Morphology and development of the *Drosophila* eye

II. *In vitro* development of ommatidial bristles

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The morphological changes which take place in the differentiation of the adult *Drosophila* eye from the larval imaginal disc have been described from a histological and cytological standpoint by Shatoury (1956, 1963) and Shatoury & Waddington (1957). In addition, Gottschewski (1960), Gottschewski & Querner (1961), and Schneider (1964) have studied these processes in eyes and brains *in vitro*. These latter workers removed eye-antennal anlage with associated structures, and placed them in culture media. Under these conditions, the imaginal discs can apparently undergo most of the normal differentiation processes, finally attaining nearly all of the adult morphology.

The imaginal eye disc can be found in the *Drosophila* larva as early as the larva’s emergence from the egg (Kaliss, 1939), and its size is known to increase during the larval instars primarily through cellular division which, in turn, ceases almost entirely shortly after metamorphosis begins (Hadorn, 1965). The onset of differentiation of the eye disc apparently occurs earlier than that of the other imaginal discs (E. Hadorn, personal communication). In the late larval and early prepupal stages, groups of cells are set aside which will eventually become the definitive ommatidia (Chen, 1929; Shatoury & Waddington, 1957). Waddington & Perry (1960) have described, by use of the electron microscope, the morphological changes which occur in the eye components of the late larval and some pupal stages. The various morphogenetic movements of the eye disc and the cerebral hemisphere (and later the optic lobe) have been described by several investigators (Shatoury, 1956; Gottschewski, 1960; Gottschewski & Querner, 1961; Schneider, 1964). The adult size and shape of the eye are reached between 40 and 50 h after puparium formation, while ommochromes first appear at 45 h and pteridines at 65 h when raised at 25 °C (Hanly, Fuller & Stanley, 1967).

It is interesting to note that none of these papers describes the differentiation of the bristles (hairs) associated with the eye surface, although Waddington &

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Perry (1960) describe in detail the hair-nerve cell group from which the bristles grow. Furthermore, some controversy exists in the literature regarding the origin of the tissue connecting the optic lobe and imaginal eye. The following report will attempt to clarify these points.

**MATERIALS AND METHODS**

The methods of timing pupal development, obtaining tissue and establishing and maintaining organ cultures have been previously described (Hanly et al. 1967). One exception to these methods is that all *Drosophila* stock which were used as tissue sources were maintained in an aseptic condition so that the sterilization steps in culture preparation were not so critical. Under these circumstances contamination in culture was extremely rare. All *Drosophila* stock and organ cultures were maintained at 26 ± 0.5 °C.

Ages given throughout this publication indicate time elapsed from puparium formation and include the prepupal as well as a portion of the pupal periods. Anatomical terms are taken from Shatoury (1956).

At the outset a series of eyes attached to hemispheres were removed from pupae and placed in hanging-drop cultures every half hour beginning at 22 h after puparium formation and continuing until 31 h of age. The cultures were then examined every hour for an 18 h period, these results being used for comparison to experimental cultures. At the end of the 18 h period some of the cultures were fixed as described below and sections were prepared for microscopic examination.

Various tissue arrangements were used for examining inductive processes. Eyes and optic lobes were sometimes separated and cultured either together or individually. Furthermore, entire brain complexes, including cerebral hemispheres, optic lobes and attached eyes were observed in vitro.

In order to establish the *in situ* steps in differentiation, timed pupae were dissected in *Drosophila* Ringer's solution and examined with both a dissecting microscope and a phase contrast microscope. Freshly removed eyes attached to cerebral hemispheres (or optic lobes, depending on age) were fixed for 2 h in Bouin's solution, embedded in paraffin and serial sections were cut 5 μ thick. These were stained with a modified Mallory's triple stain and examined for normal histological appearance.

