THE DEVELOPMENT OF A SIMPLE PATTERN: SPACED HAIRS IN *ONCOPELTUS FASCIATUS*

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SUMMARY

Adults of *Oncopeltus fasciatus*, the milkweed bug, are covered with an evenly spaced mat of hairs. Each of these hairs develops from a single selected epidermal cell which divides and differentiates *in situ*. The process which picks out the particular epidermal cell is a simple example of pattern formation. Using a measure of uniformity of distribution an attempt was made to analyse the development of the hair pattern in time and space. Three methods were used: (i) Direct mapping of epidermis undergoing hair differentiation which allowed analysis of the spatial and temporal order in a single developing pattern: (ii) The injection of an agent which killed cells in DNA synthesis and which allowed the development of only those hairs that had completed DNA synthesis at the time of injection. Thus adults developed in which only the first-formed hairs differentiated. (iii) Treatment with the insect moulting hormone ecdysone which led to premature deposition of cuticle and curtailed hair development.

Results from these 3 approaches showed that the first hairs, far from being a random sample of the complete hair population in the normal adult, were a highly dispersed group. It is concluded that the hair pattern is built up in a precise order, with the centre of the largest available spaces being the sites where differentiation begins.

INTRODUCTION

Pattern formation in multicellular organisms is little understood. One reason is that the patterns themselves are complicated; another that the time of pattern determination is often unknown. The insect integument offers examples of simple 2-dimensional patterns, which develop at a defined stage of metamorphosis. Here pattern formation may begin with a homogeneous population of epidermal cells; then, cells occupying particular sites in that population are selected as bristle mother cells, and each divides to generate the bristle-forming cells. One typical result is a dispersed bristle pattern, where the chosen cells are always mutually separated, even though the exact sites are not identical in different individuals. The process which generates even this very simple pattern is mysterious.

There have been several spatial analyses of dispersed bristle patterns (Wigglesworth, 1940; Clever, 1958; Claxton, 1964; Lawrence, 1967, 1969) but there has been no attempt to analyse the construction of such a pattern in time. In this paper we have tried to discover how the dispersed hair pattern of adult *Oncopeltus* is constructed in time and in space.

The area chosen for analysis was the third abdominal sternite. The fifth larval stage

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bears about 150 innervated *bristles*/mm^2, which are evenly dispersed over the region, while the adult, in addition to these bristles, is clothed with 2000 non-innervated *hairs*/mm^2. During the last moult cycle the epidermal cells first divide (*proliferative* divisions) and then, progressively, particularly sited epidermal cells transform into hair mother cells, which each undergo *differentiative* divisions, to form a quartet of hair-forming cells (Lawrence, 1966). One of these cells forms the shaft of the hair, another the socket, and the remaining 2 degenerate. The bristle-forming cells of the fifth-stage larva secrete cuticular bristles once again so that the adult third sternite is covered with dense, but evenly spaced hairs and bristles (Fig. 6). The basic task was to discover which of these hairs develop first, and from this to approach the problem of spacing. There are 2 extreme hypotheses: (i) the first hairs might be formed in the centre of the largest available spaces in which case the uniformity of distribution of hairs will be maintained; and (ii) the first hairs might develop as close to each other and to the bristles as they are observed in the completed pattern, that is, they will be a random sample from the final hair population. Under this hypothesis the first hairs will rapidly decrease the uniformity of distribution of the pattern.

Three methods were used, none of which proved wholly satisfactory: (i) direct mapping of fixed material; (ii) the use of hydroxyurea as an inhibitor of DNA synthesis which was injected during the differentiative divisions; and (iii) the injection of the insect moulting hormone, ecdysone, in order to accelerate cuticle deposition and curtail hair genesis.

Taken together the results suggested that the first hairs were a highly dispersed group, and that later developing hairs progressively filled in the available spaces.

GENERAL METHODS

Oncopeltus fasciatus were cultured at 29·5 °C (Lawrence, 1966). In the following results and discussion it is assumed that the distribution of hairs in the adult cuticle results directly from the pattern-forming process occurring in the epidermis of the fifth-stage larva. Does the hair pattern undergo significant distortion between the time of its construction and when it is observed in the adult cuticle?

