DEVELOPMENT OF THE *DROSOPHILA* RETINA

Thesis by

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We have left undone those things
which we ought to have done; and
we have done those things which
we ought not to have done.

Book of Common Prayer, 1789

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ABSTRACT

Pattern formation in the Drosophila retina proceeds by the recruitment of cells, along a morphogenetic front, into a lattice. At the advancing front, marked by a dorso-ventral furrow in the eye imaginal disc, cells are organized into ommatidial precursors, each containing cells destined to become photoreceptors 2, 3, 4, 5, and 8. Behind the front, a mitotic wave produces photoreceptors 1, 6, and 7, plus the remaining cells needed to complete the ommatidia. During the third larval instar, the front sweeps anteriorly across the eye disc, leaving a highly ordered pattern in its wake. Preceding the dorso-ventral furrow is a groove that bisects the eye disc into dorsal and ventral halves and presumably plays a role in establishing the equatorial symmetry line.

Cell lineage plays little role in pattern formation in the eye. Genetic mosaics show that the cells of each ommatidium are not derived from a single mother cell; the cells appear to be recruited at random at the morphogenetic front. Similarly, the mirror symmetry above and below the equator is not established by a clonal mechanism; a single clone can contribute cells to ommatidia on both sides of the equator.

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CHAPTER I

GENERAL INTRODUCTION

Approximately half a billion years ago, nature discovered how to build a compound eye. The work in this thesis (taking somewhat less time) is an attempt to understand that process.

Several comprehensive reviews of compound eye anatomy and physiology have recently been published (Goldsmith and Bernard, 1974; Eakin, 1972; Trujillo-Cenóz, 1972). This introduction will review the cellular pattern of the compound eye and its development.

Anatomy of the Compound Eye

Only insects and crustaceans have compound eyes. The eyes of other arthropod classes differ considerably in structure. Even the well developed lateral "compound" eyes of Limulus bear only superficial resemblance to the true compound eyes of insects and crustaceans.

The ommatidium, the basic unit of the compound eye, is strikingly similar in all insects. In each ommatidium, a refractive apparatus formed by six cells serves to gather light and focus it onto a group of photoreceptors, usually eight in number. Each group of photoreceptors is optically isolated by a sheath of pigment cells which are far more variable in number.

Natural selection has elaborated this highly conserved unit into specialized forms suited to the particular needs of each insect. Thus, a consideration of the different structures of compound eyes must take into account the different ways insects make their living.

Externally, the most obvious specialization of compound eyes is the number of ommatidia composing the eye. This number is strongly correlated with the freedom of movement and visually guided behavior of the insect. Proturans and Diplurans, primitive wingless insects which live in moist soil or under logs and stones are completely devoid of eyes. Collembola (springtails), also apterygotes, are negatively phototactic and characteristically have only eight ommatidia per eye (Paulus, 1975). Dragonfiles, which display elaborate visually guided behavior may have over 10,000 ommatidia per eye (Goldsmith and Bernard, 1974).

Regional specialization within the compound eye is another externally obvious adaptation. Whirligig beetles (Gyrinidae) which swim on the surface of ponds and streams have separate dorsal and ventral eyes enabling them to see above and below the water simultaneously (Bott, 1928). A more subtle form of dorso-ventral specialization is seen in the size of facets. Many dragonflies and flies have "divided" eyes in which dorsal facets are up to twice as large as the ventral ones (Exner, 1891; Deitrich, 1909). In both cases the large size of the dorsal facets appears

to be related to the ability of these insects to mate and catch prey in flight. In Diptera, divided eyes are usually characteristic of males (Dietrich, 1909). According to Downes (1969) this type of eye occurs in species where males swarm in stationary flight and mate with females flying past. In species such as Drosophila, where courtship is performed on a solid footing, males do not have divided eyes. The females of some dipteran species also show enlarged dorsal facets. In these cases the females are predacious, catching other insects in flight (Downes, 1958). Robber flies (Asilids), which hover in stationary flight and dart foward to capture prey, have enlarged facets at the anterior of the eye (Downes, 1969). However, Musca also possesses enlarged anterior facets; their significance for this species is uncertain (Braitenberg, 1967).

Facets are usually arranged in a regular hexagonal lattice. In Drosophila this arrangement is extremely orderly, giving the eye a "smooth" appearance. In skipper butterflies (Horridge et al., 1972) and desert ants (Brunnert and Wehner, 1973) small "rough" patches of disarranged facets occur in an otherwise smooth eye. Unfortunately, no comprehensive survey of eye lattices is available. In divided eyes, the transition from small to large facets within an eye is mediated by facets of intermediate size and shape. In Musca a zone of medium-sized square facets lies between small hexagonal facets at the posterior and

large hexagonal facets at the anterior (Braitenberg, 1967). A similar zone occurs between the large dorsal and small ventral facets of the dragonfly (Trujillo-Cenóz, 1972).

In many insects, the surface of each facet is thrown into an array of small protuberances called corneal nipples (Bernhard et al., 1970). Bernhard et al. (1968) showed that an orderly array of such nipples could decrease reflection and increase transmission through the cornea, a potential advantage for nocturnal insects. Corneal nipples are well developed only in some caddisflies (Trichoptera) and some Lepidoptera. Among Lepidoptera, they tend to be best developed in nocturnal species. Drosophila, like most Diptera, possesses only rudimentary but fairly regular corneal nipples.

The cornea is a chitinous, extracellular, laminated cuticle which appears to be secreted by the microvilli of the primary pigment cells (Gemne, 1971). It is very similar to cuticle found elsewhere in the body (Locke, 1974). Waddington and Perry (1960) claim that in Drosophila the cone cells (see below) also contribute to the secretion of the cornea; however, this may rest upon an erroneous interpretation of electron micrographs. In some insects, particularly horse flies, deer flies, and long-legged flies, the alternating corneal layers of high and low refractive index serve as interference filters. These produce bright, metallic eye colors and may enhance the contrast of colored

objects (Bernard and Miller, 1968).

Beneath the corneal lens, four cells form a transparent cone which optically connects the lens to the photoreceptors. In eyes where the photoreceptors extend the length of the ommatidium (apposition eyes, see below) the interposed cone cells form a squat, truncated cone. In eyes where the photoreceptors lie deep in the retina (superposition eyes, see below), the cone cells may form a long, thin "crystalline tract" which serves as a light pipe. Cone cells frequently extend thin processes down between the photoreceptors and terminate in pigment-filled feet (Apis, Goldsmith, 1964; Aedes, Brammer, 1970; Locusta, Horridge, 1966). In Drosophila, Waddington and Perry (1963) found this swollen foot complex just above the basement membrane, surrounded by the eight axons of the photoreceptors.

Grenacher's (1879) survey of compound eyes recognized four distinct cone structures: (1) acone, in which the cone cells occupy their characteristic positions but do not form a specialized refractile body, (2) eucone in which the cone cells form a hard, transparent, intracellular "crystalline cone," (3) pseudocone, in which cone cells secrete a clear, gelatinous fluid into the space beneath the lens bounded laterally by the primary pigment cells and proximally by the cone cells, and (4) exocone, in which the cone is a parabolic inward extension of the cornea. Cone cells in an exocone eye may form a crystalline tract. Cone types

are distributed with no obvious system throughout insect orders. Drosophila has a pseudocone eye.

The photoreceptors or retinula cells are similar ultrastructurally. Each retinula cell is a monopolar sensory neuron. Its membrane is folded into a close-packed array of microvilli 20 to 100 nm in diameter and 500 to 5,000 nm in length (Goldsmith and Bernard, 1974). Together, the microvilli form a rod-like structure, the rhabdomere, which contains the visual pigments (Langer and Thorell, 1966). Microvilli are usually parallel within a single retinula cell, but in certain insects, such as the firefly Photuris (Horridge, 1969) and the scarab beetle Repismus (Horridge and Giddings, 1971b), they may change orientation. Rhabdomeres with parallel microvilli are dichroic (Langer, 1965), and it has been suggested that insects may exploit this property to detect polarized light.

Rhabdomeres have a higher refractive index than the surrounding tissue or extracellular space and act as optical waveguides, increasing the probability that a photon entering a rhabdomere will be absorbed by the photopigment (Seitz, 1969). Retinula cells of Musca (Kirschfeld and Franceschini, 1969) and several other insects (Walcott, 1975) contain small pigment granules of high refractive index which can modulate light propagation along the rhabdomere by changing the refractive index distribution at its boundary. In the light-adapted eye these granules lie close along the side

of the rhabdomere, making total internal reflection less efficient, thus "bleeding" light from the waveguide. In the dark, the pigment granules move away from the rhabdomere, allowing light to propagate farther. In Drosophila these granules provide a useful marker for the genotype of the cell.

Adjacent retinula cells within an ommatidium are generally stitched together along their length by a zonula adherens. Presumably these aid in maintaining the characteristic geometry of the assembly of rhabdomeres, the rhabdom.

All insects, except Hymenoptera and certain Lepidoptera, have eight retinula cells (with the possible exception of Repismus, see below). Reports in the early literature of fewer than eight retinula cells must be taken with reservation. For example, Hesse (1901) found seven retinula cells in ommatidia of the sugar mite Lepisma. A reexamination of the Lepisma eye with the electron microscope (Paulus, 1975), however, revealed an eighth, basal retinula cell. This cell lacks a rhabdomere, but does send an axon into the optic ganglion. Similar rhabdomereless, basal retinula cells have been found in the firefly Photuris (Horridge, 1969), the dragonfly Aeshna (Eguchi, 1971) and Locusta (Horridge, 1966). In an EM study of the scarab beetle Repismus, Horridge and Giddings (1971b) found only seven retinula cells. This claim should be confirmed with serial thin sections. Hymenopteran eyes so far examined all contain nine retinula cells (desert

ant, Cataylyphis, Brunnert and Wehner, 1973; European ant, Formica, Menzel and Lange, 1971; bee, Apis, Perrelet, 1970). Among Lepidoptera, butterflies with apposition eyes (see below) have eight retinula cells. Moths and butterflies with superposition eyes are far more variable. Skipper butterflies (Horridge et al., 1972) and the cabbage white butterfly Pieris (Meyer-Rochow, 1971) contain nine retinula cells per ommatidium. The moths Ephestia (Horridge and Giddings, 1971a) and Bombyx (Eguchi, 1962) both contain eleven. The significance of these variations is unknown.

Exner's (1891) division of insect eyes into apposition eyes, characteristic of diurnal species, and superposition eyes, found in nocturnal species, remains a roughly valid classification of compound eyes, despite certain anomalous cases. The rhabdom of an apposition eye extends almost the whole length of the ommatidium and is typically small in cross-sectional area compared to that of a superposition eye. Usually, the rhabdomeres meet along the central axis of the ommatidium to form a closed or fused rhabdom. However, in Diptera (Dietrich, 1909) and some Hemiptera (Oncopeltus, Shelton and Lawrence, 1974; Rhodnius, Müller, 1970; Lethocerus, Walcott, 1971; Gerris, Schneider and Langer, 1969), the rhabdomeres remain unfused at the center, forming an open rhabdom.

Superposition eyes are specialized for nocturnal vision by their ability to sum into one image the light gathered

by many facets (Exner, 1891). They have short rhabdoms which usually occupy only the proximal third of each ommatidium. Also, the pigment granules in the pigment cells surrounding each ommatidium move distally in the dark. Consequently, in the dark-adapted superposition eye a large, clear zone exists between the corneal lens and the rhabdoms. Exner reasoned that this clear zone would permit the images formed by each corneal lens to be superposed on the rhabdoms, producing a single, erect image. Although the detailed optics proposed by Exner have been questioned (Horridge, 1969), it is generally agreed that dark adaptation in the superposition eye provides increased sensitivity with some loss of acuity (Goldsmith and Bernard, 1974). In the light, the pigment sheath moves proximally, optically isolating each rhabdom.

The rhabdom has been the major focus of specialization in compound eyes, as witnessed by the great diversity of rhabdom structures. Few generalizations can be made concerning the structure of insect rhabdoms (see Fig. 1). In some cases, rhabdom structure is understandable in terms of the ecology of the insect. However, in most instances, the role of a particular rhabdom type is not understood. Even within a single order, great variations can occur. For example, the tiered rhabdom of Lepisma has elegant symmetry while a closely related Collembolan, Orchesella has an irregular, asymmetric rhabdom (Paulus, 1975). The stratified rhabdom of Lepisma in which four cells form a distal rhabdom

Fig. 1. Rhabdom structures (not to scale).

a) Lepisma, Collembola, after Paulus, 1975.

b) Orchesella, Collembola, after Paulus, 1975.

c) Periplaneta, Orthoptera, after Trujillo-Cenóz and Melamed, 1971.

d) Repismus, Coleoptera, after Horridge and Giddings, 1971.

e) Mastotermes, Isoptera, after Horridge and Giddings, 1971.

f) Lethocerus, Hemiptera, after Walcott, 1971.

(i) Rhabdom of occasional isolated ommatidia.

(ii) Usual rhabdom (Meinertzhagen, 1975).

g) Aeshna, Odonata, after Eguchi, 1971.

h) Photuris, Coleoptera, after Horridge, 1969.

i) Apis, Hymenoptera, after Grundler, 1974.

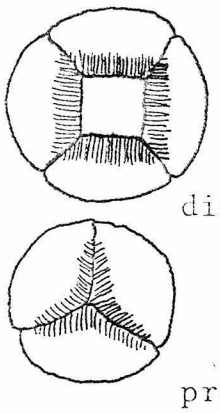
(i) Rhabdom orientation in dorsal half of eye.

(ii) Rhabdom orientation in ventral half of eye.

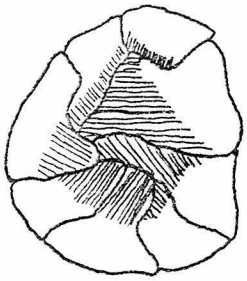
j) Drosophila, Diptera.

(i) Rhabdom in dorsal half of left eye.

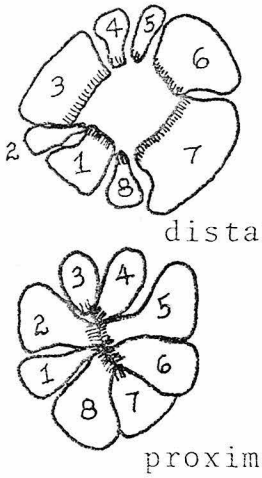
(ii) Rhabdom in ventral half of left eye.



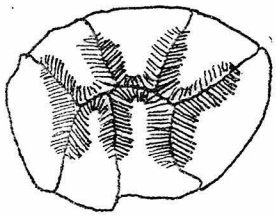
a) Lepisma



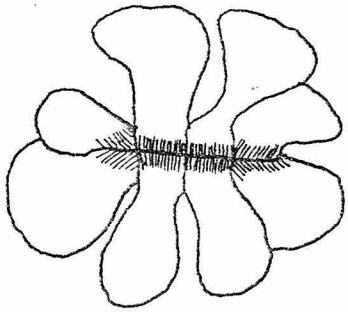
b) Orchesella



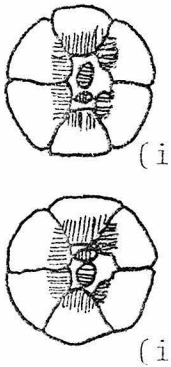
c) Periplaneta



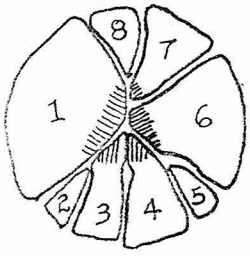
d) Repismus

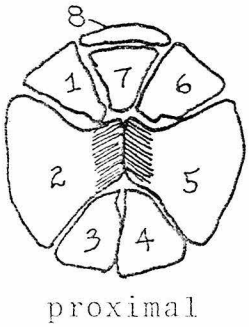


e) Mastotermes

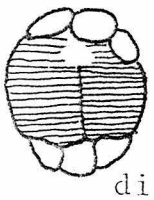


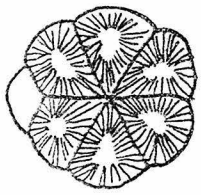
f) Lethocerus

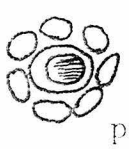




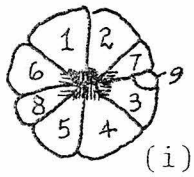
g) Aeshna

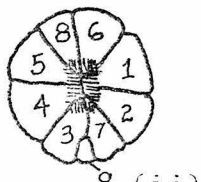




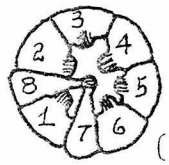


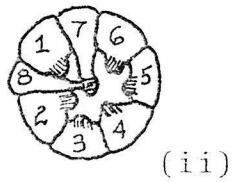
h) Photuris





i) Apis





j) Drosophila

Table 1

Rhabdom Symmetry

Radial	Radial/Bilateral	Bilateral	Bilateral/Asymmetrical	Asymmetrical
Lepisma	Photuris	Mastotermes	Apis	Drosophila
	Aedes	Dytiscus	Ashna	Periplaneta
	Oncopeltus	Gerris	Ephestia	Repismus
	Rhodnius	Entomobrya	Ishnura	Orchesella
	Wilhelmia	Allacma	Lethocerus	Erebus
		Skipper butterflies		

while three form a distinct proximal rhabdom is unusual. More frequently, the contribution of a rhabdomere to the rhabdom varies along its length. This produces the semi-stratified rhabdom found in Odonata and Orthoptera. In most eyes the rhabdomeres extend the full length of the rhabdom, with the exception of the central cells.