The culture medium was the 'complete' type described by Schneider (1964), containing 10 % fetal bovine serum. Studies of inhibition of bristle development were initiated using Actinomycin D which was obtained from Merck, Sharp and Dohme.
RESULTS

Normal in vitro development. All eyes which were cultured were examined for the following points while in culture: primary inversion of the eye, subsequent eversion of the eye, integrity of the tissue (including the optic lobe), extension (growth and lengthening) of the ommatidia, growth of the optic lobe and separation into distinct glomeruli, growth of the optic nerve, bristle formation, and finally pigment deposition. Nearly all eyes with attached optic lobes removed from pupae 22 h old or older will continue to develop in culture until adult level of differentiation and morphology is reached by most structures. The events following removal and culture can best be described as follows:

(1) Eversion. If the eye with attached optic lobe is removed from the pupa 22 h old or older, it is generally inverted as a result of dissection technique. Between ages 27 and 28 h the eye apparently has a greater natural tendency to assume this position than it does during ages on either side of this period. Plate 1, fig. 1 shows a typical eye in this inverted state. It is interesting to note that this unnatural situation usually corrects itself in culture as described below in connexion with nervous tissue growth.

(2) Optic lobe and optic nerve. The distal portion of the optic lobe begins to elongate and become differentiated into the adult outer optic glomerulus. This process has been described by Shatoury (1956) from in vivo observations. Optic lobes cultured alone must be at least 28 h or older to demonstrate the extension of the outer optic glomerulus in vitro (Plate 1, fig. 2). Development of the outer glomerulus is accompanied by elongation of the optic nerve although the growth of the nerve is not greatly retarded by earlier removal time. This has been demonstrated by isolating eyes and attached optic nerves (without the optic lobe) from 24 to 29 h old pupae. During the first 12 h in culture the optic nerve in many of these experiments was seen to double in size. As a result of the growth of the outer optic glomerulus and the optic nerve, the center portion of the inverted eye is pushed out while the edges of the eye gradually wrap around the growing glomerulus until the normal everted position is obtained. This extension of the optic lobe and nerve will be apparent within 4–6 h after removal if the pupa was approximately 27 h old at dissection, and will require about 12 h for completion. If the pupa was younger, the optic lobe differentiation begins later, continues longer, and is not as complete.

Frequently, when eyes and attached optic lobes are cultured in vitro, ramifications of the optic nerve can be observed between the extending outer glomerulus and the ommatidial layer. The relatively clear area in Plate 1, fig. 3, which demonstrates this ramification is apparently an artifact of culture, since it is missing in situ. This artifact is occasionally accentuated when an eye apparently everts very tightly around the outer glomerulus. Fluid accumulates in the space between the outer glomerulus and the ommatidial layer and the eye balloons out to nearly twice the size of the optic lobe. Under this condition, stretched nerve
tracks can be followed from the outer glomerulus to each ommatidium, demonstrating the extensive network necessary for innervation of the ommatidia. Plate 1, fig. 4 shows these nerves in a thin section of a relatively normal eye-optic lobe arrangement.

(3) Ommatidia. Within 1–2 h after removal, the sharp definition of the ommatidia is lost, especially in eyes from younger (22–25 h) pupae. This appears to be due to 'shock' following dissection and placement in different environmental conditions. The inversion of the eye itself might add to the haziness of the ommatidia by distorting them slightly. Sharp definition of these structures is usually regained within 4–6 h, as the eye begins to evert.

At the time of removal (between 24 and 30 h after puparium formation) a layer of tissue overlies the ommatidia in the place where the ommatidia will develop later. The ommatidia themselves, however, are relatively short structures seen as a translucent layer on the outer portion of the eye. Each ommatidium elongates with the passage of time, until it reaches that point demonstrated in Plate 1, fig. 5. This is an eye which was removed when 27½ h old and has been in culture for 8 h. During this time, a very dense layer of tissue can be seen in the basal portion of each ommatidium forming a dark line. This darkening is much more intense in vitro than in vivo and does not represent ommochrome deposition. According to Shoup (1966) this area of the ommatidia would be occupied primarily by the secondary pigment cells.