During the first 60 h after ecdysis the insect expands as it feeds and the integument of the third sternite is stretched until it becomes as large as the adult sternite that it will later form. Hair development does not begin until after this period of expansion (Lawrence, 1966, 1969). Subsequently the epicuticle is deposited over the epidermal cells and the hair-shafts, expands in the antero-posterior axis and is thrown into folds. These folds become partially relaxed after ecdysis to the adult, which may lead to some distortion in the hair pattern. However, we have detected little anisotropy in the adult pattern and thus do not think this could affect the experimental results and conclusions.

Analysis of the patterns

The cleaned cuticle was mounted flat in Euparal, and the hair pattern was recorded on paper by means of a projection microscope. The uniformity of distribution of the hairs was then estimated according to the method of Clark & Evans (1954) who derived an expression, *R*, which is equal to the product of twice the square root of the density (ρ) and the mean of the distances of each unit to its nearest neighbour ($\bar{r}$). Thus $R = 2\bar{r}\sqrt{\rho}$ and $0 < R \le 2\cdot15$; when $R = 1\cdot00$ the units may be randomly distributed, and *R* increases with uniformity of distribution.

INDIVIDUAL METHODS AND RESULTS

Direct mapping

Method. Camera lucida drawings were made of whole mounts of differentiating epidermis. In these preparations the epidermis contains, at any one time, cell groups in all stages of development. It was assumed that advanced cell groups (such as those with 4 cells) developed from hair mother cells which had been determined earlier than the less advanced cell groups (such as those with only 1–2 cells). The site of the future hair was *estimated* from the position of the presumptive tormogen cell and marked on the drawing. Thus, one drawing contains several stages in the development of the hair pattern. For analysis the coordinates and the age of each point were taken and the pattern examined with the help of a computer. The characteristics of patterns produced by progressive random removal of the cell groups were also calculated.
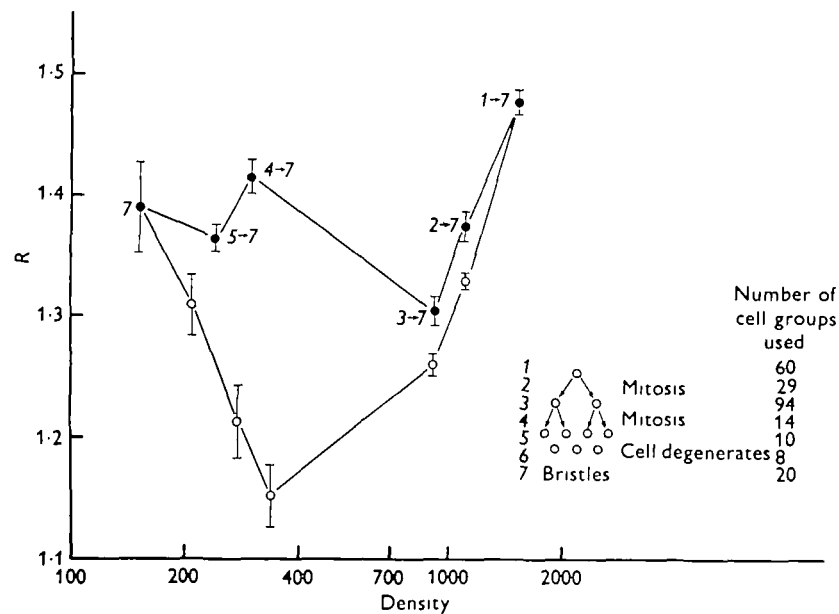

Fig. 1. The relationship of uniformity of distribution (R) and density (number/mm^2) during development of a hair pattern. The numbers next to the closed circles indicate which of the 7 cytological stages are included in the measurement at each point. The cellular events demarcating each stage are shown on the right. The closed circles represent the mean R obtained by using different boundaries. The open circles mark the mean of R estimated by removing the developing hairs in several random sequences. Vertical lines show ± s.e. of the mean.

