pigment in this band were very similar to those of the pigment in band II as described above. However, the amounts in these mutants were very much less

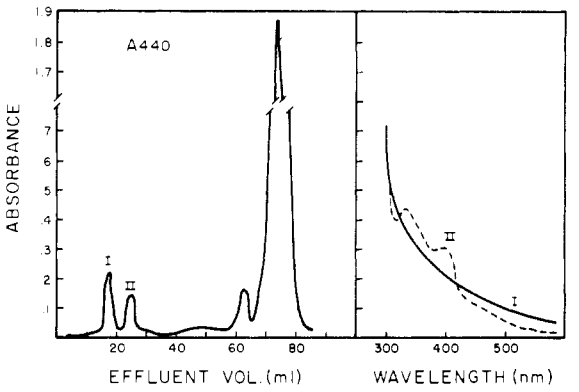

FIGURE 2: Sephadex column profile of a mutant blocked in brown pigment synthesis (*v* or *st*). The absorption spectra of the bands are shown at the right. The two peaks at 76 and 62 mL of effluent are due to drosopterins and other pteridines (mainly sepiapterin), respectively.

than in flies containing brown pigment. Upon extraction of this pigment into chloroform and addition of antimony trichloride a rapidly fading pink color (diagnostic for carotenoids) was noted. It is therefore probable that this band contains a carotenoid–protein complex but it is not clear why its amount should be so much less in mutants lacking ommochromes, unless it is preferentially associated with brown pigment granules.

An estimation of the molecular weights was made on the Sephadex G-75 (fine) column (1.6 × 55 cm) using comparative elution volumes of the following markers: BSA, ovalbumin, lipase, myoglobin, and cytochrome *c*. This showed that the protein in band I had an average molecular mass greater than BSA (>66 000 daltons), band II protein was approximately 23 000 daltons, while band III protein was about 19 000 daltons.

When other detergents were used to break open the pigment granules, brown pigment was, in general, eluted from the Sephadex columns in the high molecular weight regions, although some inconsistencies appeared. The anionic detergent sodium dodecyl sulfate ($NaDodSO_4$), the nonionic detergent Triton X-100, and digitonin were used. If the pigment granules were extracted with CTAB, $NaDodSO_4$, or Triton X-100 and the column was not equilibrated with detergent, no high molecular weight pigmented fraction was found. After equilibration with any of these detergents, brown pigment eluted from the column in the high molecular weight region. However, the pigmented fractions from the column were turbid using $NaDodSO_4$ or Triton X-100, but were completely clear with CTAB. When digitonin was used on a column not previously equilibrated with any detergent, the brown pigment eluted from the column in the high molecular weight region although the fractions were again turbid.

The behavior of the pigmented material was similar using a Bio-gel agarose column. However, using Sepharose (4B or 6B), no pigment was eluted in the high molecular weight region.

Various other reducing agents were substituted for β-mercaptoethanol in the extraction procedure using CTAB. These were 5 mM ascorbic acid, 5 mM potassium borohydride (KBH_4), and 5 mM sodium metabisulfite. Using the pigment-granule extract from *Ore-R* flies with CTAB and KBH_4, three bands similar to those already noted were found. When either sodium metabisulfite or ascorbic acid was used only one pigmented band (apparent molecular mass approximately 23 000 daltons) was found. With bisulfite the pigment faded rapidly at 10 °C in the dark; however, the pigment was stable

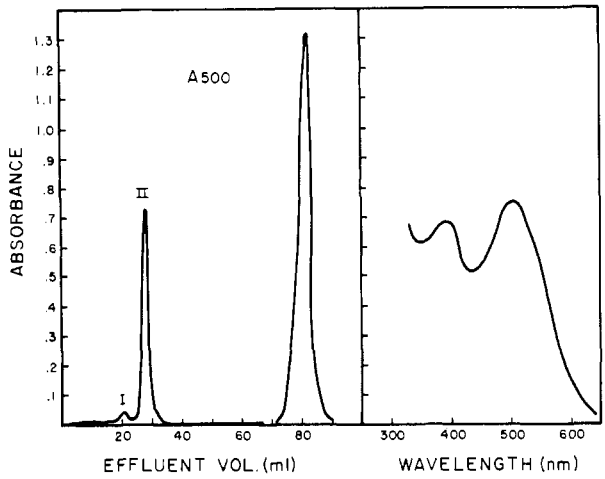

FIGURE 3: Sephadex column chromatography of CTAB extract of *Ore-R* heads in presence of ascorbic acid. The adsorption spectrum of band II is shown on the right. The large peak of absorption at 80-mL effluent volume is due to drosopterins. The smaller preceding peak due to other pteridines (Figure 2) is not seen here because measurements were made at 500 nm.