A major theme found in most species is the division of retinula cells into two distinct classes. One group, usually six in number, bears similar rhabdomeres. These cells produce the "short" visual axons which terminate in the first optic ganglion, the lamina. The second group, usually two cells, have distinctive rhabdomeres which frequently occupy the center of the rhabdom. These form the "long" axons which penetrate the lamina and terminate in the second optic ganglion, the medulla. This pattern is best seen in the open rhabdom of Diptera (Cajal and Sanchez, 1915; Braitenberg, 1967) and Hemiptera (Walcott, 1971; Horridge, 1968; Meinertzhagen, 1976), but the 6+2 pattern is also found in Photuris (Coleoptera, Horridge, 1969) and in numerous other orders (Goldsmith, 1964). Orders with more than eight retinula cells per ommatidium also show two classes of receptors with "short" and "long" axons (Meinertzhagen, 1976). A distinct functional role has often been postulated for the minority class of retinula cells. Such specialization has been shown most clearly in Drosophila by Harris et al. (1976) who found distinct spectral

properties in the central cells.

One of the most puzzling questions of compound eye anatomy is the significance of the symmetry properties and orientation of rhabdoms (see Table 1). The radial symmetry of the Lepisma eye (Paulus, 1975) is rare. More often, the basic structure of the rhabdom, formed by the six similar rhabdomeres is radially symmetric, but the placement of the rhabdomeres of the two central cells makes the entire rhabdom either bilaterally symmetric or asymmetric. Bilateral symmetry is the basic structure of many rhabdoms. Again, the placement of the central cells may confer asymmetry on an otherwise bilateral rhabdom.

The orientation of rhabdoms with respect to the body axes may be highly ordered, as in Diptera (Dietrich, 1909), somewhat ordered, as in the desert ant Cataglyphis, where rhabdoms may be rotated up to 30° from the standard position (Brunnert and Wehner, 1973), or disordered, as in skipper butterflies (Horridge et al., 1969). Little comparative information is available since most papers describe a single ommatidium, usually without reference to the body axes. This problem is aggravated by the finding that ommatidia may twist along their length. Thus, in Apis, neighboring ommatidia have similar orientation at their distal ends but this pattern is lost proximally (Grundler, 1974).

Mirror-symmetrical forms can be detected in some eyes with asymmetric rhabdoms. The most spectacular example of

this occurs in Diptera, where the dorsal and ventral halves of the eye are mirror images which meet along a sharp equator of symmetry (Dietrich, 1909). In divided eyes of Diptera the equator coincides with the transition from dorsal to ventral facets (Dietrich, 1909). Mirror symmetry of dorsal and ventral halves has also been described in Hemiptera (Notonecta, Bedau, 1911). In other insects, mirror symmetrical ommatidia have been reported to be interspersed with no obvious pattern to their distribution (Pieris, Meinertzhagen, 1976; Lethocerus, Meinertzhagen, 1975). However, considering the history of the equator in Diptera, which was clearly described by Dietrich (1909) but overlooked by later investigators (Danneel and Zeutzschel, 1957; Waddington and Perry, 1960), it seems reasonable to reserve judgment on these until a systematic study is done. Interestingly, the dorsal and ventral halves of the Apis eye have orthogonally oriented ommatidia which meet along an "equatorial zone" of ommatidia with intermediate orientation. Each half of the eye contains mirror symmetric ommatidia (Grundler, 1974).

Although all insect ommatidia contain two primary pigment cells, the number of accessory pigment cells surrounding each rhabdom is one of the most variable features of compound eyes. Numbers ranging from nine in Drosophila (Shoup, 1966) to twenty-four in Baetis (Ephemerida, Zimmer, 1898) have been reported. The membranes of pigment cells

wrap so tortuously around pigment granules that it is often difficult to trace the outlines of individual cells, even in the electron microscope. Thus, early descriptions of pigment cell number and arrangement must be regarded as tentative. An example of the confusion regarding pigment cells is seen in the history of accounts of basal pigment cells. Such cells have frequently been described, but it now seems most likely that they are only the pigment-filled feet of the cone cells. While the accessory pigment cells end on the basement membrane of the eye, some pigment may be found proximal to the membrane (Nolte, 1950). The origin of this pigment is unknown. The secondary pigment cells may play a role in organizing the lattice of the eye. The highly ordered ommatidial array of Drosophila is embedded in a regular matrix of pigment cells (Waddington, 1961). Oncopeltus, with a less orderly arrangement of facets, has a less regular lattice of pigment cells (Shelton and Lawrence, 1974).

Two major families of pigment, the ommochromes and pteridines, form the screening pigments of insect eyes (Goldsmith and Bernard, 1974). Generally, the pigments are associated with protein to form small (~0.5 μm) granules. In Drosophila the accessory pigment cells contain both ommochrome and pteridine granules while primary pigment cells, cone cells and retinula cells contain only ommochromes (Shoup, 1966).

Migration of pigment granules plays a major role in light- and dark-adaptation in compound eyes. The mechanism underlying pigment migration is unknown.

Small hairs, presumably mechanoreceptors, occur at facet vertices in some compound eyes. In Drosophila, bristles occur at alternate vertices. Each bristle is associated with a complex of four cells: (1) the trichogen, a bristle forming cell, (2) the tormogen, a bristle-socket forming cell, (3) a sensory neuron and (4) a glial cell which forms a sheath around the axon of the sensory neuron (Waddington and Perry, 1960). Bee (Apis, Wehner, 1972) and ant (Cataglyphis, Brunnert and Wehner, 1973) eyes possess corneal hairs, but their arrangement is much less orderly than that of Drosophila. Musca completely lack corneal hairs. Unfortunately, no comparative study of corneal hairs has been published.

Anatomy of the Developing Compound Eye

In view of the highly conserved structure of the compound eye, it is not surprising that developmental patterns are remarkably similar in all insect eyes. Most differences in eye development are superficial, reflecting different modes of metamorphosis.

Only the broad outlines of eye development have been sketched in most insects. In addition to the work in this thesis, only Waddington and Perry (1960) using Drosophila,

and Melamed and Trujillo-Cenóz (1975) using Musca have studied eye development with the electron microscope.

a) Hemimetabolous insects - In hemimetabolous insects, the compound eye begins development in the head ectoderm during embryogenesis. Ommatidia are first evident at the posterior margin of the eye anlage, and the eye grows by addition of ommatidia to the anterior margin. Upon hatching, these insects possess small, well developed compound eyes (Lüdkte, 1940; Bodenstein, 1953). While the eye grows continuously by adding ommatidia throughout nymphal stages, the overlying cuticle enlarges only at molts. To accommodate this disparity, the epidermis at the growing edge of the anlage detaches from the cuticle and folds inward. New ommatidia are thus added along a furrow at the anterior end (Lüdkte, 1940).

In most hemimetabolous insects, the adult eye is simply an enlarged version of the eye formed during embryonic development. However, some eyes change abruptly in the molt from the last nymphal stage to the adult. For example, the compound eye of the Aeshna nymph has facets of uniform small size. In the last molt, the nymphal eye is absorbed and the dorsal and ventral halves of the adult eye develop from two distinct anlagen (Lew, 1933). The dorsal part of the adult eye also has larger facets. The separate dorsal and ventral eyes of Gyrinus also develop from distinct anlagen. However, Bott (1928) found that these anlagen arise as a single

anlage which subsequently divides.

b) Holometabolous insects - In holometabolous insects, the compound eyes develop after embryogenesis. In Coleoptera, Lepidoptera, and primitive Diptera, the head ectoderm which forms the compound eye lies externally. In higher Diptera and Hymenoptera the eye anlagen lie internally in the eye discs, which are outpocketings of the pharyngeal ectoderm.

Holometabolous insects have larval photoreceptors formed during embryogenesis. These stemmata or larval ocelli (not to be confused with adult dorsal ocelli) may be very similar in structure to ommatidia, as in Lepidoptera (Dethier, 1942) or may be relatively unspecialized groupings of photoreceptors, as in Coleoptera (Wigglesworth, 1972).

In holometabolous insects with externally developing compound eyes, stemmata lie just posterior to the eye anlage (Bott, 1928; White, 1961; Wachmann, 1965). In insects with internally developing eyes, larval photoreceptors lie at the mount of the pharyngeal outpocketing, far removed from the eye anlage (Bolwig, 1946). The role of stemmata in the development of the compound eye is unknown. Since the axons of the first ommatidia run along the stemmatal nerve, it has been suggested that they may guide ommatidial axons into the brain (Meinertzhagen, 1973). Generally, larval photoreceptors degenerate during metamorphosis.

Throughout most of larval development, cells in the eye anlage proliferate without forming a pattern. In Aedes, a wave of mitosis spreads anteriorly across the eye anlage converting thin head epidermis into a thickened, but otherwise undifferentiated optic placode (White, 1961). In Drosophila three mitotic waves traverse the second instar eye disc (Becker, 1957).

Retinal differentiation begins late in larval life and ends shortly after pupation. As in hemimetabolous insects, ommatidial precursors first appear at the posterior of the anlage and are subsequently added at the growing anterior margin. Interestingly, the growing edge of the retina is again marked by a dorso-ventral furrow, similar to that seen in hemimetabolous insects, even when folding of the anlage is not dictated by the mechanical constraint of an overlying cuticle. For example, the Ephestia anlage contains a furrow (Wachmann, 1965) even though the anlage detaches completely from the overlying cuticle and rotates inward, so that it lies in a plane orthogonal to the cuticle (Umbach, 1934). Similarly, eye discs of Musca (Melamed and Trujillo-Cenóz, 1975) and Drosophila (see below) have no obvious mechanical constraints, yet the anterior margin of the growing eye is marked by a furrow. The morphogenetic significance of the furrow is not known.

In hemimetabolous insects and holometabolous insects with externally developing eyes, differentiation of ommatidia,

including pigmentation, begins shortly after their formation at the furrow. In eye discs, however, differentiation is delayed until pupal stages; ommatidial precursors in the eye disc are not easily visualized. This may account for the conflicting observations by early workers on the structure of the mature Drosophila eye disc. Krafka (1924) described ommatidial rudiments of "four terminal cells and six basal cells arranged around a deeply staining axis" in the third instar disc. Enzmann and Haskins (1938) reported a totally different precursor pattern in the second instar disc of "rod-like elements, each of which contains from one to three nuclei." Bodenstein (1938), however, claimed that ommatidial precursors are not evident until after pupation. Pilkington (1941) found the mature optic disc to consist "of a mass of cells several layers thick but apparently homogenous." In the same year, Steinberg (1941) found the clusters described by Krafka but could only count four cells in each cluster. Waddington and Perry (1960), using the electron microscope, reported that "the cells of a cluster are packed tightly together, and each bundle of cells extends proximally into a rope of narrow processes which seem usually to be seven or sometimes eight in number." However, they continued, "the number of cells composing each cluster cannot be precisely determined from the sections available." Waddington and Perry concluded that since cell division is not observed between the late larval and the adult stage,

"it seems most probable that each cell cluster contains all the cells which will differentiate into a complete ommatidium." Hopefully, the results presented in this thesis will resolve the question of the structure of clusters in the Drosophila eye disc.

Bernard (1937) described ommatidial cluster formation in the ant Formicina. Based on sections of developing eyes, he depicted a series of cell divisions, occurring anterior to the furrow, converting one stem cell into each cluster. Recent experiments in Drosophila, Oncopeltus, and Periplaneta contradict this model (see below). It is thus highly unlikely that it applies to Formicina.

Shortly after pupation, the eye discs of Drosophila evert through the pharynx and assume their final position, forming the major part of the head epidermis (Bodenstein, 1953). Twenty-four hours later, cells in the retina have assumed characteristic positions which make them easily recognizable (Waddington and Perry, 1960). Major differentiative changes begin about forty-eight hours after pupation, occurring simultaneously over the entire eye. The cornea, previously a continuous sheet, becomes faceted by the secretion of the primary pigment cells (Pilkington, 1941). Ommochrome synthesis begins, giving the eye a peach color (Shoup, 1966). Retinula cells begin to infold their membranes into rhabdomeres (Waddington and Perry, 1960). A corneal hair develops from each four-cell bristle complex;

these complexes are especially prominent at this stage (Waddington and Perry, 1960). Interestingly, this is also the time at which retinula cell axons in the lamina begin to move toward their final targets (Hanson, 1972). Three days after pupation, cone cells begin to secrete the pseudocone (Pilkington, 1941) and drosopterin synthesis begins (Shoup, 1966). During the third and fourth days, the ommatidia elongate, tripling the thickness of the retina (Waddington and Perry, 1960). Differentiation is completed on the fourth and final day of pupal life.

Experimental Analysis of Compound Eye Development

Experiments on developing compound eyes have dealt primarily with three questions: (1) What tissue is competent to form an eye? (2) How is eye development initiated? and (3) How does development proceed once initiated? Such a separation may be artificial, since, in all probability, they reflect different aspects of a single developmental mechanism. Nevertheless these topics are treated separately below.

Not all insect epidermis is competent to differentiate into retina. In Aedes, extra-optic head epidermis transplanted into the eye anlage did not participate in eye formation (White, 1961). Similar results have been reported in Periplaneta (Shelton et al., 1976) and Aeshna (Mouze, 1975). On the other hand, Green and Lawrence (1975) found

that a small piece of adult extra-optic epidermis, transplanted into the Oncopeltus eye anlage, produces ommatidia. Shelton et al. (1976) suggest that since Green and Lawrence took donor tissue from a region close to the eye, competence to form retina extends somewhat beyond the border of the eye. Hyde (1972) claimed that prothoracic tissue of Periplaneta transplanted into the eye anlage differentiates into retina. However, Shelton et al. (1976) have been unable to replicate Hyde's result. Similar experiments by Mouze (1975) in Aeshna and by Green and Lawrence (1975) in Oncopeltus have also given negative results. It thus seems likely that competence to form retina is restricted to the eye anlage and nearby tissue.

The initiation of pattern formation is also a controversial question. By analogy with the "differentiation center" in the insect blastoderm, at which major differentiative changes are initiated (Seidel, 1936), several authors have proposed a "differentiation center" for eye development. Seidel (1936) found that ultraviolet irradiation of the posterior margin of the Aeshna eye anlage completely prevents eye development; irradiation elsewhere in the anlage produces only local defects. Wolsky (1949) reported similar results with microcautery of the Ephestia anlage. However, Wolsky considered almost the entire anlage posterior to the furrow as the "differentiation center." Therefore, cauterization of this region would amount to

destruction of most of the eye which had so far been formed. In White's (1963) finding that microcautery of the stemmatal region of freshly hatched mosquito larvae completely prevents eye development, this problem does not arise, since the lesion is made long before retinal differentiation begins. Cauterization had not destroyed the competence of the epidermis, since a fragment from a normal anlage transplanted to the cauterized host anlagen could induce retinal differentiation (White, 1963). Unfortunately, this experiment provides no information as to what in the transplant causes induction of the host eye. In fact, no controls are reported to indicate that inducing activity is expressed uniquely in the eye anlage. The importance of such controls is shown by Wachmann's (1965) experiments on the developing Ephestia eye. Totally ablated eye anlagen of late larval Ephestia heal over with a simple epidermis that does not differentiate into an eye. However, this epidermis can be induced to form an eye by a wide variety of transplants. Eye anlage fragments from several species of insects, including orders other than Lepidoptera, induced development of an Ephestia eye. Wing disc tissue was also an inducer, even after being soaked in acetone for twenty hours! Head epidermis from outside the prospective eye, however, did not act as an inducer. This rules out the possibility that induction was triggered non-specifically by the surgical trauma of transplantation. Thus, the existence of a specialized

"differentiation center" in pattern formation is equivocal.

The signal that induces competent tissue to differentiate into retina is intrinsic to the anlage, as witnessed by the normal development of anlagen floating free in the hemocoel (White, 1961) or in vitro (Gottschewski, 1960; Kuroda, 1970). The arrest of eye development by a strip of noncompetent tissue transplanted into the anlage ahead of the furrow suggests that only competent cells can propagate the signal (White, 1961). The direction of this propagation is not intrinsic to the epidermal cells, however, since a wave of retinal differentiation will sweep across a 180° rotated piece of anlage as though the piece had not been rotated (White, 1961). The stimulus for differentiation is clearly associated with the growing edge of the eye. White (1961) found that only a few cells from the anterior margin are sufficient to induce the anlage to form new ommatidia.