Results. The relationship between uniformity of distribution (R) and density in one insect is plotted in Fig. 1. In the area drawn the very small sample of larval bristles was distributed unusually poorly ($R = 1\cdot39$), but the first hairs to develop (stages *4–6*, Fig. 1) did not significantly change this distribution. In these stages the tormogen cell could be identified and the site of the future hair estimated fairly accurately. For the younger cell groups (stages *1–3*, Fig. 1) the exact site of the future hair could not

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be predicted; moreover when these stages are included in the calculations, the density of units in the patterns is high and the mean nearest neighbour distance correspondingly low. In these conditions small inaccuracies in predicting the hair site become more important. This artifact may well explain the fall in R when stage *3* is included in the pattern, and the last 2 values (*2–7* and *1–7*) could also be underestimates.

The other curve in Fig. 1 is the relation if the *same hairs* are added in random order. The discrepancy between the 2 curves is most noticeable at the beginning—indeed these 2 curves make it quite certain that the first hairs are not a random sample of the final population, but a highly dispersed group.

Hydroxyurea

Method. Hydroxyurea was used in a dose which completely inhibited mitosis without affecting either the successful secretion of a normal cuticle, or the detailed appearance of non-dividing cells; 1 μl of insect Ringer, containing 20 μg of hydroxyurea was injected via the metathoracic leg into fifth-stage larvae of known age (dose = about 400 μg/g). Two of the chief effects of 20 μg of hydroxyurea (HU) are illustrated in Fig. 2. When injected early in the moult cycle, ecdysis was delayed but the insect moulted into a typical adult with normal hair density. When the inhibitor was injected at 60, 72 and 84 h there was no delay in ecdysis but there was significant inhibition of hair development. In some individuals very few hairs developed even though the bristles grew normally.

Outgrowth of the trichogen cells of both bristles and hairs occurs simultaneously at about 96 h, when HU had no effect. It seems likely therefore that HU is acting on the differentiative divisions alone and does not influence other processes essential to hair or bristle development. In adults with only very few hairs the surviving ones were of normal structure. HU is known to be an effective inhibitor of DNA synthesis *in vivo* (Philips *et al.* 1967) and it was hoped that in these experiments HU would block all cell divisions but allow all previously completed hair cell groups to differentiate normally and secrete a hair.

To learn more about the cytological effects of HU on *Oncopeltus* epidermis fifth-stage larva which were in the middle of proliferative divisions (48 ± 6 h) were injected with 20 μg of HU and fixed after different intervals. Examination of slides showed that effects could not be detected until 4 h after injection, when cells began to accumulate in prophase, while cells in metaphase, anaphase and telophase were lacking. In *Oncopeltus* the *S*-period extends into prophase and the period after the end of *S*-phase but before the beginning of metaphase is about 3 h (Lawrence, 1968). Slides fixed at 8 and 12 h after injection showed that many of these cells in prophase degenerated into chromatic droplets (Wigglesworth, 1942) which were absorbed by neighbouring cells. These results suggest that HU does not affect cells in the post-synthetic part of prophase, which go through mitosis normally during the next 3–4 h, but that cells in *S*-phase at the time of injection are inhibited from further DNA synthesis and killed. Metaphases were again found when insects were fixed 24 h after injection, although some cells were still dying. The effects of this dose of HU thus

