The morphology and development of *Drosophila* eye

I. *In vivo* and *in vitro* pigment deposition

By E. W. HANLY,¹ C. WILLIAM FULLER¹ & M. S. MILLAM STANLEY²

From the Department of Molecular and Genetic Biology,
The University of Utah

The development of pigment in the eye of *Drosophila melanogaster* and other insects has been the subject of many studies and much controversy. It has been established that the red color of eyes of wild-type *D. melanogaster* is due to the presence of two classes of pigments, ommochromes and pteridines (Ziegler, 1961). The relationships among the various members of each class are still obscure; the biosynthetic pathways are yet to be elucidated. No specific enzyme involved in the synthesis of any member of either group has been isolated or characterized. It has been suggested, however (Hadorn, 1955), that these metabolic pathways may involve several organs, including the eye, but that the final deposition and conversion occur only in the eye. The recent development of a satisfactory technique for the culture of *Drosophila* organs (Schneider, 1964) has made possible the study of pigment development in the isolated eye and in eyes associated with selected organs. Thus it is possible to test the hypothesis that organs interact in the formation of eye pigments.

**MATERIALS AND METHODS**

Wild-type (Oregon-R) *Drosophila melanogaster* were used as tissue explant sources in all experiments. These were raised on the usual cornmeal–molasses–agar medium at 25 ± 0.5 °C and were transferred daily to fresh food. Prepupae were removed from the bottles and placed in Petri dishes on sterile filter paper moistened with sterile saline (Waddington’s modified Ringer’s solution, I. Schneider, personal communication). The characteristic prepupal stage (soft and white with everted anterior spiracles) has a duration of no more than an hour; thus the development can be timed very accurately.

¹ Authors’ address: Department of Molecular and Genetic Biology, The University of Utah, Salt Lake City 84112, Utah, U.S.A.
² Author’s address: Insect Pathology Laboratory, USDA Agricultural Research Center, Beltsville, Md. 20705, U.S.A.
When organs were required for culture, pupae of known age were rinsed twice in sterile saline and washed for 5 min in 70% ethanol. They were again rinsed twice in sterile saline and dissected under aseptic conditions in the tissue-culture medium.

The medium used was the complete type devised by Schneider (1964). It contained 50 units of streptomycin sulfate and 50 units of penicillin G per ml. After sterilization by Millipore filtration 10% fetal bovine serum was added aseptically. Both the culture medium and the saline were stored at —10 °C.

Eyes to be cultured were dissected as the imaginal disc attached to a single cerebral hemisphere. Ring glands were not included. When a culture was to be supplemented with a second organ, both eyes were dissected from one pupa and one was used as an unsupplemented control. Tissues and organs to be cultured were transferred from the dissecting chamber to a hanging drop (0.05 ml. of medium) by means of forceps or a small pipette. The cultures were sealed with a warm mixture of paraffin and beeswax (1:1) and incubated at 25 ± 0.5 °C. They were observed at appropriate intervals for changes in differentiation and pigmentation.

The initial determination of the pigments present in each eye was based on a subjective assessment of the eye color. In order to standardize this technique several colors corresponding to shades and hues typically found in the developing eye were selected from A Dictionary of Color (Maerz & Paul, 1950). Early in the study a series of colors was designated for the in vivo developmental sequence. When possible these colors were used in evaluating the in vitro cultures. In several instances it was necessary to use other colors.

Cultures were either discarded after several days of observation or were squashed directly on to no. 1 Whatman chromatograph paper and developed in suitable solvents. Chromatograms for pteridines were usually developed for 6 h in n-propanol:5% ammonia (2:1). Ommochromes were detected using several solvent systems as described by Butenadt, Schiedt, Biekert & Kornmann (1954). All chromatograms were processed at 23 ± 2 °C.

RESULTS

In vivo pigment formation

In order to determine the normal sequence of pigment development timed wild-type pupae were dissected and the heads chromatogrammed directly. Table 1 correlates the appearance and concentration of ommochromes and fluorescent pteridines with the pupal age and the eye color. Ommochromes appeared at approximately 44 h following pupation. Drosopterins, on the other hand, do not make their appearance until approximately 65 h. These results correspond well with measurements of other investigators (Hadorn & Mitchell, 1951) and with observations of the mutants scarlet (st) and brown (bw) as shown in Tables 2 and 3. The mutant st lacks the ability to synthesize ommo-
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chromes while bw cannot synthesize pteridines. The first faint traces of ommochromes appear in bw at 44 h and the first drosopterins in st at 65 h.