Bernard's (1937) claim that a single stem cell produces each ommatidium might suggest that propagation of development at the anterior margin of the anlage is accomplished by the sequential triggering of pre-programmed stem cells. In addition to the study of the Drosophila eye reported below, several recent papers by Lawrence and his co-workers have also examined the role of cell lineage in eye development. Shelton and Lawrence (1974) and Green and Lawrence (1975) transplanted pieces of eye anlagen from white-eyed Oncopeltus nymphs to normal, red-brown eyed hosts. In the

adult, mosaic ommatidia were found along the borders of the transplant. Similar experiments in Periplaneta by Shelton et al. (1976) also produced mosaic ommatidia. These experiments clearly show that an ommatidium can be formed by the progeny of more than one cell. However, the interpretation of this result is complicated by the use of surgery to produce the mosaic eyes. If ommatidial stem cells did exist and had divided prior to surgery, the incision could divide clonally produced ommatidial precursors. Such remnants might assemble at the transplant borders to produce mosaic ommatidia. An additional problem in extrapolating these results to normal development is the severe disruptions that were observed in the eye lattice at the transplant borders.

Patterson (1929) first noted that X-irradiation of Drosophila larvae heterozygous for white produced mosaic eyes. Foreshadowing Bernard, he stated that, in these eyes, "At the margin between a white area and the surrounding red region, a given ommatidium is seen to be either all red or else all white." Similarly, Baker (1963) claimed that in mosaic eyes, "Each facet may be treated as a unit and the question asked, does it or does it not produce any pigment." Shoup (1966) also found that, "Ommatidia are either completely pigmented or unpigmented." As shown in this thesis, pigmentation of ommatidia does not follow such a simple all or none rule.

Becker (1957) demonstrated conclusively that mosaicism produced by X-irradiation of heterozygous larvae was due to somatic recombination. Larvae carrying white on one X chromosome and white-coral, an allele of white, on the other X, were irradiated. The resulting mosaic eyes often contained three distinct regions of pigmentation, (1) a light coral background corresponding to the initial white/white-coral genotype, (2) a pigmentless white patch, corresponding to homozygous white and (3) an adjacent, deep coral patch corresponding to homozygous white-coral. The latter two "twin spots" of homozygous tissue could arise only through somatic recombination (Stern, 1936). Using this technique, Becker conducted an extensive investigation of the patterns of cell proliferation during the early development of the Drosophila eye disc (discussed below). This classic study is marred only slightly by Becker's over-interpretation of his data concerning the boundaries of the patches.

CHAPTER II

Development of the *Drosophila* Retina, A Neurocrystalline Lattice

by

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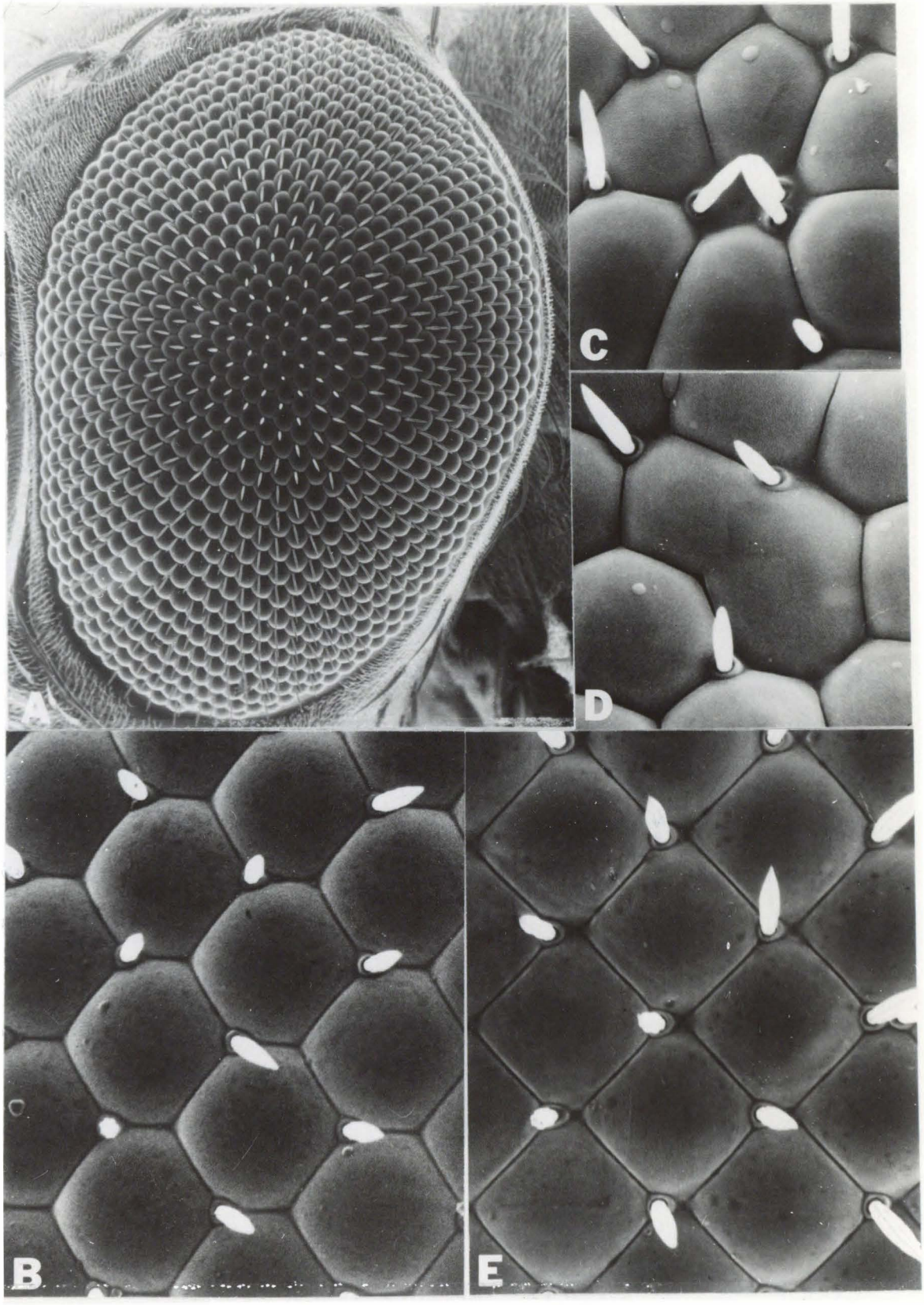
INTRODUCTION

Pattern formation in neural systems arises under the direction of the genes, by mechanisms yet unknown. Genetic analysis of mutants which perturb normal pattern formation may be instrumental in unraveling these mechanisms. The compound eye of Drosophila is well suited to such analysis. In it, some 800 unit eyes, or ommatidia, are arranged in a remarkably precise hexagonal array (Fig. 1A). Inside each ommatidium, there are eight photoreceptor cells arranged in an asymmetrical trapezoid pattern. The axons of these photoreceptors project to higher-order neurons in the optic ganglia in an exact, repetitive pattern (Trujillo-Cenóz and Melamed, 1966; Braitenberg, 1967). Above a horizontal equator, the pattern of photoreceptors and their connections to higher-order neurons is constant from one ommatidium to the next. Below the equator, this pattern is a mirror image. The entire ensemble thus constitutes a neural array of almost crystalline precision.

The formation of the regular structure of the eye is under genetic control. In Drosophila melanogaster there are over 100 different genes known, all of which must be normal to produce a proper array; mutation of any one causes perturbations in development that give the eye a "rough" appearance, due to lattice abnormalities such as interstitials, vacancies, dislocations, or fusion of ommatidia (Figs. 1B-E).

Fig. 1. Drosophila melanogaster eyes. (A) Left eye of a normal (C-S wild type) female. 228×. Anterior is to the left. (B) Normal eye at 1410×. (C) ro (rough) mutant eye. Facet vacancy at center is surrounded by three hairs. (D) Another area of the same ro (rough) mutant eye, showing fusion of two facets. (E) ey^R (eyeless-Russian) mutant eye, with square facet array (see Hartman and Hayes, 1971). In this mutant, the secondary pigment cells along the horizontal axis fail to elongate properly.


A
C
D
B
E

The repetitive structure of the normal Drosophila eye might suggest that it is formed by strict clonal mechanics. For instance, one cell could divide into two daughters of opposite symmetry, one generating the upper half of the eye, the other the lower half. The eight photoreceptors of each ommatidium could be produced by three divisions of a single precursor cell, as suggested for the ant by Bernard (1937). The clusters of eight could then be assembled into a hexagonal lattice by simple closest packing, as in a set of soap bubbles.

The evidence in this paper shows that this scheme is incorrect. The eight receptor cells within an ommatidium do not represent a clone. This became evident in eyes that were mosaic for a receptor degeneration gene rdgB (Benzer, 1971). Mosaics also show that the equator does not separate two sharp clonal compartments. Rather than developing in a clonal sequence, the photoreceptor clusters and the equator are formed from available cells along an advancing edge, as in crystal growth.

We have studied the anatomy and development of the wild-type Drosophila eye in some detail, as a prelude to the analysis of mutants which perturb normal development. Some of these results have already appeared in preliminary reports (Hanson et al., 1972; Ready, 1973; Benzer, 1973). Recently, Campos-Ortega and Gateff (1976) and Hofbauer and Campos-Ortega (1976) have obtained results similar to those reported

here. Shelton and Lawrence (1974) have also demonstrated the nonclonal origin of ommatidia in Oncopeltus. The development of the eye disc of C. phaenicia, studied by Melamed and Trujillo-Cenóz (1975), has many features in common with those given here for Drosophila.

MATERIALS AND METHODS

Drosophila melanogaster were raised on cornmeal-yeast-agar medium at 25°C. Normal flies were of the Canton-Special (C-S) wild-type strain. The recessive, X-linked mutant white (w) was chosen as a genetic marker for clonal analysis. Heteorzygous white larvae, used in mitotic recombination experiments, were collected at 4-hr intervals. At appropriate times, the larvae received 1200 rad of X-irradiation, at a rate of 325 rad/min (50 kV, 20 mA at 13 cm, through 1 mm Al). After development to the adult stage, serial 2.5-μm sections of the mosaic eyes were made to score cell genotypes.

The fixation protocol described by Poodry and Schneiderman (1970) was used. Adults and pupae were decapitated and the heads were bisected in half-strength Karnovsky's fixative (1965) (2.5% glutaraldehyde, 2.0% formaldehyde in 0.1 M cacodylate buffer, pH 7.3) for 1 hr. After two 15-min washings in 0.1 M cacodylate buffer plus 10% sucrose, tissue was transferred to an osmium postfix (1% OsO_4, 10% sucrose in 0.028 M veronal acetate buffer, brought to pH 7.3 with

HCl) for 4 hr. After osmication, specimens were dehydrated in ethanol, cleared in xylene, and embedded in Epon. All steps were performed at room temperature. Sections were cut on a Sorvall MT-2B ultramicrotome. Eye imaginal discs were treated in the same way, except that the larvae were injected with fixative before dissecting out the discs.

Serial thin sections of eyes and eye discs were stained with Reynold's lead citrate (1963) and examined in a Zeiss EM9S electron microscope. For scanning electron microscopy, discs were fixed and dehydrated as above, then critical point dried, coated with gold, and examined in an ETEC scanning electron microscope.

For autoradiography, mid-third-instar larvae were injected by Dr. H. K. Mitchell with ~0.1λ of a sterile 2×10^{-5} M aqueous solution of [methyl-^{3}H]thymidine (50 Ci/mmole, New England Nuclear, Boston, Mass.). At intervals, eye discs or eyes were fixed and embedded as described. Serial 1-μm sections of the eyes of discs were collected on glass slides and dipped in Ilford K-5 emulsion (Ilford, Essex, England) diluted 1:2 with H_2O. Coated slides were exposed in a desiccated, light-tight box at 4°C for 6 weeks, developed (Kodak Dektol developer, diluted 2:1 with H_2O), and fixed (Kodak Fixer). The slides were then stained with methylene blue.

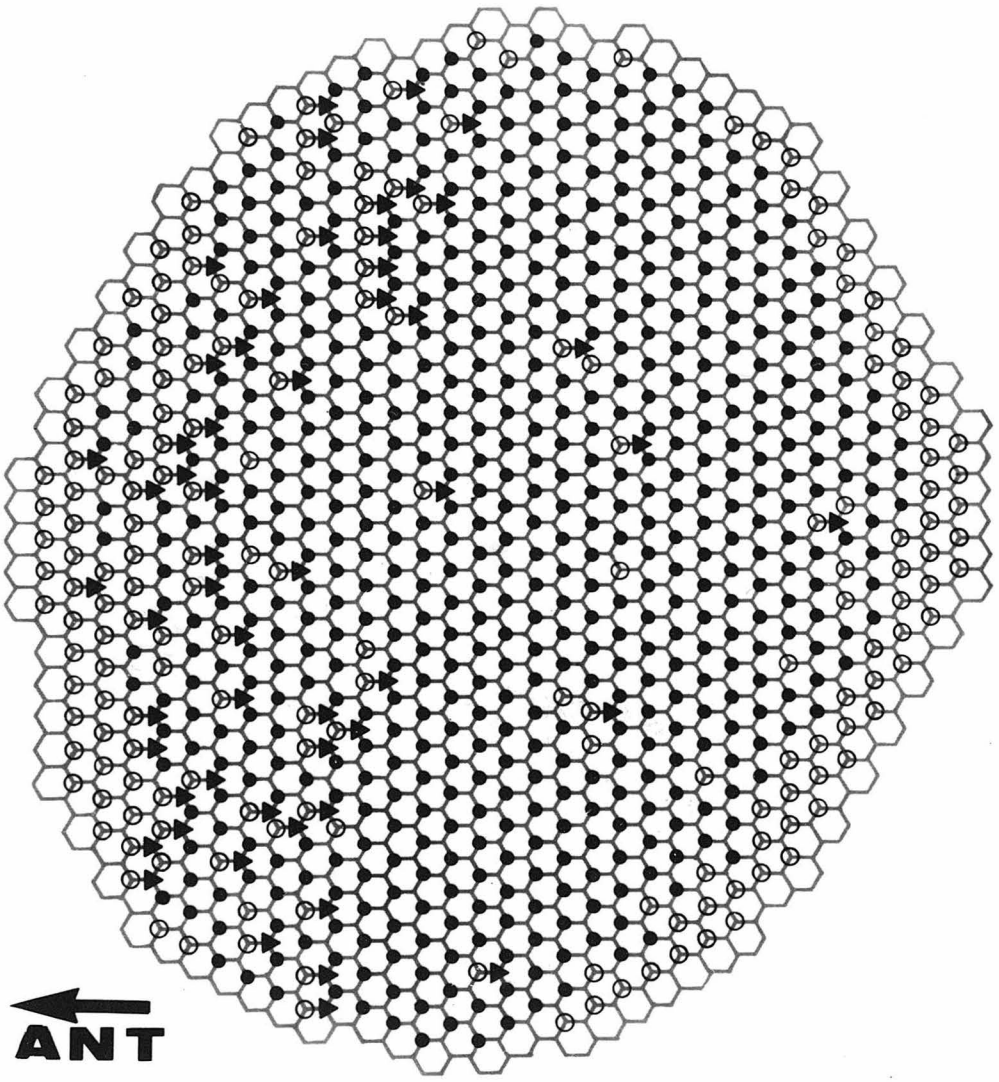
RESULTS

Structure of the Normal Eye

Many authors have described the anatomy of the dipteran compound eye, including Dietrich (1909), Wolken et al. (1957), Trujillo-Cenóz (1965), and Boschek (1971); for a review, see Meinertzhagen (1973). In the normal Drosophila eye, the hexagonal facet array is extremely accurate; only rarely does a perturbation occur. However, the number of facets varies from eye to eye. In six left eyes from C-S females, this number ranged from 745 to 828 with a mean of 776. There were between 32 and 34 vertical columns per eye. The number of facets in each vertical column also varied slightly (see Fig. 4). This variability was somewhat greater at the anterior end of the eye.

The eye also has an array of sensory hairs normally located at the anterior ends of the two horizontal faces of each hexagonal facet. The hairs thus occur at alternate vertices of the hexagons. This arrangement is fairly exact in the central area of the eye, with occasional errors in position (Fig. 2). Most commonly, the nature of the misplacement is such that the hair appears at the posterior end of a horizontal face, leaving a vacancy at the anterior end. The frequency of such errors increases at the anterior of the eye. Hairs are typically absent around the edges of the eye.

Fig. 2. Facet and bristle array of the normal eye shown in Fig. 1A. A solid circle represents a bristle in normal position, at the leading end of each horizontal face. An open circle indicates absence of a bristle from a normal position. A triangle represents a bristle in an abnormal position. Note that for each bristle that is misplaced (i.e., at the posterior end of a horizontal face), there is one absent from the anterior end of the same face.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Internally, an ommatidium contains eight photoreceptor cells. Each photoreceptor cell has a rhabdomere, a rod-like element produced by multiple infolding of the cell membrane into stacks of microvilli. The rhabdomeres of cells 1 through 6 are arranged in an asymmetrical trapezoid pattern, with their cell bodies radially placed. The rhabdomeres of cells 7 and 8 are borne on stalks extending inward from their cell bodies, at angles 90° apart, and occupy a central position, 7 above 8. In Fig. 3, the cut is at an outer level, so that rhabdomere 7 is seen, while 8 is not. The trapezoids in the dorsal and ventral halves of the eye are mirror images which meet along an equator running horizontally across the eye (Dietrich, 1909).

Histological sections show that the course of the equator is similar, but not identical, from eye to eye. Although, in general, the equator takes alternating dorsal and ventral steps, occasionally it will step twice or more in the same direction (Franceschini and Kirschfeld, 1971; Meinertzhagen, 1972). Such jogs may occur anywhere along the equator, but they are most frequent toward the anterior and posterior of the eye (Fig. 4). Over most of its course, the equator comes close to dividing each vertical column in half.