began to wane after about 16 h. Most insects fixed 48 h after injection showed retarded development as compared with controls, but normal cytology. In one individual damage to hair cells was still occurring, although the epidermal cells were again dividing normally. It would seem that the differentiative divisions are more sensitive to HU, perhaps because of their shorter cell cycle (Lawrence, 1968).
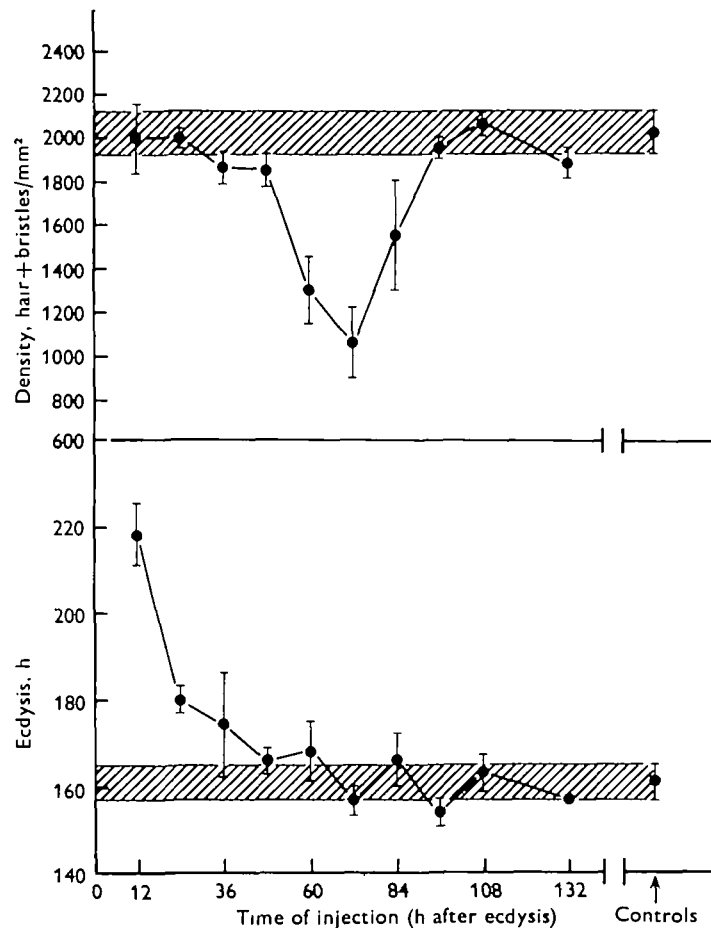

Fig. 2. Effect of hydroxyurea on density of hairs on adults and times of ecdysis to adults plotted against the time of injection measured in h after ecdysis to the fifth larval stage. The hatched area includes the mean ± s.E. of normal adults. The vertical lines on each point show ± s.E. of the mean.

A series of insects in differentiative divisions (70 h after ecdysis) were also injected with HU and fixed after different intervals. Individuals fixed 70 min after injection looked normal, but by 4 h most mitoses had ceased and the differentiating hair cells, but not the epidermal cells, were undergoing chromatolysis. Individuals fixed 12 h after injection revealed devastation of the hair-forming cells, while the majority of epidermal cells appeared unscathed (Fig. 7).

In summary these cytological observations suggest that HU kills all cells in *S*-phase

at the time of injection. When injected at 48 h after ecdysis there is still sufficient time for the epidermis to recover and differentiate, while at 70 h cells undergoing differentiative divisions are destroyed, mature hair groups are unaffected. There is no evidence that any hairs differentiate subsequent to injection at this later time.
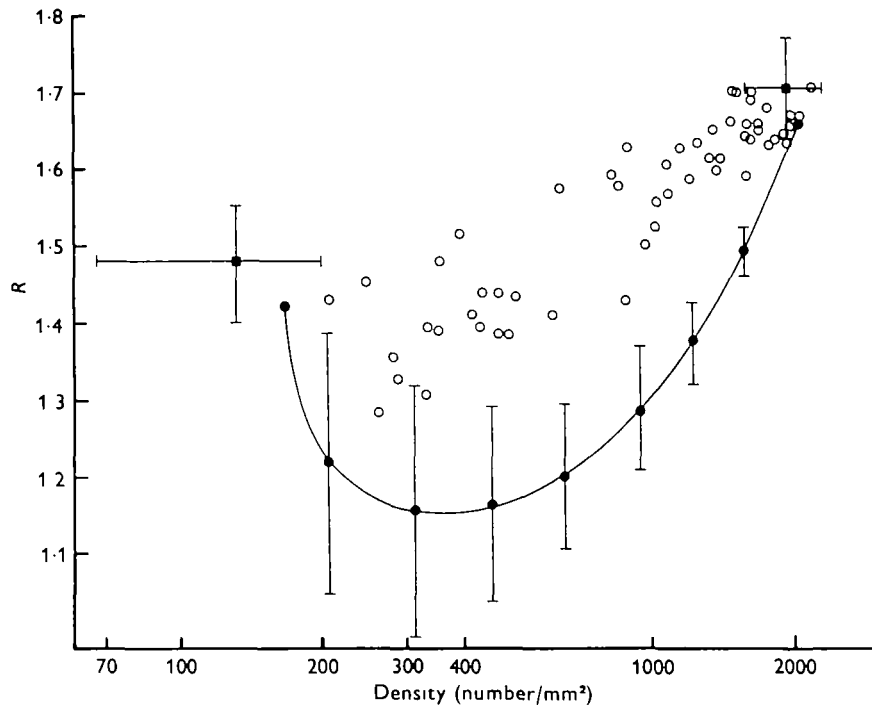