At approximately 45 h wild-type and bw eyes were faint ‘marguerite’. This color is apparently due to the deposition of one of the two general groups of ommochromes, the ommatins. The eyes darkened through ‘mustard’ until at approximately 50 h they became ‘topaz’. ‘Topaz’ apparently results from the deposition of a second group of ommochromes, the ommins. These were described by Butenadt, et al. (1954). The ommatins are yellow-orange while the ommins are brown. A red-violet color appears in the eyes of wild type at 69 h and in bw at 91 h. Its occurrence did not correlate with that of any of the substances identified chromatographically. It may result from an ommochrome-pigment granule interaction.

Table 1. The sequence of pigment development in vivo in heads of Oregon-R Drosophila melanogaster

Symbols are as follows: +, small amount present; ++, moderate amount present; ++++, large amount present; tr, trace; P, present; A, absent; Dros., drosopterins; IX, isoxanthopterin; XP, xanthopterin; HB, the HB compounds (see text).

<table>
<thead>
<tr>
<th>Color</th>
<th>Typical age pupal (h)</th>
<th>Dros.</th>
<th>IX</th>
<th>XP</th>
<th>HB</th>
<th>Yellow</th>
<th>Brown</th>
<th>Red-violet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash</td>
<td>89</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Goya Hollyberry</td>
<td>85</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Red banana</td>
<td>78</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Cardinal Algerian</td>
<td>75</td>
<td>++</td>
<td>tr</td>
<td>tr</td>
<td>+</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Oxheart</td>
<td>69</td>
<td>+</td>
<td>+</td>
<td>tr</td>
<td>+</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Maroon Rubaiyte</td>
<td>65</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>Coptic</td>
<td>60</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Mummy coffee</td>
<td>55</td>
<td>0</td>
<td>+</td>
<td>tr</td>
<td>.</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>Hazel</td>
<td>50</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>Topaz</td>
<td>47</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>Mustard</td>
<td>44</td>
<td>0</td>
<td>tr</td>
<td>0</td>
<td>0</td>
<td>P</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Marguerite</td>
<td>40</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>P</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>White</td>
<td>27</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>White</td>
<td>18</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

As early as 18 h following pupation traces of isoxanthopterin are found in both wild-type and st eyes. In wild type the concentration increases during early pupal development but decreases near the time of ommochrome deposition. It is continually present, however, in varying amounts until the imago emerges. The HB compounds (2-amino-4-hydroxypteridine and biopterin) appear first between 50 and 55 h after pupation. Xanthopterin, on the other hand, appears
at approximately the same time that the drosopterins appear. It is of interest to note that some pteridines do appear in various organs of the \textit{bw} mutant. The concentrations of these are not as high as in wild type.

\textbf{Table 2.} \textit{The sequence of pigment development in vivo in organs of the \textit{bw} mutant of \textit{Drosophila melanogaster}}

Symbols as in Table 1.

<table>
<thead>
<tr>
<th>Color</th>
<th>Pupal age (h)</th>
<th>Head</th>
<th>Mal. tube</th>
<th>Testes</th>
<th>Head ommochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IX</td>
<td>XP</td>
<td>HB</td>
<td>IX</td>
<td>XP</td>
</tr>
<tr>
<td>Copper brown</td>
<td>91-96</td>
<td>+ +</td>
<td>+ +</td>
<td>0</td>
<td>tr</td>
</tr>
<tr>
<td>Saona</td>
<td>80</td>
<td>+</td>
<td>+ +</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Copper leaf</td>
<td>75</td>
<td>tr</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Sorrell</td>
<td>70</td>
<td>tr</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Hazel</td>
<td>64</td>
<td>tr</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Buff-topaz</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Mustard</td>
<td>52</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Marguerite</td>
<td>46</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>White</td>
<td>44</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

\textbf{Table 3.} \textit{The sequence of pigment development in vivo in heads of the \textit{st} mutant of \textit{Drosophila melanogaster}}

Symbols as in Table 1. In the absence of an appropriate named color the code designation from a \textit{Dictionary of Color} by Maerz & Paul (1950) was used.