The arrangement of the pigment cells is also highly regular (Waddington, 1961). Around the cone are two pigment cells, one collaring the anterior half, the other the

Fig. 3. Retinal array. Tangential section of left eye. 2575×. Anterior is to left. The equator runs horizontally, slightly below the middle of the figure; there is mirror symmetry above and below it. A jog in the equator occurs at the left. In this plane of section, rhabdomere 7 is evident at the center of each ommatidium, surrounded by the series 1 through 6. In one ommatidium (second from top in right-hand column) retinula cell 8 can be seen extending its rhabdomere between cells 1 and 2. Small pigment granules occur directly adjacent to the rhabdomeres. The pigment cells contain larger granules. Some have been lost or broken during sectioning.

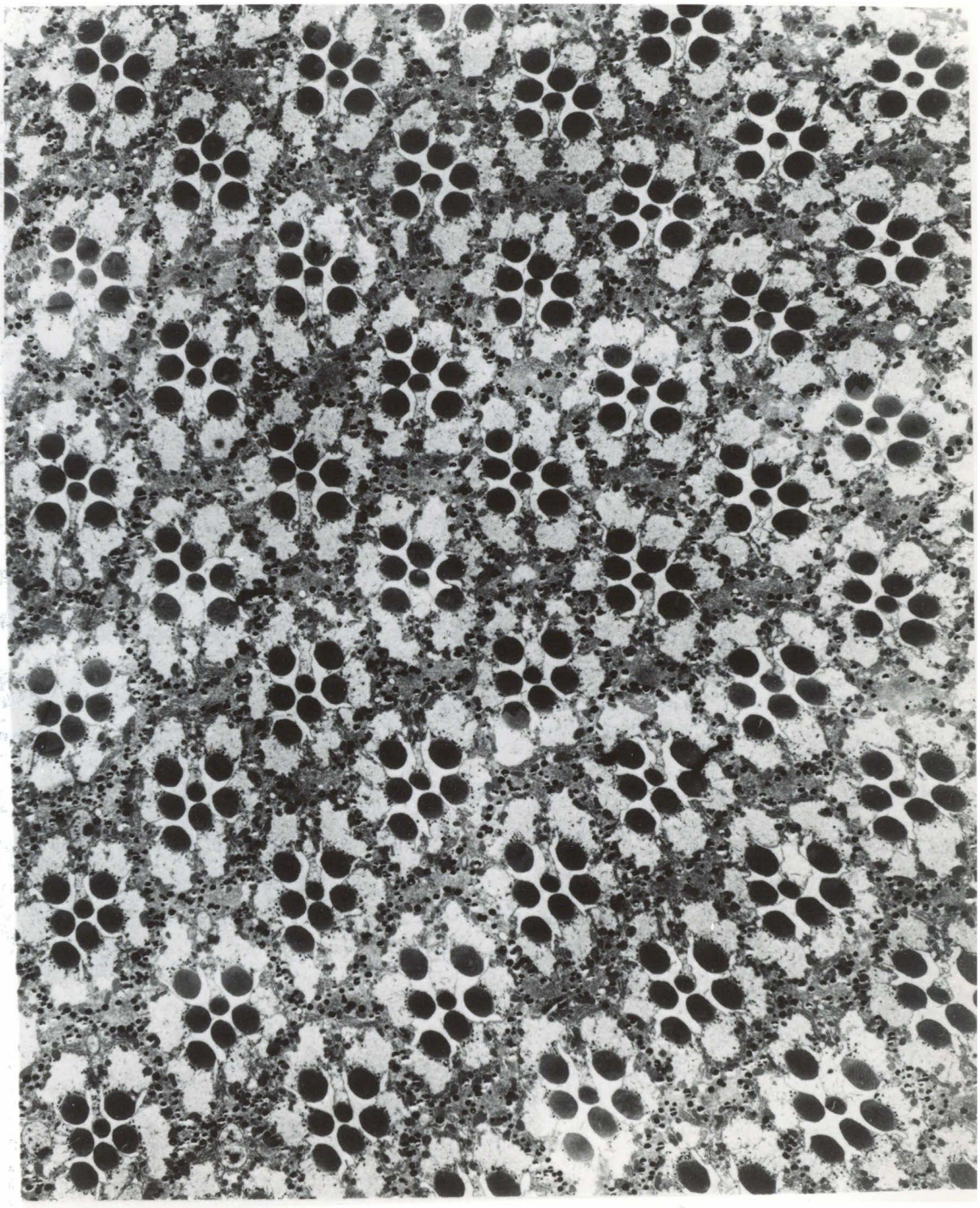
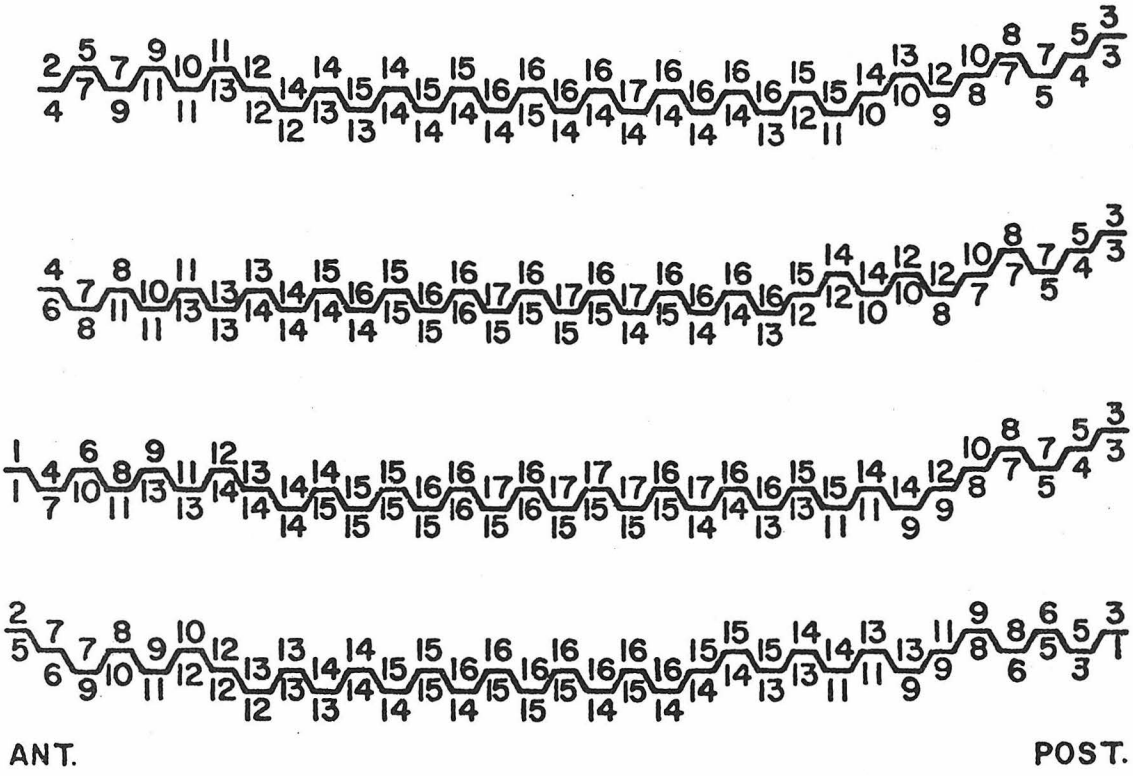


Fig. 4. Division of the eye by the equator, scored by histological sections. The course of the equator is shown for four left eyes from normal (C-S) females. Numbers of ommatidia above and below the equator are given. Note that from eye to eye there are variations in the number of columns and the heights of the columns. The equator comes close to dividing columns in half.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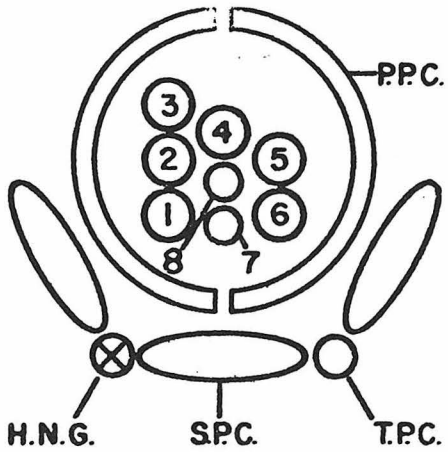
posterior. These are termed primary pigment cells; they belong to a single ommatidium. The photoreceptor cell group is surrounded by six secondary pigment cells; each is shared between two adjacent ommatidia as a common face. A tertiary pigment cell occurs at the posterior end of each horizontal face and is shared by three ommatidia. At each anterior vertex, there is a sensory hair group (except when the hair is absent or misplaced, in which case there is a tertiary pigment cell). Each group of these consists of the classical set of four cells: bristle, socket, sensory neuron, and glial sheath (Wigglesworth, 1953). The axons of these sensory neurons course beneath the basement membrane of the eye and join a nerve running to the subesophageal ganglion.

To count the total number of cells in the repeat unit of the lattice (Fig. 5), one hair complex of four cells, one tertiary, and three secondary pigment cells may be assigned to each ommatidium. Together with four cone cells, two primary pigment cells, and eight photoreceptor cells, the "unit cell" of the lattice thus contains 22 cells. An eye of 750 ommatidia represents a highly ordered structure of about 16,500 cells.

Development of the Eye

The eye is derived from the eye-antennal disc which arises from about 20 cells of the embryonic blastoderm

Fig. 5. Repeat unit of the lattice. Numbered circles, photoreceptors; HNG, hair-nerve group (four cells); PPC, primary pigment cells; SPC, secondary pigment cells; TPC, tertiary pigment cell. The four cone cells are not shown. The orientation of the photoreceptors corresponds to the upper portion of left eye, with anterior to the left. The repeat unit for the lower half of the eye is a mirror image in the horizontal plane. A normal eye composed of 800 such units requires about 100 additional secondary pigment cells at the edges to complete the eye.


P.P.C.
3
4
2
5
1
6
8
7
H.N.G.
S.P.C.
T.P.C.

(Garcia-Bellido and Merriam, 1969). This disc, like others, is formed by invagination of the blastoderm to produce a flattened sac of epithelium enclosing a lumen that remains continuous with the outside. One surface comprises the disc proper, while the other surface forms a peripodial membrane. Later, during metamorphosis, the sac will evert, the surface of the disc proper becoming the outside of the eye. By the third instar, the eye portion of the disc contains approximately 2,000 cells (Becker, 1957). Throughout larval life, a stalk at the posterior of the eye disc connects it to the brain.

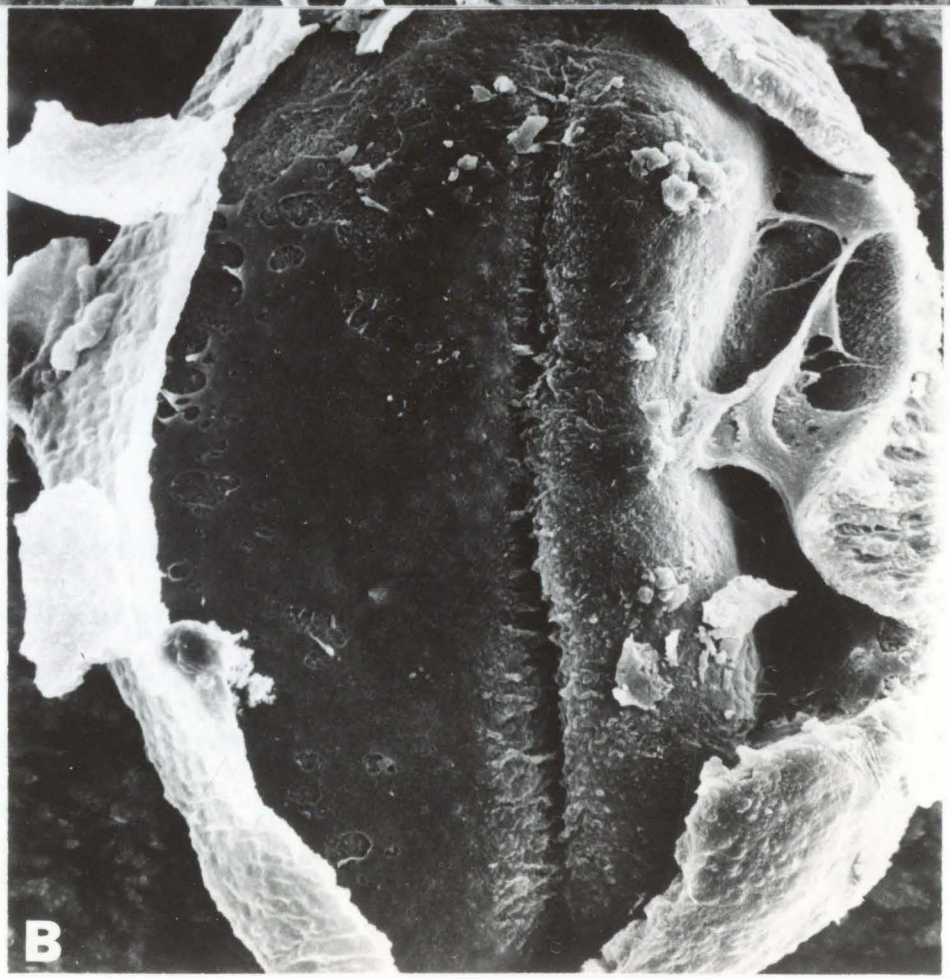
The orientation of the disc with respect to the body axes of the larva is the same as the final orientation of the eye in the adult, i.e., the posterior part of the disc is destined to become the rear of the eye, etc. Figure 6A shows the overall shape and position of the disc. In Fig. 6B, the peripodial membrane has been partially removed to expose the lumen and the eye disc proper below.

Reconstruction of the disc from serial thin sections shows it to be a monolayer of columnar epithelium; cells extend from the basement membrane to the surface of the disc facing the lumen. At the lumen surface, each cell is joined to its neighbors by a distinct zonula adherens. When a cell in the epithelium divides, it loses its attachment to the basement membrane, rounds up at the lumen surface, and divides in the plane of the disc. After division, each

Fig. 6A. Eye-antenna disc in situ, third instar larva. 270×. Anterior is to the right. The eye portion of the disc is connected by a stalk to one of the cerebral hemispheres. The ventral ganglion curves away to the left; fibers are nerves and tracheae. At the right, several macrophase-like cells can be seen adhering to the peripodial membrane of the antennal portion of the disc.

Fig. 6B. Eye disc with peripodial membrane partially removed. 517×. Anterior is to the right. The exposed surface will become the outside of the eye. Some debris remains. The deep cleft is the dorso-ventral morphogenetic furrow.


A


B

daughter again extends a foot to the basement membrane. Poodry and Schneiderman (1970) have described a similar epithelium in the leg disc of Drosophila.

During the middle of the third instar, a pattern first becomes evident as a clustering of cells at the posterior end of the disc (Fig. 7). Krafka (1924) first noted these ommatidial precursors in Drosophila. They were also described by Medvedev (1935), Steinberg (1943), and Waddington and Perry (1960), and Melamed and Trujillo-Cenóz (1975) found similar clusters in C. phaenicia. Each cluster contains eight cells destined to mature into the photoreceptors of an ommatidium. The clusters form a square array. With time, the boundary of this patterned field extends anteriorly, the advancing border being marked by a dorso-ventral furrow (Figs. 6B and 7). Melamed and Trujillo-Cenóz (1975) described a similar furrow in the eye disc of C. phaenicia. The temporal sequence of pattern formation is laid out along the anterior-posterior axis of the disc; it is possible to reconstruct its development by examining the cell arrangement at various distances from the dorso-ventral furrow. The area anterior to the furrow is rich in dividing cells over the entire surface, but lacks any obvious pattern. Immediately posterior to the furrow, "preclusters" of cells are evident, with a characteristic core of five cells usually recognizable in each (Fig. 8). As shown below, by cell marking and autoradiography, these five cells correspond to

Fig. 7. Eye-antennal disc seen with Nomarski optics. Late third instar. 400×. Anterior is to the right. Mature photoreceptor clusters may be seen in a square array at the posterior end of the eye disc (left). Anterior to the clusters, the morphogenetic furrow is visible as a vertical dark line. A small piece of nondisc tissue is present at the lower center of the photograph. Inset shows square array at 646×.