Fig. 3. The relationship between uniformity of distribution (R) and density of hairs in different adults after treatment with hydroxyurea (open circles). Means of normal adults and larvae are shown $\pm$ 2 S.D. The curve (closed circles) shows the average effect on R of removing the hairs in random order from a typical adult's pattern. This was done 8 times and the vertical lines represent $\pm$ 2 S.D.

Results. If HU killed all hair cell groups in cell divisions but did not affect mature hair cell groups, then only these mature hair cells should continue development. Ideally therefore the distribution of hairs in each affected adult insect should represent *a stage in pattern construction*. Many insects with reduced hair density were obtained (Figs. 8, 9) and the uniformity of distribution of the hairs (R), and the density was measured in each case and the relationship plotted (Fig. 3). The points can be compared with the curve produced when the adult hairs are added in random order (Lawrence, 1969, and Fig. 3). The consistent discrepancy between the 2 curves again shows that there is spatial order in the sequential placing of hairs.

There is, however, independent evidence that HU caused some spatial disruption of the developing hair pattern: examination of these reduced hair patterns has shown that sometimes hairs are found closer to each other than ever occurs in a normal pattern. This disruption probably stems from the presence of some overlap between proliferative and differentiative divisions and it is likely that death of epidermal cells

which are not replaced leads to spatial rearrangement of neighbouring cells. This effect will be less serious at later stages of differentiation because very few epidermal cells are undergoing proliferative divisions then.
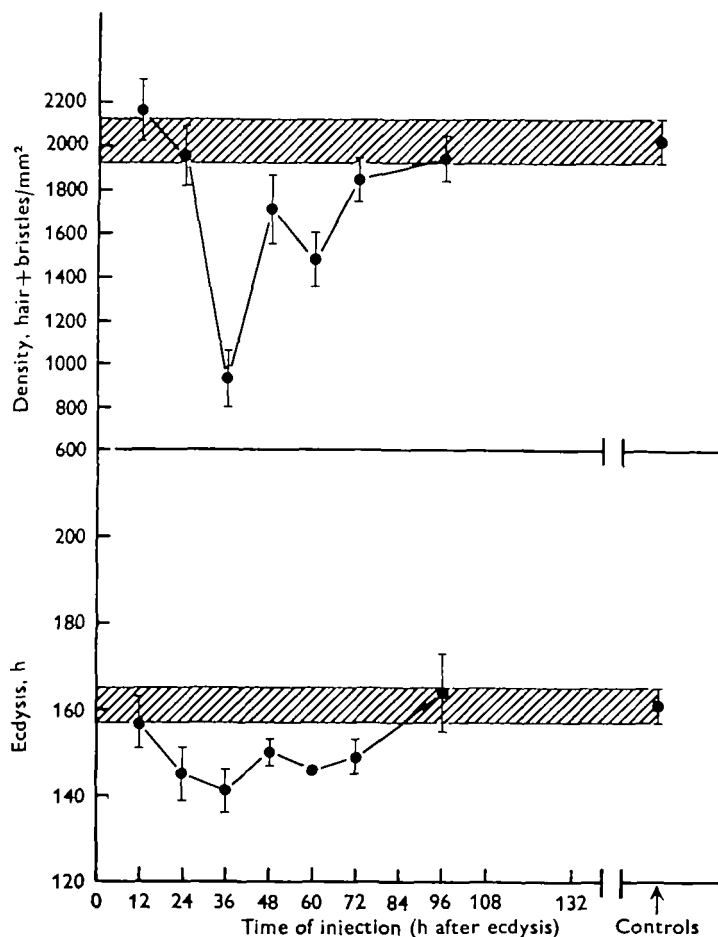

Fig. 4. Effect of ecdysterone on density of hairs on adults and times of ecdysis to adults plotted against the time of injection measured in h after ecdysis to the fifth larval stage. The hatched area includes the mean ± S.E. of normal adults. The vertical lines on each point show ± S.E. of the mean.

