

Cell Clones and Pattern Formation: On the Lineage of Photoreceptor Cells in the Compound Eye of *Drosophila*

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Summary. The genealogical relationships of photoreceptor cells within the compound eye of *Drosophila* have been studied using cell labelling, with either ^3H -thymidine or recessive mutations, during the third larval stage. It has been found that photoreceptor and secondary pigment cells arise from different precursor cells. Under the present experimental conditions, precursors of receptor cells give rise to about 8 elements which differentiate as R cells of two different groups. One of the cells differentiates as R7 and the remaining as any one of the R1 to R6. The last cells behave initially as equivalent, and can differentiate within the same or within different, but neighbouring, ommatidia. The class of R1 to R6 cell in which each one of these elements differentiates, seems to depend on the time of its origin. The implications of these findings for the formation of the ommatidial pattern are discussed.

Key words: Compound eye — Development — Cell lineages — Genetic mosaics — *Drosophila*.

Cell diversity within an organ can be thought to arise by one of two developmental mechanisms which are, perhaps, in a relationship of causality. The first mechanism is that of *segregating mitoses*, which implies that some kind of genetic determinism progressively restricts the developmental abilities of proliferating cells after divisions, thus giving rise to prefixed cell lineages. The second involves *positional information* and assumes the existence of morphogenetic fields in which originally equipotent cells differentiate according to the position they occupy in a field (Garcia-Bellido, 1972).

The ommatidia of the compound eye of *Drosophila*, like those of other insects, are multicellular systems composed of 4 morphologically and functionally different types of cells: cone cells, primary and secondary pigment cells and photoreceptor (or retinula, R) cells. There are 8 photoreceptor cells in each ommatidium, arranged regularly to form a peculiar pattern. Because of

the constancy of this arrangement, an individual R cell can be identified and categorized according to its position in the ommatidium. Retinula cells R1 to R6 can be distinguished from R7 and R8 cells by anatomical and physiological criteria (Melamed and Trujillo-Céno, 1968; Kirschfeld, 1973). The combination of cell diversity with an extreme regularity in the arrangement of cells, makes the compound eye of *Drosophila* an excellent object for developmental studies.

Many years ago Bernard (1938) studied the development of the compound eye of the ant *Phormicina flava* with conventional histological techniques, and postulated that the 8 R cells of one ommatidium originate from the same precursor cell by 3 consecutive mitoses. Accordingly the R cell population of each ommatidium would constitute a clone whose members have differentiated in close contiguity. However, Bernard's hypothesis has recently been challenged by two studies, in *Drosophila* (Hanson, Ready and Benzer, 1972; Benzer, 1973) and *Oncopeltus* (Shelton and Lawrence, 1974), using genetic mosaics. Both studies demonstrate convincingly that ommatidia situated at the boundaries between phenotypes are composed mosaically of cells with different phenotypes and therefore of different lineages. One of these studies (Shelton and Lawrence, 1974) concludes that "cell determination (of "particular classes of retinal cells") within ommatidia is in no way connected with their lineage", and that "the fate of the cells must be decided depending on the position they occupy in the developing ommatidium" (loc. cit. page 351).

We performed a statistical analysis of the composition of mosaic ommatidia along the borders between *w/w* and *w/+* tissues in mosaic compound eyes of *Drosophila* (Hofbauer and Campos-Ortega, 1976) and found that the distribution of phenotypes among the constituent R cells of mosaic ommatidia is not entirely random. Certain groups of cells within individual ommatidia tend frequently to have the same phenotype and thus the same clonal origin. One group consists of the cells R2, R3, R4 and R5, i.e. cells in a polar position within the ommatidium. R1, R6 and R7, cells in equatorial position, form a second group. We can call these cell groups polar and equatorial because of the regular arrangement of ommatidia in the eye. At the level of the equator the ommatidia undergo a mirror-image inversion so that the 2 groups of retinula cells are oriented either towards the equator or towards the poles (north or south), irrespective of their position within the dorsal or the ventral half of the eye. The 2 groups, polar or equatorial, were found to be significantly more prevalent, in our statistical evaluation of mosaic ommatidia, than any other association of R cells. This tendency for either polar or equatorial cells to belong to the same clone might be explained by assuming that the members of a given group arise from related mitoses. (This is a priori not obvious because the patches analysed were very large ones, induced during the first instar; Hofbauer and Campos-Ortega, 1976). If this interpretation turns out to be correct, then it would imply that there is some kind of regularity during the proliferation of R cells, and this would be difficult to reconcile with determination of R cells solely as the result of their position.

The idea that cells of a particular group within the ommatidium represent a cell clone may be tested by labelling dividing cells during the third larval stage, when they perform their last mitoses prior to morphological differentiation.

Two different kinds of labelling can be used: 1) ^3H -thymidine injected into third instar larvae, followed by identification of the labelled cells by means of autoradiography of adult eyes; 2) genetic labelling by means of X-ray-induced somatic crossing-over and conventional electron microscopy for the identification of the resulting clones.

Results and Discussion

^3H -thymidine Autoradiography

Incorporation of tritiated thymidine in the eye imaginal disc of the third instar of *Drosophila* reveals that the presumptive ommatidial field is traversed by 2 clearly defined waves of cell divisions, as inferred from the S-phases evidenced with the ^3H -thymidine. The 2 waves proceed from the posterior to the anterior of the larval primordium during this stage.

Priority for the discovery of these mitotic waves in *Drosophila* is given to Ready, who described their behaviour in three different abstracts (Ready, 1973, 1974, 1975; see also Campos-Ortega and Gattef, 1976). A full account of his findings is in preparation. However, the description of the pattern of proliferation of the third instar eye imaginal disc of *Drosophila* offered here is based on our own material and observations, and it sufficiently confirms Ready's findings. Furthermore, it provides additional information lacking in his publications, these omissions most probably being due to the obligatory brevity of such abstracts.

At the end of the second instar the first of the waves starts posteriorly and spreads out through the anlage, reaching the anterior border within 10–15 h. Approximately 10 h later the second mitotic wave is detectable in the posterior part of the anlage. The two waves have different widths. At any given time during the last two thirds of the 3rd instar, incorporation of ^3H -thymidine can be detected in a posterior, very sharply defined, vertically orientated zone of the eye, with a width of 2–3 ommatidial rows, and in a broad zone, beginning 4–5 rows in front of the posterior band and extending to the anterior margin of the eye (Fig. 1). If progressively older larvae are injected, the position of the bands can be observed to move forwards, at a rate of one vertical row of ommatidia every 2 h, with a constant distance between the anterior and posterior zones of labelling. At the end of the third instar the activity of the anterior wave has elapsed and only the posterior wave is present. About 10 h after pupariation the last wave has finally reached the most anterior margin of the eye primordium and no further cellular proliferation involving R cells occurs within the eye disc. Nevertheless, mitotic activity is still present within the eye anlage, though this affects only pigment and cone cells.