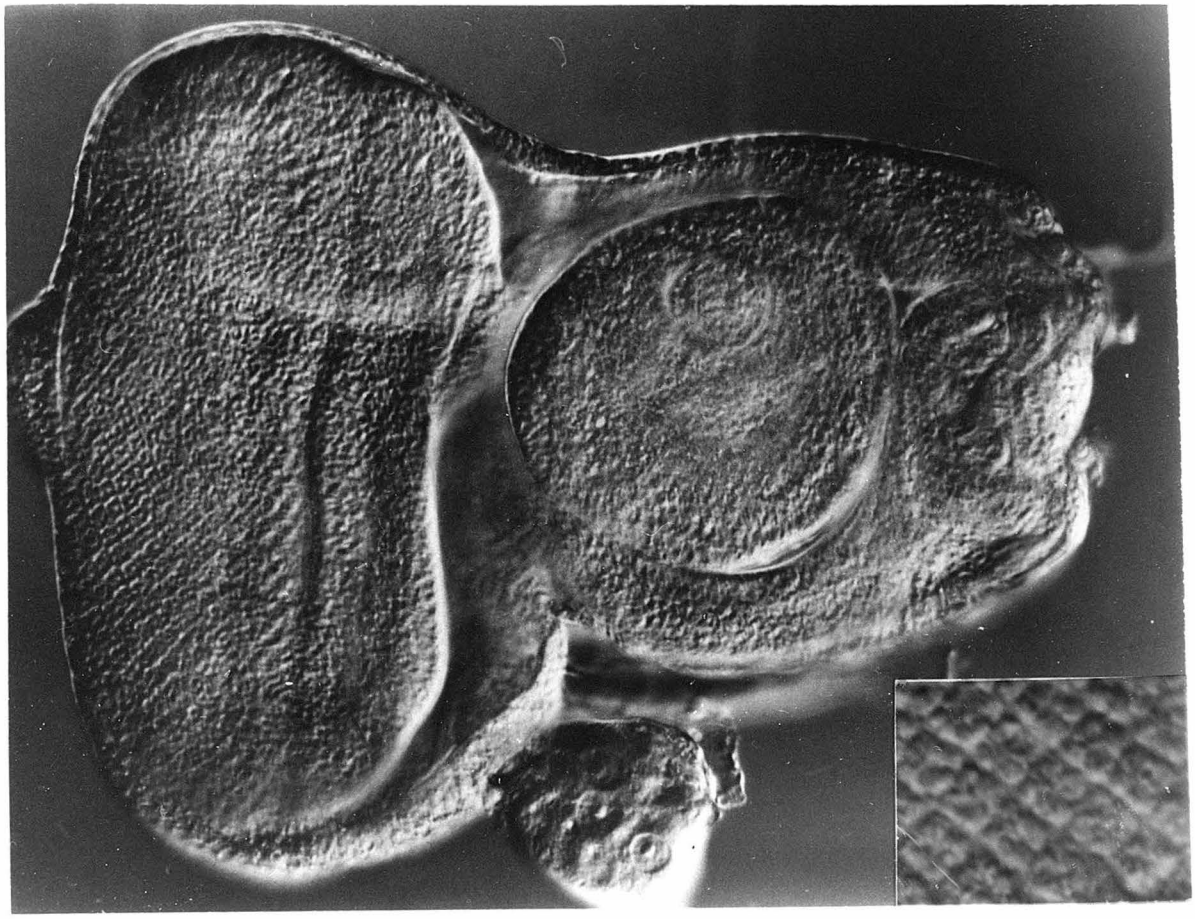
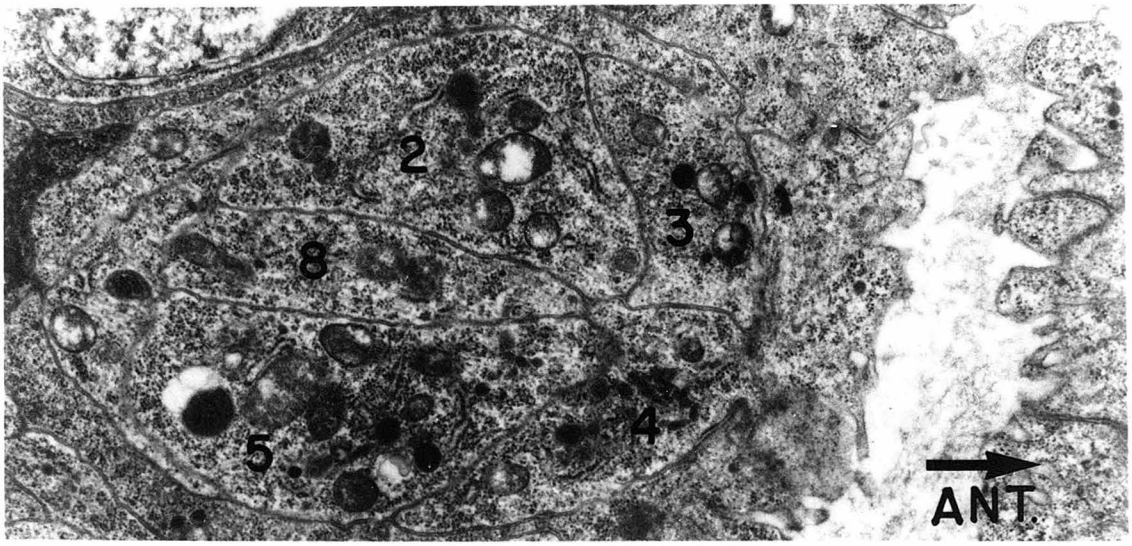


Fig. 8. A precluster. Eye disc, late third instar. 14,070×. Anterior is to the right. This precluster is just posterior to the dorso-ventral morphogenetic furrow, which appears as a clear space just anterior (right). Numbers indicate the photoreceptors that the cells are destined to form, inferred from their positions in the clusters.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photoreceptor cells 2, 3, 4, 5, and 8. They undergo no further divisions.

More posteriorly, one finds eight-celled clusters having a characteristic shape and organization (Fig. 9). The clusters are at the lumen surface and are not staggered into two layers, as Waddington and Perry (1960) and Waddington (1961) had indicated. By following subsequent stages, one can see that neighbor relations of the cells in a cluster are maintained during subsequent development; each cell can be correlated with the photoreceptor it is destined to form. Each cluster contains a central cell, surrounded by seven others which contact the central cell along most of its length. From each mature cluster, a bundle of eight axons runs posteriorly into the optic stalk, with seven axons in each bundle arranged about the axon of the central cell. Figure 10 is a sagittal section of a developing disc, illustrating the dorso-ventral furrow. Figure 11 is a tangential section of the dorso-ventral furrow region, illustrating the abrupt transition from unpatterned cells on one side of the furrow to preclusters and clusters on the other.

The cells filling the spaces between the mature clusters are difficult to identify, since they lack obvious differentiation at this stage. Counts of these cells show approximately 14 cells per cluster, the number required to form a complete ommatidium.

Fig. 9. A mature cluster. Cutaway diagram shows contact faces between cells. Numbers correspond to photoreceptor cells in adult ommatidium. Eight photoreceptor axons extend inward from the bottom of the cluster.

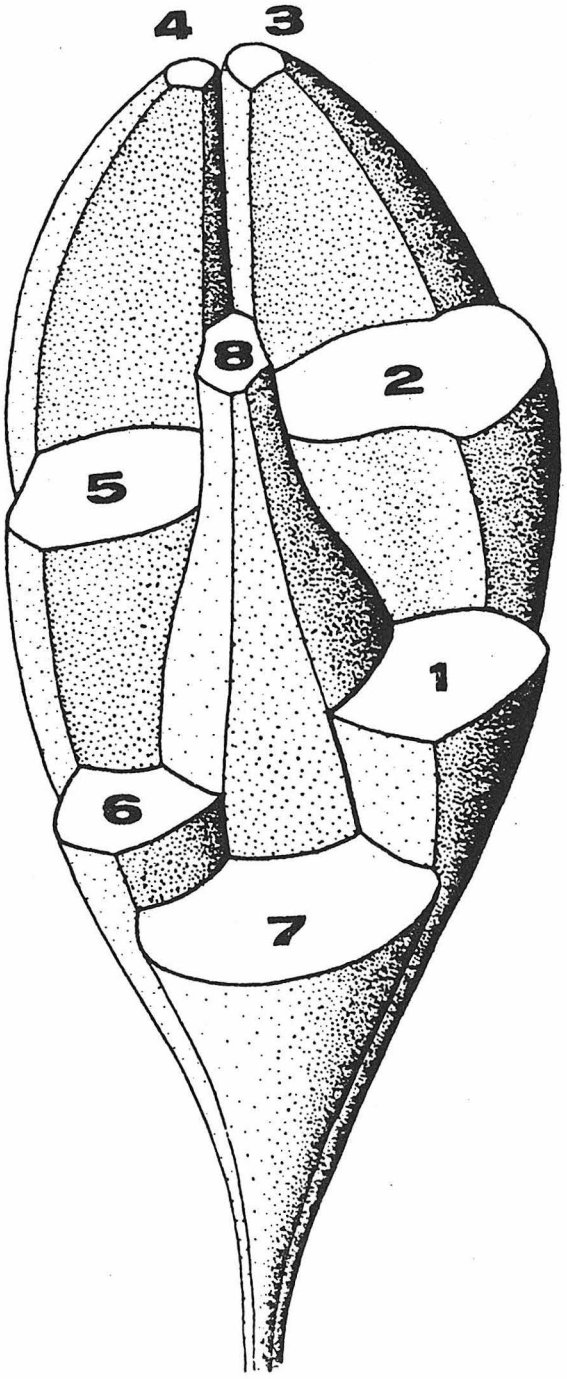
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Fig. 10. Sagittal section of eye disc. Late third instar. 945×. Anterior is to the right. The peripodial membrane which normally covers the entire disc is broken off posterior to the furrow. Note that cells bow out to either side of the furrow. Small holes are fixation artifacts.

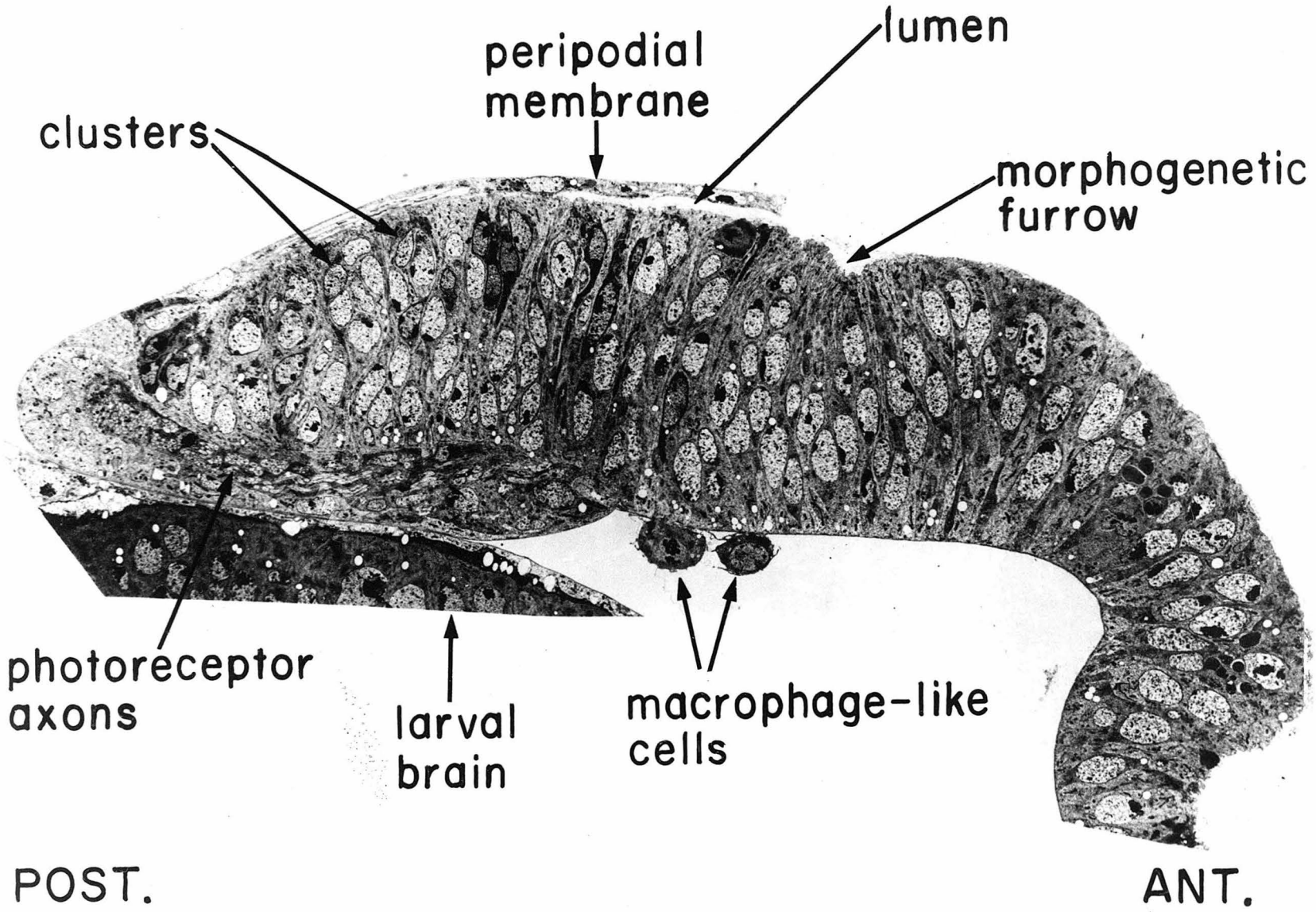
peripodial membrane
lumen
clusters
morphogenetic furrow
photoreceptor axons
larval brain
macrophage-like cells
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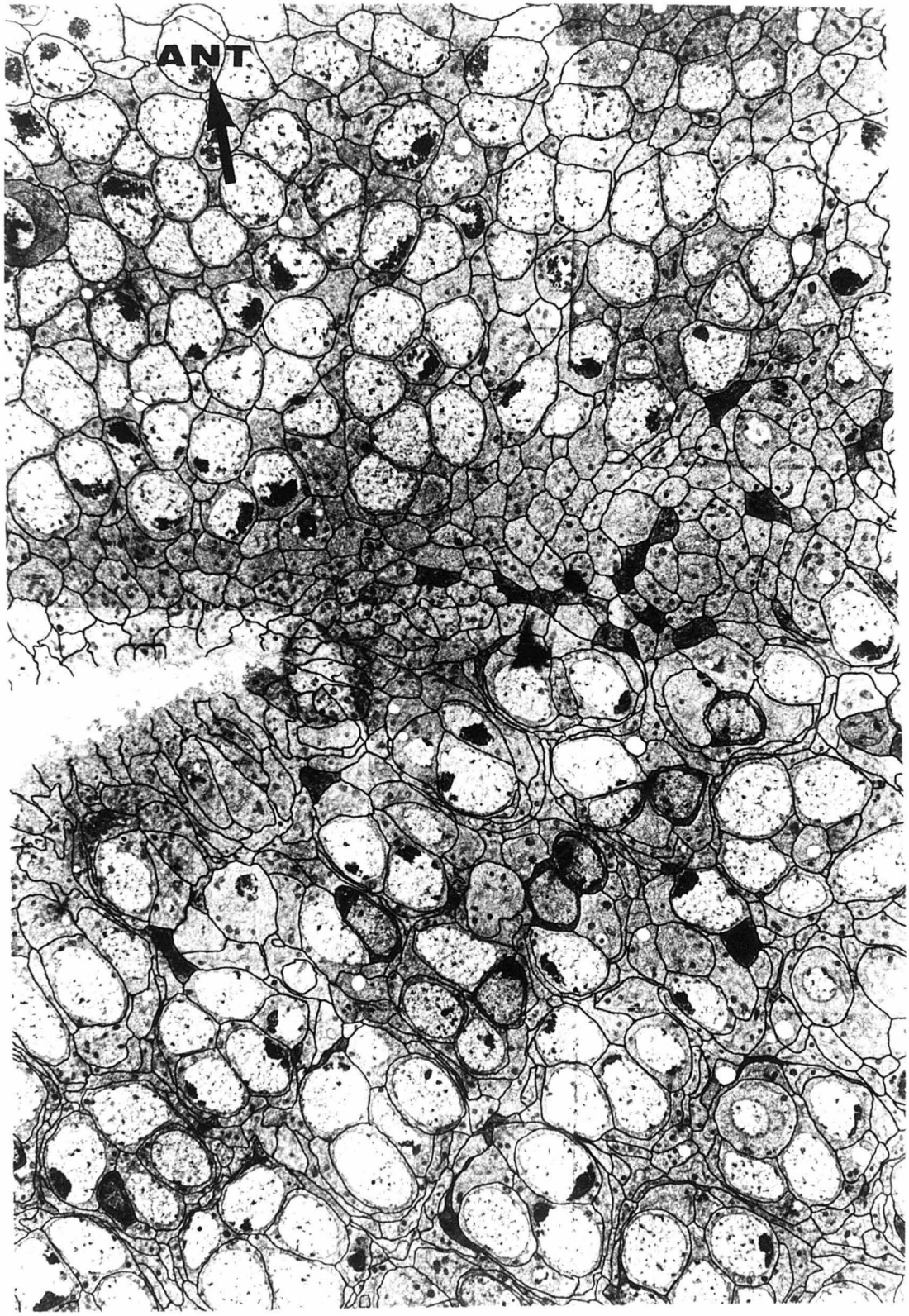
Fig. 11. Tangential section of a late third instar eye disc, showing cells on both sides of the morphogenetic furrow. 3064×. Cell membranes have been inked for emphasis. The dorso-ventral furrow appears as a clear space at the middle left and continues to the right, where the section cuts more deeply into the disc tissue. Anterior to the furrow (upper area) the cells are unpatterned. Posterior to the furrow (lower area) preclusters and clusters, girdled by other cells, can be discerned.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Well behind the furrow, where the mature eight-cell clusters have formed, a line of symmetry can be identified. Clusters in the ventral half of the disc are mirror images of clusters in the dorsal half. These antisymmetric forms meet along a zig-zag equator; mirror-image partners face each other across corners in a square array (Fig. 12). During subsequent development, the peripheral portion of the secondary pigment cell at each corner of the square array will elongate along the anterior-posterior axis. This converts the square array of the disc into the hexagonal array in the outer eye. At the basement membrane, however, a square array is retained (Fig. 13).