Ecdysone

Method. It was found that 1 μg of ecdysterone (Rohto Pharmaceuticals) injected in 1 μl of 10 % ethanol greatly advanced the time of adult ecdysis without affecting the appearance of the cuticle or the viability of the adult (dose = about 20 μg/g).

Williams (1968) reported that scaleless adults of *Samia cynthia* resulted from the injection of large doses of the insect moulting hormone, ecdysone, during the last moult cycle. Premature deposition of the cuticle had curtailed the scale differentiative divisions. In *Oncopeltus*, injection of 1 μg of ecdysterone had an equivalent effect (Fig. 4)

resulting in adults which had both reduced hair densities and underwent premature ecdysis.

It seemed reasonable to hope that in these insects the surviving hairs were those that began development early, so that they reached a sufficiently mature stage when, owing to the truncated moult cycle, cuticle deposition cut short the development of
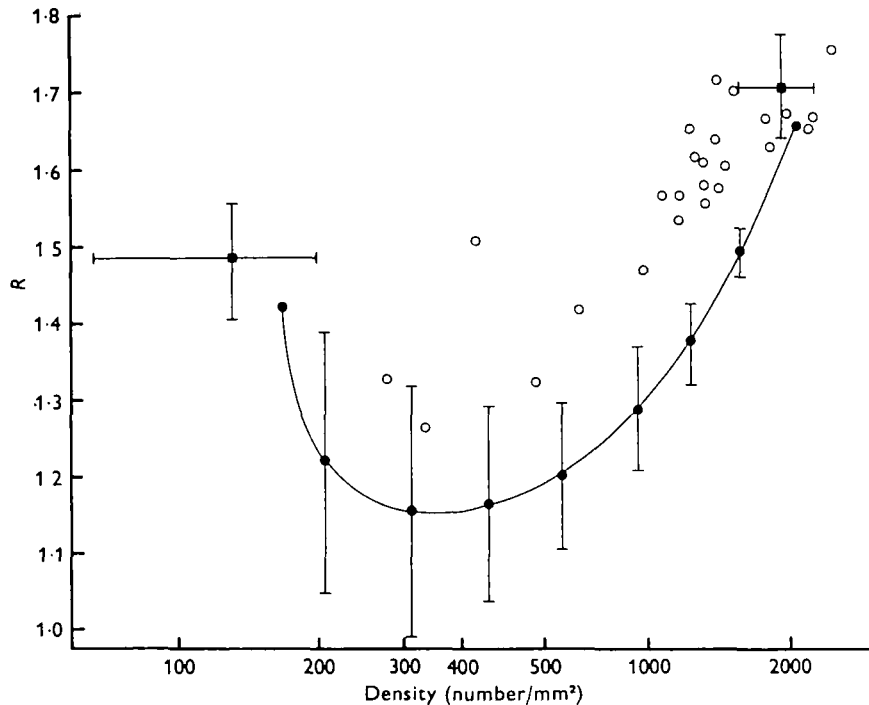

Fig. 5. The relationship between uniformity of distribution (R) and density of hairs in different adults after treatment with ecdysterone (open circles). Means of normal adults and larvae are shown ± 2 S.D. The curve (closed circles) shows the average effect on R of removing the hairs in random order from a typical adult's pattern. This was done 8 times and the vertical lines represent ± 2 S.D.

the remainder. However, it will be noted from Fig. 4 that the hair density is most sensitive to moulting hormone around 36 h; which is long before hair differentiation occurs. It is possible therefore that ecdysone affects the process of hair determination in a more indirect manner.