Serial section reconstructions of flies injected with tritiated thymidine during the third instar provide us with material for the study of the labelling behaviour of the 8 R cells. This labelling behaviour is not identical in the anterior and posterior zones. Whereas the anterior zone includes labelled representatives of all 8 R cell classes, the posterior band contains only labelled R cells from the equatorial group, R1, R6 and R7 (Fig. 1; Table 1). A statistical analysis

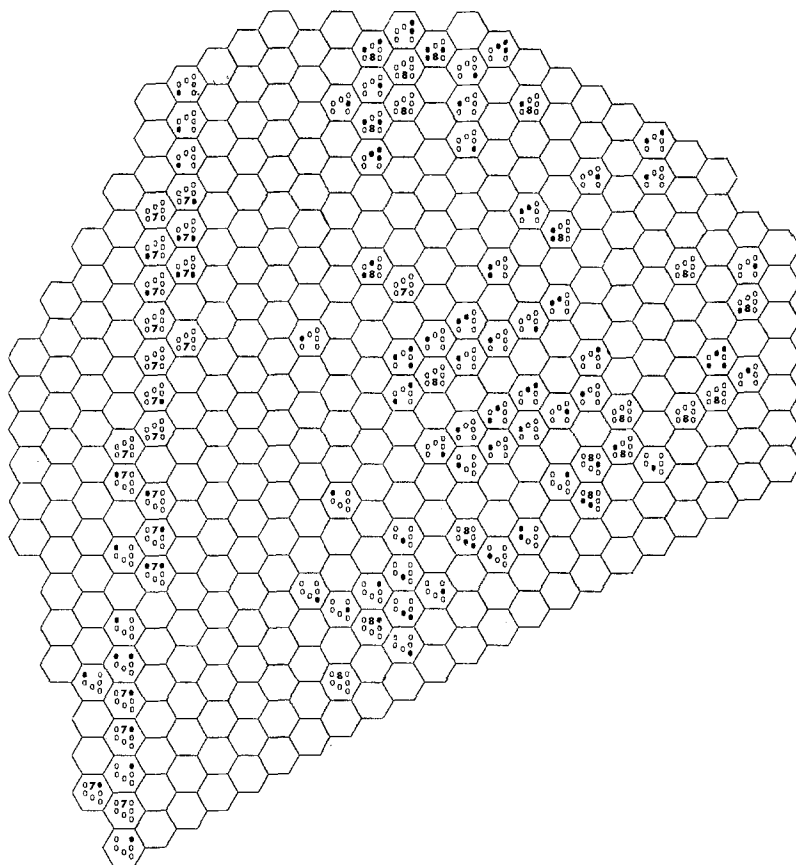


Fig. 1. Localization of ^3H -thymidine labelled receptor cells within the right compound eye of a fly injected with $1\ \mu\text{l}$ of tritiated thymidine (spec.act. $21\ \text{Ci/mmol}$, conc. $1\ \text{mCi/ml}$) approximately 20 h after the beginning of the third larval stage. This eye was reconstructed from a complete series of $2\ \mu\text{m}$ sections to about 2 thirds of its total extent; the ventral one third of the eye could not be reconstructed because the sections were too oblique for identification of labelled cells. Posterior of the eye is at the left hand of the drawing. Labelled R1 to R6 cells are marked in black and indicated by their position within the ommatidia; R7 and R8 cells indicated by numbers. Labelling is restricted to a posterior band of ommatidia containing labelled R1, R6 and R7 cells, and an anterior band, in which labelled representatives of all R1 to R8 receptor cells are present. See text for further explanation

of labelled R cells from both zones shows that cell pairs from either the polar group, R2 to R5, with R8 cells in addition, or the equatorial group, R1, R6 and R7, tend to be labelled simultaneously within the same ommatidium (Fig. 2). Considering the distribution of label within each band separately, the relationships between cell pairs are even clearer, at least so far as the equatorial cells are concerned (Table 1).

These results indicate that the polar cell group and the equatorial cell group originate at different times and, very probably, from related mitoses during the third instar. Although the present evidence shows conclusively that polar

Fig. 2. Comprehensive analysis of ³H-thymidine labelled receptor cells. The numbers 1 to 8 signify R1 to R8 cells. The Figure represents data obtained by pooling the labelled cells from both labelling bands, anterior and posterior. The lines connecting the cells represent the frequency with which both cells of a particular pair are labelled within the same ommatidium. There is a clear tendency for polar, R2 to R5, and R8 cells on the one hand and for equatorial R1, R6 and R7 cells on the other hand, to be labelled simultaneously within the same ommatidia. The percentages expressed by the interconnecting lines are relatively low. This is due to the fact that a high proportion (48.5%) of all ommatidia analysed contained a single labelled receptor cell

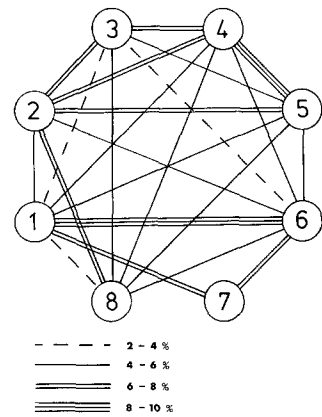


Table 1. Frequency of simultaneous ³H-thymidine labelling of the different receptor cell pairs. Analysis of the frequency of concomitant labelling of receptor cell pairs within the same ommatidia in separate zones

Receptor cell pairs		Frequency of simultaneous labelling (%)		
		anterior zone	posterior zone	both zones
R1	R2	5.9	0	4.9
R1	R3	4.1	0	3.3
R1	R4	5.9	0	4.9
R1	R5	5.4	0	4.5
R1	R6	6.8	25.0	9.8
R1	R7	2.7	31.8	7.5
R1	R8	4.1	0	3.4
R2	R3	7.7	0	6.3
R2	R4	8.6	0	7.1
R2	R5	9.0	0	7.5
R2	R6	6.3	0	5.3
R2	R7	1.8	0	1.5
R2	R8	7.7	0	6.4
R3	R4	8.6	0	7.1
R3	R5	5.4	0	4.5
R3	R6	3.6	0	3.0
R3	R7	0.9	0	0.8
R3	R8	5.4	0	4.5
R4	R5	10.4	0	8.7
R4	R6	6.8	0	5.6
R4	R7	1.4	0	1.1
R4	R8	5.0	0	4.1
R5	R6	5.9	0	4.9
R5	R7	0.0	0	0.0
R5	R8	5.9	0	4.9
R6	R7	2.3	25.0	6.0
R6	R8	6.3	0	5.3
R7	R8	0.9	0	0.6

Number of ommatidia analysed: anterior zone: 222, posterior zone: 44, both zones: 266

and R8 cells originate exclusively from the mitotic activity in the anterior wave, it is not yet possible to determine whether, conversely, the equatorial cells originate only from the mitotic activity of the second wave. Furthermore, no proof of common ancestry of elements from either group can be derived from the study of ^3H -thymidine autoradiographs.

Genetic Mosaics

Irradiating third instar larvae from the cross $w/w \times rdgB/Y^+$ with an appropriate dose of X-rays, induces mitotic recombination in some cells of the eye anlage. Three types of clones containing R cells can be collected. When somatic crossing-over occurs between the $rdgB$ locus (1-42.7, Harris et al., 1976) and the centromere of the X-chromosome, a twin-spot consisting of a w/w clone and a $rdgB/rdgB$ clone is produced. w/w clones alone may result from mitotic recombination between the w (1-1.5) and the $rdgB$ loci, and also from chromosome mutations produced at the time of irradiation (Becker, 1957; Haendle, 1971). Similarly, $rdgB/rdgB$ single clones arise from concomitant recombination between both loci and between the $rdgB$ locus and the centromere, or from some chromosome aberrations as well (see Fig. 3).