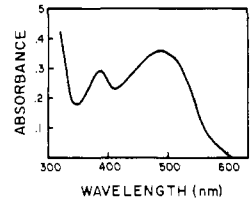

FIGURE 4: Absorption spectrum of dihydroxanthommatin from *bw* fly heads.

under the same conditions using ascorbic acid. The spectrum in the latter case was identical to that of dihydroxanthommatin (Figure 3). Again, this pigment was found in all flies expected to have brown pigment but was essentially absent in flies (*v* and *cn*) lacking brown pigment. Similar results were found with supernatant fraction of fly heads.

The amount of protein in fractions from the CTAB and ascorbic acid column was estimated by 280-nm absorption and the technique of Lowry et al. (1951). The results showed only one peak of protein at the void volume of the column. Surprisingly, fractions containing pigment were protein free. It appeared that the high molecular weight pigment contained no protein. To confirm this result, the supernatant fraction was incubated overnight with proteinase K and then CTAB and ascorbic acid were added. Pigment still appeared in the high molecular weight fractions. Further confirmation of this anomalous result was obtained by working with dihydroxanthommatin (natural or synthetic).

Dihydroxanthommatin extracted from *bw* fly heads (Ryall & Howells, 1974) exhibited a typical absorption spectrum (Figure 4). The red precipitate was soluble in phosphate buffer in the presence of CTAB and β-mercaptoethanol or CTAB and ascorbic acid. The resulting solutions applied to a Sephadex G-75 (fine) column behaved exactly as described for head extracts; i.e., pigmented fractions were obtained in the high molecular weight eluate from the column.

Synthetic dihydroxanthommatin (Butenandt et al., 1954), dissolved in buffer in the presence of either CTAB and BME or CTAB and ascorbic acid, was applied to Sephadex columns as before. With BME present, two pigment-containing fractions were obtained, one in the high molecular weight region (23000) and the second, in rather small amount, in the low molecular weight region—consistent with the molecular weight of xanthommatin. The apparent high molecular weight

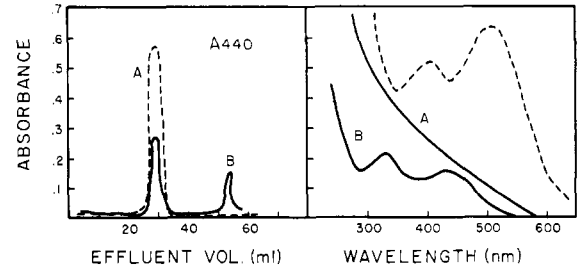

FIGURE 5: Sephadex column profile of extracted (– – –) or synthetic (—) dihydroxanthommatin in the presence of CTAB and β-mercaptoethanol. The absorption spectra of bands A and B are shown on the right.

pigment was similar to band III normally found with β-mercaptoethanol-treated material from heads; the low molecular weight pigment was identical with pure xanthommatin (Figure 5). With ascorbic acid present, the dihydroxanthommatin behaved exactly as pigment from fly heads; i.e., it eluted in the high molecular weight region, and no pigment eluted in the low molecular weight fractions.

Actinomycin D was dissolved in the phosphate buffer with CTAB and ascorbic acid and applied to the Sephadex G-75 (fine) column. It also eluted in the high molecular weight fractions.

Sucrose gradients were run of both the reduced and the oxidized fractions from the supernatant of *bw* flies. Cytochrome *c* was run on another gradient as a marker. The cytochrome *c* equilibrated inside the gradient while both reduced and oxidized pigments remained on top of the gradient.

Discussion

Ajami & Riddiford (1971) described the isolation of ommochrome proteins from the eyes of saturniid moths. Using essentially the same methods, we were able to demonstrate the presence of three pigmented fractions in the high molecular weight region of the effluent from columns of Sephadex G-75 or Biogel agarose after application of a detergent-treated extract from *Drosophila* heads. Two of the fractions (bands I and III) appeared to be ommochrome proteins (on the basis of their absence in head extracts of mutants blocked in ommochrome synthesis) and the third (band II) appeared to be a carotenoid-containing protein (*Drosophila* rhodopsin?). However, when tests were made to determine if indeed the pigmented fractions contained protein associated with pigment, discrepancies began to appear. Specifically, after treatment of fractions from band III with proteolytic enzymes and rechromatography, the pigment appeared at the same place in the effluent as before. Since the pigment had been identified as dihydroxanthommatin, this compound, prepared synthetically or from *Drosophila* heads, was then run on the column under identical conditions. Again, the pigment behaved as a high molecular weight material. We then used a different compound, actinomycin D (molecular weight 1255), which, however, contains the same phenoxazinone ring system as the ommochromes. It also behaved as a high molecular weight compound. There seems little doubt that compounds of this type do not behave "normally" on columns of Sephadex or agarose. The reason for this is not understood, although it may be related to the presence of a large planar ring structure, or to the polar nature of the molecule, or to the use of detergents at all times on these columns, or indeed to a combination of all three. On the other hand, xanthommatin may behave normally on such columns as indicated by the experiment in which synthetic dihydroxanthommatin (behaving

as a high molecular weight compound) was separated on a Sephadex G-75 column from a small amount of xanthommatin which behaved as expected.