Result. As before the hair patterns on affected insects were examined and the uniformity of distribution (R) plotted against density, and these points compared with the random-order curve (Fig. 5). Again it is clear that those hairs that do develop are not a random sample of a complete hair pattern.

DISCUSSION

Wigglesworth (1940) regarded extant bristles as absorbing some essential growth substance and thereby inhibiting nearby cells from transforming into new bristles. This model has been examined elsewhere (Wigglesworth, 1959; Claxton, 1964;

Waddington, 1962; Lawrence, 1969, 1970 *a, b*) but its application to hair pattern development requires further discussion.

In the adult integument, hairs and bristles are mutually separated by similar distances and can therefore be regarded as equivalent units in the final pattern. The Wigglesworth model asks that the epidermal cells respond to a certain threshold level of a morphogenetic substance by developing into a hair (Lawrence, 1969). If the extant bristles absorb this substance at a fixed rate while the epidermal cells continually produce it, an increase in cell number will lead to an overproduction of the substance and its accumulation in regions distant from bristles. Here the concentration may pass the threshold level and lead to hair differentiation. Thus we can regard the bristles as surrounded by inhibitory areas; outside these areas the cells become competent to form hairs.

At metamorphosis in *Oncopeltus* the development of dense hairs requires a change in one of two components of the model: either the threshold of the epidermal cells to the postulated bristle-forming substance must fall, or the output of substance from the epidermal cells must increase. The pattern of hairs in insects which are a mosaic of larval and adult patches, shows that within the terms of the Wigglesworth model, it is the threshold which falls (Lawrence, 1969). This fall in threshold decreases the size of the inhibitory areas around bristles. If this reduction in size of inhibitory areas was completed prior to the introduction of hairs, so that without further change the adult density of hairs could be reached, then the first hairs with their associated inhibitory areas would be a random sample of the final hair population. Moreover, the relationship between the distribution (R) and density should follow the random order curve (shown on Figs. 3 and 5). Alternatively, the inhibitory areas could be large initially and fall progressively during the addition of hairs; in this model the first hairs added would be a *highly dispersed* sample of the final hair population.

The results lead to one main conclusion; there is non-random order in the spatial development of hairs. Direct observation has shown that the first hairs are a highly dispersed sample. Computer simulations have revealed that even after large numbers of hairs have been added in a non-random sequence, the curve of R against density rapidly decays to the random curve when remaining hairs are again placed in random order. Thus, the continued discrepancy between the 3 sets of experimental results and the random order curves (Figs. 1, 3 and 5) suggests that order is maintained throughout the whole process of hair addition.

These results do not agree with earlier experiments made with mitomycin C, another inhibitor of DNA synthesis and mitosis (Lawrence, 1969). However, they supersede the earlier results, as the current experiments show order, which must be present in the system. Any degree of randomness can be due to artifact and side effects of the agent used. Indeed, mitomycin does not affect the epidermis evenly; its action being more extreme near the site of the injection (Lawrence, 1969). It is certain that hydroxyurea also causes some disruption of the pattern; for this reason the current observations do not tell us how precisely the hairs are added to the developing pattern, they only define a lower limit.

Although the evidence is indirect, it all suggests that the inhibitory areas begin very large and shrink steadily as hairs are introduced. This conclusion is also consistent with the effects of juvenile hormone on the hair pattern in *Oncopeltus* (Lawrence, 1969). It was argued that at metamorphosis the inhibitory areas contract and that injected juvenile hormone could stop this process at different points and thus generate hair patterns of varying densities but even distribution.

These results are consistent with the ideas that diffusible morphogens lead to pattern formation (Wigglesworth, 1940; Child, 1941; Crick, 1970).