Cells with a w/w genotype are characterized phenotypically by a complete lack of pigmentation. The mutation affects all otherwise pigmented cells, i.e. primary and secondary pigment cells and R cells. Cells with a $rdgB/rdgB$ genotype can be easily identified in the adult eye by their degenerative appearance (Benzer, 1971; Harris et al., 1976), which includes shrinkage, darkening, fragmentation and phagocytosis by secondary pigment cells (see Fig. 7). The allele $rdgB^{KS222}$ used here only affects the R1 to R6 cells, while the remaining cells of the ommatidium are phenotypically wild-type. Both genes, w and $rdgB$, are strictly cell-autonomous in their expression (Hofbauer and Campos-Ortega, 1976).

Some comments are required on our evaluation of clones. Since the cells at the third stage are able to divide only a few times the resulting clones are correspondingly small (see Fig. 7, Tables 3 and 4). Generally, the labelled cells are restricted to the same ommatidium, and the neighbouring ommatidia consist of phenotypically wild-type cells. These cases do not pose any difficulties in their interpretation since all labelled cells are probably derived from the same precursor. However, one would expect that dividing R or pigment cells are able to migrate across one, or in some extreme cases a maximum of 2 rows of ommatidia at differentiation (Hofbauer and Campos-Ortega, 1976), and therefore derivatives from the same precursor may be found among the elements of different, immediately neighbouring ommatidia. Thus we considered as members of the same clone those labelled cells within an area of seven ommatidia, one central, containing the majority of labelled cells and 6 immediately neighbouring. The diagnosis of $rdgB/rdgB$ R1 to R6 cells can be performed very easily at every level of the ommatidium, but not that of w/w R cells. They represent a further source of error. The pigment grains within $w/+$ or $+/+$ R

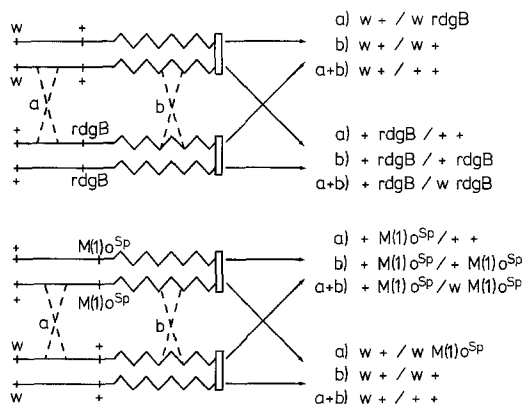


Fig. 3. Genotype and position of possible recombination events within the first chromosome of the flies used for the present investigations. See text for further explanation

cells are irregularly distributed. They are more abundant at the most distal parts of the R cells, decreasing proximally (taking the centre of the head as point of reference). This irregularity in the distribution of pigment grains is even more pronounced in R7 cells, which contain a considerably lower absolute number of pigment grains than R1 to R6 cells. To minimize errors we limited our observations to the most distal parts of the ommatidia and in some questionable cases we tried to confirm our diagnosis by observations of the same cell in some of the neighbouring thin sections with the electron microscope. This was particularly necessary for assessing the phenotype of R7 cells. A single pigment grain visible within a R cell was sufficient to assign that cell a pigmented phenotype, as *w/w* cells are *completely* devoid of pigment grains.

The total frequency of clones which can be induced during the third instar, as well as their position within the compound eye, depends on the one hand on the dose of X-ray used and on the other hand on the age of the larvae at irradiation. We used 2 different X-ray doses, 600 *r* and 1000 *r* (15 mA, 100 KV, 1.5 mm Al filter). Irradiating with a dose of 600 *r* the total frequency of R cell clones is very low, 6.2 ± 1.6 clones per eye. Since the characteristics of the clones obtained in both series are qualitatively the same, we preferred to use the higher dose yielding to a total frequency of 13.6 ± 4.9 R cell clones per eye, in spite of increasing the risk of taking as members of the same clone cells which actually belong to different clones (see below).

The characteristic pattern of cell proliferation in eye imaginal discs during the third instar reveals itself again in these mosaics. Figure 4 illustrates the position of clones found in 4 different specimens which were irradiated as third instar larvae of increasing age. The 2 mitotic waves which cross the eye disc during this stage explain that clones induced at the beginning of the instar are restricted to the posterior part of the adult eye, expand in their localization throughout the whole eye with later irradiations, and are restricted to its anterior part after irradiations at the end of the third larval stage or at the first hours after pupariation. The composition of the clones is, furthermore, also reminiscent of the ^3H -thymidine labelling behaviour of R cells: provided that the posterior wave of cell divisions was already active in the eye disc at the time of irradiation, the clones from the posterior part of the field of labelling contain only genetically labelled R1, R6 and/or R7 cells whilst those from the anterior parts are composed of R cells of all classes.

A similar distribution of clone sizes is obtained after irradiation at any given time during the third stage with the exception of the first few hours. During the first 10 h of the third larval stage, relatively large clones can be found within the anterior part of the compound eye. They differ essentially from those of the rest of this stage, and are very similar in frequency, size and cellular composition to the clones from the second stage. Most probably they arise from divisions within the last of the three mitotic waves found by Becker (1957) in squash preparations of the second stage eye disc, which overlaps between the second and the third instars. In a similar way, the divisional activity of the third stage, i.e. the 2 mitotic waves described in the previous section with ^3H -thymidine autoradiography, extends into the pupal stage. Thus, when speaking of third stage clones, we refer to those induced during the time between 10 h after the beginning of the third instar and 10 h after pupariation.

This mosaic material is particularly suitable for the analysis of possible cell lineages during ommatidial development, since in each clone all the cells are genealogically linked. We have analysed the composition of such spots, concentrating our attention on the receptor cells R1 to R7. We did not consider R8 cells which are located proximally in the ommatidia, in order to avoid serial sectioning through very large parts of the eye.