(4) Bristles. At about the time that optic lobe extension has reached the point shown in Plate 1, fig. 5, very fine, short, bristles can be seen growing from alternate corner junctions of the ommatidia as diagrammed in Text-fig. 1. The outer optic glomerulus continues to enlarge and the ommatidia elongate until a structure similar to Plate 1, fig. 6, is obtained. The bristles are by this time only somewhat shorter than the size of the imaginal bristles.
Of most interest in this last series of events is that bristles do not develop if the eye and optic lobe are removed from the intact pupa prior to 27 h (Table 1). If the complex is removed when the pupa is between 27 and 28 h old, the bristles most frequently develop no further than fine, short hairs. On the other hand, the growth of the outer glomerulus and the optic nerve continues until adult shape is attained, finally resulting in an imaginal eye with rudimentary or no bristles. When the eye alone is cultured, bristles will not develop if removal was prior to 28–28 1/2 h. Culturing the eye and optic lobe together but cut from each other yields identical results, i.e. the eye differentiation behaves as if no optic lobe were present.

The later acquisition of competence by the eye alone to initiate and maintain bristle development can be clearly demonstrated by removing and culturing an eye with attached lobe from a 28 h old pupa and comparing its development with its sister eye cultured alone. The former will develop ommatidial bristles while the latter most often will not.

Many entire brain-eye complexes without the antennal discs and ventral ganglia were also cultured. A much greater percentage of these cultures developed normally. Almost every eye everted, the optic lobes and ommatidia grew, and if the complexes were removed after 25–25 1/2 h, bristles were formed (Table 1). This is 1–2 h earlier than when culturing only optic lobes and attached eyes and 2–3 h earlier than single eyes cultured alone. If the complete complexes were allowed to remain in culture long enough, some of them developed the full complement of ommachromes and drosopterins, reaching the adult level of wild-type pigmentation.

It should be pointed out at this time that bristles begin to appear on the eye in vivo between 31 and 32 h of age. In only a very few cases did bristles appear this
early in vitro, and most of these were from eyes removed from older pupae. In general, the developmental process is retarded in vitro. The reasons for this can be many and have been adequately discussed by Schneider (1964). The most obvious one—that of an unsatisfactory medium—should be again emphasized.

Inhibition studies. With the possibility in mind that the induction processes (gene activation?) for the various developmental steps might occur at different times for different structures, Actinomycin D was used in anticipation that bristle development could be inhibited without disrupting ommatidial growth, optic lobe extension and nerve enlargement. Therefore, eyes and attached optic lobes were removed and cultured in medium containing various concentrations of Actinomycin D. Continuous exposure to Actinomycin D in concentrations of 0.01 μg/ml completely kills the tissue within 2-3 h (the earliest time examined). However, 0.001 μg/ml Actinomycin allows practically normal development of all structures, including bristles.

Table 1. The capability of various tissue combinations to produce ommatidial bristles when removed from pupae of various ages and placed in culture

The age column represents the time of pupal dissection. The whole complex includes both cerebral hemispheres, both optic lobes and both attached eyes. The eye+lobe includes a single optic lobe and its attached eye. The detached eye includes the optic lobe and eye separated from each other but in a single culture drop. The eye alone includes only the eye. The overlap in time within each column of the ability to develop bristles reflects in part the difficulty in establishing exactly the pupal age.

<table>
<thead>
<tr>
<th>Age</th>
<th>Whole complex</th>
<th>Eye + lobe</th>
<th>Detached eye</th>
<th>Eye alone</th>
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<tr>
<td></td>
<td>Not present</td>
<td>% present</td>
<td>Not present</td>
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<td>4</td>
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Pulse experiments were also run by dissecting the pupa (between 27 and 29 h old) in normal medium, removing one eye with attached lobe to medium containing Actinomycin where it was left for an appropriate period of time and
then washing it in approximately 4 ml of normal medium. It was then placed directly into the hanging drop. The other eye with attached lobe served as a control in another hanging drop. Preliminary results from these experiments indicate that 5–7 min exposures to 0.05–0.1 μg/ml Actinomycin allows normal eversion, optic lobe and nerve enlargement, and ommatidial elongation, but retards bristle appearance and development.