<table>
<thead>
<tr>
<th>Color</th>
<th>Typical age pupal (h)</th>
<th>Dros.</th>
<th>IX</th>
<th>XP</th>
<th>HB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scarlet</td>
<td>90</td>
<td>+ + + +</td>
<td>+ +</td>
<td>+ + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Mandarin red</td>
<td>86</td>
<td>+ +</td>
<td>+ +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Burnt orange</td>
<td>82</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10-D-9</td>
<td>80</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Apricot</td>
<td>72</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Jacinthe</td>
<td>70</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Sweetmeat</td>
<td>68</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Cream</td>
<td>65</td>
<td>tr</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>White</td>
<td>60</td>
<td>0</td>
<td>+ +</td>
<td>0</td>
<td>tr</td>
</tr>
<tr>
<td>White</td>
<td>50</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>tr</td>
</tr>
<tr>
<td>White</td>
<td>18</td>
<td>0</td>
<td>tr</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textbf{In vitro pigment development}

Cultured wild-type eyes developed approximately the same colors as did the \textit{in vivo} eyes although the rate of pigment deposition was considerably retarded in all cases. Fifty-nine per cent of the eyes dissected from pupae younger than 40 h were capable of developing 'marguerite' or 'mustard' coloration (cf. Table 4). The other 41% remained white. These colors result from the deposition of
ommatins. Eyes from pupae between 40 and 44 h old, dissected when white, were capable of attaining the 'hazel' stage. This corresponds to an in vivo age of about 55 h and involves the deposition of the yellow and brown ommochromes. This level of pigmentation was usually reached within the first 24-48 h after dissection. If the eyes remained in culture for 3 or 4 more days, the color generally faded. No drosopterins were detected in eyes cultured from pupae of this age.

The in vitro pigmentation of eyes of older wild-type pupae was somewhat variable. Six of 48 dissected from 'marguerite' eyed pupae (45-48 h old) developed traces of drosopterins. The percentage of eyes depositing drosopterins increased with the age at dissection until all eyes from pupae 65 h or older formed drosopterins. Most of the eyes which formed drosopterins showed a normal pattern of pteridine formation. Five, however, exhibited unusually high concentrations of the HB compounds.

Several st eyes were also cultured. All those removed after 45 h of pupation developed at least a pale yellow color. This color may have been that of the cornea; no pigment was identified on the chromatograms. Three of nine eyes cultured at 52 h formed appreciable amounts of drosopterins. Seven eyes 71-74 h old were cultured. At this age some drosopterins are already present; these eyes were capable of depositing further pigment until the adult scarlet color was attained. One of these lacked completely the HB compounds.

Eyes removed from the bw mutant behaved exactly as did the wild-type cultures except that no drosopterins were deposited.

Table 4. Frequency and extent of pigment development in vitro of wild-type eyes explanted at various ages from pupae.

<table>
<thead>
<tr>
<th>Age at explant</th>
<th>No. eyes</th>
<th>Supplemented</th>
<th>% no color</th>
<th>% yellow (marguerite)</th>
<th>% yellow-orange (mustard)</th>
<th>% brown (coptic)</th>
<th>% red-violet (rubyiate)</th>
<th>% Drosopterins</th>
</tr>
</thead>
<tbody>
<tr>
<td>35-39 (white)</td>
<td>51</td>
<td>No</td>
<td>41</td>
<td>41</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40-44 (white)</td>
<td>8</td>
<td>No</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45-48 (marguerite)</td>
<td>31</td>
<td>No</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>84</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>49-52 (topaz)</td>
<td>17</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>76</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>53-60 (hazel)</td>
<td>39</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>69</td>
<td>23</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>10</td>
<td>32</td>
<td>29</td>
</tr>
</tbody>
</table>

Eye cultures supplemented with other organs

Testes and Malpighian tubules were selected as supplements to eye cultures because it is known that they contain pigmented and unpigmented pteridines (Hadorn, 1958; Hadorn, Hanly & Gandolla, 1962). The results of supplementation of wild-type eye cultures with organs removed from the eye donor and from older pupae are presented in Table 4. No statistically significant differences
were noted between supplemented and unsupplemented cultures. The results of
the numerically few supplemented cultures suggest that eyes grown in the pre-
sence of testes or Malpighian tubules may deposit pigments at a later develop-
mental stage than do eyes grown separately. Similar results were found in
supplemented cultures of \(st\) and \(bw\) eyes.