Cell Lineages

The first question which can be answered by means of this technique is whether the secondary pigment and R cells, which are in close proximity in the ommatidia, arise from separate cell lineages and, if so, at which stage in development segregation into these lines takes place. A prediction can be made: if both

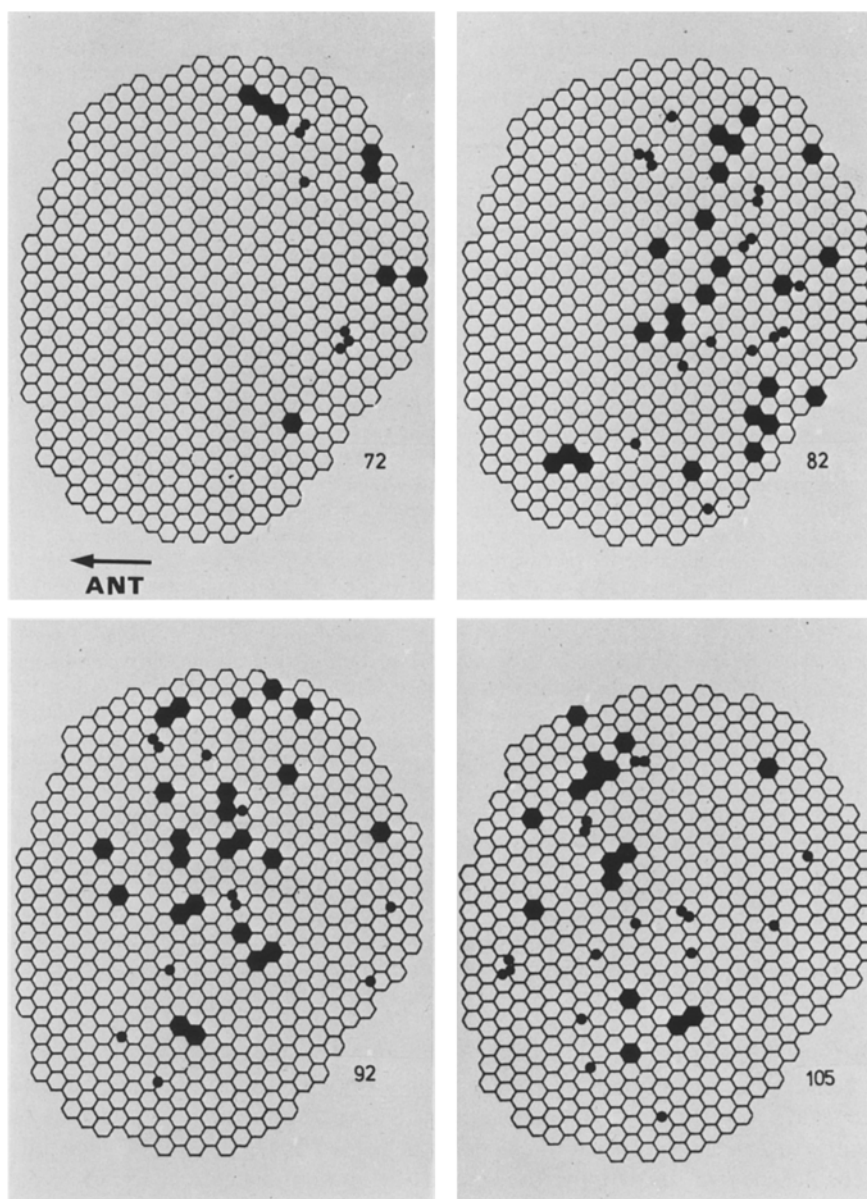


Fig. 4. Illustrates the frequency and distribution of twin-spots and single clones within the left eyes of 4 flies irradiated at different times during the third larval instar. Developmental time is calculated from oviposition. Black ommatidia contain one or more genetically labelled R1 to R7 cells, dots represent single *w/w* secondary pigment cells. The phenotype of R8 and of primary pigment cells was not scored. All the eyes used for these investigations were sectioned in series of alternating thick and thin sections. The position of the clones within the eyes represented in the Figure was marked on composite electron micrographs of 1 thin section from each group of sections. The clones identified were then translated onto standardized drawings of the compound eye, taking care to preserve the reciprocal topographical relationships between the different clones. The clones from the remaining experimental eyes were scored under direct electron microscope observation and photographed at the same magnification

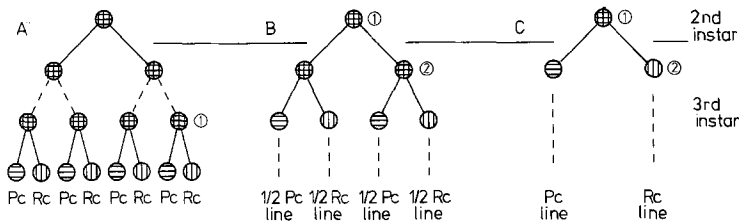


Fig. 5. Three models illustrating the segregation of receptor (Rc) and secondary pigment (Pc) cell lines. Model A assumes that segregation takes place at the last mitosis. Each division gives rise to one R cell and to one secondary pigment cell. Thus inducing mitotic recombination at 1 invariably results in mixed clones. The actual composition of the clones analysed for the present study demonstrates that this model is not applicable. B shows that segregation occurs after the first mitosis of eye anlage cells in the third instar. Mitotic recombination at 2 results in clones of R cells and of secondary pigment cells containing the highest figures of both elements. In model C segregation occurs between second and third instar. Mitotic recombination at 2, or at lower levels, results in clearly separated clones of R cells and of secondary pigment cells. Our results can be interpreted as indicative that model C is realised. However, a decision between alternatives B and C is not possible. See text

types of cells were derived from the same precursors, i.e. if segregation between lines was not yet accomplished at the time of irradiation, the largest *w/w* clones should invariably include pigment and R cells. We consider the largest clones preferentially in this case because they are most likely to be derived from cells with the highest potential for division at irradiation, and therefore the highest probability of giving rise to cells of both types. On the other hand, if pigment and R cell lines have already segregated at the time of irradiation, clearly separated clones of R cells and of pigment cells should be found (see Fig. 5).

Mitotic recombination induced during the second stage gives rise to clones which are *always* composed of both pigment (primary and secondary) and receptor cells (Becker, 1957; and own observations). However, the analysis of spots induced during the third instar shows that the largest *w/w* clones *do not invariably contain* both types of cells (see Table 2). From a total of 757 R cell single clones and twin-spots, 39 were found to contain both R and secondary pigment cells. Since mixed clones are present only in a few cases, it seems clear that R cells and secondary pigment cells must have different precursors (see Fig. 5). However, the time of segregation cannot be determined from the present material for the following reason: in fact there is no clear relationship between the size of R cell *w/w* clone and the concomitant labelling of pigment cells. On the one hand only 1 single clone with 5 *w/w* R cells was found. This clone carried 1 *w/w* secondary pigment cell. If we assume that this clone is derived from a cell with the highest divisional capability at the time of irradiation, and that segregation of lines had not yet taken place at that time, one should expect that the number of secondary pigment cells derived from it is also close to the maximum of clonally related secondary pigment cells (this number has been found to be 4, i.e. the largest *w/w* pigment cell clones contained 4 cells). On the other hand, 4 of 12 *w/w* single clones

Table 2. Clones of receptor and secondary pigment cells (spe)

Number of clones	Number of R cells/clone	Number of s.p.c./clone	Phenotype of R cells	Total number of R cell clones
Single clones				
1	5	1	w	1
1	4	4	w	12
2	4	3	w	
1	4	2	w	
2	3	3	w	31
1	3	3	rdgB	
1	3	2	w	
1	2	4	w	119
1	2	3	w	
2	2	2	w	
1	2	2	rdgB	
2	2	1	w	
1	2	1	rdgB	
2	1	3	rdgB	362
1	1	2	w	
3	1	1	rdgB	
7	1	1	w	
Twin-spots				
1	8	1	5 w, 3 rdgB	4
1	7	1	3 w, 4 rdgB	10
1	5	1	3 w, 2 rdgB	21
2	4	3	2 w, 2 rdgB	46
1	4	1	3 w, 1 rdgB	
1	3	3	2 w, 1 rdgB	64
1	3	2	2 w, 1 rdgB	
1	3	1	2 w, 1 rdgB	

with 4 R cells showed a mixed population of R and pigment cells. Again in this case the numerical relationships between both types of cells are not clear. The same argumentation applies to the twin-spots carrying *w/w* pigment cells (Table 2). We tend to believe that the mixed clones found are the result of double-hits in close proximity, i.e. their constituent cells actually derive from two different precursors having undergone mitotic recombination simultaneously.