In the developing disc, in addition to the dorso-ventral furrow associated with the advancing boundary of cluster formation, the area ahead of this boundary is indented along the anterior-posterior axis. This groove can be seen most easily in the early third instar disc (Fig. 14). It presumably plays a role in the formation of the equator. With time, the groove advances anteriorly, tracing a path corresponding to the future equator, suggesting that preclusters may be assembled in mirror-image forms in its wake. It is difficult to determine with certainty whether the preclusters have opposing orientations in areas dorsal and ventral to the groove, due to the variability of cell shapes just behind the dorso-ventral furrow.

Fig. 12A. Six mature photoreceptor clusters. Late third instar eye disc. 6383×. See diagram in Fig. 12B.

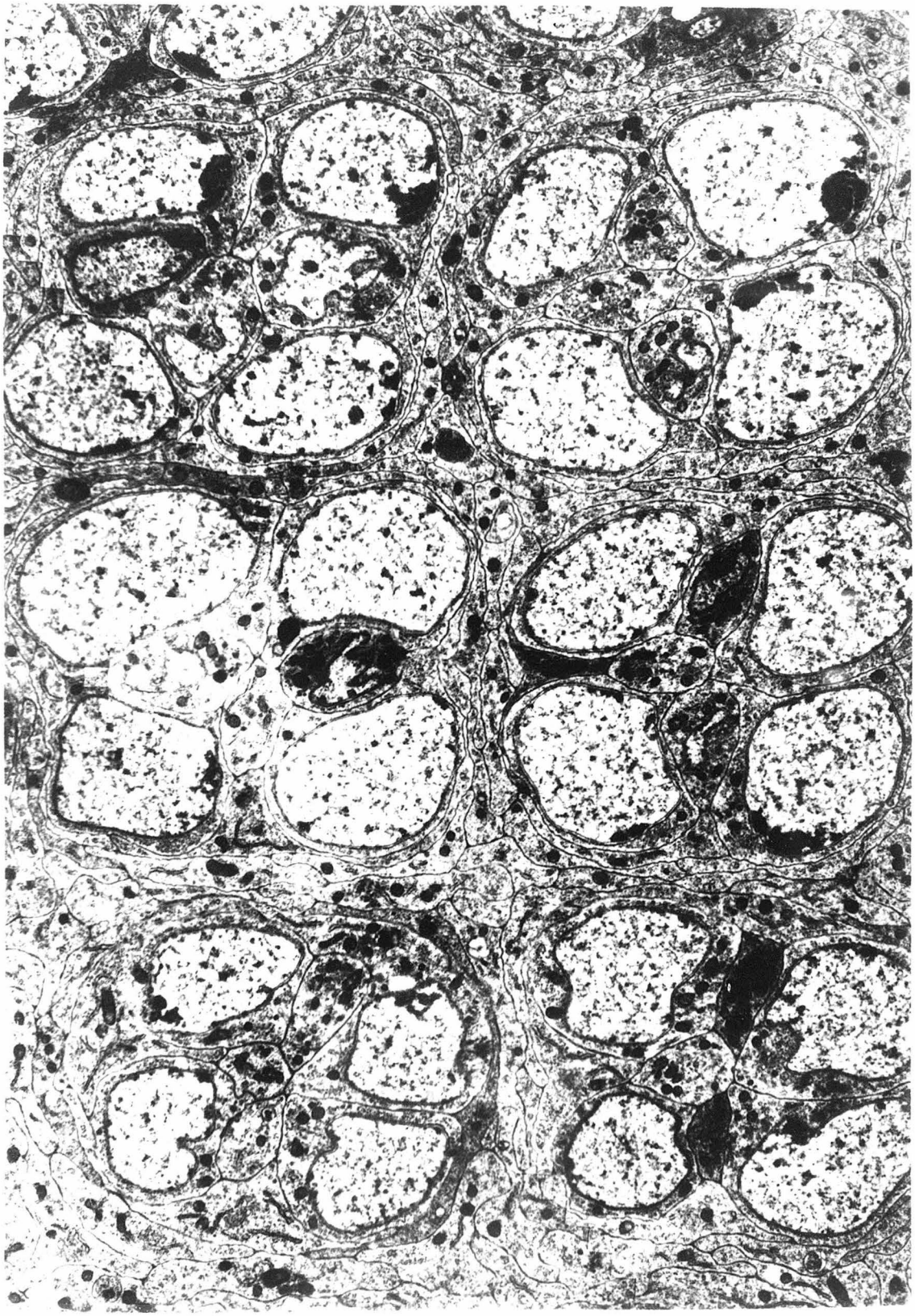

Fig. 12B. Diagram of photo in Fig. 12A. Numbers designate the photoreceptors that the cells are destined to form. Clusters have opposite spin above and below the equator, which is indicated by the dashed line. At this stage, mirror-image partners face each other across corners in a square array. The equator at this stage thus has a 90° sawtooth configuration. In this particular field, however, a jog occurs at the upper right.

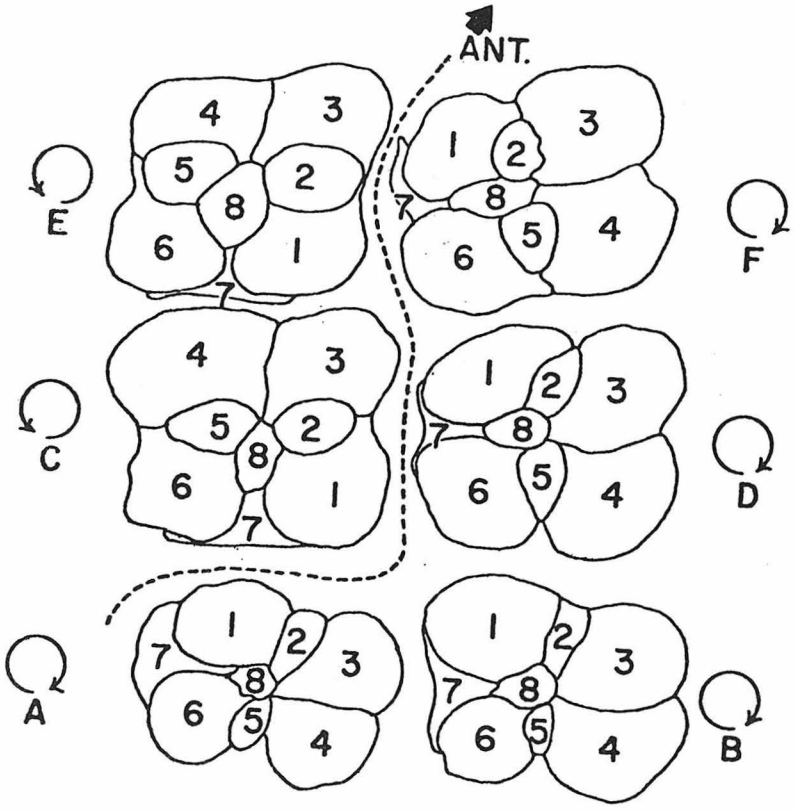
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Fig. 13. Sketch illustrates transition from square array at base of mature ommatidium to hexagonal array at top.

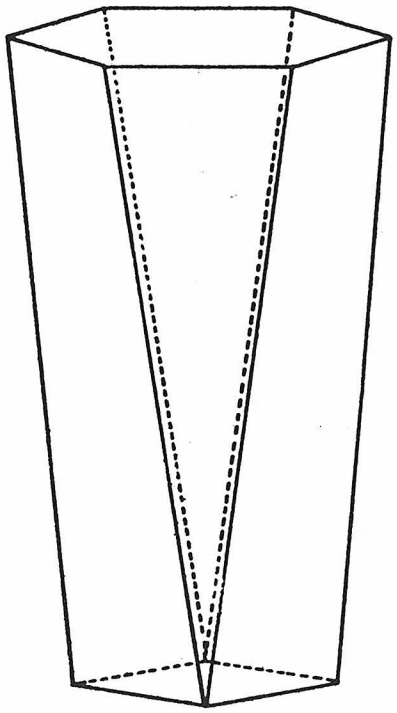
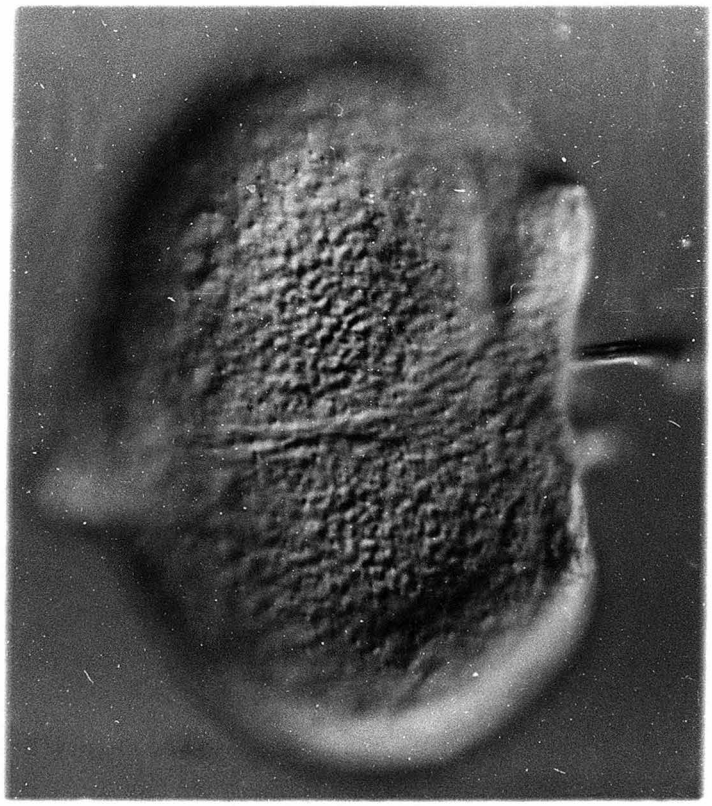


Fig. 14. The equatorial groove, as seen with Nomarski optics in an early third instar eye disc. 575×. Anterior is to the right. The groove runs horizontally in advance of the morphogenetic furrow (not yet visible). The antenna disc has been removed.



As described by Fristrom (1969), the normal mature eye disc contains few degenerating cells. Thus, cell death does not appear to be a factor in pattern formation in the normal eye.

Cell Divisions Traced by Thymidine Labeling

To study the relation of cell division to formation of the pattern, larvae were injected with [^{3}H]thymidine in the late third instar, at which stage the dorso-ventral furrow has advanced halfway across the eye disc. Development was allowed to proceed for various times after injection; then the labeled discs or eyes were fixed, sectioned, and coated with emulsion.

Autoradiographs of discs fixed soon (about 4 hr) after the injection of [^{3}H]thymidine showed label distributed broadly over the area anterior to the furrow. Behind the furrow, only a tight band, parallel to the furrow, was labeled (Fig. 15). The sharpness of the postfurrow band of label indicates that the thymidine is quickly incorporated after injection; the preparation is, in effect, pulse-labeled. It is difficult to identify the labeled cell types at this stage because the preclusters immediately behind the furrow are variable in shape and the anterior area lacks clusters.

In discs fixed 12 to 14 hr after injection of label, anterior movement of the furrow has increased the size of

Fig. 15. Autoradiograph of a late third instar eye disc fixed shortly after injection of [^{3}H]thymidine into the larva. 394×. Section is tangential to the disc surface. The position of the morphogenetic furrow is evident from the indentations at the top and bottom. Two areas of label are evident, one a band posterior to the furrow, the other a more diffuse labeling of the anterior part of the disc.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patterned field. The labeled cells which earlier had been close behind the furrow now are in a region of mature photoreceptor cell clusters, in which the individual cells are identifiable. Among the photoreceptor cells, only 1, 6, and 7 are found to be labeled, both above and below the equator. In addition, label is found in nonphotoreceptor cells. These do not appear to be in any consistent positions relative to particular photoreceptor cell neighbors.

Shortly after pupation, the furrow reaches the anterior end of the eye. Larvae were allowed to develop (after labeling in mid-third instar) until 48 hr after pupation. By then, the ommatidia have assumed an essentially adult form, and cells can be readily identified by their characteristic positions. Autoradiographs of these eyes reveal two vertical bands of label, separated by an unlabeled band several ommatidial columns wide. In the narrow posterior band, only photoreceptor cells 1, 6 and 7 are labeled, both above and below the equator, but cells 2, 3, 4, 5, and 8 are not (Fig. 16). Some of the cone, pigment, and bristle cells of these ommatidia contain label, but in no obvious pattern (Fig. 17). In the broad anterior band, any ommatidial cell type, including any of the photoreceptor cells, may be labeled. In adult eyes, the pattern of labeling is identical to that found in 48-hr pupae.

Engelhaaf, Berndt, and Küthe (1975) have demonstrated two similar mitotic waves in Ephestia. However, they did

Fig. 16. Autoradiograph of the eye of a pupa which had been injected with [^{3}H]thymidine as a late third instar larva. 2829×. Anterior is to the left. Grains can be seen over the nuclei of photoreceptors 1, 6, and 7. Among the photoreceptor cells, only the nuclei of 1, 6, and 7 show grains; label is absent from photoreceptors 2, 3, 4, 5, and 8. Some of the primary pigment cells (PPC) are also labeled. The nuclei of secondary pigment cells, tertiary pigment cells, and cells of the hair group are at a lower level; cone cell nuclei are in a higher section. Some of these contain label also.

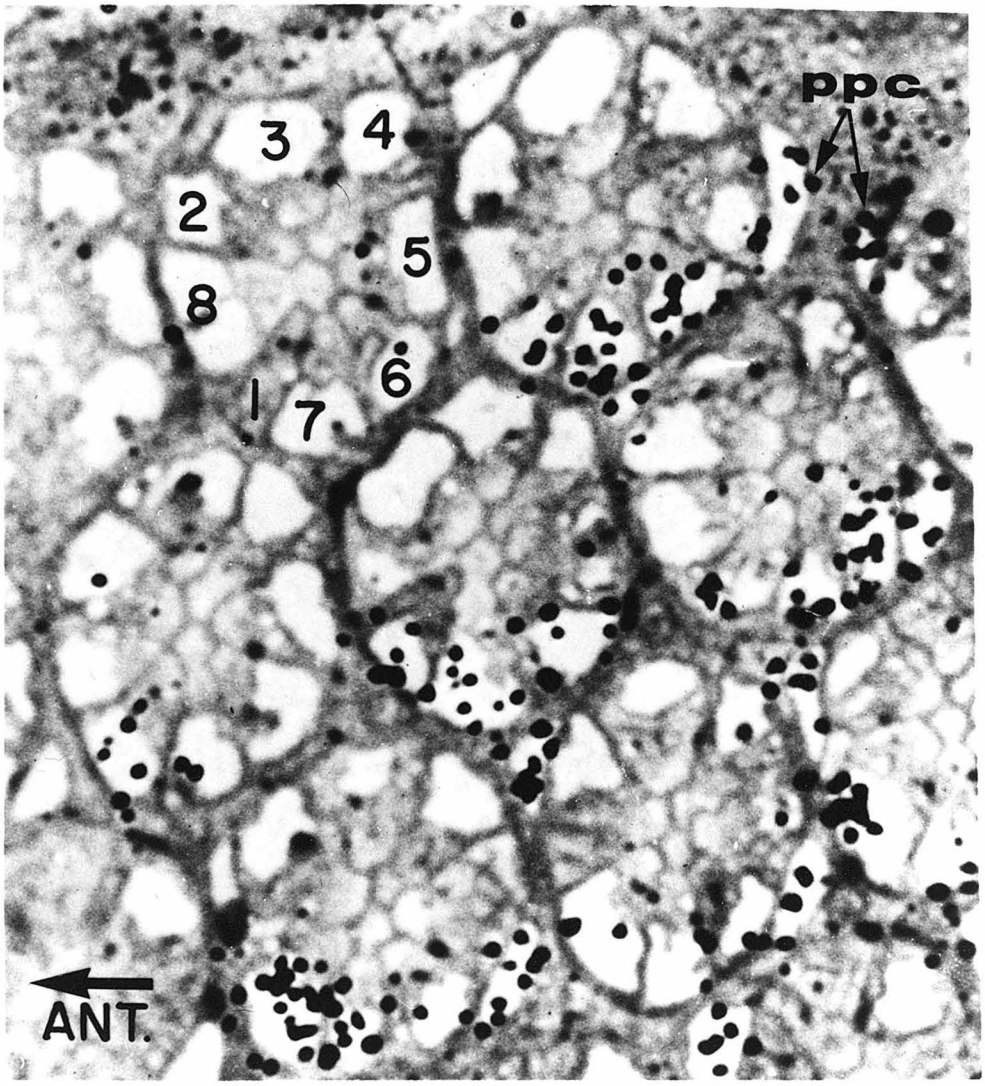
ppc
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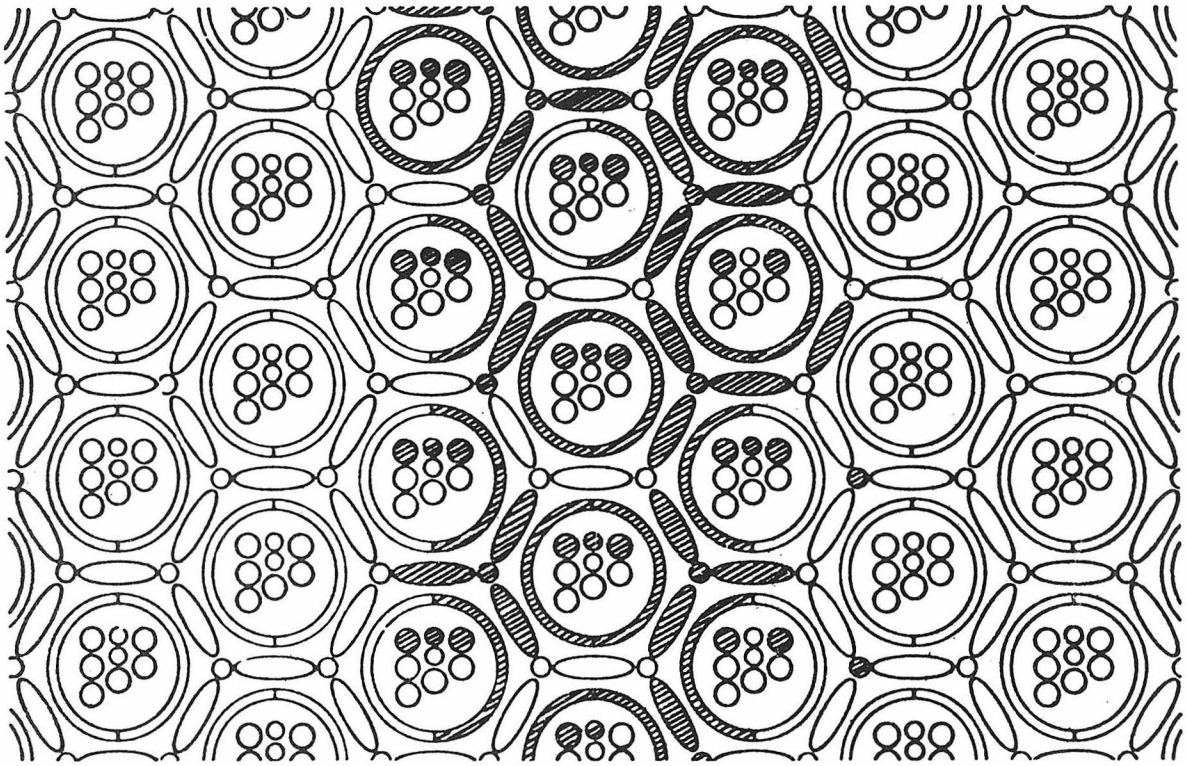
Fig. 17. Autoradiograph score sheet, showing the mitotic band posterior to the morphogenetic furrow. Anterior is to the left. [^{3}H]Thymidine was injected into a late third instar larva and cells were scored in the 48-hr pupa. Shading indicates cells that had grains over the nucleus in two or more sections. The sensory hair group (circle at anterior end of each horizontal secondary pigment cell) was scored as a unit. Note absence of label over photoreceptors 2, 3, 4, 5, and 8.