Ajami and Riddiford used gel electrophoresis as an additional method to identify their "pigment-protein". We have not been able to repeat their results with extracts from *Drosophila* heads. We have never observed pigment associated with protein bands after electrophoresis under a variety of conditions. Similarly, in sucrose gradients, no pigments, oxidized or reduced, entered the gradients, although cytochrome *c*, used as a control, entered as expected.

We conclude that there is no evidence for an ommochrome–protein complex in *Drosophila* heads under the conditions described, and it is reasonable to suppose that the same is true in saturniid moths. Of course, since the methods used always included detergents, one cannot rule out the possibility that these reagents disrupt a pigment–protein complex. However, in view of the insolubility of xanthommatin and dihydroxanthommatin (and the more complex ommins), it may well be that the association of these pigments with proteinaceous material in pigment granules is essentially nonspecific.

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Leghemoglobin Biosynthesis in Soybean Root Nodules. Characterization of the Nascent and Released Peptides and the Relative Rate of Synthesis of the Major Leghemoglobins†

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ABSTRACT: There are two major forms of leghemoglobin (Lb) in soybean root nodules. In mature nodules, LbF (the electrophoretically fast-moving component) exists in larger quantity than LbS (slow moving), in a ratio of about 1.5:1. Analysis of their relative rates of synthesis both in vivo and in vitro indicates that in mature (>3-week-old) root nodules LbS is made at a higher rate than LbF, but in young nodules (<12 days) LbF synthesis predominates. Analysis of Lb synthesized in vitro using DEAE-cellulose chromatography showed that LbF can be resolved into two components, identical with those designated as Lbc_1 and Lbc_2 for unlabeled preparation of Lb by Appleby et al. [Appleby, C. A., Nicola, N. A., Hurrell, J. G. R., & Leach, S. J. (1975) *Biochemistry 14*, 4444]. However, Lbb, a minor component, appears to be an artifact of protein isolation and fractionation or generated due to some ligand binding. There is no precursor–product relationship between Lb components. LbF can be detected by in vitro translation of nodule polysomes 6 days after infection with *Rhizobium* and is followed by the appearance of LbS and nitrogenase activity on the 7th and 10th days, respectively. Accumulation of LbF over LbS during nodule development appears to be due to different half-lives of the two components. In vitro synthesis of Lb is initiated by methionine which is cleaved before completion of the peptide chain. The completed and released peptide of LbS has valine as its amino terminus, which corresponds to its known sequence. Lack of a precursor and its vectorial discharge into membrane vesicles suggests that Lb does not cross the membrane envelope enclosing the bacteroids and remains in the host-cell cytoplasm after translation.

The presence of leghemoglobin (Lb)[1] in legume root nodules, developed due to the symbiotic association of legume roots with *Rhizobium*, is directly correlated with their effectiveness in fixing atmospheric nitrogen (Virtanen et al., 1947; Smith, 1949; Jordan & Garrard, 1951; Graham & Parker, 1961; Johnson & Hume, 1973). The role of Lb in facilitating the diffusion of oxygen at a relatively low oxygen tension (Appleby, 1969, 1974; Bergersen et al., 1973; Wittenberg et al., 1974; Bergersen & Turner, 1975) is well established. Owing to such oxygen tension, Lb may prevent the rapid diffusion of oxygen to the bacteroids where nitrogenase, an oxygen-sensitive enzyme, is located (see Verma et al., 1978).

Most legumes have several chromatographically distinguishable leghemoglobins in their root nodules. In soybean there are two major forms, LbS and LbF, which are distinguishable electrophoretically (Ellfolk, 1972). However, on DEAE-cellulose chromatography, while LbF can be resolved into two subcomponents, three other minor species have been

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[1] Abbreviations used: Lb, leghemoglobin; LbS and LbF, electrophoretically slow-moving and fast-moving components of Lb on polyacrylamide gels (these components correspond to Lba and Lbc_1 + Lbc_2 of Appleby et al., 1975); DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; poly[A(+)] RNA, RNA containing poly(adenylic acid); $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; δ-ALA, δ-aminolevulinic acid; Cl_3AcOH, trichloroacetic acid; $K_3Fe(CN)_6$, potassium ferricyanide.

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