For these reasons, we deduce that segregation into a R cell line and a secondary pigment cell line takes place between the second and the third larval stages or at the latest after one of the first divisions on the third instar anlage cells (see Fig. 5).

The Lineage of Receptor Cells

Granted the existence of separate cell lines and thus distinct precursors for receptor and secondary pigment cells, the question which logically follows is: how many R cells can be derived from each precursor? In principle, this number

should be equal to the number of R cells in the largest twin-spots. However, the answer to this question can only be approximate for the following reasons: (i) Although somatic crossing-over is a relatively rare event, and the limits between different clones are generally well defined, it is still possible that 2 neighbouring precursors could undergo mitotic recombination simultaneously and their descendants intermingle with each other. In this case a distinction between members of each clone would be impossible (see above the discussion on mixed clones). (ii) The gene *rdgB* only affects R1 to R6 cells and therefore R7 cells cannot be scored with this marker. (iii) Cell death occurs within the eye anlage during normal development (Fristrom, 1969) and we have no exact information about the extent of this phenomenon. (iv) Our estimates are subject to the error of not considering R8 cells which naturally must originate from the same precursors.

Cell death produced by X-irradiation of third instar larvae is a further factor to be taken into account. Becker (1957) showed that after irradiation of third instar larvae, imago hatch which each have characteristic, longitudinally oriented zones of distortion of the ommatidial arrangement in both eyes. The extent of such a zone depends on the X-ray dosage. After 1000 *r*, the eye roughening extends through about 5–6 vertical rows of ommatidia. The number of ommatidia affected within this region varied between 11 and 46 in six cases where larvae of different ages were irradiated. With a dosage of 600 *r* only a few ommatidia are affected. Histological observation of such eyes shows that ommatidia within the rough region are defective in some cells. Representatives of every cell type may be missing. In order to account for this phenomenon, which could have an influence on the results, we only considered cell clones in normally patterned ommatidia, situated outside the region of roughening.

For these reasons we cannot make an accurate assessment of the exact number *produced* by each precursor, but we can make good estimates about the number of cells which actually *succeed in differentiating*.

The size of the twin-spots induced during the third instar is illustrated in Table 3.

Groups were formed according to the size of the twin-spot, and the number of R cells of classes R1 to R7 present within the twin-spot was tabulated. Example: the upper case of Table 3 shows that 140 twin-spots with 2 or 3 cells were found. 55 of these contained a single R1 cell, 21 contained 2 and 1 contained 3 different R1 cells. A total of 100 R1 cells are therefore present within 76 twin-spots with 2 and 64 twin-spots with 3 cells. The values from all the groups are summarized in the lower case. The Table shows that several R1 to R6 cells may be found within a twin-spot, whereas the R7 cell occurs only once (see below). Two further factors are striking in this Table: the very small number of R7 cells and the very large number of R1 and R6 cells. The very small number of R7 cells can be explained taking into account that one of the markers used, *rdgB*, does not label R7 cells. Assuming that as many *rdgB/rdgB* R7 cells were present within the twin-spots studied as *w/w*, this would duplicate the number of these cells, thus reaching values close to those of R1 to R6 cells. The very high numbers of R1 and R6 cells cannot be explained satisfactorily. The figures for these two cells should actually be slightly higher than those of the remaining cells, because they originate from mitotic activity within both the anterior and the posterior waves and they arise as the last elements of the lineage (see below). Therefore the number of targets giving rise to R1 or R6 cells is higher. We believe that these figures are partly artifactually produced, although we are unable to find a convincing reason for this artifact. This supposition finds support when these values are compared with those given in Table 5 for the same R cell classes.

The largest twin-spots we found consist of 9 cells, but taking the factors mentioned above into account this figure is probably too high. The composition

Table 3. Composition of *w rdgB* twin-spots. Analysis of the size and composition of twin-spots induced during the third instar

Number of R cells/ twin-spot	Class of R cells	Number of each R cell class/twin-spot					Total number of twin-spots ()=number of R cells
		1	2	3	4	Σ	
2-3	R1	55	21	1	0	100	76 (2)
	R2	42	4	0	0	50	64 (3)
	R3	32	1	0	0	34	140
	R4	33	2	0	0	37	
	R5	26	2	0	0	30	
	R6	58	8	2	0	80	
	R7	13	0	0	0	13	
4-7	R1	42	21	6	0	102	46 (4)
	R2	45	7	0	0	59	21 (5)
	R3	31	6	0	0	43	9 (6)
	R4	39	8	1	0	58	10 (7)
	R5	39	7	0	0	53	86
	R6	36	15	2	1	76	
	R7	22	0	0	0	22	
8-9	R1	6	1	0	0	8	4 (8)
	R2	3	1	0	0	5	2 (9)
	R3	3	2	1	0	10	6
	R4	4	1	0	0	6	
	R5	4	1	0	0	6	
	R6	2	3	1	0	11	
	R7	4	0	0	0	4	
2-9	R1	103	43	7	0	210	232
	R2	90	12	0	0	114	
	R3	66	9	1	0	87	
	R4	76	11	1	0	101	
	R5	69	10	0	0	89	
	R6	96	26	5	1	167	
	R7	39	0	0	0	39	

Table 4. Composition of single (*w* and *rdgB*) clones. Analysis of the size and composition of single *w/w* and *rdgB/rdgB* R cell clones

Number of R cells/clone	Class of R cells	Number of each R cell class/clone			Total number of clones ()=number of R cells
		1	2	Σ	
1-5	R1	109	3	115	1 (5)
	R2	60	4	68	8 (4)
	R3	54	3	60	21 (3)
	R4	50	1	52	72 (2)
	R5	68	2	72	245 (1)
	R6	85	4	93	347
	R7	31	0	31	

of these clones is such that they would best be explained as arising from a mixing of the descendants of 2 different precursors: we collected 2 different twin-spots of 9 cells each and both consisted of 7 *w/w* and 2 *rdgB/rdgB* cells. Since the largest single clone found has only 5 cells (see Table 4), we believe that the twin-spots of 9 R cells are the result of double-hits. A more reasonable number seems to be 8, judging from the composition of these twin-spots. Whatever the exact number is, 8 or 9 (disregarding R8 cells, naturally), it suggests that R cell precursors are able to produce a maximum number of cells which is close to the number of elements necessary for building one ommatidium.