**DISCUSSION**

The interaction of tissues in embryonic induction processes is a well-described phenomenon, but its mechanisms are little understood. That an undifferentiated tissue can become determined in one direction obviously involves gene activation and deactivation. The imaginal discs in *D. melanogaster* were long thought to be determined from early embryonic life onward. However, as Waddington (1942) and Hadorn (1965) have demonstrated, this determination is considerably more flexible than previously believed. The latter’s work shows, for instance, that cells from wing discs can be ‘transdetermined’ from wing components to leg components and vice-versa. Most of the imaginal discs have no internal organization resembling the adult organ until after the beginning of metamorphosis (Bodenstein, 1950). This does not seem to be the case for eye discs, inasmuch as packets of four cells each, presumably precursors to the ommatidia (Chen, 1929; Shatoury & Waddington, 1957), can be distinguished in the third instar larva. Hadorn (personal communication) has, during his transdetermination work, reached the conclusion that eye discs are rigidly determined earlier than most of the other imaginal cell groups.

Mention of the ommatidia, cornea, and tissue surrounding the eye is very common in the literature, but little mention has been found concerning the ommatidial bristles. Bodenstein (1950) suggests that these bristles appear during the pupal stage at about the same time that pigment begins to appear. However, his findings do not agree with findings in this laboratory where bristles appear *in situ* between 31 and 32 h after puparium formation, while ommochromes put in their first appearance at 45 h at 25–26 °C. Furthermore, it is obvious that the bristles are not determined within the eye until approximately 29 h. Histological sections indicate that the bristle-forming cells appear in the ommatidia somewhat earlier as completely separate units. They are certainly well developed at 48 h (Waddington & Perry, 1960).

Beadle & Ephrussi (1936) describe the morphology of the eye after it has developed from a transplanted disc in the abdomen of a host. They mention the ommatidia, pigment and the larger bristles which are present on the tissue adjacent to the eye, but do not make note of the ommatidial bristles. Nor does Schneider (1964) describe these structures when discussing the morphology of the cultured eye discs and brain complexes. It appears that these bristles have been overlooked by most observers.
The time differences shown in Table 1 indicate that a 'determination current' composed of one or many substances is probably moving from the central portion of the brain outward to the eye. A similar process—'Determinationsstrom'—has been suggested by Wolsky & Huxley (1936) and Gottschewski (1960) for the differentiation of ommatidia, cornea and pigment formation earlier in pupal development. At 25 or 26 h after puparium formation, the brain complex with the attached eyes is competent to form ommatidial bristles. However, at this time the optic lobe itself is not yet capable of initiating and sustaining this process of determination. Some 'switch' is triggered at approximately 27½ h in the optic lobe either in the form of a new 'determination current' or sufficient concentration of a previously existing one, so that removal of the optic lobe and attached eye after that time will still allow development of ommatidial bristles. Again, however, this triggering event has not reached the eye itself until about 28½ h.

The nature of the 'determination current' is at this time unknown, although two explanations for the data may be offered. It could be material which requires the integrity of the nervous tissue for transmittal. This would be indicated (but only indicated) by the fact that culturing the optic lobe and eye separated from each other within a single drop gives results identical to culturing the eye alone. However, these data do not preclude a diffusible substance which, under the conditions of separating the eye and optic lobe, is diluted to an ineffective concentration in the relatively large volume of the culture drop.

A second explanation, possibly related to the first, could be pictured as sequential gene switching in different organs. A certain substance appearing in the central portion of the brain at 25 h could diffuse to the optic lobe and trigger a series of separate reactions which, in turn, could cause a new set of reactions in the eye (cf. Hadorn, 1955). Further work is under way toward elucidating the nature of the inducing substance(s).