not relate the waves to the formation of specific photoreceptors.

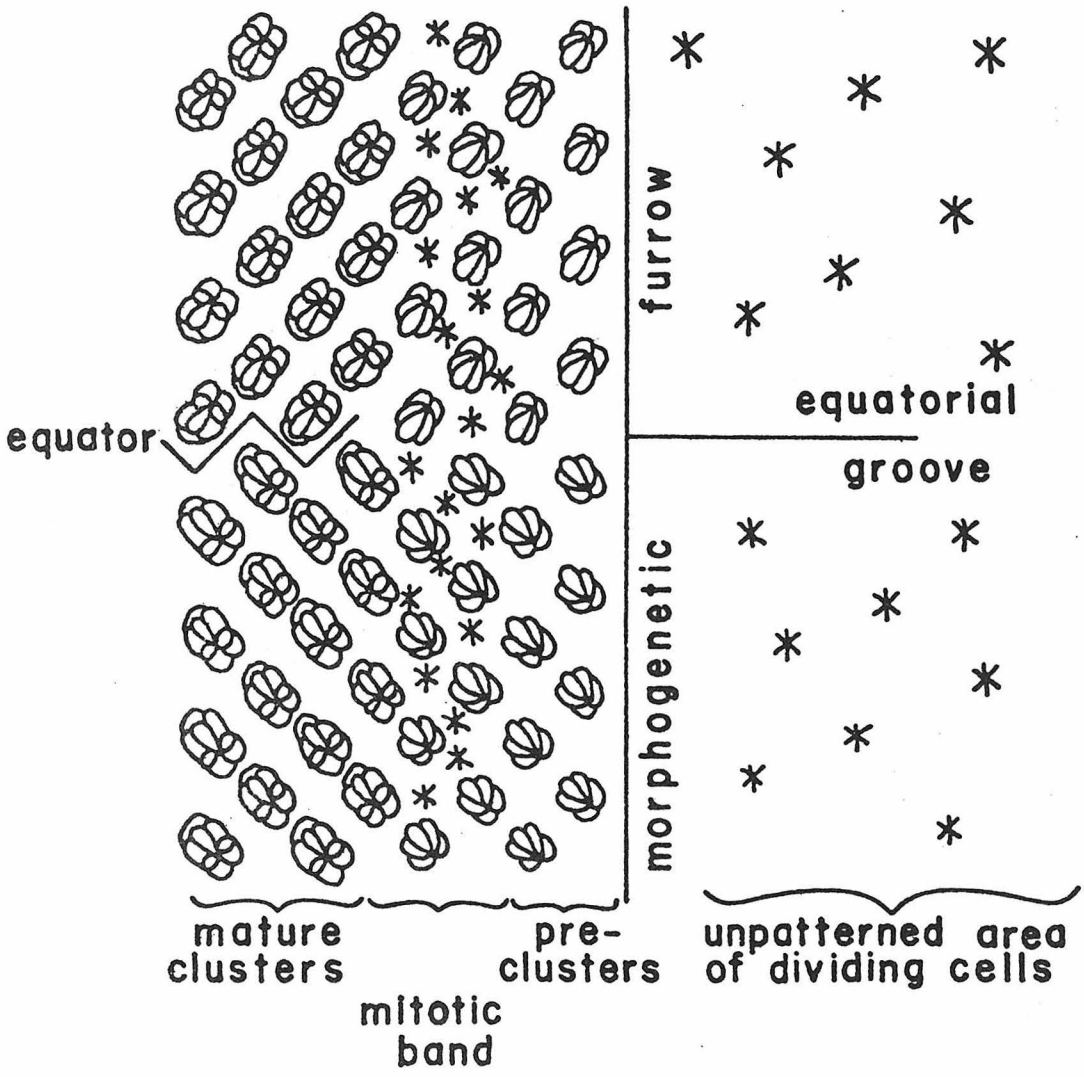
The overall sequence of pattern formation is shown schematically in Fig. 18.

Clonal Relations Deduced from Mosaics

The role of cell lineage in pattern formation can be studied by using genetic markers. Mosaicism can be induced at a specific stage of development, thus tagging the progeny of subsequent cell divisions. Any group of cells derived from a common ancestor should not show mosaicism (Stern, 1936, 1968; Becker, 1957). For example, if the equator represents the boundary between two clones which form the dorsal and ventral halves of the eye, marked clones should never cross the equator, provided they are generated after the initial determination has occurred. Similarly, if a photoreceptor cluster develops from a single mother cell, mosaicism induced before the formation of the mother cell should always produce clusters of unmixed genotype.

The sex-linked recessive mutation *white* is well-suited to such mosaic studies in the *Drosophila* eye. Homozygous normal and heterozygous *white* flies contain pigment granules in all pigment and photoreceptor cells. Homozygous *white* cells lack pigment. Clones marked with *white* can be initiated at any time during development of the eye by X-irradiation of larvae heterozygous for *white*. This treatment

Fig. 18. Schematic diagram showing the sequence of events in pattern formation. Asterisks indicate dividing cells. With time, the equatorial groove advances anteriorly to the right, followed by the morphogenetic furrow. Behind the furrow are the preclusters and the mitotic band. The area of mature clusters expands anteriorly at the expense of the unpatterned area.


furrow
equatorial
groove
equator
morphogenetic
mature clusters
pre-clusters
unpatterned area of dividing cells
mitotic band

induces, with a low probability, mitotic recombination between homologous chromosomes of a cell about to divide. Such recombination can result in one homozygous white daughter cell and one homozygous normal daughter. The progeny of the white daughter will appear in the adult eye as a pigmentless "white" patch against a red background of homozygous normal and heterozygous tissue. The autonomy of the white marker is indicated by the all-or-nothing pigmentation of cells in mosaics; on the borders of large clones, normal and white cells are indistinguishable from those in totally normal or white eyes. The white cells completely lack pigment, even when surrounded by fully pigmented cells. The mosaic eyes can be sectioned and each retinula and pigment cell can be scored unambiguously as white or normally pigmented. The role of clonality in the formation of the pattern should be revealed by the nature of such white clones.

1. The ommatidia. Mosaicism was induced by X-irradiation of larvae in the late first instar, at which time the eye primordium contains about 20 cells (Becker, 1957). A white patch resulting from somatic crossing-over at this stage will thus include many ommatidia. If ommatidia are formed from "ommatidial mother cells," such mother cells would therefore have to arise during cell divisions subsequent to the crossing-over event. Ommatidia formed from them should thus contain cells all of the same genotype.

A portion of a mosaic eye is shown in Fig. 19. Note that some ommatidia are mosaic; both white and pigmented cells can constitute a single ommatidium. Three other marked clones are shown in Fig. 20.

Among ommatidia showing mosaicism involving the photoreceptors and the pigment cells, one can ask whether any obligatory clonal linkages exist. Examination of the examples in Fig. 20 shows that any pair of cells in an ommatidium can occur in different genotypes. For example, the two primary pigment cells may differ (Fig. 19, inset). Furthermore, the anterior one may or may not be of the same genotype as the tertiary pigment cell directly in front of it. Similarly, a secondary pigment cell need not be of the same genotype as a neighboring pigment or photoreceptor cell. Any photoreceptor cell can occur in a different genotype from any other. Although certain combinations are observed less frequently than others (cf. Hofbauer and Campos-Ortega, in press), the absence of firm restrictions on mosaic combinations, either within an ommatidium or from one ommatidium to another, suggests that formation of the eye pattern is not dependent on cell lineage.

2. The equator. The shape of a marked clone induced by mitotic recombination depends somewhat upon its position in the eye. The dorsal or ventral edge of a patch often runs horizontally near the middle of the eye. This suggests the possibility that the descendants of early cells tend to

Fig. 19. Mosaic eye with a clone of unpigmented cells produced by X-ray induced mitotic recombination. (A) Outer tangential section showing the matrix of secondary and tertiary pigment cells. In pigmented areas, six secondary pigment cells can be seen, corresponding to the faces of each ommatidium. (Some of these cells appear split because the plane of section does not pass through the entire cell.) Tertiary pigment cells appear as dots. 1219×. Anterior is to the left. Inset: Section through the primary pigment cells in a mosaic ommatidium. Nomarski optics. One (semicircular) cell is pigmented; the other is not. 1699×. (B) Tangential section deeper in the eye, showing photoreceptor mosaicism. Nomarski optics. 2324×. Note the small ommochrome granules along the rhabdomeres of normal photoreceptor cells; these are absent in white cells. (Photoreceptor 7 must be scored in a higher level section because its pigment granules are more distal.) The equator runs horizontally across the bottom third of the picture. In three places, a white tertiary pigment cell can be seen at a vertex where three normal secondary pigment cells meet.