Minute⁺ clones in a Minute background

We then asked the following question: how many R cells can be derived from a *Minute⁺* R cell precursor which grows in a *Minute* background? *Minute* flies are mutants, autonomous in their expression, showing among other features a characteristic delay in development as compared with wild-type flies (Lindsley and Grell, 1968). It has been shown (Morata and Ripoll, 1975) that individual cells within a *M* anlage can be changed to a *M⁺* condition by means of mitotic recombination. The resulting *M⁺* cells can easily be labelled at the same crossing-over event with a cuticle marker and thereby their descendants can be distinguished among the progeny of the remaining, *M*, cells. This technique has been used extensively in the mesothoracic anlage and has contributed decisively to the discovery of so-called developmental compartments (Garcia-Bellido et al., 1973; Crick and Lawrence, 1975). Within the *M* wing, *M⁺* clones induced at any given developmental stage up to the third instar have been found to be considerably larger than those induced on a *M⁺* background. This is explained on the basis that the *M⁺* cells are able to perform more mitoses, and thus give rise to more descendants than the *M* cells growing within the same anlage.

Irradiation of female larvae from the cross $+/M(1)o^{Sp} \times w/Y^{+}$ leads to somatic crossing-over in some cells of the eye anlage. When mitotic recombination occurs between the *M* locus (1-56.5) (Lindsley and Grell, 1968) and the centromere (Fig. 3) the result is a *w+/w+* cell and a $+M(1)o^{Sp}/+M(1)o^{Sp}$ cell, which is supposed to die because the mutation has been described to be cell-lethal under homozygous conditions (Stern and Tokunaga, 1971). Since the former cell is supposed to divide faster than the remaining cells, it should also be able to divide more frequently than it would when growing in a *M⁺* background, as the time it has at its disposal is now longer. The total delay in development of *M(1)o^{Sp}* flies has been calculated to be 35 ± 6 h, which seems to be confined to larval stages (Morata and Ripoll, 1975).

For this experiment $+/M(1)o^{Sp}$ females, mated with *w/Y⁺* males, were allowed to lay eggs on Petri dishes containing medium for a period of 3 days. After withdrawal of the parental flies, the larvae were allowed to continue development on the same Petri dishes until the first pupae appeared. At this time irradiation with approximately 440 r (15 mA, 100 kV, 1.5 mm Al filter) was performed. After irradiation, the pupae from the first 24 h were discarded, and those arising during the following 48 h collected in special containers where

Table 5. Composition of M^+ w clones. Analysis of the size and composition of w/w R cell clones induced during the third instar in *Minute* flies

Number of R cells/clone	Class of R cells	Number of each R cell class/clone			Total number of clones () = number of R cells
		1	2	Σ	
1-4	R1	56	3	62	4 (4)
	R2	32	0	32	10 (3)
	R3	23	2	27	47 (2)
	R4	22	2	26	117 (1)
	R5	28	0	28	178
	R6	46	2	50	
	R7	32	0	32	

the imagos hatched. The eyes of flies of the phenotype $+/M(1)o^{Sp}$ were prepared for electron microscope observations. The frequency of w/w R cell clones under these conditions was 5.1 ± 3.3 per eye.

Table 5 shows the distribution of clone sizes and the composition of w/w R cell clones collected after irradiation of third instar larvae. The largest R cell clones found have 4 cells. Secondary pigment cell clones contained maxima of 3 cells. This is a very surprising result since these numbers closely correspond with those of the w/w grown in a M^+ anlage (see above), and $w M^+/w M^+$ clones induced in a M background at a given stage of the second instar have on average a size of 29 ± 12.2 ommatidia (M. Waitz and J.A. Campos-Ortega, unpublished) whilst the size of second instar control w/w clones in M^+ animals induced at approximately the same developmental stage is 5.4 ± 1.9 ommatidia (unpublished observations from our laboratory: see Becker, 1957).

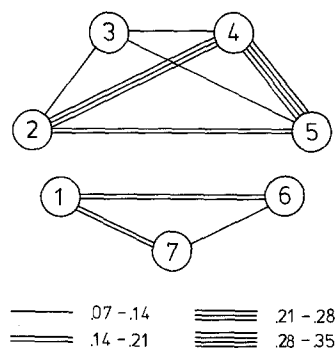
A possible explanation for the size of third instar w/w clones growing in $M(1)o^{Sp}$ flies is that the somatic crossing-over event leading to the production of w/w cells did not succeed in taking out the M gene simultaneously from these cells (Fig. 3). In this case the clones under consideration would actually be $w M(1)o^{Sp}/w+$ and not $w+/w+$ as they are supposed to be; thus their cells would be subject to the same slow growth as the remaining, non-labelled cells of the eye anlage. However, this possibility appears to us to be very implausible. Judging from the size of w/w clones induced during earlier instars in flies of the same genetic constitution, compared with that of control clones in a M^+ background (refer to Garcia-Bellido et al., 1976), the elimination of the M locus from the $+M(1)o^{Sp}/w+$ cells at their labelling occurs in the proportion of about 6 to 4. A similar proportion would be expected for the third instar, as there is no reason to believe that somatic crossing-over during this stage proceeds differently from any other developmental stage.

Assuming that this is correct the size of the clones under consideration could be interpreted as an expression of an autonomous production of R cells by precursors which, in that case, would be independent of developmental time.

Composition of Clones

Returning now to the twin-spots, we asked: which classes of R cells do the clones contain? The small size of the twin-spots and of the single clones should

Fig. 6. Analysis of the distribution of genetic label in clones. Coefficients of correlation above the level of significance (0.07) for different cell pairs of the ommatidium are shown. R8 cells are not taken into account. This coefficient expresses the probability that one cell of a particular cell pair will be genetically labelled when the other cell of the pair is labelled. For the calculation of these coefficients the number of genetically labelled R cells was compiled from twin-spots, single *w/w* clones and *rdgB/rdgB* clones irrespective of their location within different ommatidia



enable us to study the genealogical relationships between the different types of R cells.

First of all, we analysed the distribution of genetic label in twin-spots and single clones by establishing coefficients of correlation for different pairs of R cells, based on the frequency with which both cells of the pair had the same genetic label. We found that cells from either the equatorial or the polar group frequently have the same phenotype (Fig. 6), so confirming the results already obtained with the analysis of large clones induced during the first instar (Hofbauer and Campos-Ortega, 1976) and the results of ^3H -thymidine autoradiography (Fig. 2). These data therefore support the hypothesis that the members of these cell pairs are frequently derived from the same mitoses.

The composition of the twin-spots indicates, secondly, that the cells from the equatorial and from the polar groups are related by ancestry (Fig. 7B, C and D), but that they generally belong to different branches of the same genealogical tree; R7 cells have invariably been found to be sisters of R1 or R6 and never seem to originate together with R2, R3, R4 or R5, although these represent the "cousins" of R7 cells. In spite of this relationship, R1 and R6 cells, conversely, do not invariably arise together with R7; in some cases they have been found to be partners of R2, R3, R4 or R5 cells (see Fig. 7A), or even of another R1 or R6 cell (Tables 3 and 4). It must be emphasized that we are not dealing with cells from a single ommatidium, but rather with R1 to R7 cell *classes* which may be, and often are, located in different neighbouring ommatidia.