Shatoury (1956) describes the tissue between the imaginal outer glomerulus and the ommatidial layer as a probable derivative of the brain itself, in contrast to Pilkington (1942), who contends it originates from the developing eye. The data reported here indicate that the major contribution to this interconnecting layer of tissue comes from the optic nerve, although a small portion is provided by the outer glomerular extension. The optic nerve is present, however, at least as early as first instar during larval development (Waddington, personal communication). If the optic nerve derives from the brain as Shatoury describes, then all of the interconnecting tissue is nerve tissue.

A word of caution is necessary when interpreting in vitro results as if they occurred in vivo. It is possible, for instance, that the 'growth' observed in both the optic nerve and outer glomerulus in vitro results from fluid engorgement by the tissues. It should be noted, however, that extension of the outer glomerulus alone from an isolated optic lobe will not occur in vitro if dissection was made prior to 28 h, while growth of the optic nerve (attached to eye) occurs without
the presence of the optic lobe following much earlier dissection. Since outer glomerular extension occurs in young cultures if the eye is attached, it appears that initiation and maintenance of this growth is dependent upon intimate association with the eye or nerve or both, in contrast to the interpretations of Gottschewski (1960).

Since it was expected that bristle induction and growth would involve differential gene action, it was not surprising to find that Actinomycin D retarded this portion of morphogenesis without inhibiting that previously induced. It was surprising, however, that such very low concentrations of Actinomycin were effective in inhibiting all growth and differentiation of the eye. Two explanations of this phenomenon can be offered. One possibility is that the eye and outer glomerulus are extremely permeable to the antibiotic, as indicated by the fact that both these structures rapidly turn yellow in relatively low concentrations of Actinomycin while the remaining portion of the optic lobe is left uncolored. A second explanation involves the similarity between Actinomycin and naturally occurring pigments of the eye, the ommochromes. This similarity is shown in Text-fig. 2. Those portions of the Actinomycin chromophore which are almost identical to the ommochrome chromophore have been suggested as the active sites for Actinomycin inhibition (Reich & Goldberg, 1964).

If precursors to ommochromes are already present in the eye at the time of bristle development, the concentrations of these natural compounds added to the exceedingly low concentrations of the related Actinomycin may be sufficient to surpass the tolerance level of the tissues. The apparent high permeability of the eye and outer glomerulus to Actinomycin may be explained by the necessity of this tissue to incorporate precursors to ommochromes.
The development of ommatidial bristles (hairs) in vitro has been studied by culturing the eyes of *Drosophila melanogaster* with various tissues attached. If the entire brain complex (cerebral hemispheres, optic lobes and attached eyes) is removed into culture from flies 25-25½ h after puparium formation, bristles will develop on the ommatidial surfaces. Earlier removal of the complex prevents bristle development. On the other hand, removal of the optic lobe and attached eye, without the cerebral hemisphere, must be delayed until 27-27½ h in order for bristles to appear in vitro. Eyes cultured alone, or with the optic lobe detached, will develop bristles if removed from the intact pupa only after 28-28½ h. These bristles appear in situ between 31 and 32 h after puparium formation when grown at 26 °C. The possibility of the movements of a ‘determination current’ from the central portion of the brain out to the eye is discussed.

The differentiation of the nervous tissue connecting the outer optic glomerulus and the ommatidia of the adult has been studied. This tissue appears to originate primarily from the optic nerve, with a small contribution from the outer optic glomerulus.

Studies on the inhibition of the bristle development with Actinomycin D indicate that very small concentrations of the antibiotic will affect the tissues. Possible reasons for this low concentration effect are discussed.

**ZUSAMMENFASSUNG**

*Die Morphologie und Entwicklung des Drosophila-Auges. II*


Die Differentiation des Nervengewebes, welches den imaginalen Medulla externa (outer optic glomerulus) und die imaginalen Ommatidien verbindet, wurde untersucht. Dieses Gewebe scheint von allem von optischen Nerv gebildet zu werden, und in geringerem Masse von der Medulla externa.

This research was supported in part by grant GB 2915 from the National Science Foundation and in part by a grant from the University of Utah Research Foundation.

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(Manuscript received 4 November 1966)