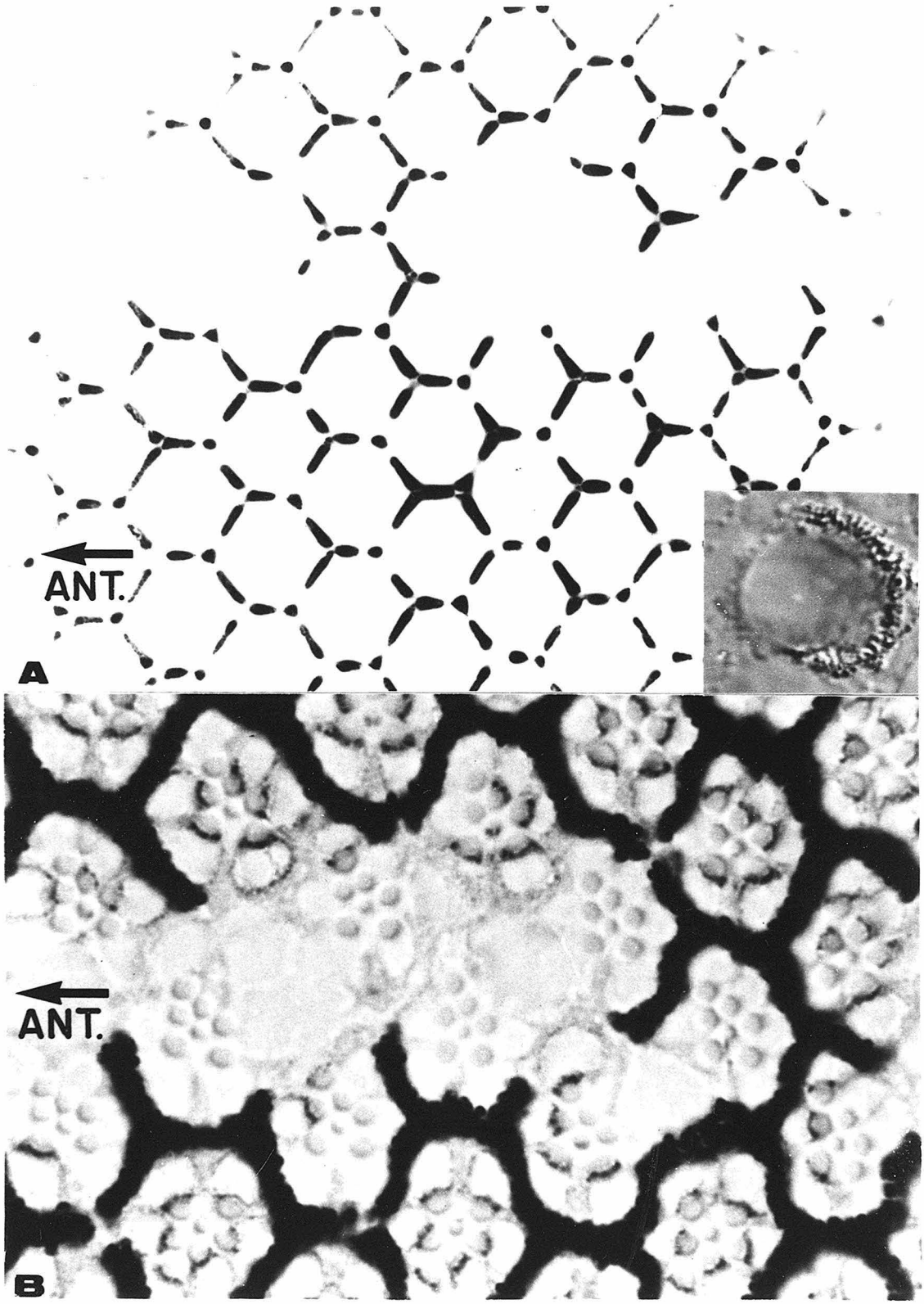

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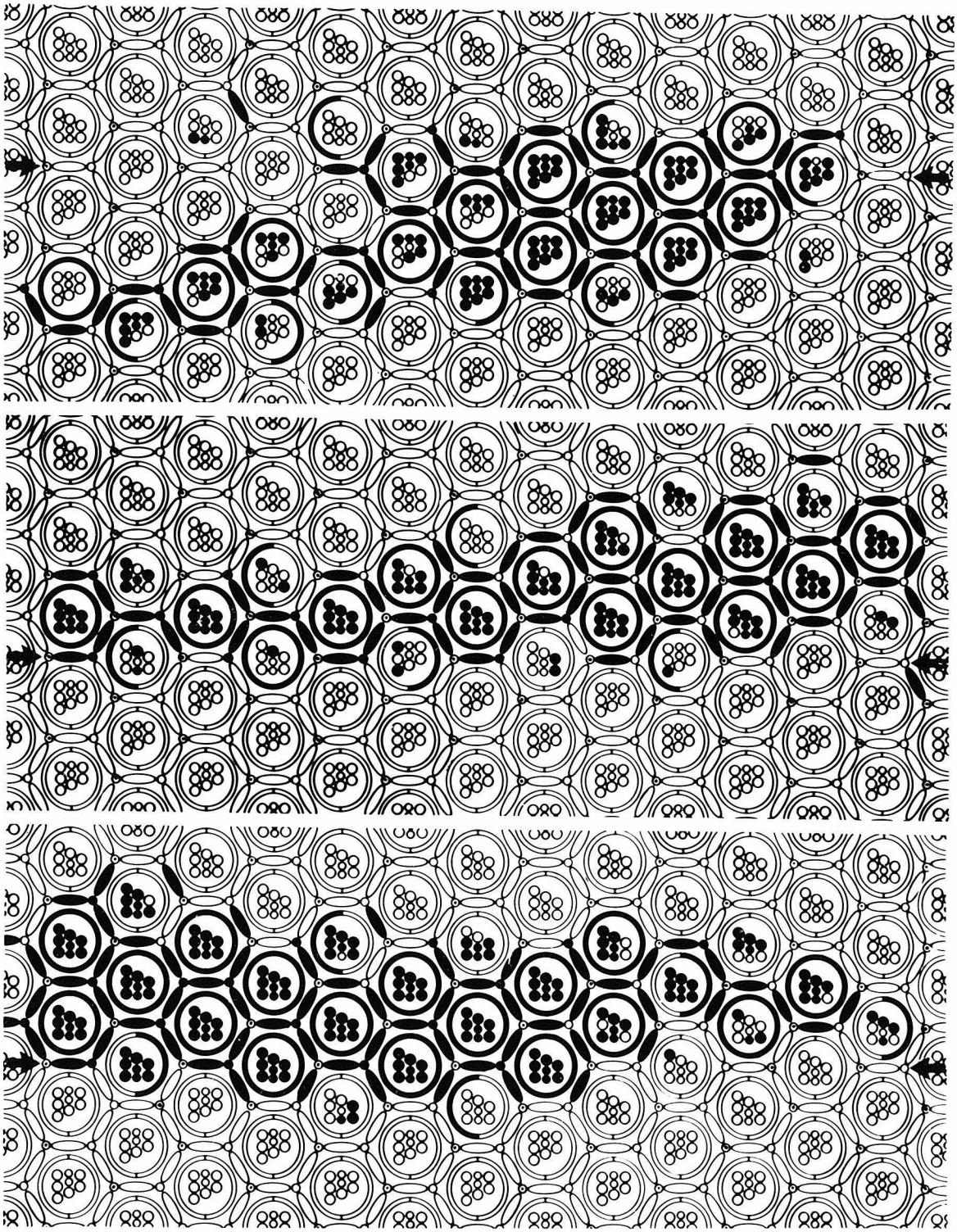
Fig. 20. Three marked clones. Black indicates unpigmented mutant cells arising via mitotic recombination induced by X-rays in late first instar. Top figure includes an entire marked clone. In the other two, the patches extended slightly beyond the areas scored. Arrows at edges indicate equators. Dotted circles indicate sensory hairs (unscored). Anterior is to the left.



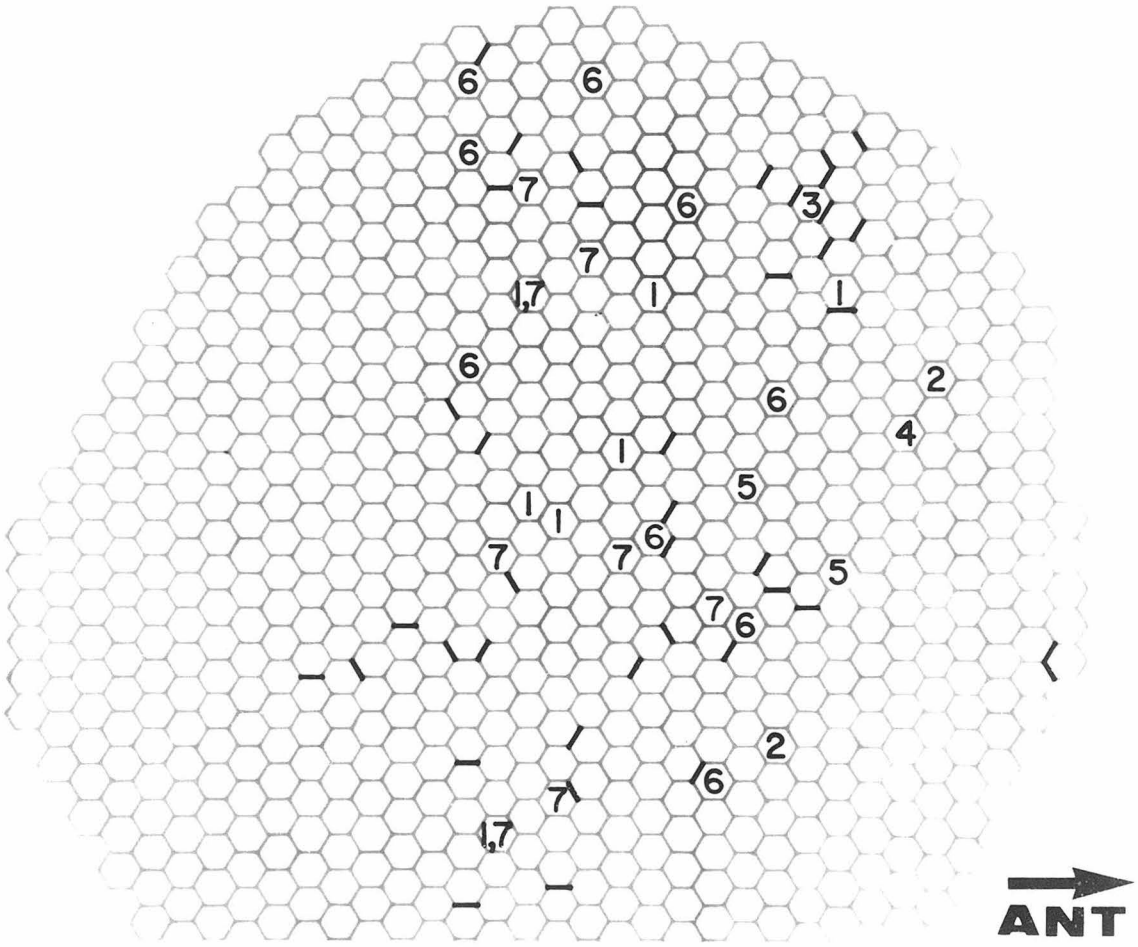
populate either the dorsal or ventral halves of the eye (Becker, 1957).

External observation of the eye, with gross scoring of ommatidia, is inadequate to characterize accurately the edges of a clone. By histological examination, every cell can be scored. It is then found that, while the cells of a white clone tend, by and large, to form a continuous patch, some marked cells lie removed from the main body of the clone. Such outlying cells are rarely more than one ommatidium removed from the main patch. This indicates that little cell migration occurs.

Three mosaic patches that occurred near the equator are shown in Fig. 20. They were induced in the late first instar and reveal that a clone marked at that stage can contribute cells to both sides of the equator (Hanson et al., 1972; Benzer, 1973). Thus, although the equator often lies near a clonal boundary, it is not determined by such a boundary.

3. The developmental scheme. According to the developmental scheme of Fig. 18, exposure to X-irradiation in the late third instar should have different effects in different areas of the disc. In the posterior part, the mature clusters should be immune to marking with white, since their cells are not destined to divide again. In the mitotic region just posterior to the furrow, photoreceptor cells 2, 3, 4, 5, and 8, already assembled into preclusters,

Fig. 21. Cell types marked by mitotic recombination induced during the late third instar, revealing the pattern of mitosis during formation of the eye. Marked photoreceptors are indicated by numbers. A heavy line along the side of a facet indicates a marked secondary pigment cell. Toward the anterior of the eye, any cell type may be marked. In a more posterior zone, only photoreceptor cells 1, 6, 7, and other nonphotoreceptor cells are marked. More posteriorly, where cell division has been completed at the time of irradiation, no cells are marked. Only photoreceptors 1, 2, 3, 4, 5, 6, 7, and secondary pigment cells were scored. Due to curvature of the eye, tertiary pigment cells and photoreceptor 8 are difficult to score over large areas. The ventral portion of the eye was not scored.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should be immune. However, the remaining photoreceptor and pigment cells, formed in the postfurrow wave, should still be subject to recombination. Ahead of the furrow, in the broad mitotic area, all cell types should be candidates for marking. An actual example is shown in Fig. 21. It indeed reveals three such regions, consistent with the developmental scheme.

DISCUSSION

Pattern formation in the Drosophila retina is intimately coupled with polarized growth. A morphogenetic front sweeps anteriorly across the disc, the leading edge marked by a dorso-ventral furrow. Ahead of the furrow, cell division occurs over the entire region, producing a supply of cells. Along the advancing front, unpatterned cells are first assembled into "preclusters" consisting of photoreceptor cells 2, 3, 4, 5, and 8, which undergo no further divisions. Behind the furrow, other cells, surrounding the preclusters, divide in a mitotic wave, producing the remaining photoreceptor cells 1, 6, and 7, as well as others needed to complete the ommatidial set. Pattern formation thus occurs in two main steps, the transition from unpatterned cells to the final lattice being mediated by a transient, intermediate pattern. The extremely precise array of the eye arises, not by a clonal sequence, but by recruitment of cells into a lattice.

The existence of the equatorial groove, an indentation of the developing disc directly ahead of, and in line with, the elongating equator, suggests that the groove might present a mechanical constraint that tends to inhibit, but not completely prevent, wandering of cells from one side to the other. This would cause the equator to appear as a weak clonal border reminiscent of the developmental "compartment" boundaries described by Garcia-Bellido (1974).

A compartment contains the descendants of a number of progenitor cells and is defined by borders that they will not cross. For example, clones on the dorsal side of the wing, if initiated after a certain stage of development, may spread as far as the wing margin, but not contribute even a single cell to the ventral surface (Garcia-Bellido, 1974). Baker (unpublished) and Waitz (unpublished) have looked for compartmentalization in the Drosophila eye, using the Minute technique (Morata and Ripoll, 1975) to expand the size of marked clones. They found that while the edges of clones are often parallel to the equator, they are not necessarily coincident with it. As Fig. 20 clearly shows, clones can cross the equator, so that it cannot be considered as an absolute boundary, at least for clones initiated in late first instar.

Becker (1957) first demonstrated that mosaic patches frequently had edges running horizontally near the middle of the Drosophila eye. Without histology, however, he could

not relate these borders to the equator. By superimposing outlines of many mosaic eyes, Becker drew a set of preferred clonal borders radiating from the posterior of the eye, dividing it into sectors of characteristic shape. Such superposition of patches, based on external examination of the eye, involves difficulties. Variations in the number of ommatidia from eye to eye and the absence of firm landmarks make the superposition of eyes somewhat arbitrary. The ragged edges of clones make their boundaries ambiguous. A hand-drawn outline can be accurate only within a few ommatidia. These drawbacks make the documentation of Becker's sectors difficult, since they are only a few ommatidia wide.

Occasional patches in the current experiments agreed with Becker's sectors, particularly long, narrow patches which ran horizontally across the middle of the eye (Becker's sector I). These have also been observed by F. A. Zihler (unpublished). Most patches, however, did not correspond to single sectors or to simple combinations of sectors. D. Kankel (personal communication) has also been unable to reconcile mosaic patches with the boundary restrictions suggested by Becker's sectors. While the prevailing shapes of patches presumably reflect a tendency of clones to grow radially from the posterior of the eye disc, as Becker suggested, the evidence is not convincing for strict constraints on clonal boundaries. The tendency toward elongated clones is characteristic of the development of other Drosophila

discs as well (Bryant and Schneiderman, 1969).

Cell differentiation in the eye appears to be unrelated to cell lineage. The final cell type may thus be determined according to the lattice position into which the cell is recruited. This is reminiscent of the growth of a crystal, in which the leading edge of the pattern serves as a template on which new elements are incorporated. Rather than being required to assess its position independently, a cell joining the ensemble could use the information available at the growing edge to determine its role.

This assessment may be based on combinatorial cell contacts, the information being mediated by surface molecules, as in the "antigen-antibody" model of Tyler (1947) and Weiss (1947). According to such a scheme, an undetermined cell ahead of the furrow might display a set of antigens. "Antibodies" on cells at the leading edge would bind a specific subset of these antigens, thus informing the newly added cell of its role and causing it to display an appropriate set of antigens in turn, propagating the pattern. Combinatorial interactions of cell surface enzymes and substrates, as proposed by Roseman (1970) would work in a similar way.

Such a model would be consistent with White's finding (1961, 1963) that development of the mosquito eye could be arrested by a narrow band of extraoptic epidermis placed ahead of the morphogenetic front. A small portion of the

advancing edge was sufficient to propagate the pattern. Hyde (1972) found that the cockroach eye also grows by recruitment of cells along the anterior margin. The studies of Wachmann (1965) on Galleria and Mouze (1975) on Aeshna show similar phenomena. Shelton and Lawrence (1974) and Green and Lawrence (1975) demonstrated recruitment in the compound eye of Oncopeltus and also showed that recruitment can extend to cells of the head epidermis outside the normal limits of the eye field, which would not be accounted for by the Tyler-Weiss model. Indeed, initiation of the first column of clusters at the rear of the Drosophila eye would also require an additional mechanism, such as patterning forces from the adjacent epidermal cells. The grafting experiments of Lawrence and Shelton (1975) in Oncopeltus indicated that the polarity of adjacent epidermis can influence the orientation of developing ommatidia.

In the amphibian Xenopus, pattern formation in the retina begins at the center of the eye (Jacobson, 1968); new cells are added to the growing edge behind a ring of high mitotic activity (Straznicky and Gaze, 1971). Jacobson (1968) found that the axes of the entire eye, with respect to projection of fibers to the optic tectum, are specified at the time that the first retinal ganglion cells are formed. This suggests that cells subsequently added are recruited in register with neighboring cells already fixed in a pattern.

The regularity of the photoreceptor array in the chick retina led Morris (1970) to suggest a clonal mechanism of pattern formation. By analysis of the timing of divisions, however, she subsequently showed that a clonal sequence was unlikely (Morris, 1973). The results were consistent with the possibility of sequential induction of cell types at the growing edge.

Other vertebrate structures, such as limb (Saunders, 1948; Wolpert et al., 1975) and somites (Cooke, 1975), grow along a morphogenetic front, preceded by a region of high mitotic activity. The development of feather patterns (Sengel, 1975) is a striking example. Similarly, neurons in the vertebrate brain are formed in the wake of mitotic waves (Angevine, 1970). Thus, pattern formation by recruitment of cells along a growing edge may be a general mechanism in systems with polarized growth.

CHAPTER III

EPILOGUE

The work presented provides the framework of events during normal development of the eye of normal genotype.

Of the one thousand Drosophila genes catalogued by Lindsley and Grell (1968), fully twenty percent, when mutant, perturb eye structure. Many of these phenotypes are due to non-specific effects such as generalized cell death in the eye disc (Fristrom, 1969) or to defective maturation of well-patterned ommatidial precursors (Waddington and Pilkington, 1943; Clayton, 1954). Others may result from defective assembly of clusters at the furrow. Mosaic analysis of mutants of the latter type may reveal clues to the nature of the morphogenetic process. For example, if a genetically normal facet is disrupted by a neighboring patch of mutant tissue, this would suggest the involvement of a diffusible morphogen. This would be especially interesting if the effect were produced only by mutant patches occupying certain regions, such as the equator or margins of the eye. At a finer level, single cell mosaic patches may be produced by somatic recombination. For example, suppose that centrally placed photoreceptor #8 plays a pivotal role in cluster organization. If this cell were mutant the ommatidium might be severely disrupted, while no effect might occur if other photoreceptors were mutant.

The many mutations in Drosophila perturb development of the eye in a large variety of ways and it might be possible to detect biochemical correlates of these defects. The great sensitivity and resolution of new techniques such as two-dimensional electrophoresis of proteins (O'Farrell, 1975) should facilitate this. Of particular interest would be surface glycoproteins that might be involved in specific cell interactions.

Given that a particular relevant protein can be identified, the technique of segmental aneuploidy developed by Lindsley et al. (1972) can be used to screen the Drosophila genome for the locus coding for that protein. Once the locus is found, it should facilitate the isolation of various mutations of the same gene, including temperature sensitive ones. These would be desirable since important proteins may be lethal if defective throughout development. After shifting such mutant larvae to the restrictive temperature at various stages of eye development, examination of eye discs and the resultant eyes may reveal the time at which the protein acts in pattern formation. Notch and shibire^{ts} provide striking examples of such mutations. Notch flies raised at 21°C have rough eyes. However, if third instar Notch larvae are shifted to 29°C, ommatidia formed during the shift are normally arranged (Foster and Suzuki, 1970). Just the opposite pattern of temperature sensitivity occurs in shibire^{ts}. At 21°C, normal facets

develop, but facet formation is defective at 29°C. Shifts to the restrictive temperature during the third instar thus produce vertical scars in the eye (Poodry et al., 1972). Unfortunately, in neither case has the temperature-sensitive molecule been identified.

Since eye discs can be cultured in vitro (Kuroda, 1970), a direct attack on the recruitment process may be possible. For example, lectins directed against specific glycoproteins, or enzymatic alterations of particular membrane proteins may lead to changes in clusters recruited at the morphogenetic furrow. If completion of a precluster at the furrow is signalled by the binding of a class of surface glycoproteins, lectins might produce clusters with fewer cells than normal. Conversely, protein modifications which diminish binding might produce clusters with a large number of cells.

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