A further striking result of this analysis is that all the twin-spots and single clones we collected and studied, including those grown on *M* flies, contained a maximum of one R7 cell but a variable proportion of R1 to R6 cells (Tables 3, 4 and 5). In spite of the fact that one of the markers we used, *rdgB*, does not label R7 cells, this observation is very suggestive for some kind of difference between the presumptive R1 to R6 cells and the presumptive R7 cells. Cells which are going to differentiate as R1 to R6 elements seem to be equivalent at birth since they can apparently adopt alternative positions in the ommatidium. In some extreme cases (Table 3, 4 and 5), sister R1 to R6 cells have been found to differentiate as representatives of the same R cell class within different neighbouring ommatidia. Given that conditions are similar for presumptive

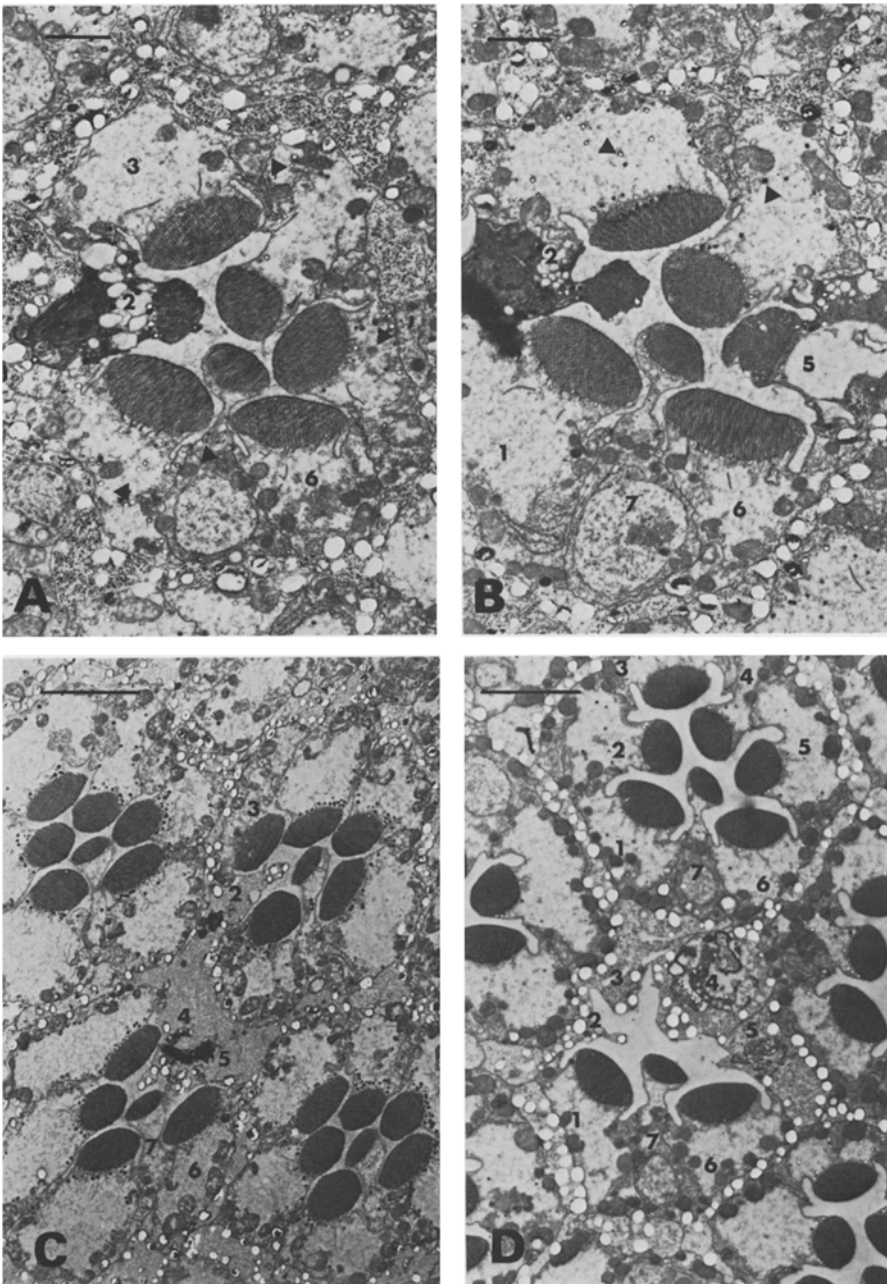


Fig. 7. Examples of 4 different twin-spots induced during the third instar. In A) cells R3 and R6 are *w/w*, cell R2 is *rdgB/rdgB*. The triangles point to pigment granules within wild-type cells. This picture documents that R1 or R6 cells (in this particular case R6) may also arise from the mitotic activity within the anterior zone. In B) cells R2 and R5 are *rdgB/rdgB* and cells R1, R7 and R6 are *w/w*. This case shows that equatorial and polar cells from the same ommatidium may actually be related by ancestry. The bar in A and B represents 0.5 μm . C) The members of this twin-spot are distributed through two neighbouring ommatidia. The clone consists of six cells, three *w/w* and three *rdgB/rdgB*. D) shows a twin-spot with 7 elements: polar cells R2 to R5 are *rdgB/rdgB* and equatorial cells R1, R6 and R7 are *w/w*. This picture illustrates the main conclusion of our study, namely that precursors of receptor cells are able to produce all R cells necessary for the formation of 1 ommatidium. The ommatidium in the upper part of Figure 7D shows the normal pattern of receptor cells R1 to R7. R8 cells, not visible at this level of sectioning, are restricted to the most proximal third of the ommatidia. The bar in C and D represents 1 μm

R7 cells, one would expect that they would behave at differentiation like R1 to R6 cells. However, our data indicate that R7 cells, on the contrary, are unable to differentiate as any other element in the R cell cluster.

Conclusions

We should like to summarize the main conclusions of our work. The results described above indicate that separate lineages for (secondary) pigment cells and for R cells segregate during the proliferative phase of eye development (the reader is referred to the paper by Garcia-Bellido and Merriam, 1971, where similar findings are described for the mesothoracic disc). Furthermore, it has been shown that under the present experimental conditions precursors of R cells are able to give rise to 8 cells. However, since this number may actually be subject to slight variations owing to difficulties in the interpretation of the results, we must accept that precursors may even produce 9 different R cells (disregarding R8). The results of clone size analysis on *M* flies suggest that precursors produce *autonomously* that number of cells. Our data indicate that among the descendants from each precursor, one cell differentiates as R7, another as R1 or R6, and the remaining ones as some of the R1, R2, R3, R4, R5 and R6 cells. The final position within the ommatidia, and therefore the nature, of these last cells can be interchanged, but not that of the former two which, we repeat, will give rise to one R7 and one R1 or R6. Finally, the data from the ³H-thymidine autoradiography allow an inference about the time of origin of photoreceptor cells. The polar cells, R2 to R5, and R8 originate as the first elements of the ommatidium; R7 originates, together with one R1 or one R6, as the last R cell of the ommatidium.

These results strongly suggest that fixed cell lineages are involved in the development of the ommatidia of *Drosophila melanogaster* and that during the third instar *determination of photoreceptor cells is not exclusively based on positional information*.