**DISCUSSION AND CONCLUSIONS**

Although many scores of eye color mutants are recognized and mapped in
*D. melanogaster*, very little is known about the biochemical synthesis of the
contributing pigments. A single enzyme, xanthine dehydrogenase, which deals
directly with eye pigments has been studied in some detail (Hubby & Forrest,
1959; Forrest, Hanly & Lagowski, 1961; Glassman, 1965). The activity of this
enzyme is apparently responsible for the conversion of 2-amino-4-hydroxypteri-
dine into isoxanthopterin as well as the formation of uric acid from purine
precursors. These reactions are not, however, in the biosynthetic pathway of
drosopterins. The relative concentrations of isoxanthopterin in wild-type and
\(st\) eyes *in vivo* correspond quite well to the variation of xanthine dehydrogenase
concentrations during development (Handschin, 1961; Glassman & McLean,
1962).

Several mutants are known which affect xanthine dehydrogenase activity and
drosopterin concentrations as well as the concentration of 2-amino-4-hydroxy-
pteridine and isoxanthopterin (Forrest *et al*., 1961; Glassman, 1965). The mutants
rosy and maroon-like indicate a relationship between xanthine dehydrogenase
and drosopterin concentrations. On the other hand, the mutant *lix* (*little
isoxanthopterin*) does not show this relationship (Hubby, 1962). The fact that
some cultured eyes showed very marked variation in HB and isoxanthopterin
concentrations and that these variations did not correlate with drosopterin
concentrations suggests that a system is operating which is more complex
than that suggested by Hubby & Forrest (1959) involving the production of
reduced nicotine adenine dinucleotide (NADH).

Schneider (1964) has shown that both ommochromes and pteridines can be
deposited in cultured eyes removed as early as the third larval instar. Her
experiments involved, however, the removal of the entire brain complex
including both cerebral hemispheres, the ventral ganglion, both eye-antennal
disc complexes, probably the ring gland, and associated structures such as aorta,
trachea, etc. These results have been verified in this laboratory (Hanly &
Burdette, unpublished results). It is significant therefore that very little pigment
developed in single eye-cerebral hemisphere complexes cultured before 40 h of
pupal age. Supplemented cultures of such young eyes were not attempted.
Ephrussi & Beadle (1937) and subsequent workers (Beadle, Anderson & Maxwell,
1938; Ephrussi, 1942) have demonstrated the ability of ommochrome precursors
to diffuse from supplemental organs into the eyes of transplantation experi-
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The explantation experiments reported here indicate that the necessary substances are not present in the 39 h old pupal eye in sufficient concentrations to allow the development of a major amount of pigment. Furthermore, those substances necessary for drosopterin deposition are not present in adequate quantities in the eye itself until 45 h after pupation.

Thus it is clearly established that organ interaction plays an essential role in the development of Drosophila eye pigments. The exact nature of this interaction remains obscure. The fact that intact larval brain complexes are able to develop color under similar culture conditions indicates that the medium provides adequate nutrition and that young eye anlage are competent to develop pigment in vitro. Since endocrine organs are included in the brain complex, it is possible that hormonal activity may be involved. Furthermore, essential precursor compounds or enzymes potentiating the synthetic pathways may be produced by organs other than the eye. The equivocal results of the supplementation studies may only indicate that the organ interactions are very complex. Further studies utilizing a wider range of organs and organ combinations accompanied by hormone or hemolymph supplementation might elucidate these relationships.

SUMMARY

The independence of pigment formation in the insect eye was tested using in vitro cultures of eyes of wild-type Drosophila melanogaster. Although larval eyes cultured attached to the entire brain complex and associated organs can form both ommochromes and drosopterins, it was found that similarly cultured isolated eyes attached to but a single cerebral hemisphere only become competent to form most of these pigments after 40 and 45 h of pupal development (25 °C) respectively. This indicates that organ interaction is involved in the development of the eye pigments. Supplementation of cultures of developing pupal eyes with testes and Malpighian tubules yielded inconclusive results.

Pigment development was evaluated using chromatography for fluorescent pteridines and for ommochromes and a visual comparison of the eye with color standards. The sequence of development of each of these intact wild-type, scarlet, and brown D. melanogaster pupae is described. Marked variation in the concentrations of 2-amino-4-hydroxypteridine, biopterin, and isoxanthopterin formed in cultured eyes suggests that the synthetic pathways involved in pteridine synthesis are exceedingly complex.

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