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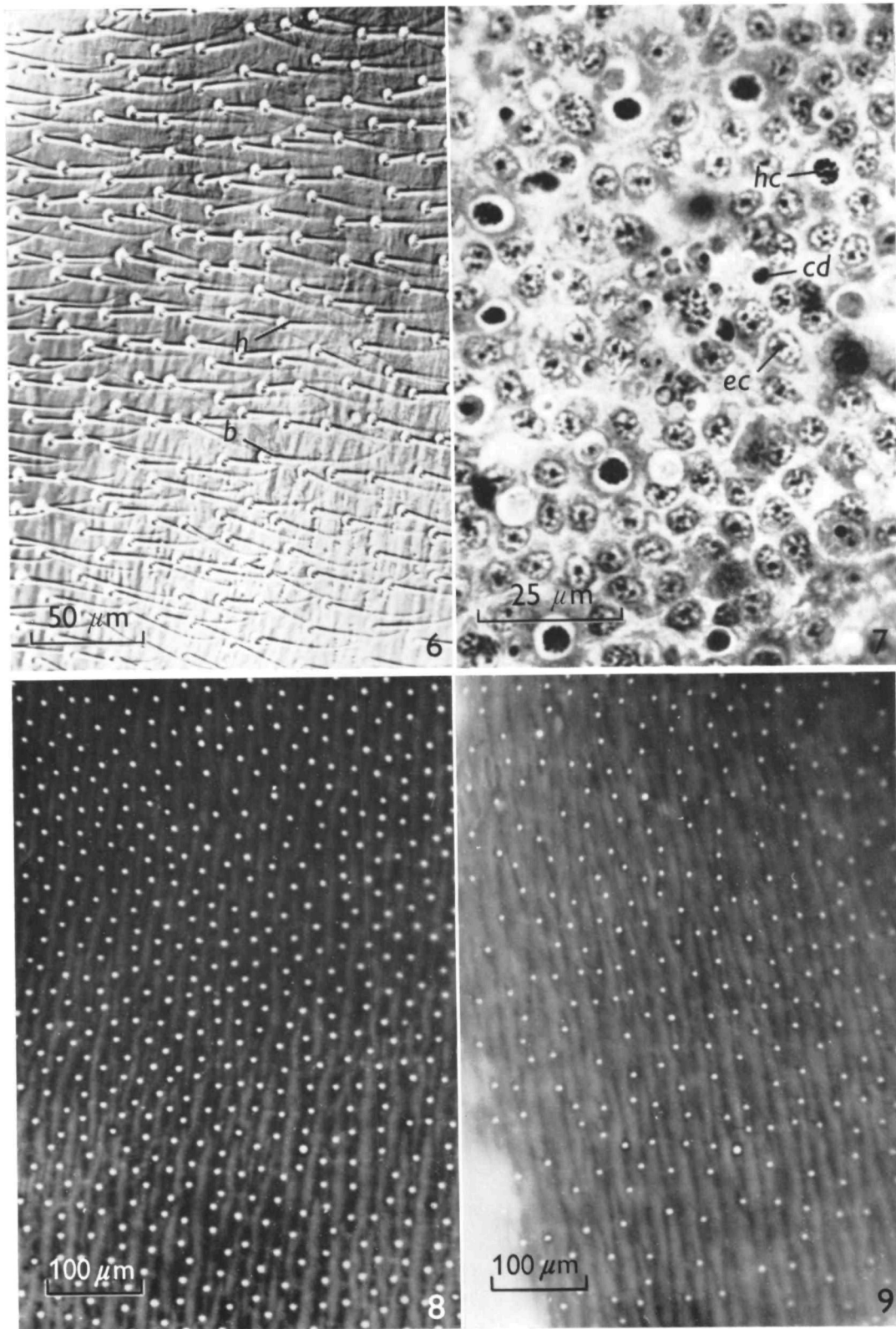
Fig. 6. A region of adult sternite of *Oncopeltus* to show orientation and spacing of hairs. Nomarski interference contrast; *b*, bristle; *h*, hair. × 350.

Fig. 7. Region of differentiating epidermis fixed 12 h after injection with hydroxyurea. Note degenerating hair cells (*hc*), epidermal cells of normal appearance (*ec*) and chromatic droplets (*cd*). × 800.

Fig. 8. Central region of third sternite of normal adult. Note density and spacing of hairs. × 150.

Fig. 9. Same region as in Fig. 8, but after injection of hydroxyurea at 60 h after ecdysis to the fifth larval stage. Note the reduced hair density. × 150.

Figs. 6–9. For legend see previous page.