Pattern Formation within the Ommatidium

The degree of genetic determinism in the production of R cells that is indicated by the present study seems to be of the order of cell groups; that is, each precursor has been found to produce a single R7 cell and a variable proportion of R1 to R6 cells. The number of elements from the R1 to R6 group might vary between 7 and 8. This variability would not argue against fixed cell lineages since cell death occurs during normal development (Fristrom, 1969); if the extent of cell death varies from region to region of the imaginal disc, the number of genealogically linked R1 to R6 cells as calculated with the present technique would also be expected to vary. It is possible, although we have no data, that a single R8 cell is also derived from each precursor.

The formation of the final pattern seems to depend on the position that the different R1 to R6 cells, arising from the same or from different precursors,

assume in a developing ommatidium. Initially R1 to R6 cells have been found to behave as equivalent, their ultimate fate being conditioned by the position they occupy in the ommatidium. It has been shown by Ready (1975) that ommatidium formation in *Drosophila* is accomplished in steps by means of clustering of presumptive receptor cells (refer to Campos-Ortega and Gateff, 1976). The present material confirms that the process of R cell clustering follows a sequence. For example, the very high correlation of R4 and R5 cells indicates a frequent common mitotic origin; the posterior mitotic wave exclusively produces equatorial cells; and the fact that these cells are the last elements to meet the preommatidial clusters, as deduced from their position within the posterior, chronologically second band of divisions, all speak in favour of a stereotyped succession of events. Given a fixed sequence in the process of R cell clustering, which in purely speculative terms could be conditioned by, for example, the progressive acquisition of a given polarity by single cells in the clusters, the position that R1 to R6 cells assume within the developing ommatidium would be a consequence of the time at which they meet the assembling group. This would explain why R1 to R6 cells are present more than once within some of the clones we studied: simply because they originated at a time when positions corresponding to the R cell class under consideration were simultaneously available in different but adjacent preommatidial clusters.

Our data so far do not distinguish between several alternative clustering sequences. At the present time we can only affirm that R8 and polar cells are the first elements of the sequence, and that equatorial cells are the last. To this we can add that all the cells which in an adult animal are organized to form a single ommatidium, may or may not be members of the same lineage. On the one hand, our data show those elements which arise from genealogically linked mitoses tend to be incorporated in the same ommatidium in a high proportion of cases, and on the other hand that this is not a necessary prerequisite for the final pattern. We should like to make a speculative prediction about how the final ommatidial pattern is generated in the eye disc: (i) Precursor cells give rise autonomously to at least 8 cells belonging to three different groups; these cells differentiate as one R8, some of the R1 to R6, and one R7. (ii) Cells associate to form an ommatidium according to a fixed sequence. (iii) After clustering the polarity of the cells is fixed and thereby the pattern becomes unmodifiable.

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References

- Becker, H.J.: Über Röntgenmosaikflecken und Defektmutationen am Auge von *Drosophila* und die Entwicklungsphysiologie des Auges. Z. indukt. Abstamm. u. Vererb.-L. **88**, 333–373 (1957)
- Benzer, S.: From the gene to behavior. J. Am. Med. Assn. **218**, 1015–1022 (1971)
- Benzer, S.: Genetic dissection of behaviour. Scient. Am. **229** (12), 24–37 (1972)

- Bernard, F.: Recherches sur la morphogénèse des yeux composés d'arthropodes. Bull. biol. Fr. Belg. (Suppl.) **23**, 1–162 (1937)
- Campos-Ortega, J.A., Gateff, E.A.: The development of ommatidial patterning in metamorphosed eye imaginal disc implants of *Drosophila melanogaster*. Wilhelm Roux's Archives **179**, 373–392 (1976)
- Crick, F.H.C., Lawrence, P.A.: Compartments and polyclones in insect development. Science **189**, 340–347 (1975)
- Fristrom, D.: Cellular degeneration in the production of some mutant phenotypes in *Drosophila melanogaster*. Molec. Gen. Genetics **103**, 363–379 (1969)
- Garcia-Bellido, A.: Pattern formation in imaginal disks. In: The biology of imaginal disks (eds., H. Ursprung and R. Nöthiger), pp. 59–91. Berlin. Heidelberg, New York: Springer-Verlag 1972
- Garcia-Bellido, A., Merriam, J.R.: Parameters of the wing imaginal disc development of *Drosophila melanogaster*. Dev. Biol. **24**, 61–87 (1971)
- Garcia-Bellido, A., Ripoll, P., Morata, G.: Developmental compartmentalisation of the wing disk of *Drosophila*. Nature New Biology **245**, 251–253 (1973)
- Garcia-Bellido, A., Ripoll, P., Morata, G.: Developmental compartmentalization in the dorsal mesothoracic disk of *Drosophila*. Dev. Biol. **48**, 132–147 (1976)
- Haendle, J.: Röntgeninduzierte mitotische Rekombination bei *Drosophila melanogaster*: I. Ihre Abhängigkeit von der Dosis, der Dosisrate und vom Spektrum. Molec. gen. Genet. **113**, 114–131 (1971)
- Hanson, T.E., Ready, D.F., Benzer, S.: Use of mosaics in the analysis of pattern formation in the retina of *Drosophila*. An. Rep. Div. Biol. Cal. Inst. Technol. **40** (1972)
- Harris, W., Stark, W., Walker, J.: Genetic dissection of the photoreceptor system in the compound eye of *Drosophila melanogaster*. J. Physiol. (Lond.) **256**, 415–439 (1976)
- Hofbauer, A., Campos-Ortega, J.A.: Cell clones and pattern formation: Genetic eye mosaics in *Drosophila melanogaster*. Wilhelm Roux's Archives **179**, 275–289 (1976)
- Kirschfeld, K.: Das neurale Superpositionsauge. Fortsch. Zool. **21**, 229–257 (1973)
- Lindsley, D.L., Grell, E.H.: Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. 627 (1968)
- Melamed, J., Trujillo-Céno, O.: The fine structure of the central cells in the ommatidia of Dipterans. J. Ultrastr. Res. **21**, 313–334 (1968)
- Morata, G., Ripoll, P.: Minutes: mutants of *Drosophila* autonomously affecting cell division rate. Dev. Biol. **42**, 211–221 (1975)
- Ready, D.F.: Pattern formation in the retina of *Drosophila*. An. Rep. Div. Biol. Cal. Inst. Technol. **203** (1973)
- Ready, D.F.: Cell proliferation and pattern formation in the developing eye of *Drosophila*. An. Rep. Div. Biol. Cal. Inst. Technol. **106** (1974)
- Ready, D.F.: Prepatterns in the developing *Drosophila* eye. An. Rep. Div. Biol. Cal. Inst. Technol. **73** (1975)
- Shelton, P.M., Lawrence, P.A.: Structure and development of ommatidia in *Oncopeltus fasciatus*. J. Embryol. exp. Morph. **32**, 337–353 (1974)
- Stern, C., Tokunaga, C.: On cell lethals in *Drosophila*. Proc. Natl. Acad. Sci. USA **68**, 329–331 (1971)

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Note Added in Proof. After this paper went to press, a full description of the findings of the group at CalTech has been published [Ready, D.F., Hanson, T.E., Benzer, S.: Development of the *Drosophila* retina, a neurocrystalline lattice. Dev. Biol., **53**, 217–240 (1976)]. Both papers are completely in agreement as far as the ³H-thymidine autoradiography findings are concerned.